Characterisation of Coagulase-negative *Staphylococcus* Isolates Recovered from Blood Cultures of Patients in a Haematology and Bone Marrow Transplant Unit

Rahila Chaudhry

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Thesis submitted by Rahila Chaudhry for the degree of Doctor of Philosophy
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SUMMARY

Infections are the most important cause of morbidity in patients with haematological malignancies and those undergoing haematopoietic stem cell transplantation. Coagulase negative *Staphylococcus* (CoNS) species are among the most frequently encountered isolates in this hospitalised bacteraemic patient group. The common therapeutic agent for the treatment of these clinically significant isolates is vancomycin. However, the emergence of resistance to other commonly used antibiotics and a shift towards the predominance to multi-resistance has resulted in new challenges to these opportunistic pathogens. Therefore, new therapeutic agents are required to combat the emergence of resistance for optimal clinical outcomes. This thesis is a study designed to characterise CoNS using phenotypic and genotypic methods together with determining the presence of important virulence factors, antibiotic-resistance including the magnitude of susceptibility to vancomycin and some of the newer class of antimicrobials used for the treatment of bacteraemic patients hospitalised for haematological malignancies. Characterisation and analysis was carried out on a total of 100 CoNS strains consecutively isolated from blood cultures of patients hospitalised in the haematology and Bone Marrow Transplant (BMT) unit of the Queen Elizabeth Hospital from April 2010 to March 2012. Comparative identification and susceptibility testing was also performed on 100 CoNS skin colonising strains obtained from a pre-admission patient (control) group. This study addresses the issue that skin colonisers could be regarded as the putative source of opportunistic bacteraemia in haematology patients. The key question being whether the CoNS strains as the aetiological agents of bacteraemia, originated from random carriage on the skin of the patients or were a result of distinct strains with enhanced virulence due to antibiotic pressure. Conventional and molecular techniques identified major phenotypic and genotypic differences with low-level similarities within and between in-patient blood culture isolates of CoNS and those skin-colonisers which could be regarded as inducers of bacteraemia. Biotyping revealed a total of eleven CoNS species of which *Staphylococcus epidermidis* was the most prevalent followed by *Staphylococcus hominis* and *Staphylococcus haemolyticus* in both patient groups. Likewise, slime the main virulent factor, was prevalent amongst both patient group *S. epidermidis* isolates. Interestingly, resistance to principal classes of commonly used anti-staphylococcal drugs was also species-specific with *S. epidermidis* demonstrating a propensity for both methicillin-resistance and multi-drug resistance. Significantly high levels of teicoplanin resistance among both *S. epidermidis* and *S. haemolyticus* in-patient isolates were also likely to express low-level or heteroresistance to vancomycin. Of the newer antibiotics evaluated, daptomycin demonstrated broad-spectrum *in-vitro* activity. The study focussing on clinically relevant combinations of vancomycin with rifampicin, gentamicin and daptomycin demonstrated either an additive or indifference effect with no antagonistic activity. Conversely vancomycin, daptomycin and rifampicin, individually and in combination demonstrated antagonistic interactions against *S. epidermidis* biofilm-embedded cells. Hence, these combinations may not be considered as potential adjunctive agents for the treatment of BSIs caused by biofilm-producing strains. Phenotypic and genotypic characterisation to ascertain the likelihood of skin-colonising strains as aetiological agents of bacteraemia revealed the presence of diverse heterogenic populations amongst all CoNS species-specific isolates of both patient groups. The findings of this study therefore do not support the hypothesis that endogenous skin-colonisers could be the potential source of CoNS-associated bacteraemia in the haematology patient group.

Key Words: CoNS, bacteraemia, antibiotic resistance, virulence factors, biofilm

Dedication

For my Son Amil

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Ethics Approval

Not applicable

List of Tables

List of Figures

List of Abbreviations

AST	Antimicrobial Susceptibility Testing
BHI	Brain heart infusion
BMT	Bone marrow transplant
BSIs	Blood stream infections
BSAC	British Society for Antimicrobial Chemotherapy
CoNS	Coagulase negative staphylococci
CBA	Columbia Blood Agar
CFU	Colony Forming Units
CLSI	Clinical and laboratory standards institute
CVC	Central Venous Catheters
DNA	Deoxyribonucleic acid
EUCAST	European committee on antimicrobial susceptibility testing
EMA	European Medicines Agency
FDA	Food and Drug Administration
GIS	Glycopeptide-Intermediate *S*taphylococci
GRSA	Glycopeptide-Resistant *Staphylococcus aureus*
GRD	Glycopeptide Resistance Detection
HPA	Health Protection Agency
hGIS	hetero-resistant glycopeptide-intermediate *Staphylococcus*
hGISA	hetero-resistant glycopeptide-intermediate *Staphylococcus aureus*
hVISA	hetero-resistant vancomycin-intermediate *Staphylococcus aureus*
ISTA	Iso-sensitivity test agar
PHE	Public Health England
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
MHA	Muller-Hinton Agar
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MDR	Multi-Drug Resistant
MDR-CoNS	Multi-Drug Resistant Coagulase negative staphylococci

MR-CoNS	Methicillin Resistant Coagulase negative staphylococci
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
NCCL	National Committee for Clinical Standards
PAP-AUC	Population Analysis Profile Area Under the Curve
PCA	Pearsons-Principle component analysis
PFGE	Pulse-field Gel Electrophoresis
rep-PCR	Repetitive sequence-based Polymerase Chain Reaction
UHBT	University Hospital Birmingham NHS Trust
UPGMA	Unweighted Pair-Group method using Mathematical Averages
VISA	Vancomycin-Intermediate *Staphylococcus aureus*
VSSA	Vancomycin-Sensitive *Staphylococcus aureus*
VRSA	Vancomycin-Resistant *Staphylococcus aureus*
VRE	Vancomycin-Resistant *Enterococci*

Chapter 1 General Introduction

1.1 Coagulase Negative Staphylococci

Staphylococcus is a genus of aerobic or facultatively anaerobic Gram-positive bacteria which under the microscope appear as round (cocci) in a grape-like cluster formation (Ryan *et al*., 2004). These organisms can survive and persist in environments which include acidic conditions, high sodium concentrations and wide temperature variations. The heterogenous group of coagulase-negative *staphylococcus* (CoNS) are defined by their lack of production of the secreted enzyme coagulase hence, their inability to clot plasma (Hubner and Goldmann, 1999). This diagnostic feature fulfils the clinical need to differentiate between the pathogen *Staphylococcus aureus* (*S. aureus*) due to its prominent role in infection and other staphylococci that were historically classified as less or non-pathogenic (Becker *et al*., 2014).

As a group, CoNS are the major component of the skin and mucous membranes, functioning mainly as commensals or saprophytic organisms, having a benign symbiotic relationship with the host (Otto, 2010). The conversion of these innocuous members of the skin microbiota into potential pathogens is dependent on the skins capacity to resist infection and the hosts effective defence response (Otto, 2009; Piette and Verschraegen, 2009). However, in susceptible patient groups with impaired host response functions, these colonising CoNS strains act as a key source of endogenous infections (Cogen *et al*., 2008).

Despite being commensals, the ability of CoNS to cause a variety of infections are dependent on strain-specific features at a subspecies level and their association and interaction with host-specific characteristics. Thus, non-virulent skin colonising strains of CoNS may cause infection, if associated with co-factors such as immunosuppression or the presence of indwelling medical devices (Yuen *et al*., 1998; *et al.,* von Eiff *et al*., 2002; Coello *et al*., 2003; Zitella, 2003).

The prevalent bacterial communities on the skin are known to consist mainly of saprophytic CoNS strains. These strains are significantly heterogenic in both pathogenicity and habitat preference and have emerged as significant nosocomial pathogens in hospitalised immunocompromised patients due to changes in patient demographics and advances in interventional medical procedures (Kloos and Bannerman, 1994; Piette and Verschraegen 2009; Becker *et al*., 2014). The increasing significance of CoNS infections associated with this high-risk patient group has highlighted the importance of accurate identification of CoNS isolates to the species level for diagnostic purposes, as this can have both clinical and epidemiological implication (Tacconelli *et al*., 2003).

CoNS are a diverse group of organisms, as such that in the smaller arena of opportunistic human infections where commensal CoNS colonising strains abound, minor difference between related isolates need to be identified. This is in order to make a differential diagnosis of the infective organism, the species type, and the environmental distribution of an epidemic strain. Previously, taxonomists like Kloos and Schleifer (1975) grouped these diverse organisms into classes or species with common properties on the basis of phenotypic characterisation and DNA homology. This was based on the Baird-Parker scheme, whereby a small number of easily definable key characteristics were used to identify isolates at the species and subgroup levels. Conversely, the most recent taxonomy and classification status, using advanced molecular techniques such as 16S rRNA (rDNA) gene sequence analysis, demonstrates that the genus *Staphylococcus* comprises of more than 41 recognised taxa (Takahashi *et al.,* 1999). These include at least 47 species and 23 subspecies of which, nine have two subspecies and one has three subspecies designations. Among the heterogenous group of CoNS subsets, 21 species of these have been identified as common to humans (Euzéby, 2007; Becker *et al.,* 2014). As native flora of the skin and mucus membranes, all these CoNS species types are known to be capable of causing opportunistic infections, particularly when natural host barriers are breached either by cutaneous-puncture or by plastic biomaterials device placements (Zitella, 2003; Falcone *et al.,* 2004).

1.2 Species-specific CoNS isolates

The key source of endogenous infections by recognised strains of CoNS are those that are normal physiological colonisers of different areas of the skin and mucous membranes (Cogen *et al*., 2008; Otto, 2010). Research on healthy adult human nasal microbiota is composed of more species rich and diverse microbe communities with fewer staphylococci being harboured in the nares in comparison to other areas of the body (Frank *et al.,* 2010). Among the eighteen predominant CoNS known to colonise humans, *Staphylococcus epidermidis* and *Staphylococcus hominis* are the most abundant of the CoNS species, accounting for 60-70% and 22% respectively, of the total staphylococci species recovered from individuals (Kloos and Bannerman 1994; Widerström *et al.,* 2012). As an integral part of normal skin flora, different species of CoNS predominate in preferential host ecological niche areas. *S. epidermidis* are particularly prevalent in areas such as the anterior nares, axillae inguinal and perineal areas, reaching population density of up to a million colony forming units per centimetre square (Kloos and Schleifer 1975; Becker *et al.,* 2014). Likewise, *S. hominis* and *Staphylococcus haemolyticus* have a predilection to colonise areas of the skin high in apocrine glands such as axillae and pubic areas. Other species such as *S. capitis* predominate in areas surrounding sebaceous glands whereas *S. auricularis* are exclusive to the external auditory meatus region (Kloos and Schleifer 1975; Widerström *et*

al., 2012). *S. haemolyticus* an important nosocomial pathogen on the other hand, predominating in the perineum and inguinal areas is the second most clinically isolated CoNS after *S. epidermidis* (Karchmer, 2000; D'Mello *et al.,* 2008; Kristóf *et al*., 2011).

Other commonly found species of CoNS that are indigenous to humans include *S. caprae*, *S. warneri, S. simulans, S. saccharolyticus, S. lugdunensis, S. cohnii, S. xylosus* and the recently identified skin coloniser strain, *S. pettenkoferi* (Kloos *et al.,* 1994; Becker *et al.,* 2014). Among the colonising strains of CoNS, *S. epidermidis, S. haemolyticus, S. hominis, S. caprae*, *S. warneri, S. capitis and S. lugdunensis* are the only seven species commonly implicated in nosocomial spread (Widerström *et al*., 2012). Cases of nosocomial infection caused by some of these indigenous CoNS species are commonly reported in patients with depressed immune systems, undergoing severe hospital chemotherapies. In such specific clinical situations these CoNS isolates are therefore regarded as exceptional opportunistic pathogens (von Eiff *et al*., 2002; Tashiro *et al.,* 2015).

1.3 Emergence of CoNS as nosocomial pathogens

CoNS are important and frequently encountered pathogens in the healthcare setting and account for the majority of nosocomial infections, presenting in indolent rather than in acute infections (von Eiff *et al.,* 2002). The increasing importance of recognising CoNS as a group of opportunistic pathogens may be partly due to radical changes in the hospitalised patient populations with multiple co-morbidities as well as the development and increased use of transient or permanent medical devices (Coello *et al*., 2003). A variety of CoNS infections associated with the widespread use of intravascular catheters and prosthetic devices in the severely immunocompromised, for example, patients with cancer, transplants or those in intensive care have been confirmed by numerous clinical studies (Worthington *et al*., 2000; Zitella, 2003; Falcone *et al.,* 2004). Other factors, such as malignancies and aggressive anticancer treatments together with the use of broad-spectrum antimicrobial therapies dramatically shift the incidence of CoNS related infections (Pagano *et al.,* 1997; Costa *et al.,* 2006; Chen *et al.,* 2010).

The enhanced risk of nosocomial CoNS infections acquisition is further increased by cross-transmission of isolates which can occur between patients, healthcare personal or the hospital environment colonised or infected with resistant isolates. Commensal CoNS isolates are defined by the immunological tolerance afforded to them by an immunocompetent host. In clinical conditions such as immune suppression arising from the disease state and associated treatments such as in patients with haematology malignancies undergoing immuno-modulation therapies received during haematopoietic stem cell transplantation may shift the demarcation between innocuous skin commensal and disease-

causing pathogen. CoNS, as typical opportunists comprise one of the major nosocomial pathogens and the most common cause of blood stream infections (BSIs) in the intensive care setting. BSIs is a term interchangeably used with bacteraemia when referring to bacterial growth from blood cultures of patients displaying signs of infection (Diekema *et al.,* 2001; Fluit *et al.,* 2001). The increasing incidence of Gram-positive bacteria causing BSIs and the extensive emergence of multi-drug resistant strains has been associated with increased risk of morbidity, mortality and cost (Wisplinghoff *et al.,* 2003; Caggiano *et al.,* 2005; Cosgrove, 2006; Tumbarello *et al.,* 2009).

Bacteraemia remains a significant cause of life-threatening complication in patients with haematological malignancies and those undoing autologous or allogenic transplants (Raad *et al.,* 2007; Klastersky *et al.,* 2007). In this predisposed patient group, intensive immune-modulating therapies, the immune-compromised state related to indolent malignancy or graft-versus-host disease may give rise to prolonged at-risk periods for opportunistic infections (Yuen *et al.,* 1998). Gram-positive bacteria have been microbiologically documented to account for between 60 to 70% infections in haematology patient group, commonly translocating from the skin or oral cavity into the bloodstream (Hughes *et al*., 2002; Ahlstrand *et al.,* 2012). Over the last two decades, some predominant species of CoNS have been recognised as one of the most important causes of opportunistic infections worldwide, principally related to BSIs (Emori *et al.,* 1993; Pfaller *et al.,* 1999; Marin *et al.,* 2014). Most previous clinical studies do not however, differentiate between CoNS species, primarily addressing CoNS as a group or collectively as *S. epidermidis*. Therefore, infections caused by infrequently occurring CoNS species and their clinical significance may possibly have remained under-reported. Together with having an accurate diagnosis of the aetiological agent, the positive predictive value of CoNS species specific identification increases knowledge of their individual pathogenicity; in turn contributing to understanding their clinical significance and epidemiology. Earlier and recent trends in epidemiology also reflect significant CoNS species to species differences demonstrating variances in antimicrobial susceptibilities, thus markedly enhancing their clinical significance (Christensen *et al.,* 1983; Burnie *et al.,* 1997; Chen *et al.,* 2010). Additionally, the recent observed increase in antibiotic resistance among CoNS isolates causing BSIs threatens the ability to manage such infectious complications common to the immunocompromised patient group (Worth *et al.,* 2009; Chen *et al.,* 2010). Hence the overall pathogenic potential of CoNS species in nosocomial infections reflects the escalating adverse clinical impact of these isolates in the healthcare setting.

1.3.1 Clinical manifestation of CoNS

Staphylococci including CoNS are known to have an ability to form a slimy biofilm, which enables them to adhere to medical devices such as catheters. These isolates also form a protective layer around themselves, making eradication difficult either by the patient's immune response or by the administration of antimicrobial therapy (de Allori *et al.,* 2006; Arslan *et al.,* 2007; Smith *et al.,* 2008).

S. epidermidis the most intensively studied species, is the most frequent of the CoNS species to be associated with nosocomial BSIs predominantly as biofilm-associated infections on indwelling medical devices. The occurrence and frequency of *S. epidermidis* related infections however can vary considerably, dependent on geographical locations (Sader *et al.,* 2001). *S. hominis* strains colonise the skin for shorter periods of time compared to other *Staphylococcus* species, can nonetheless, sometimes cause infections in patients with abnormally weak immune systems. Likewise, the species, *S. haemolyticus* implicated as a common causative agent of vascular catheter-associated infections are also associated with serious medical complications including septicaemia in neutropenic patient (Sloos *et al.,* 2000; von Eiff *et al.,* 2002). *S. epidermidis* is a major inhabitant of the skin and mucosa and the most common clinical isolate of the cutaneous microbiota comprises greater than 90% of the aerobic resident flora of an immune-competent host (Cogen *et al.,* 2008). *S. epidermidis* over the past two decades has emerged as a frequent cause of nosocomial infections, facilitated by several extrinsic factors which contribute its conversion from an innocuous member of the resident microflora to pathogen (Otto, 2009). Extrinsic factors that cause *S. epidermidis* to become a pathogenic agent include the immunocompromised state of the host and indwelling medical devices, which act as major access ports for systemic infections (Coello *et al.,* 2003; Costa *et al.,* 2006). Infectious strains of CoNS in particular *S. epidermidis* selected from among the abundant diverse clones in the normal skin flora, have an advantage in terms of pathogenicity due to their greater ability to adhere to foreign body implants (Galdbart *et al.,* 2000; Bjarnsholt, 2013). This characteristic is an important clinical pathogenic mechanism which some species of CoNS are more adaptive than others in causing of persistent chronic infections particularly in the immunocompromised host (Otto, 2009).

1.3.2 CoNS virulence

The capacity of a bacterium to cause disease reflects in its relative pathogenicity when host natural and immune defences are compromised. Virulence is defined as a measure of the pathogenicity of the bacterium and the degree of virulence is therefore stated to be related directly to the ability of the organism to cause disease by disrupting host defence

mechanisms (von Eiff *et al.,* 2002). The expression of virulence determinants are stimulated and regulated by conditions encountered in the host which are unique to the metabolic requirements of the infective agents (Fey, 2010). In accordance with the relatively benign relationship that CoNS isolates have with their host, most, if not all the virulence factors of the species appear to have association with original functions compatible with the commensal lifestyle of the bacterial species. Of clinical importance, many of the virulence-associated infections that affect immunocompromised patient populations involve CoNS species that are part of the normal human microbiota or are ubiquitous to the hospital environment (Ziebuhr, 2006). Pathogenicity of CoNS therefore appears to stem from molecular determinants that evolved from life as skin commensals to facilitate survival during course of infection (Otto, 2009)

Enzymes and toxins produced by bacteria are usually a means by which pathogenicity is established (Cunha *et al.,* 2006). It has been recognised that various CoNS species produce a number of exoenzymes including haemolysins, several lipases and proteases which contribute to the pathogenesis of CoNS (Otto, 2004). These findings are consistent with reports of a study investigating clinically significant CoNS infections in hospitalised new-born infants. The presence of one or more virulence factors including haemolysin and lipase were reported to be prevalent in 77.8% of the CoNS isolates contributing to pathogenicity of infections in patients with underdeveloped immunity (Cunha *et al.,* 2006). Moreover, this study suggests that the presence of these CoNS virulence factors provides a selective advantage for skin colonisers in high-risk hospitalised patient groups which may also lead to an increase in the severity of infection. This, therefore, puts patients with either an under-developed immune system or are immunodeficient due to malignancy at a higher risk of subsequent CoNS related infections when submitted to invasive procedures (Klingenberg *et al.,* 2007).

Exoenzymes such as haemolysins that contribute to pathogenicity are produced by various CoNS species including *S. epidermidis, S. haemolyticus* and *S. warneri* amongst others. The haemolysins act by binding to susceptible host tissue cells causing damage and release of vital cellular content. More importantly however, haemolysin binding to host red blood cells leads to lysis, releasing free iron molecules, an essential growth factor for bacterial cell multiplication (Cunha *et al.,* 2004). Lipases are exoenzymes and their production, an adaptive response aiding persistence in fatty secretions. Lipase production has been demonstrated by *S. epidermidis* and various other CoNS species including *S. hominis*, *S. capitis*, *S. caprae*, *S. cohnii*, *S. warneri* and *S. simulans* (Otto, 2004; Longauerova, 2006). Likewise, proteases production by isolates of CoNS can also be an important virulence factor with the potential to damage functional proteins that are constituents of host tissues

associated with immune defences. Studies have suggested that proteases production and interaction with the host defence proteinaceous barriers could be a contributory factor for enhancing bacterial disease pathogenesis (Otto, 2004). Including the production of haemolysin, proteases and lipases involved with invasive diseases, CoNS species such as *S. lugdunesis* express other virulent determinants like thermostable DNAse and clumping factors phenotypically similar to those of *S. aureus* (Hellbacher *et al.,* 2006).

In contrast to the large arsenal of toxins produced by *S. aureus* causing infection in the host, relatively few toxin related virulence traits have been identified in CoNS isolates. Through *in-vitro* studies, authors demonstrate that in order for CoNS to exhibit pathogenicity, these strains predominately express virulence traits primarily associated with adhesion, translocation, or evasion of host immune responders (Cogen *et al.,* 2009). Therefore, in patients with an impaired immune system such as those with haematological malignancies, expression of such virulence traits leads to pathological changes that are clinically detrimental to the host.

1.3.2.1 Biofilms

Biofilm, also referred to as "slime" is the virulence factor associated with chronic infections in many diseases, principally characterised by extreme resistance to antimicrobial agents and excessive capacity to withstand host immune responses (Xu *et al.,* 2000; Høiby *et al.,* 2010). The production of biofilm composed of capsular polysaccharides is a universal attribute of many bacteria which allows them the adhere to and persist on almost any surface. In general, bacterial growth and proliferation occurs phenotypically as either single cells (planktonic) or as sessile aggregates (biofilm) composed of multiple bacteria forming a consortium. The primary stage in biofilm development is the binding of planktonic bacterial cells to a foreign body or host tissue. The second stage in the development of biofilm which allows intracellular aggregation is the liberations of extracellular material consisting of polysaccharides, proteins and bacterial DNA. This is followed by biofilm maturation and dispersal of the biofilm encased bacterial cells (Costerton, 1999; Otto 2008). Capsular polysaccharide synthesised by many microorganisms are high-molecular weight polymers which are associated with pathogenicity and virulence promoting factors. Of these polymers, exopolysaccharides constitute a source of carbon and energy reserves, providing cell adhesion and protection against adverse environmental conditions (Otto 2008, 2009, 2010).

Biofilms are therefore, defined as multicellular aggregated architecturally complex communities held together by self-produced extracellular matrix (slime) tolerant of most antibiotics and host defences compared with planktonic cells (Bjarnsholt, 2013). Of clinical importance, many of the biofilm-associated infections that affect immunocompromised

patient populations involve bacterial species that are part of the normal human microbiota or are ubiquitous to the hospital environment (Ziebuhr *et al*., 2006). Factors such as nutrition availability, aspects of environmental stress including temperature and osmolarity and sub-inhibitory antibiotic concentrations play a significant role in both the formation and/or repression of biofilm formation (Mertens and Ghebremedhin, 2013).

The ability to develop biofilm is one of the major pathogenic determinants of CoNS isolates. Biofilms produced by CoNS species vary, dependent on environmental condition and specific strain attributes. Formation of biofilm is highly beneficial to CoNS communities which exist within them, providing protection against host defence systems and conferring resistance to many antimicrobials. Biofilm producing CoNS are mainly associated with medical devices such as catheters or implanted material often resulting in treatment failures in chronic infections (O'gara and Humphreys 2001).

CoNS isolates like other bacterial population within biofilms exist within a wide range of adaptive physiological states. Both genotypically and phenotypically diverse populations within the biofilm express distinct biological activities and antimicrobial stress responses (Xu *et al*., 2000). A number of mechanisms contribute towards the physiological and genetically diverse heterogenic nature of this response (Singh *et al*., 2017). These include factors such as local environmental adaptation affecting antibiotic diffusion, microscale chemical gradients within the biofilm environment, stochastic genotypic expression and variation through mutation and selection (Stewart and Franklin 2008). Tolerance to antibiotic killing is therefore likely due to reduction in nutrient availability and oxygen from the periphery to the centre of the biofilm. These conditions lead to a state of starvation-induced antibiotic tolerant isolates which may be responsible for recalcitrance of biofilm infections associated with treatment failures (Radlinski and Conlon 2018).

Of all the CoNS species, the emergence of *S. epidermidis* as an opportunistic pathogen has been synonymous with the widespread use of intravascular medical devices colonised by these isolates (Otto, 2009; Bjarnsholt, 2013). *S. epidermidis*, the most ubiquitous of skin - colonisers have the inherent ability of forming mucoid biofilms on the inert surfaces of indwelling medical devices associated with persistent and chronic infections, resulting in serious clinical consequences (Galdbart *et al.,* 2000; O'gara and Humphreys 2001; Wisplinghoff *et al*., 2003)

1.3.2.1.1 S. epidermidis biofilms

The pathogenic mechanisms by which *S. epidermidis* are implicated in a range of nosocomial infections are not well established but are to a degree similar to the spectrum of infections caused by *S. aureus* (Costa *et al.,* 2006). It therefore appears plausible that these two staphylococcal species share one or more virulence determinants such as cell-wall associated adhesins and secreted proteins that are involved in pathogenesis (Fey, 2010). These include the extracellular matrix and plasma proteins which promote adherence playing a central role in colonisation of medical devices (Arciola *et al.,* 2004). As such these have been shown to be the foci of device-related infections, as large numbers of biofilm enchased bacteria have been observed to be present on these devices on removal (Costerton *et al.,* 1999; Galdbart *et al.,* 2000). Established device-related biofilm infections are difficult to treat, therefore necessitating device removal to eradicate both the organism and eliminate the source of persistent infection (Høiby *et al.,* 2015).

S. epidermidis despite their supposed low virulence, can cause devastating chronic infections in the compromised host. Such virulent strains have been observed to produce a family of pro-inflammatory peptides which have multiple functions in immune evasion and biofilm development (Otto, 2009). Diversity among *S. epidermidis* isolates distinguishes 74 clonal types of which Sequence Type 2 (ST2) has been identified as the most prevalent and invasive. Molecular analysis further shows that the ST2 contains the IS256 and *ica* genes which are correlated with *S. epidermidis* invasiveness and biofilm formation (Otto,2004, 2009; Schoenfelder *et al.,* 2010).

Immune-incompetent patients including those with haematological malignancies have a high propensity for infection by *S. epidermidis*. Together with intravascular device infections, cancer patients undergoing myelosuppressive chemotherapy with accompanying neutropenia also renders them at a greater risk of developing S. *epidermidis* associated BSIs (von Eiff *et al.,* 2002; Wisplinghoff *et al.,* 2003). Patients with haematological malignancies where both the cellular and humoral immune response is severely compromised, antimicrobial therapy eradicates planktonic cells released from biofilm but is observed to be ineffective against biofilm encased sessile populations (Mah and O'Toole, 2001). Similarly, in the immune-competent host, biofilms produced by virulent *S. epidermidis* afford the strains partial protection from both the hosts immune system and exogenous antimicrobial agents. Furthermore, virulent biofilm producing strains are also a major complicating factor in the management of *S. epidermidis* sepsis as these have a reduced affinity for common antimicrobial agents thereby resulting in treatment failures (Arslan and Özkardes, 2007). Of all the ubiquitous human commensal, *S. epidermidis* is primarily an optimal carrier and

reservoir for antimicrobial resistance genes, thus enhancing its overall pathogenic potential (Otto, 2009).

1.3.2.2 Biofilm mediated antimicrobial resistance

Biofilm producing bacteria are significant contributors to human disease accounting for over 80% of biofilm-related infections which fail eradication with standard antimicrobial treatments (Davis, 2003). The basic mechanism of biofilm resistance to antimicrobial therapy is due to failure of the antimicrobial agent penetration to the full depth of the biofilm. The diffusion of the antimicrobial agent is known to be retarded by the polymeric substances which are part of the biofilm matrix (Stewart and Costerton, 2001; Stewart, 2002). Reduced biofilm susceptibility to particular antimicrobial agents is dependent of several factors. These include diffusion limitation due to the molecule size of the agent, deactivation of the agent in the outer layers of the biofilm and antimicrobial tolerance (Mah and O'toole 2001; Otto, 2004, 2008, 2009). The glycoprotein and polysaccharide components of the biofilm limits the transportation and activity of the antimicrobials resulting in sub-inhibitory activity. Antibiotic resistance is further supported due to the bacterial cells transitioning from the exponential to either slower or no growth and an adaptative 'persister cell' phenomena (Singh *et al*., 2017). Nutrient-starved slower growing or stationary-phase cells in the deeper layers of biofilm are also less susceptible to antimicrobial agents. This adaptive phenotypic response for survival is characteristic of biofilm encased bacterial cells (Stewart, 2002; Stewart and Franklin 2008). Furthermore, a review publication by Stewart, (2002) of antibiotic penetration limitation in biofilm indicated that biofilms formed by beta-lactamase producing bacteria prevent the penetration of penicillin through the biofilm due to antibiotic reaction-diffusion interactions. Similarly, the penetration of positively charged aminoglycoside agents such as gentamicin, are retarded by the active binding of these molecules to the negatively charged biofilm matrix polymers. Reduced antimicrobial susceptibility in biofilm producing bacteria has also been proposed due, not only to phenotypic adaptive responses, but additional genotypic mutational events within the biofilm. These genetic changes allow the emergence of high-level persister cells present within the biofilm that remain refractory to antimicrobial challenges despite removal of antibiotic pressure (Costerton *et al.,* 1999; Stewart and Franklin 2008).

It has been postulated that pathogenic strains of CoNS are reliant on the presence or absence of specific genes that are involved in both the virulence and antimicrobial resistance process. Studies have demonstrated that the two most important virulence characteristics of *S. epidermidis* both sharing the same genetic pathway are biofilm formation and expression of methicillin resistance (Mack *et al*., 2007). The genes involved in the process of primary

attachment to medical devices are the *ica* gene and *alt*E gene. These genes encoding the vitronectin-binding cell surface protein and the *mec*A gene encoding the control of PBP2a synthesis in methicillin-resistant staphylococci are found on the same genetic loci (Mah and O'toole 2001; Blair *et al*., 2015). Therefore, in *mec*A positive *S. epidermidis* strains insertion of transposons at these specific regulatory gene loci, influence both the formation of biofilm and expression of methicillin resistance, thus hampering treatment opportunities against infections caused by these strains (Mack *et al*., 2007). Hence these genetic and regulatory level adaptations contribute to the evolutionary success of nosocomial strains of *S. epidermidis* as opportunistic pathogens in the immunocompromised host (Schoenfelder *et al*., 2010).

1.4 Haematological malignancies and bone marrow transplantation

Advances in medical interventions over the past few decades such as allogeneic and autologous bone marrow transplantation (BMT) part of haemopoietic stem-cell transplantation for the therapeutic management of patients with haematological malignancies have gained much importance. Autologous or allogenic BMTs are classified according to the source of the haematopoietic stem cells. Allogenic BMT are harvested and infused either from donor bone marrow or peripheral blood cells treated with granulocyte colony stimulating factor whereas autologous are cells sourced from the recipient (Yuen *et al*., 1998; Collin *et al*., 2001). These therapies have been implemented in specialist hospital haematology units for patients primarily diagnosed with haematological malignancies such as; acute myelogenous leukaemia, chronic myelogenous leukaemia, acute lymphoid leukaemia, non-Hodgkin's lymphoma and myelodysplastic syndrome amongst other multiple myelomas. The underlying disease state and therapeutic interventions related to specific humoral and cellular defects puts this patient population at a greater risk of BSIs an important cause of both mortality and morbidity (Klastersky *et al*., 2007; Cervera *et al*., 2009; Garcia-Vidal *et al*., 2018).

Severe infections are also a frequent and life-threatening complication of haematopoietic stem cell transplantation due to the abnormalities of the hosts defence system (Neuburger and Maschmeyer, 2006). Haematological disease-related and treatment induced neutropenia together with immunosuppression puts these patients at a greater risk of acquired opportunist infection (Dettenkofer *et al.,* 2003). A major complication of cancer chemotherapy is development of febrile neutropenia which may probably be the only indication of severe underlying infectious state. The extent and duration of the neutropenic state is, therefore, a key risk factor for extent of fever and hence also the development of serious infection complications (Feld, 2008; Marin *et al.,* 2014). BSIs rank first in terms of

serious infectious complication of febrile neutropenia and are associated with higher rates of morbidity and mortality (Caggiano *et al.,* 2005; Kleinhendler *et al.,* 2018).

Similarly, patients undergoing bone marrow transplantation (BMT) have illnesses associated with immunological deficiency which are further compounded and compromised by pre-treatment chemotherapeutic regimens (Bal and Gould 2007). The incidence of BSIs due to opportunistic infections appears to be higher in BMT patients in comparison to non-BMT haematology patients. This is mainly due to factors which significantly effect poor prognosis such as relapse, uncontrolled malignancy and the neutropenic state amongst others (Worth and Slavin, 2009). Ablative chemotherapeutic regimens as part of BMT is known to invariably lead to patients developing severe neutropenia particularly after the post-engraftment period. The resulting loss of immune-reactivity for varying periods of time after BMT leaves the patients extremely susceptible to bacterial infections in the early stages of post transplantation due to severe impairment of cellular and humoral immunity (Dettenkofer *et al.,* 2003). BMT patients are also known to be infected with the same primary organisms as in the general haematology neutropenic or non-neutropenic patient. Similarly, the most frequent cause of bacteraemia due mainly to the prolonged neutropenic state in BMT receipts are the Gram-positive isolates (Chen *et al*., 2010). However, treatment-induced neutropenia, long central venous catheter (CVC) dwell times, and the use of immunosuppressive agents' place BMT recipients at a higher risk of CVC related BSIs (Kratzer *et al.,* 2007).

The most common causative organisms of device related infection are usually the ubiquitous skin flora of hospitalised patients, primarily CoNS isolates (Casey *et al.,* 2007). The association of CoNS bacteraemia with the use of indwelling intravascular devices is a well-established observation. This is due the capacity of CoNS to colonise by adhering to these devices via the production of extracellular glycocalyx (slime) and adhesins (Arciola *et al.,* 2004). Other portals of organism entry include, skin catheter hubs, infusion solutions and colonised insertion sites which are a major risk factor for infection. Additional potential sources of CoNS colonisation and infection include nasal area, oral mucosa and skin lesions which occur secondary to chemotherapy and/or graft versus host disease causing loss of natural skin integrity (Wolf *et al.,* 2008). Furthermore, the infective agents from both endogenous and exogenous sources colonising long-term indwelling catheters proliferate within biofilms and disseminate into the bloodstream resulting in severe systemic infections (Raad *et al.,* 2007). The incidence of such catheter-related BSIs (CR-BSIs) has been shown to correlate greatly with the degree of immunosuppression in the haematology and oncology patient groups. CR-BSIs in cancer patients remain among the most common of observed complications, with a reported prevalence as high as 38% in this group, however a significant

increase in disease-related mortality associated with CRIs is not observed (Madani, 2000; Wolf *et al.,* 2008). Conversely, reports of increased morbidity, prolonged hospitals stays with increasing costs associated with CRIs in cancer patients make CR-BSIs the most frequent and costliest of healthcare-associated infections (Beekmann *et al.,* 2003)

Reducing poor clinical outcomes associated with bacteraemia in patients with haematological malignancies remains challenging due to the emergence of resistance to multi-drug classes used for effective therapy. In most healthcare settings the prompt implementation of broad-spectrum empiric antimicrobial therapy with the addition of vancomycin limits the progression of bacteraemia and increases the chances of a favourable outcome for the BMT receipts (Hughes *et al.,* 2002; Raad *et al.,* 2007; Zakhour *et al.,* 2016). Reports of mortality attributed to CoNS-related bacteraemia, therefore, remain low (<5% of 57% diagnosed with GP-bacteraemia) particularly in patients receiving autologous or allogenic transplants dependent on prompt infection prophylaxis, diagnosis and treatment (Collin *et al.,* 2001; Freifeld *et al.,* 2011).

1.4.1 Blood stream associated infections

Healthcare-associated infections (HAI) or nosocomial Blood stream infections (BSIs) are the most common complication in hospitalised patients especially those with terminal malignancies, resulting in increased morbidity and mortality rate of 18%-42% (Wisplinghoff *et al.,* 2004; Kleinhendler *et al.,* 2018). The progress made in modern medical care and availability of greater chemotherapeutic options with an increase in transplantations has nonetheless substantially increased the risk of CoNS infection complications in cancer patients. The overall individual incidence of BSI for haematology patients is three-fold higher in comparison to oncology patients equally related to CoNS as the predominant isolate in this patient group (Schelenz *et al.,* 2013).

BSIs have been reported to be among the most common infectious complications observed in haematology patients, the majority of which are associated with GP-organisms (57.1%) together with the significant predicator of mortality being prolonged neutropenia concomitant with nosocomial infection (Tumbarello *et al.,* 2009). The altered immune status of these vulnerable patient groups due to interference of immunosuppressive factors facilitates the risk of BSIs resulting in significant disease and morbidity particularly among transplant recipient patients. Moreover, the risk of developing nosocomial BSIs in neutropenic patients who have undergone BMT has been documented to be much greater than in non-transplanted patients with other malignancies and neutropenia. As such, investigators have reported incidence rates of 360 BSIs per 1000 neutropenic episodes in BMT patients in comparison to 11-38% reported cases of BSIs in neutropenic patients (Wisplinghoff *et al.,*

2003; 2004). This has been attributed to neutropenic patients with haematological malignancies unable to mount an adequate inflammatory response to infective agents with fever presenting as the only symptom of serious infection. Neutropenia-associated hospitalisation was also reported to be more common in haematology patients with chemotherapy-treated leukaemia (1 in 6 patients) relating to the highest incidence and cost per in-patient stay (Caggiano *et al.,* 2005) Overall, neutropenic-associated infection relating to hospital costs in patients with haematologic cancers were projected to be a fifth higher in comparison to patients undergoing chemotherapy for other cancers (62% in comparison to 22-53% respectively). In another well-documented review study, Goto and Al-Hasan (2013), report that there were a total number of 200,000 nosocomial BSI episodes with a total of 157,000 associated deaths in Europe which were a consequence of local primary or secondary immunodeficiency disorder of the immune system. This population-based study also reports an estimated occurrence of 96,000 BSI episodes totalling 12000-19000 related deaths in England alone. Furthermore, focusing on the preventative measures for reducing nosocomial BSIs in haematology and oncology patients, authors suggest including monitoring and identification of common causative organisms with timely implementation of appropriate empirical antimicrobial therapies thus reducing the overall burden of disease.

The evolution of medical care and development of potent anti-staphylococcal agents has led to the change in the predominance and clinical significance of Gram-positive associated bacteraemia mainly in the immunocompromised hospitalised patient population (Karchmer, 2000; Klastersky *et al.,* 2007; Cervera *et al.,* 2009). This is evidenced in a multi-centre pan-European observational study of sepsis occurrence in acutely ill patients reporting that the highest percentage of Gram-positive associated bacteraemia in the critical ill patient through normal and/or nosocomial acquisition, was predominantly due to methicillin sensitive and resistant strains of both *S. aureus* and CoNS (Vincent *et al.,* 2006). Infections occurring due to the progressive increment of virulent multi-drug resistant strains appears to have been fuelled by selective pressure resulting from the extensive use of broad-spectrum antimicrobials (Huges *et al.,* 2002; Trecarichi *et al.,* 2015). Therefore, in line with the changing patterns of acquired nosocomial BSIs, a life-threatening complication of cancer therapy, hospitals implement agreed treatment protocols for a rational choice of single or combination therapy, dependent on local patterns of antimicrobial resistance. These effective therapeutic protocols are for the treatment and management of febrile neutropenic patients for optimal clinical outcomes are harmonised with national recommendations (BMJ, 2017).

1.4.2 Sources of BSIs

BSI are usually representative of compromised immune system failing to contain infection at a focal site and the consequent dissemination of disease. Bacteraemia is usually the most common manifestation of CoNS infection. Several literature reviews report the global trend of increasing incidence and severity of CoNS bacteraemia, whereas previously these isolates were exclusively regarded as contaminants arising from the hosts cutaneous flora (Weinstain *et al.,* 1997; García *et al.,* 2004). It is generally believed that the CoNS isolates colonising the skin of the individual patient are usually the source of CoNS-associated bacteraemia concomitant with indwelling prosthetic device-related infections (Christensen *et al.,* 1983; Coello *et al.,* 2003; Costa *et al.,* 2004). However, CoNS as benign residents on skin surfaces and as aetiological agents of BSIs usually arise only in conjunction with specific host predispositions.

CoNS-associated bacteraemia is a well-recognised complication of intravascular medical device-related infections. These devices implanted during patient's hospitalisation are an essential requirement for continuous intravascular access (Zietella, 2003). CoNS are frequently implicated as the infective agent due to the prevalence on the skin and isolation from colonised foreign body devices and their insertion sites (Marin *et al.,* 2014). Confirmation that these devices are the source of infection remains challenging as skin contaminants can be acquired from the various sources during surgical implantation (von Eiff *et al.,* 2002; Ahlstrand *et al.,* 2012). However, a marked increase in CoNS CR-BSIs relating to endogenous sources in the critically ill has been observed over the past few decades. Studies conducted evaluating the source(s) of CoNS bacteraemia using specific defined criteria for CoNS bacteraemia, report that mucosal sites other than the skin as potential sources of infection, are likely to be an important source of CoNS bacteraemia (Costa *et al.,* 2004). Further evidence is provided by another prospective study describing the relationship between colonising and invasive strain patterns of CoNS among patients undergoing treatment for haematological malignancy. The authors also document that CoNS-associated bacteraemia in patients with haematological malignancy and those undergoing BMT are commonly due to translocation from the skin or mucosa into the bloodstream (Ahlstrand *et al.,* 2012). Therefore, disruption of skin integrity and prolonged use of catheters which are highly likely to become infected during the course of disease, increase the risk of CoNS bacteraemia in this patient group.

1.4.2.1 Device-related bloodstream infections

The majority of intravascular devices are long-term insertions in patients with haematological malignancies undergoing chemotherapy with a high prevalence of neutropenia (Falcone *et al.,* 2004; Martiínez-Morel *et al.,* 2014). Continuous intravascular access is an essential requirement for these patients in particular recipients of stem-cell transplants to secure access for blood product transfusions, chemotherapeutics, parental nutrition, antibiotics and stem-cell infusions (Zakhour *et al.,* 2016). The most commonly used devices include percutaneous non-cuffed non-tunnelled CVCs and cuffed tunnelled CVSs (Hickman, Broviac and Groshon). Long term use of CVCs together with disruption of skin integrity and treatment-induced neutropenia therefore places BMT recipients at a higher risk of CoNS device-related infection (Zietella, 2003; Martiínez-Morel *et al.,* 2014; Yamada *et al.,* 2017).

Bacteraemia remains among the top four causes of HAI commonly associated with the use of peripherally inserted central catheters especially in the intensive care setting. This is evidenced by a hospital-wide surveillance of hospital-acquired bacteraemia in English hospital between 1997 and 2001, conducted by the Nosocomial Infection National Surveillance Scheme (NINSS), reported that the incidence of device-related bacteraemia was highest among high-risk patient specialists where central lines were the commonest source of infection (Coello *et al.,* 2001).

Frequent use of long-term intravascular accesses and other implantable devices are implicated as the major source of infections caused by Gram-positive cocci. The high incidence of CoNS BSIs has been directly correlated with intensity and length of neutropenia and the higher CVC usage in cancer patients (Pagano *et al.,* 1997; Marin *et al.,* 2014). Infections in patients with haematological malignancies undergoing chemotherapy are promoted by treatment-induced disruption of both mucosal surfaces and damage to the skin. Invasive procedures such as insertions of CVCs or percutaneous biopsy procedures provide portals of entry and proliferation of opportunistic pathogens originating from the skin commensal flora (Neuburger and Maschmeyer, 2006; Ahlstrand *et al.,* 2012). The endogenous lining of the interior surface of intravascular catheters have been found to be colonised by biofilm forming microorganisms within 24 hours of insertion. The protective properties of biofilm appear to be an important pathogenic mechanism for the development of catheter-related infection (Wolf *et al.,* 2008). This, therefore, increases the risk of CoNS bacteraemia in haematological malignancies due to the prolonged use of catheters which are likely to become infected during the course of disease (Wisplinghoff *et al.,* 2003). Raad and colleagues (2007), in their study demonstrated that CVCs, localised entrance and exit sites were the major source of BSIs in patients with underlying haematologic malignancy.

Likewise, the authors of another study have also documented a significantly higher figure of CR-BSIs in a large number of BMT recipients during neutropenic state (Dettenkofer *et al.,* 2003). Poor clinical outcomes were also associated with CoNS bacteraemia in the presence of persistent neutropenia in this patient group.

Marin and colleagues (2014) in their prospective observational study also reported that an endogenous source and catheter-related incidence of BSIs were most frequent in neutropenic patients with haematological malignancies. In this study, out of the 579 episodes of BSI in neutropenic patients, 493 (85%) occurred in patients with haematological malignancies with CoNS (41.8%) identified the predominant causative isolates (Marin *et al.,*2014). Similarly, in a review study looking at 110 episodes of CVC related BSIs, in 82 patients with haematological malignancy, CoNS were the most commonly isolated organisms (Zakhour *et al.,* 2016). Several strategies to reduce catheter colonisation have been considered for efficacy such as antimicrobial lock therapies for prevention and eradication of CVC related BSIs. Catheter removal on the other hand, is a component of definitive treatment for BSIs caused by colonising strains of CoNS and other Gram-positive organisms but is highly detrimental for the clinical management of critically ill patients (Zitella, 2003, Worth and Salvin 2009). Implementation of appropriate intravenous antibiotic including catheter lock solutions and/or removal of the implanted lines remains the mainstay of treatment in most cases of CR-BSIs in patients with haematological malignancies (Zakhour *et al.,* 2016).

1.5 Diagnosis of bacteraemia

Fatal bacteraemic infections in patients with acute leukaemia were commonly due to Gram-negative bacilli in the early 1970s. However, in the past few decades, the major group of organisms contributing to a shift towards Gram-positive single-organism bacteraemia are predominantly the CoNS (Wisplinghoff *et al*., 2004; Wilson *et al.,* 2011; Zakhour *et al.,*2016). A prospective study carried out by Denttenkofer and colleagues (2003), on patients with haematological malignancies and post BMTs, documented that the highest incidence of nosocomial infection especially during the neutropenic phase was mainly due to CoNS species. Similarly, authors of another study demonstrated that in BMT recipients, risk factors for CoNS bacteraemia not only included aplasia and graft-versus-host-disease (GvHD) but the relatively high incidence of CoNS catheter-related infections resulted in exacerbation of chronic GvHD with aggravation of immunosuppressive therapy (Kratzer *et al.,*2007). Thus, the potential factors predisposing this patient group to CoNS-associated BSIs amongst other factors include immunosuppressive therapy, biofilm encased organisms in colonised invasive devices and procedures such as ventilator use, and selective pressure exerted by

antimicrobial therapy (Zakhour *et al.,*2016). Furthermore, bacteraemia associated with frank sepsis in the haematology patient group involving highly virulent strains of CoNS such as *S. lugdunensis* have been linked to fatal clinical outcomes (Pagano *et al*., 1997). Accurate diagnosis of CoNS bacteraemia is therefore essential in this patient group, dependent on both the clinical presentation and in the majority of cases, isolation of the same strain or clonal population. This is imperative when bacteraemia is associated with infections related to colonised indwelling medical devices usually implicated as the source of infection (Worthington *et al*., 2000; Zitella, 2003). Poor clinical presentation associated with ineffective therapeutics therefore necessitates removal of the device to eradicate the possible source of infection at a potential risk to the patient (Worth *et al*., 2009).

The clinical significance of BSIs due to CoNS in the critically ill patient is being increasingly recognised with the elucidation of their pathogenic nature (von Eiff *et al*., 2000). Diagnosis of CoNS-associated BSIs is, however, difficult in hospitalised patients as the clinical signs and symptom in the critically ill patient may be masked and non-specific (Longauerova, 2006). However, CoNS as ubiquitous skin commensals of low virulence and common contaminates of blood cultures can also be the cause of true BSIs in patients undergoing various surgical procedures and the immunocompromised (Piette and Verschraegen, 2009; Becker *et al.,* 2018). These factors, therefore, makes differentiating true infection from colonisation, challenging. Hence, accurate surrogate markers to differentiate between CoNS BSIs and contamination are used in conjunction with clinical, laboratory and microbiological parameters for an improved diagnostic approach (Pfaller and Herwaldt 1998). In the high-risk patient population clinical characteristics of the patients together with the microbiological evidence provides the clinicians with information to distinguish between true CoNS BSI or contamination. Nonetheless, the general approach adopted by clinicians in most healthcare settings is to treat patients with broad-spectrum empirical antibiotics prior to microbiological diagnosis to improve patient outcomes and decrease mortality due to risk factor of febrile neutropenia (Huges *et al.,* 2002; Kleinhendler; *et al.,* 2018).

Bacteraemic patients with febrile neutropenia due to their immunocompromised state and poor inflammatory response do not demonstrate localised signs or symptoms other than fever associate with infection. Temperature spiking in association with bacteraemia, has been shown to occur around an hour following the organism's entry into the blood stream. Therefore, obtaining blood cultures at the time of onset of fever has shown to greatly increases the likelihood of detecting bacteraemia (Synder, 2015). This conventional method of obtaining blood cultures for microbiologic diagnosis of BSIs in this patient group has some limitations due to lack of rapidity and sensitivity and associated factors such as previous antimicrobial therapy affecting isolation and identification of the causative organism in blood

cultures which significantly increases the morbidity and mortality risk in this patient group (Marin *et al.,* 2014). Other studies have shown that molecular techniques such as real-time polymerase chain reaction (RT-PCR) that are not affected by prior antibiotic prophylaxis have added clinical benefits for febrile patients due to their greater sensitivity, specificity and speed of diagnosis (Mancini *et al.,* 2008). Nonetheless despite the advantage of rapidity for detection of the infecting organism, antimicrobial susceptibility still remains the limiting factor as detection of resistance marker genes have yet to be fully evaluated with such molecular based techniques (Guido *et al.,* 2012). Detection of blood stream isolates in critically ill patients is therefore of both major diagnostic and prognostic importance. The management of bacteraemia is consequently undertaken in accordance with published guidelines and current local hospital policies which include sampling of blood for culture if possible, prior to initiation of broad-spectrum empirical antimicrobial therapy (Weinstain *et al.,* 1997 Chen *et al.,* 2005; BMJ, Best Practice 2017). Blood Culture specimens are a critical tool for the detection of organisms in the blood stream which may be potentially pathogenic, representing a clinically significant infection associated with a high risk of morbidity (Weinstein *et al.,* 1997). Hence, blood cultures remain the "gold-standard" for microbiological diagnosis of bacteraemia in hospitalised haematology-oncology patients enabling targeting of therapy against specific organisms. The clinical microbiology laboratories plays an important predictive role in rapid diagnosis of bacteraemia by identifying the aetiological infectious agent and guidance relating to evaluated antimicrobials, consequently resulting in effective management of critically ill patients.

Advances in microbiological diagnostic platforms and complementary assays have facilitated the identification of clinically important pathogens. In most clinical laboratories at present, blood cultures are currently performed using fully automated continuous-monitoring computer assisted systems. These include systems such as BactT/Alert-3D (bioMérieux, France) using colorimetric sensors detecting microbial growth by analysing the production of carbon dioxide. Positive signals indicating the growth of organisms are usually flagged-up by the automated system within 24 to 48 hours followed by a preliminary presumptive identification of the organism performed by Gram-stain test (Beekmann *et al.,* 2003; Mancini *et al.,* 2008). Following this, confirmation of infectious aetiology is carried out by use of rapid phenotypic tests for accurate pathogen identification and evaluation of antimicrobial susceptibilities. The provision of microbiological diagnostic results allows clinicians to implement optimal effective antimicrobial therapeutics in a timely manner limiting the development and spread of drug-resistance. Furthermore, effective therapeutics also lower the risk of possible clinical failures in severely ill patients with life threatening diseases (Wisplinghoff *et al.,* 2003; 2004).

Correct interpretation of positive blood culture may be difficult since the sources of CoNS BSIs may simply be due to skin colonising CoNS strain contaminants or derived from catheter colonisation. Therefore, a combination of both clinical and microbiological criteria are usually applied to assess the significance of CoNS and other isolates deemed to be potential skin contaminants as significant causative agents of BSIs. These criteria include continued isolation of the same isolate from multiple blood cultures and/or in conjunction with isolation of the same isolate from colonised intravenous catheters or sites (Casey *et al.,* 2007, 2007). However, similar criteria applied to assess the clinical significance of CoNS isolated from blood cultures of haematology patients remain difficult in clinical practice. In such cases clinical diagnosis together with isolation from either a single or at least two blood cultures are frequently thought to be representative of CoNS-associated infection (Marin *et al.,* 2014). Henceforth, *S. epidermidis* bacteraemia, a very frequent event in BMT recipients associated with either a single or multiple positive blood cultures are often considered as tthe significant causative agent (Ninin *et al.,* 2006). It has been noted that blood culture methodology is nevertheless limited by a lack of both rapidity and sensitivity for a targeted approach to antimicrobial therapy initiation; significantly increasing the risk of morbidity and mortality in this patient group (Marin *et al.,* 2014).

Studies utilising molecular techniques such as RT-PCR for the rapid diagnosis of BSIs in febrile neutropenic patients show superiority over conventional diagnostic systems by providing positive and negative predictive value algorithms for effective diagnosis (Mancini *et al.,* 2008). Such molecular techniques remaining unaffected by prior antibiotic prophylaxis for detection purposes, however, have their performance limited by the requirement of further testing to determine susceptibility, since rapid detection of resistance marker genes have yet to be fully evaluated with this methodology (Guido *et al.,* 2012). The application of rapid molecular techniques are however, emerging as the new standard for microbial identification and differentiation of clinically significant CoNS to the subspecies level. Therefore, rapid diagnosis using molecular techniques can prove to be a valuable contributory factor towards the clinical management of patients with haematological malignancies. Currently blood cultures remain the reference method for the diagnosis of bacteraemia in the majority of clinical microbiology laboratories for guidance of targeted treatment strategies resulting in better patient outcomes.

1.6 Identification of aetiological agents of bacteraemia

The appropriate management of bacteraemic infection is dependent upon accurate and rapid identification of the infecting organism. Despite the increased recognition of the significance of infections attributed to the heterogenous group of CoNS isolates, species level identification generally, still remains an undefined clinical requirement. The identification of CoNS as a group, is basically restricted to clonal morphology, Gram-stain characteristics, catalase production and the absence of coagulase determined by latex and tube agglutination tests. The application of these tests limit the classification of staphylococci into the two groups, the well-established pathogen *S. aureus* and the CoNS. Generally, species level identification are not thought to be necessary for the clinical management of infections in some patient groups. However, several investigators evaluating the importance of identifying CoNS to the species level from blood cultures as a predicator of clinical significance demonstrated that some species-specific strains were more commonly associated with bacteraemia than others in high-risk patient groups (Weinstein *et al*.,1998; Heikens *et al*., 2005). Accurate identification of clinically significant CoNS isolates is therefore essential for the early prediction of potential pathogenicity.

The fundamental principles of character analysis by conventional identification methods of CoNS species and sub-species are based on the original scheme proposed by Kloos and Schleifer (1975). This widely used taxonomic system for the sub-classification of CoNS is based on 13 key characteristics which include coagulase activity, haemolysis, nitrate reduction and acid production from carbohydrate substrates. Due to the improved taxonomic knowledge of staphylococci and the availability of new reliable identification methods and technologies; precise identification of strains of various *Staphylococcus* species involved in clinical infections are currently becoming the rule in routine diagnostic laboratories. These standard automated Identification methodologies, in high-throughput clinical microbiology laboratories have been shown to impact directly on improving the quality of patient care and management (Barenfanger *et al*., 1999 Funke *et al*., 2005; Patel *et al*., 2017). Moreover, molecular based commercial assays in comparison to standard identification techniques, although not as cost effective have proved to be more accurate in identifying staphylococci to the species level (Mehta *et al*., 2009).

Of the eleven different species of CoNS isolated from bacteraemic patients, *S. epidermidis, S. hominis* and *S. haemolyticus* are the three most commonly species detected, accounting for both the clinically significant isolates and contaminating skin flora (Diekema *et al*., 2001; Kristóf *et al*., 2011). The other less commonly involved species in bacteraemia include *S. capitis* and *S. lugdunensis*. *S, cohnii, S. auricularis, S. simulans, S. caprae S. sciuri and S.*

warneri, these species when isolated, are usually deemed as blood contaminant isolates but in the immunocompromised host may be significant. Epidemiological studies ascertaining the species-species strain distribution of blood culture isolates of CoNS reported the dominance of *S. epidermidis* (68.7%) and *S. haemolyticus* (14.8%) among adult haematology patients (Burnie *et al*., 1997; Tacconelli *et al*., 2003). The other species of CoNS detected included strains of *S. hyicus, S. warneri, S. cohnii,* and *S. saprophyticus.* The isolation of diverse CoNS species-specific isolates from bacteraemic patients therefore indicates the importance of species level identification, as isolation of certain species-specific strain are associated with a predilection to cause disease.

A number of commercial automated systems are widely used by routine microbiology laboratories for the accurate identification of clinically important *Staphylococcus* species. Among these, the utilisation of the VITEK®2-Gram-Positive identification card (GP-ID; bioMérieux, Marcy L'Etoile, France) has been designed to increase identification of Gram-Positive cocci (Layer *et al*., 2006). The VITEK®2 and other commercial phenotypic based identification systems such as the BD Phoenix system (Becton Dickinson Diagnostic Systems, Sparks, MD) under normal laboratory conditions, reliably identify up to 90% of the staphylococcal species in comparison to reference molecular strain identification methods (Ligozzi *et al*., 2002). However, mis-identification of *S. hominis* particularly with respect to *S. epidermidis* still remains to be improved as these strains demonstrate similar biochemical activity (Cunha *et al*., 2004; Layer *et al*., 2006). Accurate identification of CoNS species may therefore require additional verification by either molecular testing or other suggested differential tests, notably when appropriate treatment is dependent on correct species identification.

Advancing automated technology has led to the emergence of genotypic methods as a new standard for bacterial identification to both the species and subspecies level. These commercial systems utilise comparisons of both the variable and conserved regions of 16S-ribosomal RNA (rRNA) sequences for differentiation of organisms to species level (Layer *et al*., 2006; Kim *et al*., 2008). The use of phenotypic based methodologies in routine diagnostic laboratories however predominate over genotypic methodologies due to cost pressures and the availability of sensitive, cost-effective mass-spectrometry based identification systems (Patel, 2015; Patel *et al*., 2017).

1.6.1 Biotyping using VITEK®2

In the routine clinical laboratory setting, commercial automated systems such as the VITEK®2 (bioMérieux SA, Marcy-'Etoile, France) is used for the identification of CoNS by their biochemical reaction patterns ("biotyping"). This rapid phenotypic micro-method is a cost effective system for accurate identification of medically relevant CoNS species (Funke *et al*., 2005). The VITEK®2 system is based on an auto-modular format, consisting of a dilution mode, filler-sealer module, a fluorogenic technology based optical reader, incubator and a computer control module. The system utilises uniquely coded small plastic cards consisting of individual wells, containing a variety of reagents, substrates or antibiotics for identification or antimicrobial susceptibility testing.

Inoculated, sealed cards placed in the reader-incubator are scanned by light emitting diodes and phototransistor detectors every fifteen minutes, after baseline readings are taken. The changes in light transmission readings, detected by the photometer are transferred to the computer module which interprets the data determining the organism's biotype and identification. (Product Literature, 2004).

1.6.1.1 CoNS species identification utilising VITEK®2

Commercial rapid automated systems now routinely used in the clinical laboratory setting have a high degree of sensitivity and specificity for speciation of *Staphylococcus* species. The identification of most human *Staphylococcus* species and sub-species is comparable to an accuracy of 70 to >90% as these systems undergo continuous improvements with the addition of more discriminatory tests included within the identification panel (Logozzi *et al*., 2002)

The Gram Positive (GP) reagent card has 64 wells, of which 43 wells are for fluorescent and inhibitory tests that include biochemical tests measuring carbon source utilisation, enzymatic activities and resistance, pH change tests and derivatives for the detection of enzymes, amiopeptidases and –osidases. Substrates present in 21 wells, measure various metabolic activities such as acidification, alkalinisation, enzyme hydrolysis and growth in the presence of inhibitory substances. The GP card, well contents listed in Table 1-1 is used for the identification of 115 taxa of the most significant non-spore-forming Gram-Positive bacteria. (Funke *et al*., 2005)

Well	Test	Amount/Well mg/L	Well	Test	Amount/Well mg/L
2	D-AMYGDALIN	0.1875	31	UREASE	0.15
4	PHOSPHATIDYLINOSITOL PHOSPHOLIPASE	0.015	32	POLYMYXIN B RESISTANCE	0.00093
5	D-XYLOSE	0.3	37	D-GALACTOSE	0.3
8	ARGININE DIHYDROLASE 1	0.111	38	D-RIBOSE	0.3
9	BETA-GALACTOSIDASE	0.036	39	D-RIBOSE	0.15
11	ALPHA-GLUCOSIDASE	0.036	42	LACTOSE	0.96
13	Ala-Phe-Pro ARYLAMIDASE	0.0384	44	N-ACETYL-D-GLUCOSAMINE	0.3
14	CYCLODEXTRIN	0.3	45	D-MALTOSE	0.3
15	L-Aspartate ARYLAMIDASE	0.024	46	BACITRACIN RESISTANCE	0.00006
16	BETA GALACTOPYRANOSIDASE	0.00204	47	NOVOBIOCIN RESISTANCE	0.000075
17	ALPHA-MANNOSIDASE	0.036	50	GROWTH IN 6.5% NaCl	1.68
19	PHOSPHATASE PHOS	0.0504	52	D-MANNITOL	0.1875
20	Leucine ARYLAMIDASE	0.0234	53	D-MANNOSE	0.3
23	L-Proline ARYLAMIDASE	0.0234	54	METHYL-B-D-GLUCOPYRANOSIDE	0.3
24	BETA GLUCURONIDASE	0.0018	56	PULLULAN	0.3
25	ALPHA-GALACTOSIDASE	0.036	57	D-RAFFINOSE	0.3
26	L-Pyrrolidonyl-ARYLAMIDASE	0.018	58	O/129 RESISTANCE (comp.vibrio.)	0.00084
27	BETA-GLUCURONIDASE	0.0378	59	SALICIN	0.3
28	Alanine ARYLAMIDASE	0.0216	60	SACCHAROSE/SUCROSE	0.3
29	Tyrosine ARYLAMIDASE	0.0275	62	D-TREHALOSE	0.3
30	D-SORBITOL	0.0875	63	ARGININE DIHYDROLASE 2	0.27
31	UREASE	0.15	64	OPTOCHIN RESISTANCE	0.000399

Table 1-1: GP card well contents for the identification of Staphylococcal species (Adapted from VITEK®2 GP product information). Note: Non-designated well numbers are empty (control) wells.

The VITEK®2 GPC-ID panel for the phenotypic identification of CoNS, results in recording of the tests as 0 for negative results and 1 for positive. These values are compacted by the VITEK software computing system by combining triplets of positive/negative binary results into a 16 digit VITEK Bionumber. Organism identifications are made based on test probabilities of each taxon in the data base of the VITEK systems. (Bannerman *et al*., 1993; Product Literature: 2004; Layer *et al*., 2006).

Although this type of phenotypic identification system, based on the organism's metabolic activity enables identification of the bacterial isolate to the species level with ease, there are several factors that limit the degree of accuracy achieved (Spanu *et al*., 2003, Funke and Funke-Kissling, 2005). The factors that compromise accuracy of the system include, different stains of the same species exhibiting variation in phenotypic characteristics dependent on external factors such as culture media, length of culture incubation and

temperature conditions. Typically expressed biochemical characteristics of isolates may also be influenced by chemotherapeutic agents such as those recovered from critical ill patients on long-term antimicrobial therapies (Ligozzi *et al*., 2002; Kim *et al*., 2008).

1.6.1.2 Identification with VITEK®-Mass Spectrometry

Diagnostic microbiology in recent times has rapidly evolved towards the use of more streamlined automated organism identification systems such as, Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). Traditionally identification of bacteria from clinical samples has been done by examination of clinical morphology and by biochemical characterization classically using substrate and enzymatic tests (Bannerman *et al*., 1993; Kloos *et al*., 1975, 1994; Cunha *et al*., 2004). These tests routinely used in clinical laboratories remain the "gold standard" for identification but are subjective to interpretation if done manually. Inoculum density and process error in some cases of micro-dilution based automation can also be a major limitation factor. These drawbacks have however been alleviated by the development and implementation in the routine clinical laboratory of MALDI-TOF mass spectrometry, the intrinsic property of which is detection of mass-to-charge ratio (*m/z)* of the bioanalyte.

Bacterial identification using mass spectrometry application is based on the acquisition of unique protein profiles when the isolate subjected to multiple laser shots vaporises and, in the process, gains an electric charge (ionisation) (Cabonnelle *et al*., 2011; Patel, 2015). The procedure involves the conversation of biopolymer structural protein molecules present in the condensed phase into individual intact ionized bacterial macromolecules in the gaseous phase. After migration in an electric field, these ions get separated according to their molecular weight followed by detection of representative masses, via the mass analyser component of the MS system. Ion detection is characterised by the (*m/z)* ratio and relative intensity of the signal generated is displayed graphically as spectrum of peaks as illustrated in Figure 1-1. The structure of the original bacterial molecules is deduced from analysing the spectral pattern of the ionised fragments (proteomic fingerprints) via the MS-software analysis component. Interpretation of the generated sample spectra compared to a library of defined reference spectra of well characterised isolates provides high confidence level species identification (Theel, 2013).

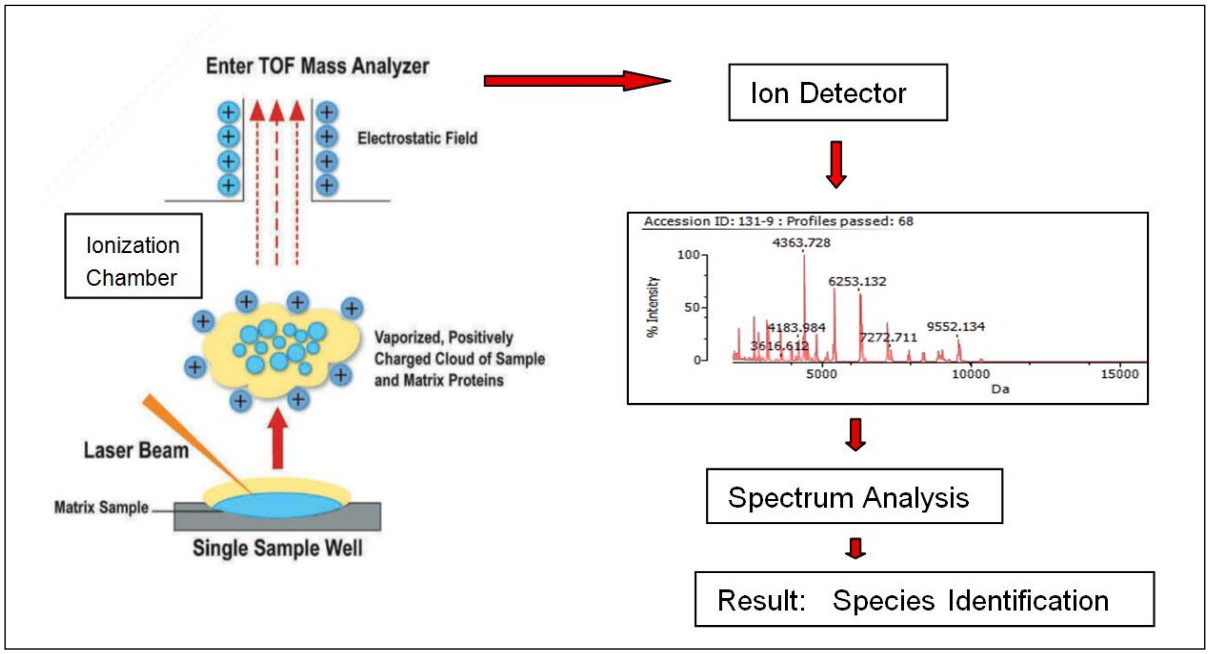


Figure 1-1: Schematic of mass spectrometry sample ionization, capture of positively charged particles, mass analysis detection of ions (Da), mass spectrum production and Spectrum analysis, Data base comparisons and identification result. (Adapted from Theel, 2013)

The application of mass spectrometry comprises accurate analysis of peptides and the determination of these peptide sequences identify and characterise the protein composition of the isolate. The unique spectral fingerprints generated and interpreted are specific to bacterial genus, species and sometime subspecies providing high-level strain identification (Carbonnelle *et al*., 2012)

MALDI-TOF MS, a fast, cost-effective and robust technique has transformed genus and species identification of clinically relevant etiological agent of infection (Carbonnelle *et al*., 2011; Clark *et al*., 2013). This versatile proteomics based technology is documented to provide highly reproducible data with good discriminatory power for identification of clinically relevant staphylococcal species (Patel, 2015). The ease of use of this method makes it especially appealing in routine laboratories for identification of clinically important isolates to the subspecies level. Undoubtedly, CoNS identification utilising the MALDI-TOF MS system is facilitated by the availability of simple and accurate procedures with sufficient database information for accurate species level classification. Retrospective study carried out using MS-technology, by Argemi and colleagues (2015), assessing the clinical significance and species distribution of CoNS in various clinical specimens demonstrated that of all the CoNS identified, *S. epidermidis* was the most frequently isolated species from clinical samples. The other predominant species of CoNS identified were *S. haemolyticus, S. hominis,* and *S. warneri* amongst others including *S. capre, S. cohnii, S. intermidius S. lugdunensis* and *S. simulans* which are isolates rarely isolated from blood cultures (Kleeman *et al*., 1993; Kim *et*

al., 2008). The extensive database of MALDI-TOF MS application also include newly identified CoNS species-specific strains such as S*. pettenkoferi, S. condiment,* and S*. piscifermentan*. The advancement, development and performance of these identification platforms showing a consistently higher degree (>99%) of correct species identification greatly improve laboratory diagnosis of infections a critical factor in ensuring maximum benefit to the patient (Widerström *et al*., 2012).

Studies evaluating rapid diagnostic testing with use of MALDI-TOF MS as a diagnostic tool together with implementation of appropriate antimicrobial stewardship, a critical component for the management of bacteraemic patients, have demonstrated positive clinical outcomes (Loonen *et al*., 2012; Patel 2015; 2017). Furthermore, the implementation of such diagnostic technologies are more importantly linked with a significant decrease in mortality rates and hence healthcare associated costs (Patel *et al*., 2017). Therefore, the implementation and use of MALDI-TOF MS as a rapid diagnostic technology for the identification of pathogens associated with BSIs, in routine clinical laboratories in conjunction with subsequent improvements; impacting on the initiation of optimal antimicrobial therapy in future, will also most likely have a role in preventing the spread of nosocomial infection.

1.6.2 Genotypic methods for speciation fingerprinting

Identification established on basis of biotype and antibiotype are used for strain delineation in most clinical diagnostic microbiology laboratories. More importantly, these phenotypic based typing methodologies have historically contributed to the understanding of epidemiology of infections caused by clinically relevant bacterial strains (Sloos *et al*., 2000). Moreover, these phenotypic characteristics are known to be an expression of the organisms underlying genotype. Therefore, analysis of the fundamental genetic make-up of the organism represents the ultimate metric for assessing strain relatedness. Hence, comprehensive studies of the genotype provides a reliable epidemiological marker in differentiating clonal lineages; in turn, enabling elucidation of sources and transmission routes of infection (Goering, 2010).

Several different epidemiological typing methods over the past few decades, have primarily been used to study specific nosocomial CoNS genotypes established as opportunistic pathogens associated with BSIs and foreign implanted devices in the healthcare setting (Björkqvist *et al*., 2002 Heikens *et al*., 2005; Hellbacher *et al*., 2006). The application of genotypic characterisation using molecular techniques, because of their greater sensitivity and specificity, demonstrate significantly higher accuracy of clonal delineation in comparison to phenotypic based typing methods. Genotypic typing methods employed for

characterisation and evaluation of staphylococcal isolate clonality include, ribotyping targeting the 16S rRNA genes, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), random amplification of polymorphic DNA (RAPD), and various other PCR-based techniques (Poyart *et al*., 2001; Miragaia *et al*., 2002; Wisplinghoff *et al*., 2003; Heikens *et al*., 2005; Casey *et al*., 2006; Ibrahem *et al*., 2008). Although the genotypic approach remains time-consuming and expensive compared to routinely employed phenotypic methods; they demonstrate greater discriminatory power in delineating clonal diversity among clinically relevant infective strains particularly those associated with nosocomial outbreaks (Heikens *et al*., 2005).

Genotyping techniques utilising electrophoresis, PCR and amplicon sequencing parts of the genome and others including analysis of the whole bacterial genome serve as a tool for species delineation (Sloos *et al*., 2000; Deplano *et al*., 2011). Despite recent advances in molecular typing techniques, PFGE has been by far the most frequently used typing method for DNA fingerprinting of clinical important strains. Considered as the "gold standard" among molecular typing techniques, PFGE provides the most discriminatory and reliable method for CoNS strain characterisation. Whole cell DNA from *Staphylococcus* isolates subjected to analysis by *Sma*I macrorestriction and PFGE is advantageous, allowing the study of large DNA fragments of up to one million base pairs in length (Miragaia *et al*., 2002). This genotyping methodology principally used to characterise CoNS species has greater discriminatory power and higher resolution in identifying clonally diverse strains of the same species with a high degree of reproducibility (Goering, 2010). The limitations of the PFGE methodology, however, include substantial time measurement and resource requirements and a lack of resolution power translating to not distinguishing fragments of near similar sizes (Sabat *et al*., 2013). To overcome these limitations a combination of PFGE and PCR-based techniques may be utilised to discriminate between unrelated strains or demonstrate genetic similarity. Techniques employing PCR amplicon-sequencing have proven to be highly specific and sensitive for species level characterisation of CoNS strains (Poyart *et al*., 2001; Heikens *et al*., 2005).

Genotyping techniques are increasingly becoming an integral part of clinical laboratories for rapid investigation of suspected healthcare-associated outbreak strains in real-time. Currently however, these detection techniques are still limited to reference laboratories, mainly due to the requirement of both specialised equipment and technical skills. Other, molecular typing systems such as repetitive-element based polymerase chain reaction (rep-PCR) for strain categorisation requiring the use of specific performance characteristics based on objective criteria are a laboratory based typing tool used to differentiate between unrelated strains for species classification (Shutt *et al*., 2011). Commercially available rep-

PCR based technology, the DiversiLab system (bioMérieux®, Marcy l'Etoile, France) utilises the amplification of non-coding, repetitive sequences that are interspersed throughout the bacterial genome as a rapid method for strain typing. This semi-automated rep-PCR system incorporates primers that target the non-coding repetitive sequence elements which are scattered in multiple regions throughout the bacterial genome. This typing system is based on PCR amplification and subsequent electrophoresis of the DNA between adjacent repetitive sequences. Separation of the amplified genomic DNA carried out on chip-based microfluidic capillary electrophoresis result in data that is automatically collated and subsequently evaluated by the DivesiLab software Bioanalyser (Healy *et al*., 2005). Virtual gel images, selectable demographic fields, dendrograms and scatter plots, generated automatically aid data interpretation and associated evaluation of clonal similarities and/or differences (Tenover *et al*., 2009; Shutt *et al*., 2011).

Although highly standardised, the limitations identified with rep-PCR are specific to this technology which are substantially dependent on variations in assay conditions affecting correct data interpretation. The resulting lack of reproducibility and reduction in resolution makes rep-PCR less discriminatory than PFGE particularly for typing of Gram-positive isolates such as MRSA and enterococci (Fluit *et al*., 2010). However, other studies have demonstrated rep-PCR to be an effective and reliable methodology for typing of clinical isolates of *S. epidermidis* associated with nosocomial outbreaks and proved useful in determining the possible source of associated infection (Dutch *et al*., 2006; Trevino *et al*., 2009). Furthermore, studies demonstrate that the rep-PCR is of more use as a screening tool rather than a typing tool for potential hospital outbreaks as additional testing is required for confirmation of genetic relatedness of specific clonal types (Tenover *et al*., 2009; Fluit *et al*., 2010). Overall, the use of molecular typing systems enable understanding of local trends in the aetiology of infection and aid in tracing the source of infection. Additionally, these epidemiological tools are useful for monitoring disease progression and measure outcomes of interventional strategies that lead to the reduction and spread of nosocomial strains particularly amongst high-risk patient groups (Heikens *et al*., 2005; Piette and Verschraegen, 2009)

Revolutionary changes in diagnostic medical microbiology are currently taking place with the emergence of next generation sequencing techniques which enable bacterial whole genome sequencing (WGS) feasible. These high-resolution sequencing technologies are gradually replacing the currently used molecular typing tools such as PFGE in research institutions for characterisation of bacterial isolates (Sabat *et al*., 2013). More importantly, for enhanced surveillance and outbreak detection, these techniques can be used to establish absolute clonality, distinguishing strains derived from the common source of infection with superior

accuracy (Otto, 2013). Furthermore, genotyping can also be used to identify clonally related species by tDNA intergenic spacer analysis from purified genomic DNA and more importantly for the detection of *ccr* and *mec* complexes which are antimicrobial resistance determinants (Argudín *et al*., 2015). These genomic based methodologies will therefore translate into improving clinical outcomes by not only unlocking the epidemiology of the infecting pathogen but also the genes conferring resistance (McArthur and Wright, 2015).

1.7 Antimicrobial therapy

The discovery of antibiotics to combat infectious diseases caused by pathogenic bacteria marked a revolution in the 20th century. Unfortunately, due to the inappropriate use of antibiotics, most bacterial isolates have developed the ability to oppose the action of antibiotics. These antimicrobial activities include either the production of enzymes which block the antibiotic action, changes in the target site or develop mechanisms whereby the antibiotic is actively removed from the bacterial cells (Rossolini *et al*., 2010). Intrinsic bacterial resistance, defined as the organism's natural resistance to specific antibiotics, forms the basis of determining the susceptibility spectrum of an antibiotic. Acquired resistance by bacteria particularly in the case of opportunistic pathogens such as the CoNS isolates, on the other hand is dependent on their ability to adapt by acquisition of genetic material or via spontaneous mutations. Antibiotic resistance dissemination in CoNS isolates can therefore occur by either clonal spread, plasmid epidemics via replicon transfer or genetically, via transposons. All three modes of resistance have been reported to be exponential in nature associated with duplication and transfer of pathogenic associated DNA material leading to greater resistance between and within genera (Hawkey 2008; Hawkey and Jones, 2009). More importantly, with staphylococcal-associated opportunistic infections although few changes in traditional aetiologies have been noted, there have, nonetheless been reports of rapid changes in the phenotype in relation to resistance development (Gordon and Lowy 2008).

Antibiotic resistance is conventionally viewed as bacteria that exhibit significantly reduced susceptibility to the tested antimicrobials in the laboratory. Mechanisms which include altered drug uptake, alteration in drug target sites and drug inactivation are known to be the major contributors of antimicrobial resistance (Hogan and Kolter, 2009). Table 1-2 (Adapted from Walsh,2000) summarises the target sites modes of action and resistance mechanisms of commonly used anti-staphylococcal therapeutics (Blair. *et al*., 2015)

Antibiotic	Target	Mode of Action	Resistance Mechanism
	Cell Wall		
ß-Lactams:		Transpeptidases/ Transglycosylases (PBPs)	ß-Lactamases, PBP mutants
Penicillins			
Cephalosporins			
Glycopeptides:			
Vancomycin		D-Ala-D-Ala terminal peptiodoglycan and of	Replacement of D-Ala-D-Ala with
Teicoplanin		Lipid II	D-Ala-D-Lac or D-Ala-D-Ser
Cyclic Lipopeptide:		Membrane potential depolarisation	Inhibition of protein, DNA and RNA synthesis
Daptomycin			
	Protein Synthesis		
Macrolides:		Protein biosynthesis at the centre of the	rRNA methylation/ Drug efflux
Erythromycin		ribosome	
Clindamycin			
	Peptidyl Transferase		
Aminoglycosides:			
Gentamicin		Protein synthesis blockage	Enzymatic modification of drug
Kanamycin			
Tobramycin			
	Peptidyl Transferase		
Tetracyclines:			
Tetracycline		Protein synthesis blockage	Proteins produced act as drug export or efflux
Tigecycline			Binding to 30S ribsome effecting entry of
	Peptidyl Transferase		amino-acyl molecules
Oxazolidinones:			
Linezolid		Protein synthesis blockage	Monoamine oxidase inhibitor/ mutation
			at 23S rRNA
	DNA Replication/Repair		
Fluroquinolones			
Ciprofloxacin	DNA Gyrase	Blockage of DNA replication	Gyrase mutations to drug resistance
Rifampicin	DNA Transcripition	Blocakage of RNA polymerase	ß-RNA subunit mutational change

Table 1-2: Principal antimicrobial-resistance strategies to the main classes of anti-staphylococcal drugs by which staphylococci nullify the action of the antibiotics

CoNS as part of the normal human microbiota are known for their low virulence compared to other bacterial species well recognised in Gram-positive infections. An increasing incidence of antibiotic resistance has however, been reported in Gram-positive associated bacteraemia in cancer patients' concomitant with an increased risk of morbidity (Neuburger and Maschmeyer, 2006). Currently, in the era of antimicrobial resistance there is a diminishing arsenal of antibiotics available for the treatment of serious infections caused by multi-drug resistant staphylococci.

The increasing antimicrobial-resistance rates in CoNS isolates that are frequently associated with bacteraemia and hospital-acquired infections therefore poses a serious problem for treatment of clinically significant infections (Koksal *et al*., 2009; Ma *et al*., 2011). One of the most common comorbid diseases associated with the presence of infection in particular CoNS line-related bacteraemia is malignancy. These device-related infections associated with phenotypic growth of bacteria as adherent biofilms play a vital role in altering the antimicrobials mode of action. This is a well-recognised phenomenon of clinical failure due to factors other than those related to the patient's clinical state (Pozo and Patel 2007).

Due to the severity of the underlying disease state and other co-morbidities, the occurrence and frequency of CoNS related bacteraemia poses a serious challenge with respect to treatment options. Therefore, adequate and rational use of antimicrobial agents is required for treatment to be both effective and efficient. Inadequate antimicrobial therapy, defined as an infection that is sub-optimally treated has been postulated to be a determinant factor in the emergence of antimicrobial resistance influencing both patient outcomes and resource utilisation (Kollef, 2000; Cosgrove 2006; Galar *et al*., 2012; Goto and Al-Hasan 2013). Adverse clinical outcomes due to infection caused by antimicrobial resistant organisms' impact more severely among patients experiencing haematological malignancies, as these are associated with increase in morbidity, increasing treatment costs and in some cases equally higher mortality rates (Wisplinghoff *et al*., 2003; Tumbarello *et al*., 2009; Islas-Muñoz *et al*., 2018). Therefore, nosocomial infections caused by such multi-drug resistant (MDR) CoNS principally related to BSIs, represent a major therapeutic challenge associated with worse clinical outcomes (Pfallar *et al*., 1999; Sader *et al*., 2001).

Inappropriate prescribing of broad-spectrum antibiotics in this high-risk patient group not only incurs unnecessary economic cost but more importantly, has epidemiological repercussion since it leads to selection and dissemination of resistant strains (Galar *et al*., 2012 Islas-Muñoz *et al*., 2018). Presentation of fever during chemotherapy-induced neutropenia may be the only diagnostic factor indicating severe underlying infection. Recommendations across most healthcare centres at present, are that neutropenic patients who present with fever be treated swiftly with broad spectrum antibiotics to cover eradication of both Gram-positive and Gram-negative pathogens (Hughes *et al*., 2002; Freifeld *et al*., 2011). The most prevalent empirical antibiotics used in patients with haematological malignancies with BSIs are the carbapenems and glycopeptides (Garcia-Vidal *et al*., 2018). This recommended practice followed by most UK based haematology units where combination therapy is initiated using aminoglycoside e.g. gentamicin and a β-lactam such as piperacillin/tazobactam with the addition of vancomycin (Cohen and Drage, 2011). Others initiate therapy with vancomycin and a carbapenem such as meropenem (Freifeld *et al.,* 2011; BMJ, 2017). However, antibiotic resistance has arisen due to excessive overuse or inappropriate prolonged use of broad-spectrum antimicrobials within most healthcare facilities (Sujatha and Praharaj, 2012). Other factors such as changes in the treatment and management of patients with haematological malignancies during neutropenia using different anti-cancer therapies can also influence antimicrobial resistance characteristics and BSI outcomes (Vardakas *et al.,* 2005). Gudiol and colleagues (2013), in their study showed that neutropenic cancer patients treated with corticosteroids, antifungal and quinolone prophylaxis in the presence of CVCs were more likely to be associated with CoNS

bacteraemia originating from exogenous sources. Additionally, in immunocompromised patients including recipients of haematopoietic stem cell transplants the use of quinolone prophylaxis is a known risk factor for selection and dissemination of MDR strains of organisms such as *E. coli* and MRSA. Although Gram-Negative bacteria are frequently documented as aetiological agents of BSIs in the U.S, Gram-Positive associated bacteraemia however, remains prevalent across most of Europe (Marin *et al*., 2014). Stefani and Varaldo (2003), in their study showed that despite marked geographic variation across areas of Europe, hospital acquired infections due to methicillin resistant CoNS isolates were proportionally higher (60-70%). Additionally, these isolates displayed an ability to accumulate antibiotic resistance determinants leaving susceptibility limited to a few anti-staphylococcal agents. Inappropriate antimicrobial therapy has been observed to be an independent risk factor for morbidity in CoNS related bacteraemia in patients with haematological malignancies (Wisplinghoff *et al*., 2003; 2004). Mucositis and soft-tissue infections as exogenous sources of BSIs are frequently due to CoNS and as such the β-lactam antibiotics and glycopeptides constitute the backbone of therapy for CoNS related infections. Both the glycopeptides, vancomycin and teicoplanin are at present known to exhibit and retain outstanding activity against Gram-positive pathogens. Selective pressure exerted by use of beta-lactam antibiotics and the underlying condition however, influences outcome in neutropenic cancer patients with BSIs (Tumbarello *et al*., 2009; Montassier *et al*., 2013).

Most clinical strains of CoNS are known to be highly resistant to penicillin due to the production of penicillinase and resistance has also emerged to other alternative anti-staphylococcal agents such as the aminoglycosides, macrolides and tetracycline (Johnson *et al*., 1990; Fluit *et al*., 2001; Martins *et al*., 2013). Furthermore, the value of methicillin and other penicillinase-stable β-lactam anti-staphylococcal agents has also been compromised by the emergence and subsequent nosocomial spread of both methicillin resistant strains of *S. aureus* and CoNS, demonstrating a worldwide prevalence of 35% and 75-80% respectively (Yang and Kerdel 2006; Piette and Verschraegen, 2009; Wilson *et al*., 2011).

1.7.1 Principal anti-staphylococcal agents

1.7.1.1 Methicillin

Methicillin, a semisynthetic penicillin was developed as an anti-staphylococcal agent to which resistance occurred soon after its use, however “methicillin resistance” is a generic terminology used to describe resistance to oxacillin and most β-lactam antibiotics (John and

Harvin, 2007). Methicillin resistant strains of CoNS and *S. aureus* have been reported worldwide since the first strains of MRSA occurred in European hospitals in the early 1960s (Diekema *et al*., 2001). The increasing problems associated with methicillin resistance soon after its introduction in *S. aureus,* have been well documented (Hiramatsu *et al*., 1997; Hanaki *et al*., 1998). Similar trends have now also been noted in nosocomial CoNS isolates becoming increasingly resistant to the semisynthetic penicillins (Livermore, 2000). Methicillin non-susceptibility has been primarily identified as intrinsic in nature and not due to destruction of the antibiotic by β-lactamase produced by the staphylococcal strains as was previously postulated (Fluit *et al*., 2001). Resistance to methicillin (oxacillin) in staphylococci is often expressed heterogeneously with only 1 of 10^4-10^8 cells, expressing the resistance trait. This phenomenon is therefore known to complicate the accuracy of determining resistance with the use of standard phenotypic test methods (Hussain *et al*., 2000; Martins *et al*., 2007). This in turn limits the use of methicillin as a treatment option for the management of CoNS infections in the immunocompromised patient group with underlying risk factors.

The emergence of methicillin resistance in staphylococci results from the acquisition of the *mecA*-encoded penicillin-binding protein 2a (PBP2a), a transpeptidase conferring broad spectrum beta lactams resistance (IWG-SCC. 2009). Similarly, many of the CoNS species display resistance to the β-lactam antibiotics, including methicillin by virtue of either β-lactamase activity or a low affinity for the penicillin-binding protein PBP2a production encoded by the *mec*A gene. This is the defining determinant of broad-spectrum β-lactam resistance in CoNS as well as in other staphylococci (Rossolini *et al*., 2010). PBP2a an inducible membrane bound DD-peptidase enzyme determines methicillin or oxacillin resistance by catalysing the transpeptidase reaction that disrupts cell wall peptidoglycan cross-linkage. Published reports indicate that increased rates of methicillin resistance observed in clinical samples from patients in the intensive care setting are due to the horizontal transfer of the methicillin resistant gene, *mecA* located on the genetic element staphylococcal cassette chromosome (SCC) between staphylococcal species (Mombach *et al*., 2007; Blair *et al*., 2015). The acquisition and insertion of the SCC-*mec* element into the chromosome of susceptible staphylococcal strain leads to the emergence of methicillin resistance clonal lineages (Zong *et al*., 2011). Such evidence, moreover, implies that CoNS could be serving as reservoir for the nosocomial spread of resistant genes which adds to their own pathogenic potential (Otto, 2009; May *et al*., 2014). This has also been noted in studies evaluating the genomic structure of *S. haemolyticus*, showing unique chromosomal regions containing frequently rearranged insertion sequences thus defining species diversification and adaptation to antimicrobial resistance acquisition (Del'Alamo *et al*., 1999; Stefani and Varaldo, 2003; Koksal *et al*., 2009; Sader *et al*., 2012). It has been further

postulated that MDR-CoNS, most notably MDR-*S. epidermidis* probably act as a reservoir of SCC-*mec* for isolates of *S. aureus.* Evidence for transfer of SCC-*mec* elements associated with methicillin resistance from CoNS in particular from *S. epidermidis* to isolates of *S. aureus* is quite strong due to the homology of DNA sequences between the SCC-*mec* Type IV elements (98-99%) present in both isolates. Methicillin resistance (MR) rates are much higher among *S. epidermidis* and *S. haemolyticus* in comparison to *S. aureus*; an observation that indicates that the transfer of SCC-*mec* elements may also originate from other CoNS prior to their transfer to *S. epidermidis* (Otto, 2013).

In addition to nosocomial CoNS strains acquiring resistance to other anti-staphylococcal agents, resistance frequency of 80% to methicillin among CoNS isolates causing BSIs has been reported (Sader *et al*., 2001; Wroblewska *et al*., 2002). A high proportion of these BSI MR-strains are also frequently reported to demonstrate resistance to other classes of antibiotics including the macrolides, aminoglycosides, fluoroquinolones and trimethoprim (Moreira and Daum 1995; Fluit *et al*., 2001; Martins *et al*., 2013). The emergence and dissemination of these highly resistant strains are considered to be due to accelerated evolution propelled by selective pressure due to the unwarranted excessive use of broad-spectrum antibiotics. (Davies and Davies 2010; Galar *et al*., 2012 Islas-Muñoz *et al*., 2018). Therefore, the current interventional strategy universally accepted for the treatment of such infections in almost all healthcare settings is the use of vancomycin (Safdar and Rolston 2006, Sader *et al*., 2012; BMJ: Best Practice 2017). Furthermore, the excessive use and at times, the unnecessary use of vancomycin has also led to the emergence of vancomycin resistant strains of both enterococci and staphylococci in the healthcare setting (Srinvasan *et al*., 2002; Howden *et al*., 2004, 2010; Tenover and McDonald, 2005; Deresinski, 2009; Edwards *et al*., 2011).

Interestingly, it had been noted in an early study by Dopeling and colleagues, (1996) that it was unnecessary to incorporate vancomycin instead of methicillin or other β-lactams empirically for febrile neutropenia with haematological malignancies associated with Gram-positive bacteraemia. Further studies have now shown that the addition of glycopeptides appears to have had little impact on clinical outcomes but have conversely been associated with adverse events including potential toxicity (Levine, 2006; Fullmer *et al*., 2014). Despite these finding, vancomycin remains the therapeutic drug of choice in most healthcare settings as the initial empiric regimen for Gram-positive infection and/or associated with persistent febrile state. Although hospital-acquired infections due to multidrug-resistant pathogens are currently the major resistance challenges among Gram-positives due to their global dissemination and clinical impact, glycopeptide resistance in staphylococci still remains uncommon. This phenomenon could likely be due to fitness issues based on the

mechanisms of evolution of the resistant phenotype (Rossolini *et al*., 2010). Conversely, some antibiotic resistance traits for widely used anti-staphylococcal drugs could compete as important drivers for the emergence of hypervalent strains of staphylococci associated with reduced susceptibility to the glycopeptides (Ahlstrand *et al*., 2012; Sujatha and Praharaj, 2012). Inadequate empiric therapy is an added risk factor associated with poor clinical outcomes in the immune-incompetent patients with MDR-CoNS associated BSIs. Hence, for severe infections, time to appropriate therapy implementation with adequate empiric antimicrobial treatment is therefore a decisive factor for survival.

1.7.1.2 Glycopeptides

In most healthcare institutions, patients with haematological malignancies are usually prescribed broad-spectrum antibiotics in the course of a neutropenic episode with presentation of fever. Empirical cover and definitive therapy of the increasing incidence of Gram-positive bacteraemia in neutropenic patients with cancer is commonly provided by administration of a glycopeptide antibiotic (Ahlstrand *et al*., 2011, 2012). Of the glycopeptides, teicoplanin has similar activity to vancomycin against both methicillin resistant and susceptible strains of staphylococci however, monotherapy for persistent bacteraemia has been noted to result in therapeutic failures, leading to superinfections (D'Antonio *et al*., 2004). Additionally, suboptimal clinical outcomes in these patients have also been reported due to emergence of resistance during therapy (Johnson *et al*., 1990; Falcone *et al*., 2006; Kristóf *et al*., 2011). Nonetheless, teicoplanin use in adjunctive therapy has proved to be efficacious in the treatment of bacteraemia in febrile neutropenic haematology patients (D'Antonio *et al*., 2004). Vancomycin monotherapy use in this patient group on the other hand has proved to have superior efficacy as it appears to demonstrate greater bactericidal activity against most CoNS species (Menichetti, 1992, Menichetti *et al*., 1994; Ma *et al*., 2011).

Both the glycopeptides exert their antimicrobial effect by inhibition of staphylococcal cell-wall synthesis. Acquired resistance to vancomycin and teicoplanin although a rare event, is however, postulated to be associated with multi-step genetic acquisition of thickened peptidoglycan constituents of the staphylococcal cell-wall. This stronger extracellular peptidoglycan structure results in trapping and preventing the high molecular weight glycopeptide compound from reaching the target site (Hiramatsu, 2001; Srinvasan *et al*., 2002). Cell-wall thickening, a major contributor to glycopeptide resistance has been

extensively studied in clinical VISA, however, the exact mechanism of glycopeptide resistance in CoNS remains to be fully elucidated (Hanaki *et al*., 1998; Nunes *et al*., 2006).

Structurally, both vancomycin and teicoplanin although similar, display differing antimicrobial and pharmacokinetic properties. The biochemical mode of resistance is based on the glycopeptides having a high affinity for the terminal D-alanyl-D-alanine residues. These residues are a ubiquitous component of the bacterial cell-wall precursor, Lipid II present in the cytoplasmic membrane which carries the building blocks of the cell-wall peptidoglycan terminating with the D-alanyl-D-alanine dipeptide (Rossolini *et al*., 2010). On the basis of their chemical composition similarity, both vancomycin and teicoplanin demonstrate a comparable mode of activity against Gram-positive microorganisms by targeting the D-alanyl-D-alanine terminus. The glycopeptides inhibit polymerisation of peptidoglycan by interacting and enclosing the terminal D-alanyl-D-alanine region of the pentapeptide side chains further inhibiting enzyme mediated peptidoglycan precursors. Vancomycin forms dimers when binding to the D-alanyl-D-alanine terminal chain of developing peptidoglycan layer whereas teicoplanin forms monomers due to lipophilic activity. Hence, blocking of the transglycosylase function subsequently prevents cross-linkage and assembly of peptidoglycan constituents hindering the second stage of cell wall biosynthesis. Inhibition of cross-bridge formation set of the peptidoglycan layer causes cell rupture (Hiramatsu, 2001)

On the other hand, plasmid-mediated resistance activity involves modification of the cell wall synthesis precursors. This comprises incorporation of D-lactate instead of D-alanine in the normally occurring peptidoglycan precursor to which the antibiotic has a greatly reduced affinity seen commonly amongst enterococci than in staphylococci species (Hanaki *et al*., 1999; Sujatha and Praharaj, 2012). Resistance is typically associated with the dissemination of gene clusters including *van*A and *van*B prevalent amongst clinical isolates mediated by conjugative or non-conjugative transposons carried by transferable plasmids (Rossolini *et al*., 2010).

This phenomenon of reduced glycopeptide resistance has been observed both in multi-resistant MRSA strains and increasingly in nosocomial MDR-CoNS strains, commonly associated with foreign body implants (Van Der Zwet *et al*., 2002; Zong *et al*., 2011). Investigators who first reported *in-vivo* resistance to vancomycin in *S. haemolyticus,* also demonstrated that *in-vitro* selection of resistant strains was associated with changes in cell wall, suggestive of decreased vancomycin penetration (Schwalbe *et al*., 1987). However, MDR-CoNS strains demonstrating increasing resistance to teicoplanin with progressively high MICs have nonetheless been shown to remain correspondingly susceptible to vancomycin *in-vitro* (Tabe *et al*., 2001; Ma *et al*., 2011). This has been noted predominantly

amongst MDR-*S. haemolyticus* strains which appear to sequester teicoplanin better than vancomycin. The phenomenon may be dependent on parameters such as cell wall composition and synthesis or alteration of sites not associated with the D-alanyl-D-alanine target (Sujatha and Praharaj, 2012). The thickening of the cell-wall in CoNS isolates in response to glycopeptide exposure that favours resistance development may also be due other mechanisms such as cell-wall metabolic alterations which affect antimicrobial activity (Palazzo *et al*., 2005). Such alternation in the peptidoglycan outer layers results in a mesh-like structure which trap vancomycin molecules, further preventing penetration of the antibiotic into the inner part of the cell-wall layers; where synthesis and assembly of new layers are formed thus reducing therapeutic efficacy (Hiramatsu, 2001; LaPlante *et al*., 2009).

In comparison to vancomycin, teicoplanin activity in CoNS isolates has also been noted to decrease with an increase in cell-wall thickness, similar to, but at much lower levels than resistance noted in MRSA isolates (Sieradzki *et al*., 1998; Cremniter *et al*., 2010). Mechanisms other than cell wall thickening such as increased transpeptidation inhibition by teicoplanin also contribute to acquisition of greater resistance in these strains. However, significant increase of teicoplanin MICs from 2 to 8mg/L are usually seen to be accompanied by only small changes in vancomycin MICs with increasing antimicrobial pressure. Despite removal of glycopeptide pressure, teicoplanin is observed to maintain its low-level or intermediate resistance activity (Hiramatsu, 2001). Hence, increase in teicoplanin resistance could be used as a potential marker for prevention of developing vancomycin resistance in clinically significant staphylococcal infections.

1.7.1.2.1 Teicoplanin therapy

Teicoplanin as an alternative drug is more frequently used in European countries and is generally considered to be a safer drug with fewer rates of documented adverse events such as nephrotoxicity in comparison to vancomycin. Teicoplanin has been shown to be effective in the treatment of febrile neutropenia and catheter-related staphylococcal infections amongst others caused by MDR-Gram-positive pathogens. It has been demonstrated that teicoplanin binds more avidly to the specified target sites in comparison to vancomycin (Kristóf *et al*., 2011). However, teicoplanin efficacy may be reduced due to its affinity for high protein binding because protein fixation impairs antibacterial activity (Bal and Gould, 2007). Additionally, teicoplanin has an advantage over vancomycin in that is has a longer elimination half-life, therefore allowing for longer dosing intervals thus reducing the potential for both ototoxicity and nephrotoxicity (Menichetti *et al*.,1994). However, serum

concentrations of teicoplanin require frequent monitoring in order to achieve appropriate therapeutic concentration rather than risk developing nephrotoxicity. The rate of therapeutic success is, nonetheless, limited with teicoplanin use for documented bacteraemia in neutropenic patients with haematological malignancies due to other factors. These include persistence of fever over a longer period of time, higher relapse incidences after treatment and although not significantly so, a greater number of superinfections post-teicoplanin treatment than noted with vancomycin use (D'Antonio *et al*., 2004). Utility of teicoplanin is therefore limited, as doubts also remain regarding daily dosage levels associated with required clinical response rates in neutropenic patients which may be variable for optimal management of staphylococcal infections.

1.7.1.2.2 Vancomycin therapy

Vancomycin, a first-generation bactericidal antibiotic is the most widely used empiric therapeutic agent. After its approval by the FDA for clinical use in 1958 for its excellent activity against Gram-positive pathogens its use continues for the treatment of serious infections especially in immunocompromised hosts (Butler *et al*., 2014). Its use became vital for the treatment of MRSA infections prevalent in many healthcare institutions worldwide more than four decades ago (Griffith,1984; Srinvasan *et al*., 2002; Steinkraus *et al*., 2007; Deresinski, 2009). Vancomycin also remains one of the critically effective drugs currently available for the treatment of life-threatening infections such as MDR-CoNS associated bacteraemia. The accelerated use of vancomycin since the 1980s has however, led to an increase in the emergence of vancomycin resistance amongst Gram-positive isolates (Levine, 2006; 2008). Resistance noted particularly amongst isolates of *S. aureus* has led to serious limitations in therapeutic options available for the treatment of severe infections due to these isolates (Lodise *et al*., 2008; Sujatha and Praharaj, 2012). Similarly, with the emergence of CoNS isolates with high rates of methicillin-resistance causing primary or secondary bacteraemia among immunocompromised patients has also accelerated the use of vancomycin as the therapeutic drug of choice (Schwalbe *et al*., 1987; Safdar and Rolston, 2006; D'mello *et al*., 2009). This in turn has also led to the rise in vancomycin resistance among clinically significant isolates of CoNS in many healthcare settings world-wide (Biavasco *et al*., 2000; Natoli *et al*., 2009; Sujatha and Praharaj, 2012). Clinical failures with vancomycin therapy now raise concerns regarding its decreased bactericidal activity and clinical efficacy.

Vancomycin resistance first described in *S. haemolyticus* species by Schwalbe and colleagues in1987, as an emerging nosocomial has been documented to develop resistance

to multiple antibiotic classes and is unique in its predisposition in also acquiring resistance to the glycopeptides (Sieradzki *et al*., 1998; Biavasco *et al*., 2000). Reports of widespread resistance to vancomycin in CoNS, however, remain relatively limited following the first reported case in Japan by Hiramatsu (2001) of extensive resistance in isolates of *S. aureus*. Prior to this, Hiramatsu and colleagues (1997; 1998) were also the first to report case of clinical strain of MRSA with reduced vancomycin susceptibility displaying heterogenous resistance. Despite, susceptibility test results showing retention of vancomycin activity against almost all Gram-positive pathogens, its increased use in the hospital environment has led to the emergence of resistance to vancomycin among strains of enterococci and heteroresistant, intermediate resistance or total resistance among strains of *S. aureus* (Jones, 2006; Howden *et al*., 2010). Heterogeneous or intermediate-glycopeptide resistance particularly to teicoplanin, among species-specific CoNS strains, has been reported to be more prevalent among isolates of *S. haemolyticus* (Sieradzki *et al*., 1998; Biavasco *et al*., 2000; Nunes *et al*., 2006). These *S. haemolyticus* strains are characterised by thicker cell-walls and increased amounts of unprocessed D-alanyl-D-alanine causing trapping of the teicoplanin molecules resulting in higher observed MIC values (Hiramatsu, 2001; LaPlante *et al*., 2009). Such isolates representing heterogenous susceptibility are poorly detected by routinely used manual or automated susceptibility test systems. Therefore, alternative diagnostic test methods are required in cases of persistent vancomycin therapy failure associated with infections caused by CoNS strains displaying heterogenous glycopeptide resistance profiles (Biavasco *et al*., 2000; D'mello *et al*., 2008). Furthermore, based on clinical data analysis it has been established that patient's exposure to vancomycin therapy relative to the isolates susceptibility to vancomycin is an effective predicator of clinical outcomes (Rybak *et al*., 2013). Hence, the use of appropriate bactericidal assays to detect isolates with reduced susceptibility to vancomycin and teicoplanin could lead to better prediction of clinical outcomes (Levine, 2008; Satola *et al*., 2011).

Vancomycin with its narrow therapeutic index and association with venous toxicity resulting in some limitations of use, therefore, necessitates close monitoring of serum concentrations (Levine, 2006; Rybak *et al*., 2009). Due to the potential of ototoxicity and nephrotoxicity associated with vancomycin use, serum trough level assays are routinely performed to allow for dose adjustments concomitant with antimicrobial efficacy for optimal clinical outcomes (Levine, 2006; Rybak *et al*., 2009). Determination of vancomycin serum trough level concentrations is the most widely used pharmacokinetic parameter for monitoring the clinical efficacy of the antibiotic. The optimal focus on both toxicity and clinical efficacy also takes into account factors such as bacterial load and decrease of *in-vivo* bactericidal activity commonly associated with the presence of biofilm on intravascular devices (Kitzis and

Goldstein 2006). Therapeutic monitoring therefore performed within a relatively narrow range minimises toxicity thus ensuring optimal therapeutic success. Additionally, vancomycin-induced nephrotoxicity in patients with haematological malignancies receiving other combination nephrotoxic chemotherapies remain at a greater risk of adverse outcomes (Vardakas *et al.,* 2005; Fullmer *et al*., 2014). Trough serum concentration assays, recommended as the most practical and accurate method for monitoring drug efficacy are performed after continuous steady-state infusion therapy for 36 to 48 hours before administration of the next dose which is adapted in line with clinical correlation after further monitoring (Rybak *et al*., 2009). Recommended target trough concentration levels are set at a therapeutic range of 15-20mg/L and dose adjustment are undertaken in conformity with national antibiotic policies (Penack *et al*., 2010).

Achieving adequate serum concentration levels are therefore fundamental to eradication of serious infections in high-risk patient groups for improved clinical outcomes. However, higher trough serum concentrations may become necessary for the treatment of staphylococcal strains displaying reduced susceptibility to the glycopeptides to avoid risk of clinical failure (Kitzis and Goldstein 2006). Conversely, continuous administration of sub-therapeutic dose levels of vancomycin increases the likelihood of MDR bacterial emergence. This is based on evidence which suggests that trough vancomycin serum concentrations of <10mg/L which is below the optimal target levels of 15-20mg/L can produce strains with vancomycin intermediate resistance-like characteristics (Rybak *et al*., 2009). Compromising infection outcomes, therefore, further increases the burden of morbidity and in some cases a potential increase in mortality rates among cancer patients with BSIs (Marin *et al*., 2014; Kleinhendler *et al.,* 2018).

1.7.1.2.2.1 Heterogenous glycopeptide resistance

The excessive use of vancomycin in the 1980s for the treatment of infections such as antibiotic-associated colitis and MRSA, has led to increasing cases of complete resistance or reduced susceptibility to vancomycin. (Levine, 2008). The phenomenon of reduced resistance or heteroresistance has been widely observed in range of Gram-positive organisms particularly in infections caused by *S. aureus* (Geisel *et al*., 1999; Fridkin *et al*., 2003; Hanaki *et al*., 1998; 2007). In general, heteroresistance is defined as resistance to specific antimicrobial agents by a subset of isolates within a homogenous microbial population that are normally considered to be otherwise susceptible. The pathogenic potential and clinical significance of heteroresistance however, has yet to be clearly defined. Hetero-vancomycin resistance is postulated to constitute a precursor stage to the emergence

of vancomycin resistance in isolate populations consisting of a prevalence of heteroresistant strains within a homogenous population (Hiramatsu, 2001).

The emergence of heterogeneously glycopeptide-intermediate staphylococci (hGIS) strains have been associated with increasing therapeutic adversities in infections caused by methicillin-resistant staphylococcal strains (Tenover and Moellering, 2007). Intermediate and heterogeneous resistance to the glycopeptides (GIS and hGIS respectively) still remains best described for *S. aureus* isolates resistant to treatment (Hanaki *et al*., 2007). These hGIS phenotypes are a small subset of the population with decreased susceptibility to vancomycin, not detected with standard susceptibility test methodologies. These subsets within a susceptible strain population have, nonetheless, been purposed to be precursors to development of intermediate vancomycin resistance due to prolonged therapeutic pressure (Hiramatsu *et al*., 1997; Hiramatsu 2001). Prolonged duration of bacteraemia despite increased dose adjusted vancomycin therapy resulting in clinical evidence of vancomycin treatment failure has been observed to be due to episodes associated with higher bacterial loads of isolates expressing vancomycin heteroresistance (Charles *et al*., 2004). Additionally, low vancomycin trough serum levels observed in patients with hVISA bacteraemic infection indicate either induction of pre-existing vancomycin resistance or selection of resistant strains due to antibiotic pressure. The use of linezolid as an alternative therapeutic agent in this case achieved an appropriate response among patients with hVISA infection where appropriate vancomycin serum levels failed to ensure improved outcomes. The association between emergence of heteroresistance and clinical failure has been further linked to the higher mortality rates observed in the immunocompromised host on prolonged ineffective vancomycin therapy (Tenover *et al*., 2001; Steinkraus *et al*., 2007; Howden *et al*., 2004, 2005,2010).

Unlike studies showing increasing glycopeptide resistance in *S. aureus* isolates associated with therapeutic failure, similar reports amongst CoNS isolates currently remain infrequent (Tenover *et al*., 2001; Walsh *et al*., 2001; Tenover and Moellering, 2007). Reduced susceptibility to the glycopeptides in particular, to vancomycin has however been reported in several species of CoNS with the frequency of expression differing among species dependent on factors such as the rate of clonal mutation occurrence under antibiotic pressure. Of the clinically relevant CoNS species, both *S. epidermidis* and *S. haemolyticus* have been found to exhibit reduced susceptibility to the glycopeptides more frequently (Biavasco *et al*., 2000; D'mello *et al*., 2008). Reports of homogenous resistance to vancomycin exhibiting MIC >8 mg/L based on BSAC classification, among strains of CoNS, continue to be rare, nonetheless, some strains demonstrating heteroresistance have been reported (Tevell *et al*., 2014). Routine screening methods for staphylococcal strains

exhibiting heteroresistance, often with vancomycin MICs ranging between 1-4mg/L and subpopulations demonstrating MICs of up to 8mg/L, remain in flux (Hiramatsu, 1997; Hiramatsu *et al*., 2001). Conversely, staphylococci isolates exhibiting such characteristics have been considered to be concomitant with either treatment failure or are likely to present as precursors of glycopeptide resistant strains (Sieradzki *et al*., 1998; Wong *et al*., 1999). Although the clinical significance of glycopeptide resistance in CoNS has yet to be clearly ascertained, a study by Nunes and colleagues, (2006) evaluating the prevalence of glycopeptide resistance amongst CoNS BSI isolates suggests that the presence of heterogeneous resistance these isolates could also be clinically relevant. In their study, authors report that out of the 106 patients with bacteraemia, a prevalence of 9(8.5%) glycopeptide resistance was found among CoNS isolates of which, *S. haemolyticus* and *S. epidermidis* were the predominant species. The observed incidence of reduced susceptibility to the glycopeptides amongst BSI strains in high risk patient populations, therefore, limits appropriate antimicrobial treatment leading to greater risk of poor clinical outcomes.

Optimal laboratory screening methods for the detection of such heteroresistant vancomycin-intermediate strains of staphylococci at present remain unclear. However, monitoring the prevalence of isolates with reduced vancomycin susceptibility is of epidemiologic importance as these strains can become endemic within the hospital environment. Therefore, transmission of these resistant staphylococcal strains among immunocompromised hospital patients would places them at a greater risk of acquiring infections that can become increasingly difficult to treat (Srinvasan *et al*., 2002). Whilst vancomycin has retained its considerable activity against MDR-staphylococci, the emergence of the heterogenous and vancomycin intermediate resistance has necessitated the exploration and use of either newer anti-staphylococcal drugs or alternative combination therapies.

1.7.2 Newer therapeutics

Patients with haematological malignancies who are subjected to both increasingly potent and intensive chemotherapeutic therapies require optimally defined antimicrobial agents and the prompt implementation of targeted therapy to reduce the risk of CoNS associated infection. The high frequency of oxacillin-resistant CoNS has rendered the empirical use of methicillin, obsolete for the treatment of such nosocomial infections. Vancomycin as part of initial empirical treatment of febrile neutropenic patients with bacteraemia and severe neutropenia with microbiologically documented infections has been recognised to lead to better clinical outcomes (Safdar and Rolston, 2006; D'mello *et al*., 2009). With increasing reports of emerging high levels of resistance to vancomycin, primarily considered the therapeutic drug

of choice for the treatment of proven or suspected Gram-positive bacteraemia in most healthcare settings the need for alternative therapeutics has therefore become of primary importance (Diekema *et al*., 2001). Although adverse effects including nephrotoxicity are known to be more common in patients receiving a glycopeptide as part of their normal regimen, meta-analysis studies by Varsdakas (2005), report that mortality rates were no different in patients treated with or without addition of glycopeptides. Despite these findings, the standard protocol in most healthcare setting and in haematology units is to administer antibiotic therapy with either cephalosporin or a β-lactam/ β-lactam plus inhibitor with the addition of an aminoglycoside and inclusion of vancomycin prior to microbiological sampling. The prompt administration of effective broad-spectrum antimicrobial therapy in haematology patients with fever and signs of bacteraemia is known to correlate well with patient outcomes (Cohen and Drage, 2011). This in turn presents another important challenge for treatment of difficult to treat staphylococcal infections in these high-risk patient groups, requiring second or third-line agents for refractory cases. The advancing shift towards more resistant pathogens with fewer options for therapy underscores the continued need for prevention and need for newer therapeutic options. Therefore, the use of alternative antimicrobial agents for clinical use with few minimal adverse effects could prove to be useful with the added advantage of reducing the threat of infection in high-risk patient groups and potential for improved clinical outcome (Raad *et al*., 2007; Chaftari *et al*., 2010; Moore *et al*., 2011).

In light of emerging antibiotic resistance, a number of new anti-staphylococcal agents have recently been introduced and approved by the United States, Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) for the treatment of severe staphylococcal infections. Daptomycin, tigecycline linezolid and the fourth generation cephalosporins, have recently been introduced as back-up drugs mainly for the treatment of MRSA infections particularly in cases of clinical failures with standard therapies (Raad *et al*., 2007; Moore *et al*., 2011). These antibiotics, appear to have greater activity against methicillin and vancomycin resistant staphylococci and may prove to be clinically efficacious due to escalating resistance to vancomycin (Yang and Kerdel 2006; Raad *et al*., 2007; Rolston *et al*., 2014)

1.7.2.1 Linezolid

Linezolid a broad-spectrum synthetic class of antimicrobials, an oxazolidinone was approved by the FDA for use in units and patients where multi-resistant enterococci or staphylococci are documented (Lomaestro, 2003). Linezolid, a weak reversible monoamine oxidase inhibitor, mode of action involves the binding of tRNA to the ribosome with the 50S subunit (V

domain of 23S rRNA) thus blocking bacterial cell protein synthesis. Resistance to linezolid is conferred due mechanisms including mutation at the 23S rRNA but this remains rare as 23S rRNA is also encoded by several other genes (Walsh, 2000; Blair *et al*., 2015). This is essentially a bacteriostatic mode of action allowing mRNA translation but inhibiting peptide elongation which appears to be effective in prevention of staphylococcal virulence factors such as coagulase and haemolysins (Livermore, 2003). Due to its structure and unique mode of action, there is at present no observation of cross-resistance with other classes of antimicrobial agents.

Although linezolid demonstrates potent activity against methicillin-resistant CoNS little is known regarding its efficacy in the treatment of patients with CR-BSIs where CoNS are commonly implicated as the source of infection (Gu *et al*., 2012). Haematology units where there is a high incidence of multi-resistant Gram-positive organisms, have however introduced the use of linezolid as a second-line agent administered either orally or intravenously against clinically important Gram-positive cocci for better clinical outcomes. Linezolid is generally associated with high levels of bioavailability and its use does not require pharmacological monitoring (Cohen and Drage, 2011). Currently there is limited data to show that linezolid is as effective as vancomycin in uncomplicated bacteraemia, as its bactericidal activity is thought to be limited in patients with immunosuppression. Nonetheless it has been suggested that linezolid showing 100% bioavailability *in-vivo* with minimal adverse effects can be used in the event of severe VISA/VRSA infections for which there are few antimicrobial alternatives (Livermoore, 2000; Fridkin *et al*., 2003; Raad *et al*., 2007).

Linezolid resistance among staphylococci remains uncommon however the development of resistance, although slow in the clinical setting for CoNS infections has been linked to previous therapeutic use (Kelly *et al*., 2006; John and Harvin 2007). Furthermore, clones of linezolid-resistant CoNS due to selective pressure may be involved in colonisation of neutropenic patients thus becoming a source for transmission in the hospital environment (Mulanovich *et al*., 2014). Prolonged treatment with linezolid (>2 weeks) has also been associated with other factors such as haematopoietic toxicity and a delay in white blood cell count recovery in febrile neutropenic patients with malignancies (Kuter, 2001). Cases of reversable bone marrow suppression with anaemia, immune-mediated destruction or platelet aggregation have been reported with the prolonged linezolid administration (French, 2003). Therefore, its routine use particularly as a monotherapy remains limited in patients with haematological malignancies with risk factors of neutropenia due to its haematopoietic toxic potential.

1.7.2.2 Tigecycline

Tigecycline belonging to the glycylcycline class of antibiotics, is an expanded spectrum tetracycline derivative, with excellent activity against a range of resistant Gram-negatives as well as Gram-positive cocci and bacilli (Livermoore, 2005). Tigecycline exerts its bacteriostatic effectiveness by binding to a single high-affinity intracellular site on the 30S-ribosome, thus preventing protein synthesis by blocking entry of amino-acyl molecules (Walsh, 2000). The commonly occurring resistance phenotypes which include MRSA, VRSA and MR-CoNS appear to remain largely unaffected by its potent antibacterial activity where resistance is due to β-lactamase and penicillin-binding proteins modifications (Rubinstein and Vaughan.2005).

Due to its potent activity against both susceptible and resistant BSI CoNS isolates, tigecycline has recently been introduced into clinical practice for the treatment of severe infections recalcitrant to commonly used antibiotics. (Raad *et al*., 2007). *In-vitro* susceptibility data for tigecycline against clinically important methicillin susceptible and resistant strains of CoNS show MIC range of 0.25 to 1mg/L and range from 0.5-2mg/L, respectively, suggests it its greater potential role in eliciting the desired therapeutic response *in-vivo* (Garrison *et al*., 2005). Additionally, this data suggests that tigecycline has comparable or better activity in comparison to other agents with a lower potential for resistant development due to its pharmacokinetic profile (Garrison *et al*., 2005). Although not a bactericidal drug, tigecycline has also demonstrated superior activity against high-biofilm producing MRSA clinical isolates in comparison to other anti-staphylococcal agents such as vancomycin, rifampicin and linezolid (Raad *et al*., 2007; Rose and Poppens 2009).

Tigecycline demonstrates excellent *in-vitro* activity against BSI *S. epidermidis* and *S. haemolyticus* isolates from BMT recipients that are resistant to methicillin (Kratzer *et al*., 2007). Its role is, nevertheless, currently directed more towards empiric treatment of infections where either both MRSA and Enterobacteriaceae or MDR-Gram-positive isolates are likely (Livermore, 2005; Kratzer *et al*., 2007)

1.7.2.3 Daptomycin

Daptomycin is a lipopeptide (cyclic glycopeptide) antimicrobial agent, used mainly for the treatment of Gram-positive infections approved for clinical use in 2003. This antimicrobial agent has a distinctive mechanism of action, exerting its bactericidal activity by disrupting the plasma membrane function and targeting anionic phospholipids without penetrating the

cytoplasm (Blair *et al*., 2015). This mode of action also involves altering the bacterial cell membrane potential by impeding calcium-dependent channels which causes disruption of the cytoplasmic membrane. The resulting loss of membrane potential, inhibition of DNA, RNA and protein synthesis, together with depolarisation leads to intracellular cell content loss and cell death (Walsh, 2000; Steenbergen *et al*., 2009; Blair *et al*., 2015).

Daptomycin demonstrates concentration-dependent killing and has been predominantly used for the treatment of BSIs, and soft tissue infections. Daptomycin retains potency against infections caused by methicillin-resistant and glycopeptide intermediate or resistant strain of *S. aureus* (Moor *et al*., 2011). Moreover, daptomycin use in clinical trials of staphylococcal related bacteraemia has proved to be just as clinically effective as vancomycin (Fowler *et al*., 2006). Definitive efficacious empirical therapy for these serious BSIs is, however, at present still provided by the administration of the glycopeptide, vancomycin in most healthcare institutions (Deresinski, 2009). Nonetheless, daptomycin in comparison, has also been shown to be just as efficacious as vancomycin in the treatment of CR-BSI and methicillin resistant CoNS in patients with cancer. Additionally, nephrotoxicity has been shown to be reduced by three-fold with the use of daptomycin instead of vancomycin for the treatment of Gram-positive CR-BSI in cancer patients with faster symptom resolution, microbiological eradication and a significantly better clinical response (Chaftari *et al*., 2010). Rolston and colleagues (2014), in their observational study, also documented improved clinical outcomes with the use of daptomycin to eradicate Gram-positive BSIs in haematology neutropenic patients. Their study results showed the superior activity of daptomycin therapy, as of the 156 patients investigated 31% failed vancomycin therapy but demonstrated better clinical outcomes post daptomycin therapy. The decline in bactericidal activity of vancomycin particularly in neutropenic patients is of concern as the administration of bactericidal agents are the preferred for treatment of this patients. The study results further demonstrate the superior efficacy of daptomycin in comparison to vancomycin as it is reported to be better tolerated with no effect on the degree of neutropenia in haematology patients during chemotherapy.

Daptomycin remains clinically effective in a variety of infections for which it is indicated including bacteraemia as well as in non-indicated infections in neutropenic patients (Rolston *et al*., 2014). Reports of daptomycin antimicrobial lock therapy with installation into the intravenous line of 2ml of antibiotic at 100-1000 times the systemic concentration further demonstrate its superior efficacy for microorganism eradication in comparison to vancomycin (LaPlante and Mermel, 2007). The efficacy of daptomycin for the treatment of severely ill patient populations with multiple co-morbidities commonly infected with MDR CoNS demonstrate highly favourable earlier clinical and microbiological responses despite its use

as a frequent second-line or salvage therapy (Kelesidis, 2011). Notwithstanding its increased use non-susceptible isolates have rarely been reported worldwide including non-susceptibility among CoNS (Sader *et al*., 2014). Furthermore, daptomycin alone, and in combination with other antibiotics such as rifampicin and gentamicin has also proved to have greater activity against staphylococci embedded in biofilm on catheter surfaces (LaPlante and Woodmansee 2009; Rolston *et al*., 2014). Increased use of daptomycin for the treatment of complex infections caused by methicillin resistant strains of staphylococcus such as VISA has resulted in a notable decrease in susceptibility. However, combination therapy with other β-lactams such as oxacillin or with cephalosporins such as ceftaroline has resulted in enhancing daptomycin activity. Restoration of daptomycin susceptibility in combination therapy has been demonstrated in effective eradication of BSI MRSA isolates that had acquired daptomycin resistance due to selective monotherapy pressure (Rose *et al*., 2012).

1.7.2.4 Ceftaroline

Recently resistance to the β-lactams has been combated by the introduction of ceftaroline, a fifth generation cephalosporin demonstrating broad spectrum activity against a range of multi-resistant organisms including MRSA, CoNS and VRE (Biek *et al*., 2010). Ceftaroline has been approved by the FDA in the United States and in Europe by EMA for skin and soft tissue infections only. The clinical efficacy of this agent has been demonstrated in the treatment of both complicated soft tissue infections and community-acquired pneumonia (Biek *et al*., 2010).

Ceftaroline has also demonstrated activity superior to vancomycin which is attributed to its higher affinity for the modified PBP2a which confers resistance to most β-lactam antimicrobials. This novel broad-spectrum cephalosporin exerts its bactericidal drug activity by binding to the PBPs inhibiting the transpeptidation reaction required for cell-wall biosynthesis resulting in lysis and cell death (Walsh, 2000; Laudano, 2011). Ceftaroline, the new anti-MRSA cephalosporin may also be appropriate for use against methicillin resistant CoNS BSI isolates (Polenakovik and Pleiman 2013). Several studies have demonstrated *in-vitro* activity of ceftaroline against MRSA, MR-CoNS and non-susceptible vancomycin and daptomycin isolates with MIC values ranging from less than 0.12 to 0.5mg/L (Ge *et al*., 2008; Biek *et al*., 2010; Guay, 2011; Sader *et al*., 2013). The positive attributes of ceftaroline with respect to treatment of CoNS associated bacteraemia in patients with haematological malignancy has yet to be evaluated in dedicated clinical studies. Its approval for use in the

clinical setting may provide a potential empirical alternative to the commonly used anti-staphylococcal therapeutic for CoNS associated bacteraemia.

1.7.3 Antimicrobial Combination Therapy

Broad-spectrum combination empiric therapeutics are commonly used to treat seriously ill patients to increase the likelihood of clinical success. Combination therapeutics targeting metabolically active bacteria or specific bacterial metabolic sites have also been utilised to combat severe staphylococcal infections. Additionally, the advantage of potential synergistic effect against the isolates, lowers the risk of resistance development which is likely to occur due to prolonged antibiotic use during the course of disease progression (Hughes *et al*., 2002; Dall *et al*., 2018). *In-vitro* studies demonstrating synergy with agents such as ciprofloxacin, gentamicin, tobramycin or rifampicin against clinical isolates of CoNS, used in combination have proved to be effective in the treatment of serious infections due to these organisms (Raad *et al*., 2007; LaPlante and Woodmansee 2009; Rose and Poppens 2009). Similarly, vancomycin has demonstrated greater clinical efficacy when used in combination with other anti-staphylococcal agents, in particular against biofilm producing methicillin-resistant staphylococcal infections (Dall *et al*., 2018). Likewise, rifampicin frequently used for the management of biomaterial related infections demonstrates superior bactericidal efficacy in eradicating biofilm enchased staphylococcal strains in combination with either tigecycline, daptomycin or linezolid (Walsh 2000; Fey, 2010). This exceptional efficacy is attributed to the antibiotic action targeting RNA polymerase of metabolically diverse biofilm encased cells leading to eradication (Mah and O'toole 2001). Conversely, although *in-vitro* activity of rifampicin may be similar to the other agents it is rarely used clinically as a mono-therapeutic; due to occurrence of single-step point-mutations in the beta-RNA subunit of the drug leading to rapid resistance development *in-vivo* (Fey, 2010).

In clinical practice, antimicrobial combinations are increasingly used for therapeutic efficacy against recalcitrant infection and controlling resistance evolution (Steenbergen *et al*., 2009). As such, for treatment of bacteraemia particularly amongst cancer patients, most healthcare facilities follow the recommended empirical treatment guidelines comprising combination therapy to include an aminoglycoside. Typically, in cases of severe bacteraemia, combination therapy includes the addition of gentamicin with a β-lactam as frontline therapy or with a cephalosporin as second-line therapy for patients non-respondent to first line therapeutics (BMJ-Best Practice, 2017). In high-risk patient groups, the application of synergistic interaction between two antimicrobials or other compounds has also demonstrated a reduction in adverse drug reactions and increased drug potency of the

antimicrobial agent that may be less effective when used as a mono-therapeutic (Bollenbach, 2015). Combinations alternatives in cases of treatment failures due to toxicity include agents such as the trimethoprim-sulfamethoxazole, quinupristin-dalfopristin, rifampicin or the next generation of cephalosporins, including ceftobiprole and ceftaroline (Cervera *et al*., 2009).

Sakoulas and colleagues (2014), in their study demonstrated excellent synergist activity of ceftaroline-daptomycin combination for the treatment of documented refractory staphylococcal bacteraemia. Successful clinical outcomes were also demonstrated with the use of ceftaroline-daptomycin combination therapy in cases of refractory *S. aureus,* MRSA, VISA, and *S. epidermidis* in bacteraemic patients with underlying co-morbidities. Daptomycin activity appears to be enhanced by the superior binding affinity of ceftaroline to the PBPs, hence resulting in its synergistic effectiveness in reducing the infection burden in bacteraemic patients (Barber *et al*., 2014). Combination therapy with ceftaroline has not only demonstrated rapid and sustained bactericidal susceptibility of daptomycin but also prevented emergence of resistance (Rose *et al*., 2012).

Dose-response characteristic of antimicrobial combination determines both dynamics of effectiveness and resistance development. Therefore, due to the reduction in mutational changes responsible for resistance emergence in response to combined antimicrobial challenges, a decrease in overall resistance evolution is observed (Bollenbach, 2015). Optimised treatment with appropriate antimicrobial combinations therefore appear to have greater potential for improving clinical outcomes in highly vulnerable patient groups with underlying disease (Climo *et al*., 1999; Dall *et al*., 2018).

1.8 Antimicrobial Susceptibility Test methods

Surveillance data shows that antimicrobial resistance is fast becoming a significant threat to both the prevention and treatment of hospital acquired bacteraemic infections particularly in the immunocompromised host. Accurate predication of antimicrobial efficacy are therefore an essential requirement for successful treatment of BSIs. Typically, genetic encoded mechanisms of antibiotic resistance determine antibiotic susceptibility during infection. Laboratory based phenotypic criteria dominates the field of antibiotic epidemiology and effectiveness. *In-vitro* antibiotic testing techniques, however, are poor predictors of susceptibility as these do not take into account the presence of extrinsic determinants of antimicrobial susceptibility prevalent during the course of infection. These factors include host-pathogen interaction, hosts immune response and environmental factors such as

bacterial heterogeneity due to the presence of biofilms and antagonising antibiotic efficacy which all contribute to therapeutic failure (Radlinski and Conlon, 2018).

In most routine clinical laboratories traditional *in-vitro* antimicrobial susceptibility test (AST) methods utilise automation based on broth microdilution principles, ultimately generating results with a relatively high level of accuracy within 18-24 hours (Ligozzi *et al*., 2000; Nonhoff *et al*., 2005). These AST quantitative dilution techniques are based on two fold dilutions of the test antibiotic in broth media, which in turn generates the MIC value of the given antibiotic in mg/L at which bacterial growth is inhibited. On the other hand, qualitative agar antibiotic disc-diffusion susceptibility categorisation may show reasonable but incomplete correlation with therapeutic outcomes due to factors affecting defined experimental conditions (Balouiri *et al*., 2016). The disadvantage of routine implemented AST techniques at present also comes at the risk of reduced sensitivity for the detection of slow-growing, low frequency heteroresistance subpopulations. This further increases the risk of underestimating the prevalence of antimicrobial resistance which in turn has a detrimental impact on clinical outcomes. In the healthcare environment, high-risk patients are generally placed on broad-spectrum therapy without waiting for microbiology AST results. This practice plays an important role in contributing to the emergence and spread of antimicrobial resistance (Montassier *et al*., 2013).

In general, antibiotic susceptibility is examined in simple homogenous conditions *in-vitro*. Antibiotic efficacy is however, known to be affected by the dynamic and complex nature of host-pathogen interaction influenced by extrinsic factors during the course of infection. In the immunocompromised host, these highly influential extrinsic factors together with the administration of inappropriate antimicrobial therapy, potentiates resistance selection resulting in treatment failure and exacerbation of disease progression (Radlinski and Conlon, 2018). In line with the changing patterns of nosocomial BSIs, a move towards optimal targeted patient therapy for the treatment of cancer patients with febrile neutropenia have been made recently by hospitals in accordance with national recommended guidelines (Penack *et al*., 2010; BMJ-Best Practice 2017). Implementation of these procedures could lead not only to the avoidance of antibiotic-associated illness but also a decrease in selection and dissemination of resistance to commonly used broad-spectrum antimicrobial agents for better therapeutic response.

Several test assay strategies performed routinely by clinical microbiology laboratories are utilised for the provision of accurate susceptibility results for treatment options, in the clinical context for a given patient population group. Conventional, manual based qualitative and quantitative AST test methodologies take up to 24 hours or more for results reporting to the

clinicians for implementation of targeted empiric therapy. Advances in technology in comparison to previously described AST methodologies have nonetheless demonstrated the clinical and financial benefits of rapid AST result reporting for the treatment and hence eradication of significant infections (Barenfanger *et al*., 1999). Therefore, AST result provision for therapeutic guidance for implementation of optimum antimicrobial therapies for effective clinical outcomes.

AST is important, not only to confirm susceptibility to the chosen empirical antimicrobial agent but also detection of acquired resistance mechanisms especially amongst species-specific *Staphylococcus* isolates (Rossolini *et al*., 2010). The MIC of antimicrobials tested, are primarily determined by the routine implemented test methodologies, which include the automated VITEK®2 and standard E-tests for confirmation. The AST result breakpoint categorisation criteria of susceptible, intermediate and resistant are based on pharmacokinetics and pharmacodynamic interactions. Pharmacokinetics refers to the dosing distribution and half-life of the antimicrobial agent that impact achievable concentrations *in-vivo* and pharmacodynamic the interaction with the organism. Therefore, the optimal interpretation of MICs are based on the pharmacokinetic/pharmacodynamics of the antimicrobial agent in terms of microbiological eradication and clinical efficacy. These are referenced in expert sources such as CLSI, BSAC and EUCAST, which are web-based expert sources providing published guidelines for interpretative criteria for MICs that relate to proven clinical efficacy of specified antibiotics for a given bacterial species (Reller *et al*., 2009).

At present *in-vitro* AST testing in routine clinical laboratories is carried out based on three basic principles, including disc diffusion, gradient diffusion and broth microdilution. Broth microdilution methods are presently incorporated in automated formats which include systems such as the VITEK®2 (bioMérieux, France). These AST microdilution-based automated techniques are performed on planktonically growing isolates generally providing reliable test results for a range of antimicrobial agents. The automated MIC systems utilise abbreviated dilutions across a narrow range generally covering the breakpoint concentration of the test drug (Ribeiro *et al*., 1999; Ligozzi *et al*., 2002; Nonhoff *et al*., 2005). Routinely used AST methodologies, therefore, effectively provide results for prediction of therapeutic success or failure in most cases of systemically spreading infections.

1.8.1 Automated AST systems

The VITEK®2 automated commercial system microdilution procedure includes the generation of MICs with the convenience of computerised generated reports. This highly automated system uses compact plastic GP-reagent cards each containing microliter quantities of the

relevant test antibiotics and test media in a 64-well format inclusive of positive and negative control wells. As previously described, the VITEK®2 system employs, 15 minutes, repetitive turbidimetric monitoring determined by fluorometric measurement of bacterial growth over an abbreviated incubation period of up to 18 hours. The quantitative antimicrobial susceptibility results are categorised according to the current installed version of the Global EUCAST/CLSI defined guidelines by the manufacturer (bioMérieux, UK). The associated VITEK®2 Advanced Expert System™ software programme analysis enables the biological validation and interpretation of antimicrobial susceptibilities and detection of resistant phenotypes. Modifications and upgrade of the VITEK®2 computer software and advanced expert system has further provided assurance of result interpretation and accuracy of reported susceptibility results to the clinician for better treatment options (Ligozzi *et al*., 2002; Product literature, 2004; Funke and Funke-Kissling 2005).

1.8.1.1 VITEK®2 GPC AST card

The VITEK 2™ Gram-Positive susceptibility AST card used with the automated VITEK®2 System (Software version 05.03–2011) is used as an i*n-vitro* test to determine the susceptibility of *Staphylococcus* species to specified antimicrobial agents (Product Information Manual: www.biomerieux.com). The AST-P578 card consists of eighteen test anti-staphylococcal antimicrobials inclusive of section well for detection of inducible clindamycin resistance as listed in Table-3. Each test antibiotic is tested across a set range of pre-defined doubling dilution concentrations. The VITEK®2 automation is equipped with a second-generation Advanced Expert Software (AES). The AES interprets AST results at specified MIC levels ensuring biological and therapeutic accuracy.

Antibiotic	**Code**	**Concentration Calling Range (mg/L)**		**Antibiotic**	**Code**	**Concentration Calling Range (mg/L)**	
		≤	≥			≤	≥
Benzylpenicillin	PEN	0.03	0.5	Teicoplanin	TEI	0.5	32
Oxacillin	FLU	0.25	4	Vancomycin	VAN	0.5	16
Gentamicin	GEN	0.5	16	Tetracycline	TET	1	16
Kanamycin	KEN	4	64	Tigecycline	TIG	0.12	2
Tobramycin	TOB	1	16	Fusidic Acid	FUS	0.5	32
Ciprofloxacin	CIP	0.5	4	Mupirocin	MUP	2	512
Erythromycin	ERY	0.25	8	Chloramphenicol	CHL	2	16
Clindamycin	CLI	0.25	4	Rifampicin	RIF	0.5	4
Linezolid	LIZ	0.5	8	Trimethoprim	TRI	0.5	16

Table 1-3: Gram-Positive AST-P578 card well contents for the susceptibility testing of *Staphylococcal* species (Adapted from VITEK®2 GP-AST product package information). Susceptibility is defined as MIC (mg/L) of ≤ falling within the concentration calling-range of MIC ≥ defined as nonsusceptibility.

Analysis and susceptibility and non-susceptibility categorisation of each antibiotic are determined in accordance with the MIC interpretative guidelines based on the Global European interpretative guidelines (CLSI, 2005/2006; EUCAST 2010/2011). The use of quantitative MIC tests, therefore, enables a targeted approach to implementation of therapeutic regimens regarding dose and dosing frequency thus improving clinical efficacy.

Studies assessing the performance of VITEK®2 system for staphylococci AST demonstrate that the system provides accurate susceptibility test results with a sensitivity/specificity of 90/96%, in agreement with reference test methods (Ligozzi *et al*., 2002; Nonhoff *et al*., 2005). The VITEK®2 test assays have however, proved to be unreliable for the detection of resistant subpopulations of cells present in an otherwise fully susceptible strain population at frequencies ranging from 10^{-6} to 10^{-7} CFU/mL. These resistant subpopulations can miss detection due to sampling errors, as such resistance detection requires use of the inoculum above the standard threshold density of 5×10^5 CFU/mL (Wootton *et al*., 2001).

1.8.2 Standard E-test antimicrobial test method

Due to the pitfalls of conventional automated AST test methodologies for the determination of reduced susceptibilities to vancomycin in particular, non-automated MIC determination by agar or broth dilution and E-test (Epsilometer-test) are an alternative test methodology for resistance detection. E-test methodology has proved have similar sensitivity but higher specificity in comparison to automated microdilution based methodologies (Tenover *et al*., 2001).

The principle of gradient test method utilising E-test antimicrobial MIC test strips is based on combining both dilution and diffusion procedures. The E-tests are a quantitative assay for the determination of both MICs and detection of antimicrobial resistance. These test strips are patented porous plastic or paper strips impregnated with a predefined 15 two-fold dilutions concentration gradient of antibiotic. Application of E-tests on pre-inoculated susceptibility agar surface results in diffusion of antibiotic agents in a continuous and exponential concentration gradient range from one end of the strip to the other. The MIC value for a specified antimicrobial-microorganism combination are therefore, determined at the point of interaction of the strip and the growth inhibition ellipse after incubation (ETEST® bioMérieux, 2012). The predefined antibiotic gradient represents an accurate and acceptable strategy for routine susceptibility testing over an extended concentration gradient such that extreme ranges of both resistance and susceptibility are covered. These gradient based applications have been shown to correlate well with those determined by qualitative broth dilution methods (Balouiri *et al*., 2016). The standardised approach of *in-vitro* susceptibility E-test susceptibility testing allows for quantification of antimicrobial efficacy and

hence, evaluation of clinically relevant results, an essential requirement for guidance of effective therapeutic selection.

The E-test technique can also be used to determine the antagonist or synergistic effect of antimicrobial agents used in combination (White *et al*., 1996). This methodology utilises E-test strips impregnated with the two test antibiotics placed in a cross formation at angle of 90° at the interaction between the scales at the respective MICs of the test microorganism. Using this combined E-test assay, synergy is detected by a decrease of the MIC of the combined antimicrobial agents by a factor of two dilutions in comparison to the antimicrobial agents tested alone. Synergy or antagonism for the two antimicrobial compound combinations are defined by calculating the fractional inhibitory concentration index (FICI) and results categorised according dependent on calculated values (Doern, 2014; Balouiri *et al*., 2016). Therefore, synergy test techniques demonstrating antimicrobial interactions allow for the use of pharmacodynamic indices to select effective agents and dosing regimens for targeted therapy of serious infections (Bollenbach, 2015).

The recent trends in epidemiology and antibiotic resistance observed in CoNS-associated bacteraemia in patients with life-threatening malignancies therefore necessitates appropriate antimicrobial interactions for accurate predication of efficacy. Hence targeted combination therapeutics for the management of the critically ill has not only been shown to significantly increase the likelihood of clinical success but also a decrease in emergence of resistance to the agents used for therapy (Bollenbach, 2015; Yan *et al*., 2018).

1.8.2.1 Detection of heteroresistant Vancomycin-Intermediate resistance

Screening for CoNS isolates with reduced susceptibility to the glycopeptides is becoming increasingly important in patients who do not respond to appropriate glycopeptide therapy. Vancomycin resistance amongst staphylococci appears not to be homogeneous characteristic, hence making the detection of heterogenous populations within susceptible and resistant cells difficult, using standard quantitative techniques. Low-level glycopeptide resistance detection requires longer incubation times as well as enhanced growth media to allow expression of the resistance determinants and visible growth (Wong *et al*., 1999; van Hal *et al*., 2011).

Subsequent to the first description of heteroresistance in clinical isolates of MRSA by Hiramatsu and colleagues (1997) in Japan, testing methodologies for the detection of heteroresistant vancomycin-intermediate strains have primarily focused on *S. aureus.* The

best accurate and cost-effective screening methods for the detection of heteroresistance in *S. aureus* isolates however, still remain unclear. Similarly, there are at present no clearly defined phenotypic screening and confirmatory test methodologies for the detection of reduced or heteroresistant susceptibility to the glycopeptides in CoNS isolates. Nonetheless, using a combination of previously described test methodologies, has shown an increase in overall accuracy of resistance detection (van Hal *et al*., 2011).

Methodologies in different parts of the world to detect intermediate or heteroresistance differ in defining breakpoints and interpretative criteria. Most of the work has been carried out on emerging isolates of *S. aureus* with reduced susceptibility to vancomycin (Walsh *et al*., 2001). The recommended simple screening method for detection of heteroresistance staphylococcal is the macro-method with E-tests which allows detection of specific resistant subpopulation phenotypes after 48hour incubation (van Hal *et al*., 2011). These subpopulations within susceptible staphylococcal isolate groups achieve optimum growth in the presence of vancomycin concentrations ≥4mg/L after prolonged incubation. Currently however, there are few systemic data available that provide evidence regarding clinical outcome in patients with such heteroresistance staphylococcal infections. There may be other factors that lead to therapeutic failures which may not be linked to bacterial response to the glycopeptides. These may include factors such as the inability to achieve sufficient serum levels of the antimicrobial to have an effective response *in-vivo*. As the definitive type of testing algorithm for use of accurate detection of hVIS has yet to be clearly established, similar test strategies used for resistance detection in *S. aureus* have been used to determine the prevalence of reduced susceptibility to vancomycin in CoNS isolates (Ahlstrand *et al*., 2011).

Simple and accurate phenotypic methods have yet to be established for the detection of glycopeptide heteroresistance as a standard in clinical laboratories. The modified population analysis profile method using the area under the concentration-time curve (PAP-AUC) is considered the most reliable methodology for detection of heteroresistance. The greater resolution of this method allows for detection of highly resistant subpopulations that are present in low frequencies. The PAP-AUC remains the gold-standard method, however it is expensive, time consuming and labour-intensive hence limiting its use to specialised referral test laboratories (Wootton *et al*., 2001). In comparison to the PAP-AUC method, the use of a combination of easy to perform test assays set up in parallel with routine susceptibility test methodologies have been shown to increase accuracy of detecting heteroresistance. These test assays include the Glycopeptide Resistance Detection (GRD) E-test, the macro-method E-test together with the use of standard methodology for comparison purposes, have been shown to increase specificity and sensitivity for detection of heteroresistant strains (Yusof *et*

al., 2008; van Hal *et al*., 2011). The GRD E-test, consisting of a double-sided predefined gradient of vancomycin and teicoplanin has been developed specifically to simplify detection of GIS and hGIS resistance phenotypes. However, these prototype GRD E-tests are at present used for research purposes only and not validated for clinical use. Therefore, optimal routine laboratory-directed approach to screen for heteroresistance still remain to be determined in cases where elevated vancomycin MICs are observed. Such observations are clinically important in patients who remain non-responsive to standard glycopeptide therapy or those at risk of developing resistance due prolonged antimicrobial therapy.

The recent trends in epidemiology and antibiotic resistance observed in CoNS-associated bacteraemia in patients with life-threatening malignancies therefore necessitates appropriate antimicrobial interactions for accurate predication of efficacy. Targeted combination therapeutics for the management of the critically ill has also been shown to significantly increase the likelihood of clinical success and decrease emergence of resistance to the agents used for therapy (Bollenbach, 2015; Yan *et al*., 2018).

1.9 Haematology and Bone Marrow Transplant unit

At the time of this study the Queen Elizabeth Hospital (QEH) part of the University Hospitals Birmingham NHS Foundation Trust, was a 1200-bed tertiary care hospital located in the West Midland region of the UK. All hospital services are now allocated in new larger block of buildings next to the former hospital building. This adult specialist referral and teaching hospital provides a wide range of general and other specialist services including trauma, neurosurgery, cardiac, liver, renal, burns and plastics. The hospital is the regional centre for cancer including haematological malignancies, bone marrow transplants and has the largest solid organ transplantation programme in England. This specialist healthcare facility was documented to have the highest concentration of immunocompromised patients and the highest ratio of intensive care unit to general beds in England (CDR, 2006). The in-patient population demographic and factors such as the high frequency use of invasive techniques, have contributed to greater levels of Gram-positive associated bacteraemia seen in this and other hospitals (Coello *et al.,* 2001). A prerequisite for infection in these high-risk patient populations is CoNS colonisation acquired either from environmental sources including healthcare staff or from endogenous translocation.

Noting the rising levels of bacteraemia frequently reported in high-risk patient groups with underlying co-morbidities, a clinical surveillance laboratory audit of all positive blood cultures was undertaken by the microbiology clinician (Dr S Larkin, Specialist Microbiology Registrar);

to determine the infective agents most frequently isolated from these patients. Audit results revealed high rates of CoNS-associated bacteraemia in all specialist units of the hospital. Overall results indicated that there was an incidence rate of 252 isolates from 206 blood cultures analysed from 173 bacteraemic patient episodes in the hospital. Of these, the highest rate was attributed to the isolation of 106 non-speciated, CoNS in comparison to 36 strains of other staphylococcal species. Similar to other healthcare facilities, therapeutic options remain limited for the treatment of CoNS-associated infections. This is predominantly attributed to factors such as the inherent ability of these CoNS isolates to express resistance to multiple antimicrobials, further complicating the management of the immunocompromised patient group. Hence, in compliance with the hospital antimicrobial policies, vancomycin currently remains the drug of choice for the treatment of staphylococcal-associated bacteraemia. The audit report also evidenced that the greatest rates of CoNS-associated bacteraemia linked with the extensive use of vancomycin therapy were in patients in the haematology and BMT unit followed by those in the renal unit. The clinical significance of episodes of CoNS-associated bacteraemia in hospitalised patients has yet to be ascertained. In the haematology patient group however, CoNS-associated bacteraemia is documented to be a risk factor linked with significant life-threatening consequences (Chen *et al*., 2010; Marin *et al*., 2014).

1.9.1 Characteristics of QEH haematology patients

The haematology patient population is comprised of patients receiving chemotherapy for a range of haematological malignancies and those receiving autologous stem cell therapy or primarily allogenic bone marrow transplantation. Detailed description of patient care on the haematology unit and all information regarding diagnosis and treatment were kindly provided by Dr Sandeep Nagra (Clinical Haematology- Senior BMT Fellow).

At the time of the study the haematology and BMT wards were located in the former QEH building. The haematology and BMT unit comprised of two separate ward units linked by a common corridor divided by push plated double doors. The ward area maps kindly provided by the microbiology infection control team, illustrate the areas accessed by all medical professionals, nursing staff and affiliated healthcare staff common to both ward areas (Figures 1-2 and 1-3).

The general haematology (Ward West 5), comprises of 16 bed unit consisting of a series of four bed cubicles linked to the BMT ward area via a single corridor separated by a set of double doors (Figure1-2). Nursing care in this specialist unit was administrated within the constraints of local infection control policies governing high-risk patient groups. Likewise, the BMT ward area (Figure1-3) comprising special environmentally controlled single bed

isolation rooms were reserved for patients undergoing stem cell transplantation. The six single isolation specialist rooms provide a protective environment reducing the risk of infection at the stage when patients are at their most vulnerable after transplantation. The in-built high efficiency particulate filtration system and positive air pressure application ensures removal of endogenous and exogenous contaminates. Compliance with the control of infection cleaning procedures in this area further minimises risk of infection in the critically ill, immunocompromised patients.

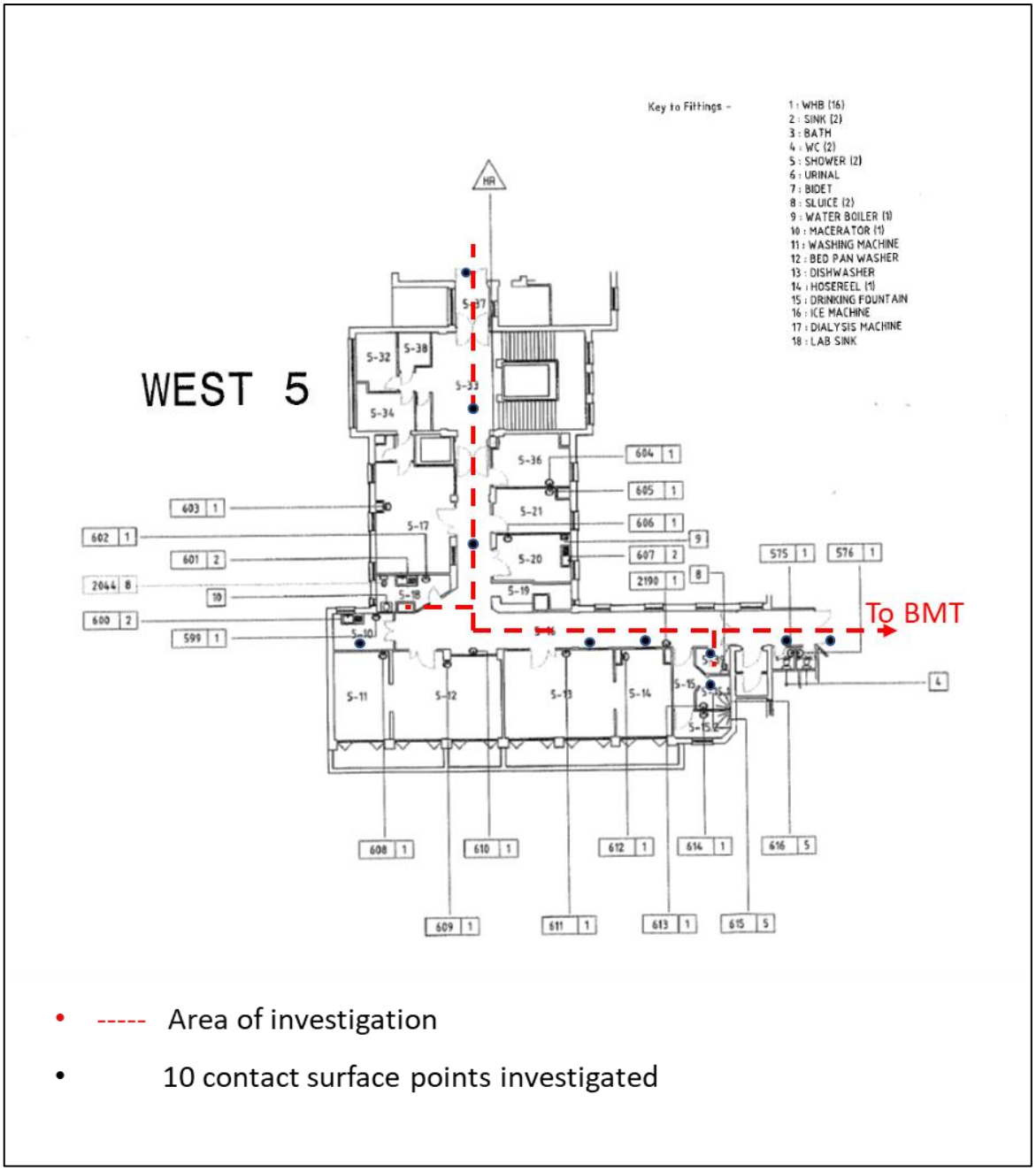


Figure 1-2: Haematology ward area map (West 5: Queen Elizabeth Hospital). This area comprises of four bed unit rooms. Arrow marks the access corridor to the BMT ward area. The 10 zones of high contact/touch points used by staff investigated for bacterial presence and load were along the area marked in red. Investigation points include the nurses workstation, portable computer system, treatment room, waste disposal room, door handles, scanner unit, drugs trolley, bed-side tables and double-door handles and push-plates.

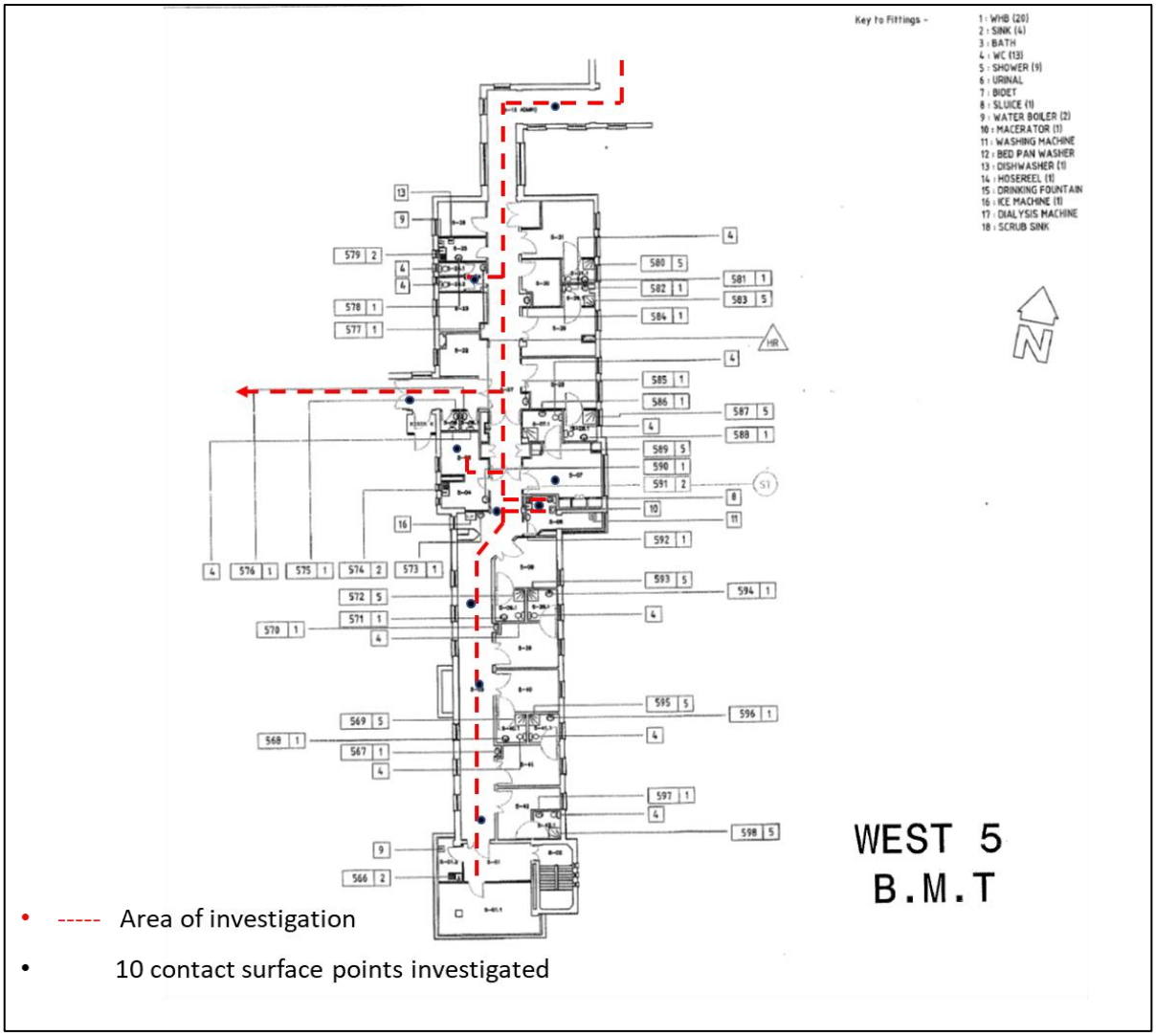


Figure 1-3: The BMT ward area map (West 5: Queen Elizabeth Hospital). This area comprises of single isolation rooms under positive pressure providing a protective environment for BMT patients. Arrow marks the access corridor to the main general haematology ward area. The 10 zones of high contact/touch points used by staff investigated for bacterial presence and load were along the area marked in red. The 10 surface areas of high-use include the clinical workstation front and rear surfaces, portable computer system located in the main corridor, treatment room work surface, macerator room door handles and door plate, side-room door handle, bed-side table and double-door handles and exit door push-plates.

The adult haematology patient population treated at the hospital, ranges from age 55 to a maximum of 60 years. Advanced technique utilisation, better supportive care and improved matching of donors for allogenic transplants resulting in lowering of transplant-associated toxicity has allowed inclusion of patients up to age 75 years. The autologous transplant population has increased from 30 to more than 100 patients over time: comprising of patients with various malignancies including those presenting with acute myeloid leukaemia, acute lymphatic leukaemia, lymphomas, myelomas, haemophiliacs and pathological conditions involving other bone marrow or immunodeficiency syndromes. Previous transplant related

mortality was about 30%, that is 1 in 3 patients died because of infections or graft-versus-host diseases. The mortality rate has currently improved, dropping to around 10% due to better clinical management regimens.

For pre-planned admission of allogenic stem cell transplantation, all patients have tunnelled central lines implanted including Hickman catheters for long term vascular access chemotherapeutic administration and stem cell infusion. Chemo and radiotherapy conditioning therapy in the first seven days of admission followed by transplantation, leaves neutropenic patients extremely vulnerable to BSIs. Early predication of bacteraemia and its associated source in neutropenic febrile patients is of importance for administration of appropriate empirical therapy to reduce risk of morbidity. Diagnosis of bacteraemia includes, fever, defined as a single oral or tympanic temperature reading of ≥38°C with neutropenia as an absolute neutrophil count of $<0.5 \times 10^9$/L expected to fall further during the course of disease. Immunocompromised patients are therefore, classified as those receiving either chemotherapy six weeks post-admission, receiving high dose steroids, post-allograft or those having received extensive field radiotherapy over the final six weeks of post-admission. Therefore, following these parameters, haematology patients at the QEH undergo microbiological investigations with prompt administration of empirical broad-spectrum antimicrobial therapy in line with the national recommended clinical guidelines for the treatment of suspected infection in febrile neutropenic patients

Initial microbiological investigations in onco-haematology patients suspected of bacteraemia, include two sets of blood cultures, one taken via peripheral venepuncture and another via Hickman line together with other appropriate specimens such as central line exit swabs. In the high-risk neutropenic patient, empirical therapeutics are initiated immediately after the first episode of fever of unknown origin. Algorithms for empirical treatment for haematology patients, with consideration given to the presence of localised signs and symptoms include meropenem given as intravenous injection three times a day with the addition of vancomycin to provide Gram-positive and Gram-negative cover. Vancomycin is administered intravenously by intermittent infusion with the aim of achieving pre-dose serum levels of between 15-20mg/L. In agreement and conforming to national recommended guidelines for clinical practice, patients with staphylococcal BSIs treated with vancomycin, have serum levels monitored for efficacy and prevention of toxicity. Although CoNS isolates commonly recovered in cultures are thought to be representative of contaminants (pseudo-bacteraemia) or true BSI; broad-spectrum antimicrobial empiric therapy is always initiated prior to microbiological diagnosis due to the haematology patients underlying clinical co-morbidities, Patients are re-evaluated on receiving microbiological diagnosis and vancomycin therapy continued if signs of fever associated with staphylococcal bacteraemia persist after 72 hours.

Although patients with aggressive malignancies and those undergoing transplantation show a higher response rate with pre-emptive addition of glycopeptide agents, cyclosporin treatment is also initiated for those displaying signs of drug induced nephrotoxicity. Line removal is also indicated in cases of persistent or proven bacteraemia with CoNS, *S. aureus* or other Gram-negative/positive organisms. However, with positive cell engraftment in place, haematology and BMT patients are discharged on recovery from the complexities of associated infections with Hickman lines in-situ for follow-on therapeutics.

1.10 Pilot study

As described previously, the general practice guidelines includes prescribing vancomycin to treat bacteraemia in combination, with a carbapenem, usually meropenem as initial antimicrobial therapy in patients displaying signs of fever in the haematology and BMT unit. It was therefore postulated that antibiotic pressure resulting in resistance selection may be due to prolonged use of broad-spectrum antibiotics, particularly of vancomycin on the unit leading to therapeutic failures. Although, *in-vitro* susceptibility tests do not predict clinical outcomes, several studies, primarily focusing on invasive *S. aureus* infections have observed higher vancomycin MICs within the susceptible range associated with treatment failures (Steinkraus *et al*., 2007; Rybak *et al*., 2009; Edwards *et al*., 2011). With prolonged use of vancomycin in this high-risk patient group there was growing concern that vancomycin may also have diminished activity predominantly against MDR-CoNS isolates. Therefore, detection of small changes in vancomycin MICs over time could be of clinical importance as these could signify development of resistance and hence explain clinical failures.

A retrospective microbiological and clinical pilot study was therefore conducted to review the incidence of CoNS-associated bacteraemia in patients in the haematology and BMT unit. A laboratory review of all CoNS-associated positive blood cultures obtained from these hospitalised patients with haematological malignancies was carried out over a nine month period. The correlating microbiology laboratory antimicrobial susceptibility data was accessed via the laboratory electronic information system. The reported susceptibility ranges of CoNS blood isolates were recorded to establish baseline characteristics of antimicrobial resistance to the glycopeptides, vancomycin and teicoplanin. For comparative purposes, over the same time period, a blind clinical review of vancomycin serum levels of all in-patients in the haematology unit, diagnosed with CoNS bacteraemia treated with vancomycin was undertaken by the clinical microbiologist (Consultant Microbiologist, Dr Deborah Mortiboy). The purpose of the pilot study was to explore the link between vancomycin reported susceptibility results and the therapeutic dose level achieved, as

assessed through vancomycin serum concentration levels in relation to CoNS bacteraemia. The predictive efficacy of vancomycin could therefore be extrapolated from correlating the laboratory susceptibility data with the results of the clinical review in association with expected clinical outcomes.

During the study period, AST results of all CoNS isolates from blood cultures obtained from the haematology and BMT patients, judged to be the causative agents of infection were reviewed using the laboratory electronic information system. Results revealed 71 blood cultures positive for CoNS in bacteraemic patients in the haematology unit (Ward 5) over a nine month period from July 2009 to April 2010. Similar retrospective analysis of reported vancomycin and teicoplanin MICs was carried out on non-speciated CoNS isolates, according to standard routine microbiology protocol over the same period. The susceptibility results were classified in accordance with the rule base of the VITEK®2 integrated software application system based on the rationale breakpoint of 2mg/L, the upper limit for reporting vancomycin susceptibility was applied. This application was in accordance with protocol based on VITEK breakpoints defined for susceptibility interpretation for all staphylococcal species (EUCAST 2010; version 2.1), as exact breakpoints for result interpretation for non-speciated CoNS isolates are yet to be defined. These VITEK®2 breakpoint rationale are for all staphylococcal isolates and remain the current defined category for inclusion of *S. lugdunensis,* the most virulent of the CoNS species for treatment purposes and reporting to the clinicians (EUCAST 2010; BSAC May 2010 and 2014). This application of a susceptible breakpoint of 2mg/L for vancomycin is consistent with a previous study detailing the *in-vitro* activity of antimicrobials against CoNS blood isolates from BMT recipients' (Kratzer *et al*., 2007).

The initial pilot study laboratory data analysis results of vancomycin MICs of bacteraemic patient CoNS isolates (*n* = 71) isolates revealed resistance rate of 1%. However, borderline vancomycin susceptibility of 2mg/L, defined to be at the upper end of susceptibility range was observed in almost half (47%) of the CoNS isolates. On the other hand, resistance to teicoplanin was observed in 13 (18%) of the isolates with borderline teicoplanin susceptibility (MIC = 4 mg/L) observed in 11 (16%) of the CoNS isolates. The following observations were therefore drawn from the laboratory data analysis of reported glycopeptide susceptibilities:

1. Almost half of the reported CoNS isolates in this bacteraemic patient group displayed vancomycin MIC of 2mg/L which are clinically, considered to be at the upper end of the susceptibility range.
2. A significantly higher level of resistance was observed to the glycopeptide, teicoplanin in comparison to vancomycin.

Overall, although resistance to vancomycin was not observed in the BSI CoNS isolates analysed, the detection of significant levels of teicoplanin resistance were considered to be of clinical importance. Due to the prolonged use of vancomycin in the haematology unit, the postulated emergence of resistance is highly likely, as observed by the prevalence of elevated MIC levels in almost half of the CoNS isolates analysed. The likelihood of resistance development is further substantiated by the observed frequency of teicoplanin resistance, an agent known to be a good marker for emerging resistance to the glycopeptides (Biavasco *et al*., 2000; Levine, 2008).

In accordance with the standard hospital policies, patients receiving vancomycin therapy have drug serum trough level assays routinely performed to monitor clinical efficacy and minimise toxicity of the antibiotic over the prescribed period. In this high-risk patient group, monitoring vancomycin drug levels also ensures avoidance of other drug related toxic effects. These may include drug induced antagonistic or synergistic effects in combination with other commonly administered chemotherapies such as antifungal and corticosteroid therapies.

In the absence of clinical correlations of serum levels with outcome or drug toxicity, a clinical data audit of vancomycin use and reported serum vancomycin levels for the patients on the haematology and BMT unit was also performed. Electronic medical records and clinical pathology laboratory database were used to access patient demographic, clinical and laboratory analytical information. This clinical audit undertaken by Dr Mortiboy, included a retrospective medical record review of 252 vancomycin serum level reports for patients receiving vancomycin therapy for staphylococcal-associated BSIs, over an equivalent nine month period from January 2010 to September 2010. The results of this clinical data analysis revealed 179(71%) of the reported vancomycin serum levels were below the target value of ≤10mg/L. On the other hand, only 54(21%) of the reported vancomycin serum levels were within the expected target value of between 10-15mg/L.

Comparative analysis of results of the clinical and laboratory audit was then undertaken to ascertain the link between higher vancomycin MICs and the therapeutic dose levels achieved in relation to the CoNS bacteraemia prevalence in the haematology/BMT patient group (Consultant Microbiologist, Dr Ira Das). Correlating the findings from clinical data analysis with the laboratory data of reported vancomycin MICs, the following points were made in conclusion and presented to the clinical microbiology team.

1. Majority of isolates exhibited high MICs to vancomycin.
2. Majority of patients were not achieving therapeutic target levels of the drug.
3. CoNS-associated bacteraemia in the unit are not treated adequately

4. Inadequate vancomycin therapy resulting in prolonged antibiotic use, also led to further unnecessary investigations and in many cases unwarranted removal of lines.
5. Prolonged sub-therapeutic levels of vancomycin also favored emergence of vancomycin resistant organisms such as enterococci that are prevalent on this unit.

On the basis of these conclusions drawn from the pilot study, it was suggested that further investigations were warranted to reliably evaluate the prevalence of antimicrobial resistance amongst CoNS isolates in this high-risk bacteraemic patient group. Currently in accordance with the present antibiotic policies governing the treatment of Gram-positive bacteraemia, vancomycin still remains the drug of choice for the treatment of febrile neutropenic patients in the haematology and BMT wards. Vancomycin serum levels routinely monitored in this patient group in order to avoid toxicity and maintain therapeutic levels indicated sub-optimal achievement. Such factor favour the selection and emergence of resistance which is an important healthcare problem in high-risk patient groups. Therefore, further exploration of the relationship between BSI CoNS species-specific isolates and prevalence of antimicrobial resistance with particular reference to the glycopeptides was also merited. Additionally, further study would necessitate assessment of other factors that could potentially influence selection of multi-resistant isolates in haematology patients with suspected CoNS BSIs associated with elevated vancomycin MICs. Informed decisions could therefore result in selection of appropriate antimicrobial therapeutics for the management of CoNS BSIs thus limiting potential clinical failures for optimum patient outcomes.

1.10.1 Study design

1.10.1.1 Source population characteristics

The study was restricted to patients hospitalised in the haematology and BMT unit with CoNS-associated BSIs. Episodes of CoNS bacteraemia over a two year period, from April 2010 to March 2012 in this patient group were retrospectively identified from archived clinical microbiology laboratory computerised database records. Over this two year period, a total of 100 CoNS isolates recovered from blood cultures of patients in the unit were identified for further investigation.

In principle, this was a laboratory-based retrospective study and was therefore carried out without requirement for and access to any patient clinical data. Hence the results of CoNS-positive blood cultures were interpreted to be probable or possible CoNS-associated bacteraemia based on the patients setting and the criteria applied for the sending blood

cultures for microbiological investigations. In accordance with ethical standards, approval by the ethics committee was therefore not required for this study as all the non-clinical patient microbiological data obtained was anonymised and analysed retrospectively

Assessment of the microbiological data obtained, consisted of analysis of the incidence of CoNS bacteraemia and antimicrobial susceptibilities, focussing particularly on reported susceptibility results of the glycopeptides, vancomycin and teicoplanin. In accordance with standard laboratory protocol, the conserved CoNS isolates were identified via laboratory freezer storage records and a small amount retrieved from each stored sample for further analysis. The study was designed to characterise these CoNS isolates using both phenotypic and genotypic techniques. The primary goal of the study was to examine the relationship between antimicrobial resistances expressed to the commonly used anti-staphylococcal agents, including vancomycin and other virulence factors which may have correlations with CoNS species-specific features. Furthermore, the aim was to explore the prevalence of reduced glycopeptide resistance (heteroresistance) amongst these CoNS isolates, which could be associated with an impaired response to glycopeptide therapy resulting in clinical failure.

1.10.1.2 Control patient population source

CoNS are atypical virulent species forming part of the normal skin microbiota but are increasingly becoming the leading cause of nosocomial infection particularly amongst high-risk patient groups. it is postulated that the source of nosocomial infection, in particular CoNS-associated bacteraemia originates potentially from cutaneous sites of CVCs or from colonised mucosa of hospitalised patients undergoing immunosuppressive therapies (Costa *et al*., 2004).

Due to the retrospective nature of this study, skin-colonising strains could not be obtained from the relevant in-patient population prior to hospitalisation for direct comparisons with bacteraemia strains. As translocation of strains from colonised sites can only be further evidenced or supported by clinical, experimental and molecular relatedness studies; skin-colonising strains deemed valid for this study were therefore obtained from cultures of swabs taken from three areas of patients skin, prior to hospital admission. Therefore, for the purpose of this study, the yield of colonising flora from the nose, groin and throat regions of 100 pre-admission patient swabs was examined prior to routine investigations for MRSA colonisation. The study was therefore directed towards evaluating the potential of predominant skin-colonising strains as the clinically significant putative source of CoNS-

associated bacteraemia in the hospitalised haematology patient group. More importantly these disseminated skin-colonising staphylococcal isolates may have the potential not only to recolonise other body sites but also act as a source of cross-infection among other patients in the hospital environment.

The increase in CoNS-associated bacteraemia in haematology and BMT patients undergoing sub-optimal antimicrobial treatment also carries significant clinical and epidemiologic implications. At present, the widespread use of vancomycin as the therapeutic drug of choice could result in emergence of resistance due to selective pressure which is a matter of great concern as this leads to poor clinical outcome for the patient. Effective screening methods utilised in this study would allow for the detection of CoNS species-specific resistance in this high-risk patient group. Additionally, subtyping of CoNS to the species level is important for verification of clonally related colonising strains that are a potential source involved in translocation, dissemination, and/or cross-transmission events subsequent to acquisition of bacteraemia.

With reports of emerging resistance to the glycopeptides, the results of the study has the potential for provision of information and clinical guidance for a more targeted approach for treating CoNS-associated bacteraemia. The use of alternative newer antibiotics and effective combination regimens together with continuous therapeutic drug monitoring would represent key options for the treatment of these life-threatening opportunistic infections in haematological patients.

1.11 Aims and objectives

The primary aim of the study was to determine the phenotypic characteristics of CoNS isolates recovered from blood cultures of patients in the haematology and BMT unit. The prevalence of species-specific characteristics were explored using both standard and advanced laboratory techniques.

The main objectives of this study were:

- To explore the phenotypic characteristics of the prevalent skin colonising strains of CoNS isolates recovered from the groin area of a group pre-admission patients for comparative purposes.
- To investigate and determine the environmental prevalence of CoNS species in areas most frequently accessed by staff and patients in the haematology and

BMT wards. The aim of this investigation was to establish phenotypic correlation of environmental CoNS isolates with those of the in-patient population through cross-infection.

- To determine genotypic differences or similarities between blood culture isolates of CoNS with those skin colonisers which could be regarded as inducers of bacteraemia in the haematology and BMT in-patient population.

- To compare and investigate the phenotypic antibiotic resistance profiles of both the bacteraemic in-patient CoNS and those skin-colonising isolates obtained from the pre-admission patient using standard laboratory protocols. The aim was to determine the prevalence of reported resistance amongst CoNS isolates to commonly used antimicrobials.

- To investigate and determine the incidence of vancomycin and teicoplanin susceptibility among the well-characterised in-patient CoNS isolates using standard MIC susceptibility test methods.

- To further evaluate the incidence of reduced activity of the glycopeptides, using susceptibility screening methods other than those in routine use. These were utilised for the detection of glycopeptide resistance expressed heterogeneously.

- To determine the activity of several new antimicrobial agents including daptomycin, tigecycline and ceftaroline against the BSI CoNS isolates.

- To investigate the incidence of four commonly expressed virulence factors in correlation with the antibiotic resistant phenotype among both the in-patient and pre-admission group CoNS isolates.

- To assess the activity of vancomycin, gentamicin, daptomycin and rifampicin applied in-vitro individually and in combination against three well characterised S. epidermidis BSI isolates representative of a range of susceptibility patterns. A specified E-test technique was utilised to assess the synergistic or antagonistic effect of these antimicrobial agents.

- To assess the biofilm-associated in-vitro activity of vancomycin daptomycin and rifampicin individually and in combinations against a well characterised S. epidermidis biofilm producing reference strain using the 96-well microtitre plate assay technique.

Chapter 2 Comparative analysis of the prevalence of coagulase negative staphylococcus species-specific strains recovered from blood cultures of patients in the haematology and bone marrow transplant unit with those comprising skin microflora.

2.1 Introduction

CoNS have emerged as significant nosocomial pathogens, most commonly isolated from clinical blood culture specimens, but as part of the normal skin flora are also common blood culture contaminants. Studies have shown that in patient with haematological malignancies and post-BMT, the highest incidence of nosocomial infection especially during the neutropenic phase was mainly due to CoNS species (Denttenkofer *et al*., 2003). In the majority of cases isolation of CoNS is said to represent contamination and not true infection (García *et al*., 2004) and hence is commonly reported generically as CoNS species without further species-specific identification. However, specific-species CoNS, in particular *S. epidermidis* have been implicated in clinically important infections representing the major cause of bacteraemia in the immunocompromised host with implanted or indwelling devices (Pagano *et al*., 1997; Pfaller *et al*., 1999; Sloos *et al*., 2000; Tashiro *et al*., 2015).

CoNS bacteraemia if treated promptly and adequately is rarely life threatening (Pfaller *et al*., 1988; García *et al*., 2004). However, in patients with haematological malignancies there is potentially an increased risk of fatal outcome especially if more virulent strains of CoNS species such as *S. lugdunensis* are involved (Pagano *et al*., 1997: von Eiff *et al*., 2002). Due to the pathogenic potential of these organisms, accurate identification and characterisation of CoNS species has becomes more important clinically. Recognition of their clinical significance in nosocomial infections allows for predication of pathogenesis, determination of clinical significance and therefore intervention with appropriate antimicrobial therapy for optimal outcome.

Hence, diagnosis of CoNS bacteraemia is dependent on accurately identifying species-specific CoNS isolates from blood cultures as a predicator of their clinical significance (Zitella 2003; Worth *et al*., 2009). In the majority of cases, the same strain or clonal population repeatedly isolated from patient blood cultures is indicative of infection (Zitella 2003; Worth *et al*., 2009). An early study carried out by Weinstein and colleagues (1998) showed that of the eleven different species of CoNS blood culture isolates, *S. epidermidis, S. hominis* and *S. haemolyticus* were the three most common species detected, accounting for both 98% of the clinically significant isolates as well as 89% of the contaminants. The other less commonly isolated species included *S. capitis* and *S. lugdunensis*. Other CoNS, including S*. cohnii, S. auricularis, S. simulans, S. caprae S. sciuri and S. warneri* when isolated are usually

considered as blood culture contaminants but in the immunocompromised host maybe clinically significant (Widerström *et al*., 2012; Becker *et al.,* 2014).

An epidemiological study of blood culture isolates of CoNS demonstrating hospital-acquired infection showed species distribution dominated by isolates of *S. epidermidis* (68.7%) and *S. haemolyticus* (14.8%) among adult haematology patients. The other species of CoNS detected were *S. hyicus, S. warneri, S. cohnii,* and *S. saprophyticus* (Burnie *et al*., 1997). The diversity of CoNS species isolated from bacteraemic patients indicates that species level identification may aid clinicians and researchers to associate certain species-specific isolates with a predilection to cause disease.

Traditionally, identification of bacteria from clinical samples has been carried out by examination of clinical morphology and by biochemical characterisation, using substrate and enzymatic tests. (Bannerman *et al*., 1993; Kloos *et al*., 1975,1994; Cunha *et al*., 2004). Commercial rapid automated systems such as VITEK®2 (bioMérieux SA, Marcy-'Etoile, France) are increasingly used in routine clinical laboratories because of their high degree of sensitivity and specificity for bacterial speciation This micro-method is a cost effective system for accurate identification of medically relevant CoNS species (Funke *et al*., 2005). The VITEK®2 systems have an auto-modular format, consisting of a dilution mode, filler-sealer module, a fluorogenic technology based optical reader, incubator and a computer control module. The system utilises uniquely coded small plastic cards consisting of individual wells, containing a variety of reagents and substrates for species identification. Inoculated, sealed cards placed in the reader-incubator are scanned by light emitting diodes and phototransistor detectors, every fifteen minutes readings. The changes in light transmission from after initial baseline readings, detected by the photometer are transferred to the computer module analytical system where organism biotype is determined through data interpretation of the biochemical reaction patterns (Bannerman *et al*.,1993; Product Literature, 2004). The identification of most human *Staphylococcus* species and sub-species with these systems has been shown to be comparable to an accuracy of 70% to 90% with the addition of more discriminatory tests included within their test panels (Logozzi *et al*., 2004).

Diagnostic microbiology in recent times has rapidly evolved towards the use of more streamlined automated identification systems such as, Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). The intrinsic property of the MALDI-TOF MS technology for isolate identification is based on the acquisition of the bioanalyte, generating a unique protein profile of the entire bacterial isolate (Cabonnelle *et al*., 2011; Patel, 2015). Data analysis and comparisons with a library of reference spectra of

these unique generated spectral fingerprints specific to genus, allows species identification to subspecies level (Theel, 2013; Argemi *et al*., 2015). This system effectively replaces conventional biochemical based bacterial identification to species level from 73 to >94% (Cabonnelle *et al*., 2011). Rapid Identification methodologies have been shown to impact directly on improving the quality of patient care and management (Barenfanger *et al*., 1999 Funke *et al*., 2005).

2.2 Aim of this current study

(a) To phenotypically characterise clinical isolates of CoNS recovered from bacteraemic patients on the haematology and BMT unit.

(b) To investigate and characterise the predominant CoNS skin-colonisers recovered from pre-admission patients (control group).

(c) To determine the distribution and prevalence of CoNS species, in the ward environment.

2.3 Materials and Methods

2.3.1 Risk Assessment

The use of all equipment, procedures, handling and disposal of bacterial cultures and other waste was undertaken according to the standard operating procedures documentation inclusive of managing risks registers both at the department of Microbiology at the Queen Elizabeth Hospital Birmingham and at Aston University laboratories.

2.3.2 Media

All pre-poured media used in this study was obtained from bioMérieux (SA, Marcy-'Etoile, France) except where otherwise stated. This includes Columbia Blood Agar (CBA) base with the addition of 5% w/v nicotinamide adenine dinucleotide (*NAD*) supplemented with 5% defibrinated horse blood and DNase Agar.

2.3.3 Microbial cultures

A total of 100 blood culture positive CoNS isolates from in-patient haematology/BMT group and a 100 predominant CoNS skin-colonising isolates from pre-admission patients (control group) were investigated together with the geographical distribution of CoNS isolates from hospital unit comprising, both the haematology and BMT ward area.

All in-patient and pre-admission (control) patient group isolates were made anonymous and serially coded with a designated differential letter and number.

2.3.3.1 In-patient BSI CoNS isolates

One hundred CoNS isolates were obtained from consecutive bacteraemic episodes from patients in the haematology and BMT unit from 11th April 2010 to 15th March 2012. (Designated isolate identifier code A1-A100).

Isolates were obtained from blood cultures processed by the BacT/ALERT® (bioMérieux, France) automated blood culture system. These CoNS isolates were identified in accordance with the recommended Public Health England (PHE), formally the Health Protection Agency (HPA) UK standards for microbiology investigations.

CoNS isolated from positive blood culture bottles were cultured on Columbia Blood Agar (CBA). Cultures were incubated in atmospheres of both 5% carbon dioxide and anaerobically at 37°C for 18 hours for further investigation.

Primary identification of CoNS was based on colony morphology, Gram staining (ProLab-Diagnostics. UK) and the absence of coagulase activity screen (PASTOREX™ Staph-Plus BioRad. UK) and confirmed with DNase Agar and tube-coagulase test (Plasma source – QEH Blood Bank; plus, double strength tryptone water (Oxoid, UK). The following control strains (LENTICULE® - PHE/ bioMérieux-UK.) were included in each test.

Positive latex and coagulase test - Oxford *Staphylococcus aureus* NCTC 6571

Negative latex and coagulase test – *Staphylococcus epidermidis* ATCC 12228

2.3.3.2 Storage of isolates

All CoNS Isolates for further investigation were saved and stored at -20°C using Microbank™ beads in cryopreservative solution (ProLab-Diagnostics.UK).

2.3.3.3 CoNS Isolate cultures for testing

A single bead removed from Microbank™ vial stored at -20°C was sub-cultured onto a blood agar plate and incubated at 37°C for 18 hours in ambient atmosphere. After recovery two subsequent subcultures of the isolates were carried out to stabilise the phenotype prior to further testing.

2.3.3.4 Establishing the purity of CoNS blood culture isolates

Different isolates of CoNS can appear morphologically similar on culture. Therefore, a preliminary evaluation was carried out to establish whether the BSI culture isolates were homogeneously pure and not representative of multiple CoNS species types.

From the time of first positivity 10 consecutive sets (aerobic and anaerobic), of blood cultures from 10 different patients were tested. These were randomly selected, sequentially from the one hundred bacteraemia episodes investigated during the first part of the study period. CoNS isolates investigated were selected from primary CBA plates inoculated with samples from positive blood culture bottles incubated in an atmosphere of 5% CO_2 at 37˚C for 18 hours.

Five colonies were randomly chosen from an inoculated CBA plates and each colony was then sub-cultured separately onto fresh blood agar. Of the 100 colonies analysed, all five selected from each bacteraemic episode were subjected to analyses for purity and phenotypic similarity by biochemical profiling and antimicrobial susceptibility testing.

2.3.3.5 Selection of control group CoNS isolates from pre-admission patients

Three screening swabs (Charcoal amies, Bibby Sterlin, Stone, UK) samples are routinely taken from the nose, throat and groin areas and analysed for MRSA carriage prior to hospital admission; in accordance with hospital and government implemented policy (HPA, 2010). Prior to analysis for MRSA,100 of these pre-admission patient screen samples were randomly selected for analysis of predominant skin-colonising isolates. The nose, throat and groin swabs from each patient were then cultured to evaluate the sample area yielding the highest growth and distribution of normal skin-colonising CoNS. Each sample site swab was cultured separately on CBA and incubated at 37˚C for 18 hours in ambient atmosphere. The test procedure for the detection and identification of predominant CoNS isolates was determined as previously described.

The groin area of the 100 pre-admission patients sampled was identified as the area with the highest density of diverse CoNS species. This area was therefore selected as representative of predominant patient skin-colonising isolates prior to hospital ward admission (Designated isolate identifier code G1-G100).

2.3.3.6 Environmental sampling of haematology patient wards to determine CoNS species distribution.

Geographically as shown previously in Figure1-2 the haematology ward unit consisting of four bed cubicles has access to the BMT ward area (Figure 1-3) via a common corridor through a set of restricted-use double doors (Chapter 1.9.1). Infection control procedures in place, including the use and disposal of personal protective equipment present at all key points of the ward areas ensure minimum transmission of potential pathogens. Ten areas within each ward unit most frequently accessed by staff members common to both ward areas were identified and sampled for investigation along the red line marking the area of investigation (Figures 1-2 and 1-3). These 'high-touch/contact' points near patients, included the nurses' stations, portable patient information computer system (PICS), treatment room where drug preparation was carried out, macerator room for waste disposable, door handles, staff hand scanner clocking-in system (SMART), drugs trolley, patient bed side tables, double-door handles and push plates access link to the other ward area.

Standard environmental HPA standard operating procedures (HPA: Environmental sampling 2010; Lemmen *et al*., 2001) were followed when undertaking the sampling of the ward equipment and specified areas. Sampling was carried out using a sterile swab (Charcoal Amies, Bibby Sterlin, Stone; U.K.) moistened with 0.5% w/v sterile saline solution. The sample swabs were cultured on CBA, streaking out well across the whole of the plate to enable colony counts of bacterial population. Culture plates were incubated at 37˚C for 18 hours in an atmosphere of 5% CO_2. Total colony counts were carried with preliminary identification of isolates based on colony morphology. Identification of CoNS isolates were carried out using previously described methodology.

2.4 CoNS isolate preparation for VITEK®2 automated Identification

Automated biochemical profiling of the CoNS isolates was undertaken using the GP-ID (Gram Positive-Identification) test card in accordance with the manufacture's recommended procedure. The inoculum using the VITEK®2 DensiCHEK™ meter (bioMérieux, Basingstoke, UK) was prepared from a fresh 18 hour culture. Using a sterile swab, a few well isolated colonies were selected to prepare a homogenous suspension in 3ml sterile saline (aqueous 0.45% NaCl, pH 7 bioMérieux, France) to a density equivalent to McFarland 0.5 standard. GP-ID card inoculated with the suspension was loaded onto the VITEK®2 automated systems for incubation and analysis, following the manufacturer's instructions (User Manual-VITEK®2 systems product information 410791). Results interpretation was carried out in accordance with the manufacturers installed VITEK®2 software version 25; 5.04

The recommended control strain *S. aureus* ATCC 29213 was included in each test batch for quality control and assurance.

2.4.1 Identification of CoNS isolates using VITEK-MS MALDI-TOF system

MALDI-TOF MS target slides were inoculated by picking up a freshly grown overnight colony of CoNS on CBA. Deposits of each colony were made in two replicates on the 48 spot target slide and 1µL of the matrix-MALDI-CHCA (α-cyano-4-hydroxycinnamic acid dissolved in 50% v/v acetonitrile and 2.5% v/v trifluroacetic acid) was used to overlay the spots. Test spots were then allowed to air dry prior to acquisition, whereby desorption and ionisation of microbial analytes through the energy of the laser occurs resulting in mass spectra production.

E. coli ATCC 8739 calibration control strain was used on each control spot section of the MALDI-TOF MS target slides as recommended by the manufacturer.

The acquisition and detection of protein mass spectra detection based on mass-to-charge ratio was performed on the VITEK®-MS instrument version 2.0 with a mass-to-charge ratio (*m/z*) of 2 to 15 KDa. For results the automated data analysis of raw spectra were matched with the spectra library in the VITEK®-MS software application version 1.1.0 which computes identification of staphylococci isolates, in accordance with applications system knowledge data base (Clinical User Manual; Ref. 161150-137).

2.5 Statistical methods

Data were analysed using the SPSS statistical software for Windows. Descriptive statistics were computed for all variables in this study. Difference testing between the CoNS isolates obtained from the two patient groups, was performed using the Chi-squared test. Differences were considered statistically significant at $p < 0.05$.

The individual biochemical results extracted for VITEK®2 were compacted in Microsoft Excel and statistical analysis; Pearsons-Principle component analysis (PCA) with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was performed using the STATISTICA version 10 (USA) Software package.

Asymptomatic Marginal-Homogeneity Test (Stuart-Maxwell Test) was used with R version 3.2.3 (2015-12-10) – "Wooden Christmas-Tree Copyright (C) 2015. The R foundation for Statistical Computing for data analysis of CoNS species identification using the two test platforms. (Data analysis assistance courtesy of Dr Martyn Wilkinson, Biomedical Scientist Statistician: Hospital Infection and Research Department QEHB. UK)

2.6 Results

2.6.1 VITEK®2 automated phenotypic Identification based on biochemical profiling of CoNS isolates

2.6.1.1 Evaluation of culture purity

The preliminary evaluation carried out to establish whether the bacteraemic culture isolates were mono-microbial and not representative of multiple strain types, showed that 99.6% of the strains were similar. A low-discriminatory power categorisation by the automated system requiring further manual tests for species differentiation was observed in <1% of the isolates tested. Overall result analysis of isolates tested from selected bacteraemic episodes showed an absence of multiple species-specific isolates, indicating that the probability of polymicrobial bacteraemic episodes was highly unlikely.

2.6.1.2 Speciation and distribution of CoNS isolates

A total of 100 haematology patient blood culture isolates of CoNS, collected from 11th April 2010 to 15th March 2012 were investigated together with a 100 CoNS skin isolates from pre-admission (control) patient group.

Automated biochemical analysis and distribution of the CoNS species isolates from the two patient groups are summarised in Table 2-1. One out of the 100 isolates of CoNS recovered from the in-patient group included in this study, was non-identifiable by VITEK®2 system. This in-patient CoNS isolate A23, was sent to the reference laboratory for identification and was confirmed to genus level as *Staphylococcus* species by PCR 16S RNA analysis, proven to be biochemically non-reactive.

CoNS Species	**Number of Species isolated from Haematology/BMT Patient Group**	**Number of Species isolated from Control Patient Group**
S. epidermidis	57%	32%
S. hominis	18%	24%
S. haemolyticus	16%	17%
S. capitis	3%	11%
S. warneri	1%	7%
S. lugdunesis	0%	5%
S. auricularis	1%	1%
S. simulans	0%	3%
Kocuria/ Dermococcus Species	3%	0%
Non-Reactive CoNS	1%	0%

Table 2-1: Comparative distribution of CoNS species-specific isolates from the two patient groups, bio-analysed for identification using VITEK®2 system GP-ID card.

In total there were eleven different CoNS species-specific isolates identified from both patient groups. *S. epidermidis* (57%) was the most frequently recovered species from the in-patient group followed by *S. hominis* (18%) and *S. haemolyticus* (16%). Similarly, in the control patient group the predominant CoNS species isolated was *S. epidermidis* (32%) followed by *S. hominis* and *S. haemolyticus,* 24% and 17% respectively.

Among the 26% of the other minor group of CoNS species-specific isolates from the control patient group, the predominantly species was *S. capitis* (11%) followed by *S. warneri* (7%), *S. lugdunesis (*5%) *S. simulans* (3%) and *S. auricularis* (1%). The range of the other CoNS species isolated from the in-patient group was however much smaller, differing considerably in comparison to the control group isolates. *S. capitis* (3%) was still the predominant species isolated followed by *Kocuria* species (2%) and 1% each of *S. warneri, S. auricularis and Dermococcus* and the non-reactive *Staphylococcus* isolate. The comparative species distribution of the CoNS is illustrated graphically in Figure 2-1.

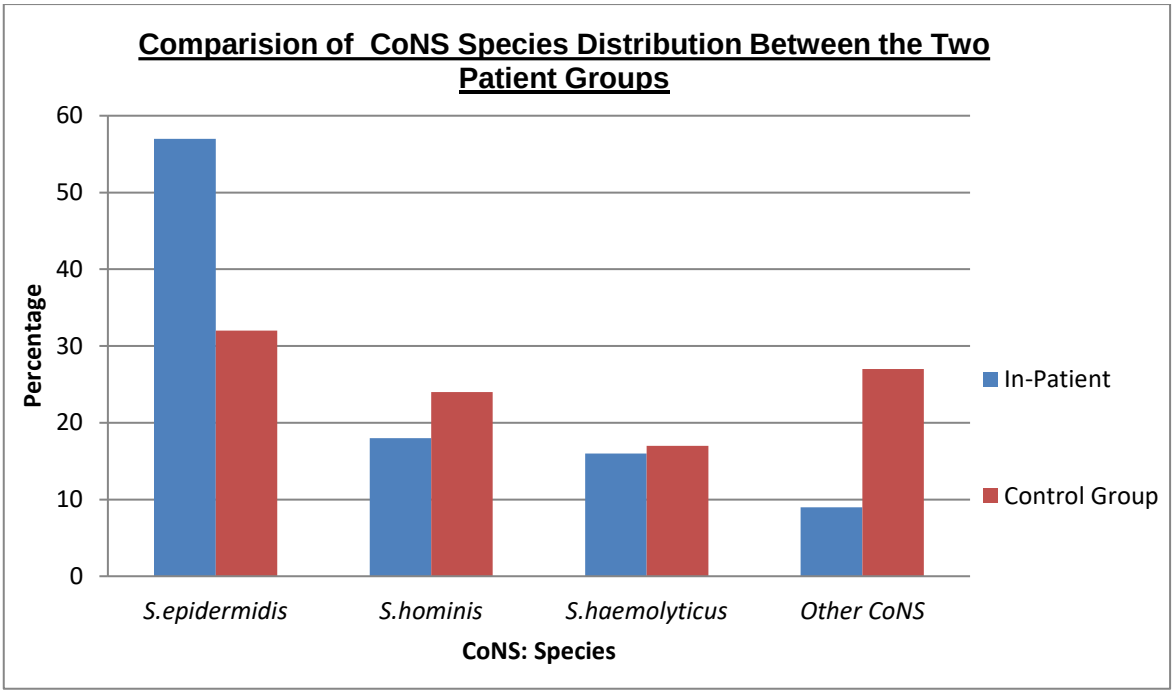


Figure 2-1: Comparison of phenotypically characterised CoNS species distribution, from bacteraemic patients in the Haematology and BMT with the pre-admission (control) patient group isolates. The other CoNS isolates from both patient groups included the following species: *S. capitis, S. warneri, S. lugdunensis, S. simulans, S. auricularis, Kocuria, Dermococcus* and non-reactive *Staphylococcus* species.

Figure 2-1 and the corresponding tabulated results for each CoNS species reveals that the in-patient group had a higher frequency (91%) of the three predominant CoNS species isolates in comparison to 73% isolated from the control patient group.

2.6.1.3 Chi-square statistical analysis: Difference in distribution of CoNS isolates categorised as the other CoNS species from the two patient groups

The Chi-square 2 by 2 tests was used to determine the significance of observed difference between the distribution and range of the CoNS isolates categorised as the other CoNS species from the two patient groups. Test analysis showed that there was a significant difference ($p < 0.0001$ with 3*df*) between the distribution of the other CoNS species isolates from the in-patient group in comparison to those of the control group. Analysis, therefore, reveals that there is significant association between the frequency and distribution of the minor CoNS species population and the patient group from which the isolates are derived.

Data analysis further revealed that there was no significant difference ($p > 0.05$) between the distribution frequency of *S. epidermidis S. hominis* and *S. haemolyticus* isolates from the in-patient and the control patient groups. Therefore, the distribution of the three predominant CoNS species was not associated to the site of isolation.

2.6.2 Data Analysis using Pearsons-Principle Component Analysis (PCA) with Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

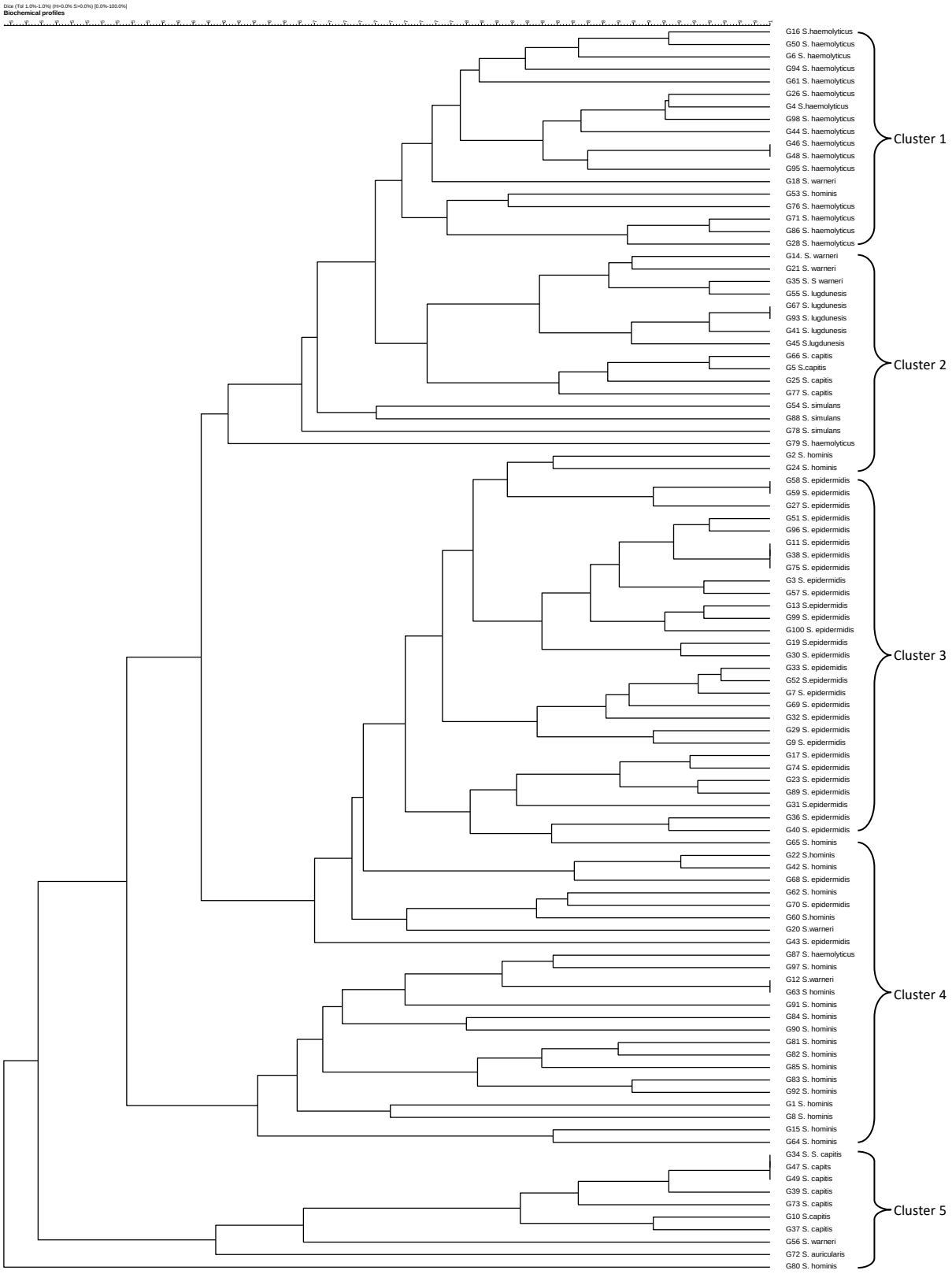
The 100 CoNS isolates from each of the patient groups were further subjected to PCA with UPGMA analysis using the STATISTICA v.10 software. Analysis was performed based on similarity or differences of CoNS strain biotype profiles and dendrograms created by means of UPGMA clustering of a similarity matrix band on band matching (Caddick *et al*., 2006).

Figures 2-2 and 2-3 detail dendrograms constructed of the 100 CoNS isolates from in-patient and control patient groups. The scale bar at the top of the dendrogram represents the percentage similarity, where the cut-off value was set at 95%. The UPGMA dendrogram analyses of biotype profiles of these CoNS species demonstrate a relatively high degree of strain un-relatedness between the isolates from each of the patient groups. In both groups the numbers of clusters identified also demonstrate the high degree of un-relatedness within species-specific CoNS isolates as well.

Isolates of *S. haemolyticus* in the in-patient group demonstrated the greatest degree of similarity as detailed in Figure 2-2, followed by isolates of *S. epidermidis* cluster number 3 and 1 respectively. Conversely in the control patient group, this degree of similarity was only apparent in isolates of *S. epidermidis* Figure 2-3 cluster number 3. The other clusters in the control patient group in comparison to the in-patient group included a higher number of isolates from different species of CoNS together with a much lower degree of similarity within species-specific isolates.

Figure 2-2: Phenotypic relatedness of 100 in-patient group CoNS: Dendrogram based on Vitek®2 biochemical analysis using Dice coefficient UPGMA method.

Figure 2-3: Phenotypic relatedness of 100 control patient group CoNS: Dendrogram based on Vitek®2 biochemical analysis using Dice coefficient UPGMA method.



2.6.2.1 Interpretation of PCA data analysis

PCA with UPGMA identified CoNS strain relatedness and clustering within the groups of CoNS based on biochemical profiling representing the degree of variance within the CoNS strains.

Although a high degree of relatedness was observed between CoNS species-specific isolates from the in-patient group, significant differences in the biochemical profiles were observed among individual species isolates. The dissimilarity between species-specific CoNS isolates was however even more evident in the control patient group whereby fewer and smaller clusters were identified compared to the in-patient CoNS.

The biochemical profiling dendrograms for both patient groups show a high degree of diverse clonal types within each species-specific CoNS group as summarised in Table 2-2.

Dendrogram Analysis	**In-patient Group**	**Control Group**
Number of different biochemical profiles within all CoNS species isolates	67 (n=100)	92 (n=100)
Number of different biochemical profiles within the predominant CoNS species.		
S. epidermidis (n=number of isolates)	51 (n=57)	27 (n=32)
S. haemolyticus (n=number of isolates)	7 (n= 16)	12 (n=17)
S. hominis (n=number of isolates)	18 (n=18)	15 (n=24)

Table 2-2: Summary of PCA dendrogram analysis comparison within the two patient groups together with differences within CoNS species groups and isolate relatedness.

CoNS species relatedness based on biochemical profiles determined by means of UPGMA dendrogram analysis (Figures 2-2 and 2-3) demonstrate a high degree of diversity amongst CoNS species in both patient groups. This was more evident in the control group of patients, as there were 92 different biotype profiles out of the 100 CoNS isolates analysed, whereas of the 100 CoNS isolates, of the in-patient group overall, 33% of the isolates showed similarity out of the 67 different biotype profiles identified.

For the in-patient group 100% phenotypic similarity was observed in 32% of the 100 CoNS profiles analysed. In the in-patient group, small clusters of *S. epidermidis* isolates were observed, of which the largest consisted of 6 and 8 isolates (Figure 2-2 cluster 1) and smallest consisted of a minimum of two isolates demonstrate 100% phenotypic similarity. Conversely, only a few isolates of *S. haemolyticus* showed 100% similarity. The diversity within CoNS species-specific relatedness was observed to be even higher in the control patient group with only 14% of the isolates showing 100% phenotypic similarity within the different CoNS species isolates. Of the 27 isolates comprising the other minor group of

CoNS species in the control patient group, only two isolates each of *S. capitis, and S. lugdunensis,* demonstrated 100% relatedness. Conversely in the in-patient group, all of the nine other CoNS species isolates demonstrated a poor degree of similarity, grouping together at <30% species-specific relatedness as illustrated (Figure 2-2, cluster 5).

2.7 VITEK-MS MALDI-TOF identification of CoNS isolates based on proteomic analysis

CoNS species identification analysis was performed by applying the recommended criteria of the VITEK® MS software calculated identification scores based on quantitative confidence values of between 60 to 99.9%, which are then displayed for significant organism group identification. As designated in the MS database, scores of ≥ 95 and 99.9% were considered as high-confidence, genus level identification. In this study all the *Staphylococcus* species tested, scored between ≥ 98.9% and 99.9%, indicating a high probability of staphylococcal isolate identity to the species level. The one isolate that was not identified by VITEK-MS MALDI-TOF, as was the case with biochemical profiling was in-patient CoNS isolate A23. As previously confirmed by the reference laboratory this isolate identified as a non-reactive *Staphylococcus* isolate was included with the minor group of CoNS for overall analysis.

All except 6% of the one hundred in-patients CoNS isolates analysed by MS MALDI-TOF presented the same CoNS species-specific identification when compared to those results obtained by biochemical profiling. The differences in species identification using the two systems are detailed in table 2-3.

	In-patient Group: Difference in CoNS Identification	
Isolate	**VITEK®2**	**VITEK-MS MALDI-TOF**
A30	*S. hominis*	*S. epidermidis*
A94	*S. hominis*	*S. epidermidis*
A98	*S. hominis*	*S. epidermidis*
A44	*Kocuria varians*	*S. auricularis*
A64	*Kocuria rosea*	*S. capitis*
A40	*S. auricularis*	*S. epidermidis*

Table 2-3: Difference in CoNS species identification with the use of VITEK®2 and the VITEK-MS MALDI-TOF systems.

Biochemically inert isolates such as *Koucria* species were identified to the CoNS sub-species level with MS MALDI-TOF system. The two isolates *Kocuria varians* and *Kocuria rosea* with proteomic analysis identified as *S. auricularis* and *S. capitis* respectively. Therefore, these

isolates were included in the other group of staphylococci for all further analysis, with the retention of their biochemically based species identification . On the other hand, using the MS MALDI-TOF system, three isolates of the *S. hominis* and one of *S. auricularis* all identified as *S. epidermidis* with confidence value scores of between 95 and 98%. These results for the in-patients group indicate that the probability of CoNS species-specific identification obtained to the sub-species level is comparatively higher with the MS methodology. Conversely, for the pre-admission patient group isolates difference in species identification was observed in 22% of isolates with concordant identification observed in 88% of the 100 CoNS analysed as detailed in Table 2-4.

	Control Patient Group: Difference in CoNS Identification	
Isolate	**VITEK®2**	**VITEK-MS MALDI-TOF**
G2	*S. hominis*	*S. epidermidis*
G22	*S. hominis*	*S. epidermidis*
G24	*S. hominis*	*S. epidermidis*
G42	*S. hominis*	*S. epidermidis*
G60	*S. hominis*	*S. epidermidis*
G80	*S. hominis*	*S. epidermidis*
G92	*S. hominis*	*S. epidermidis*
G35	*S. warneri*	*S. haemolyticus*
G12	*S. warneri*	*S. hominis*
G18	*S. warneri*	*S. haemolyticus*
G20	*S. warneri*	*S. haemolyticus*
G21	*S. warneri*	*S. epidermidis*
G56	*S. warneri*	*S. capitis*
G57	*S. epidermidis*	*S. capitis*
G58	*S. epidermidis*	*S. capitis*
G59	*S. epidermidis*	*S. capitis*
G27	*S. epidermidis*	*S. capitis*
G70	*S. epidermidis*	*S. hominis*
G46	*S. haemolyticus*	*S. capitis*
G41	*S. lugdunensis*	*S. epidermidis*
G55	*S. lugdunensis*	*S. epidermidis*
G93	*S. lugdunensis*	*S. haemolyticus*

Table 2-4: Control patient group identification differences of CoNS species distribution with the use VITEK®2 in comparison to the VITEK-MS MALDI-TOF identification system.

In the control patient group, the largest difference in species identification using the VITEK-MS^{MS} MALDI-TOF system was observed with isolates of *S. hominis* (29%) identifying as *S. epidermidis.* However, five isolates of *S. epidermidis* identified differently with MS MALDI-TOF of which four were identified as *S. capitis* and one as *S. hominis*. This resulted in an overall, 5% increased difference in *S. epidermidis* species distribution. Nonetheless, the error difference was minimal as these isolates remain the predominant species isolated in this patient group. Of the other CoNS species, six and four isolates of *S. warneri* and *S. lugdunensis* respectively were identified as one of the three predominant CoNS species-specific isolates.

2.7.1 Comparison of CoNS species distribution using two different identification methodologies

The difference in CoNS speciation using the biochemical profiling methodology VITEK®2 and proteomics based VITEK-MS MALDI-TOF in the two patient groups is illustrated in Figure 2-4 below.

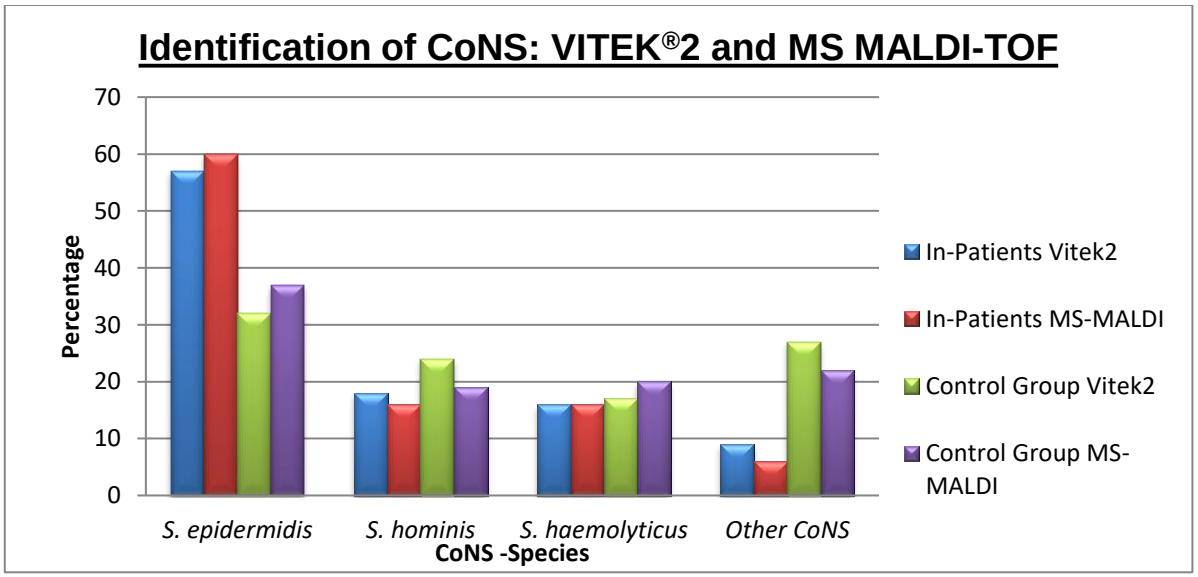


Figure 2-4: Comparison of CoNS species identification and distribution in both in-patient and control patient groups using the two automated systems for CoNS speciation. The minority group of other CoNS species group include isolates of *S. capatis, S. warneri, S. lugdunensis, Kocuria* species, *S. auricularis, Dermacoccus* species and the non-reactive *Staphylococcus* species.

Phenotypic characterisation of CoNS isolates using the conventional biochemical profiling methodology comparison with MS MALDI-TOF for both in-patient and control group showed similar differences in speciation of the predominant CoNS isolates. The exception was

observed with speciation of *S. haemolyticus* in-patient isolates, showing 100% identification result concordance. It would appear from the initial result comparisons that morphologically similar but biochemically inert isolates such as the *Kocuria* species had a more definitive staphylococcal group speciation using MS MALDI-TOF system where, analysis is based specifically on structural/ribosomal proteins of the intact bacterial colony. These differences were more apparent in the control patient group isolates, where 22% of the CoNS isolates differed in species identification with MS MALDI-TOF analysis. In both patient groups, results show that isolates of *S. hominis* largely identified as *S. epidermidis* with MS MALDI-TOF. The speciation difference observed was greater in analysis of the other minor CoNS group isolates of the control patient group. *S. warneri* and *S. lugdunensis* CoNS control group isolates mainly identified as the predominant CoNS species types, hence showing a 5% and 3% difference in identification as *S. epidermidis* and *S. haemolyticus* respectively.

Overall initial identification result analysis for the in-patient group show no difference in the predominant species isolated using the two different identification methodologies. Conversely for the control patient group isolates, using the two different identification methodologies the predominance of the second most isolated CoNS species differed significantly. Although *S. epidermidis* remained the predominant species isolated, some isolates of *S. hominis* in the control patient group were speciated as *S. haemolyticus* by MS MALDI-TOF. Further statistical analysis on both patient group isolates was carried out to verify the significance of CoNS species-specific result differences obtained using the two different identification systems.

2.7.2 Statistical Analysis: CoNS species Identification using the VITEK®2 and VITEK-MS MALDI-TOF

The main objective of this analysis was to determine the presence of significant difference in CoNS species identification with the use of the two different automated technologies. The null hypothesis proposed was that there is no significant difference in speciation dependent on the identification system used and the alternative hypothesis would be that there would be a significant difference. A significant difference would be determined according to the critical p value of <0.05.

The first analysis performed on the species identification data about using the two identification platforms based on different principles for species identification was the Stuart-Maxwell test. This was carried out using, The R Foundation for Statistical Computing version 3.2.3 (10-12-2015). The Asymptotic Marginal-Homogeneity Test was applied to the data

where the outcome variable was CoNS species identification having more than two categories. The two categories for the explanatory variable were the two automated identification systems, VITEK®2 and VITEK-MS MALDI-TOF.

Results of the first analysis carried out on both in-patient and control group of patients together and data obtained by both identification systems stratified by block where, Chi-squared value obtained was = 23.614, degrees of freedom = 8 and the p-value = 0.00266. Since the p-value obtained is less than the significance value of <0.05 suggesting that, statistically there was a significant difference in the performance of the two automated systems used for the identification of CoNS isolates.

Further analysis carried out using the Asymptotic Marginal-Homogeneity Test:

For the in-patient group:

Data analysis, identification by two systems stratified by block where Chi-squared value = 6, degrees of freedom = 4 and the p= 0.1991. This p-value exceeds the accepted significance value of ≤0.05 indicating that there was statistically no difference in identification of CoNS species with the use of either the VITEK®2 or VITEK-MS MALDI-TOF system, therefore the null hypothesis was accepted.

For the control patient group:

Data analysis, identification by two systems stratified by block where Chi-squared value = 18.174, p = 0.005813 with 6 degrees of freedom, this suggests that there was statistically significant difference in the performance of the VITEK®2 and the VITEK-MS MALDI-TOF system used for the identification of the CoNS species isolated from the control patient group. The null hypothesis would therefore be rejected in this case and the alternative hypothesis accepted.

CoNS species identification data was subjected to further statistical analysis, to double check whether there was a difference in response with the use of two different identification systems. In this case a probability of mismatch between the two identification systems for the two patient groups. Using the computing software, a binomial (logistic) regression model was constructed, where the outcome variable (probability of mismatch) was related to the explanatory variable namely the patient group. The final odds ratio: exp(ß) ~4.44 indicates a high likelihood of a significant difference in species identification dependent on which patient group the CoNS were isolated from. This compares well with the species identification data, where for in-patient group 6 out of 98 CoNS isolates mismatched whereas for the control patient group 22 out of 98 isolates were mismatched. The salient p-value of 0.00216

resulting from the statistical tests based upon the linear logistic regression model, suggests that there is a significant difference in species identification performance with the use of the VITEK®2 and the VITEK-MS MALDI-TOF systems which is also significantly dependent on the patient group from which the CoNS isolates are derived.

2.8 CoNS species distribution in the haematology and BMT wards

Result analysis of the bacteria load from each in-patient ward area sampled using previously described identification protocols are shown in Table 2-6. The overall bacterial load including the number of CoNS species isolated in the individual bed space area of the BMT ward were much lower than those isolated from the haematology ward area. Consistency in type and numbers of CoNS species or other identifiable bacterial species was not observed over the two test periods.

Most areas of the BMT ward demonstrated absence of bacterial growth or occurrence of low bacterial counts. Relatively higher bacterial loads were observed on the BMT macerator handle where healthcare staff disposed of patient waste in comparison to the low bacterial load on the macerator handle in the haematology ward area. Conversely the four bed shared ward units of the haematology ward areas had higher bacterial loads, including differences in types of CoNS species isolates. Some patient areas had a higher bacterial load per cm^2 of normal skin organisms including total number of CoNS species. The bacterial load appeared to be transient as the numbers and types of CoNS species and other isolates showed little consistency over the two time periods of testing. This was observed in areas of high access use by all healthcare workers such as the computer key board area of the PICs system in both ward areas. Healthcare workers did not wear gloves as this was not a requirement when accessing this equipment as was the case when “clocking-in/out” using the SMART hand reader equipment located outside the entrance to the haematology ward area. Common bacterial isolates associated with disease such as *S. aureus* were not identified in this environmental study, during the sampling period carried out, both in March and May 2011.

	Environmental Screening of Haematology and BMT Ward Areas					
			March 2011		May 2011	
Contact	**Site**	**Area**	**Number of Colony Forming Units /Standard Area**		**Number of Colony Forming units/Standard Area**	
Test			***Staphylococcus* Species**	**Other Isolates**	***Staphylococcus* Species**	**Other Isolates**
Points	***Haematology Ward***					
1	Bed 16 - Side table	10cm/10cm	No growth		3-*S. haemolyticus,* 1-*S. cohnii,* 1-*S. epidermidis*	8-Cols, Mcoc, NHS
2	Drug Trolley - Blue	10cm/10cm	1-*S. haemolyticus* , 1-*S. hominis*	4 -Mcoc, Baci	2-*S. haemolyticus* ,1-*S. cohnii,* 1-*S. hominis*	
3	Macerator handle	10cm/10cm	No growth			16-Coryne, Mcoc, Cols
4	Nurse Station - Left side	10cm/10cm		1-Baci, 1-Dermococcus	2-*S. epidermidis,* 1-*S.auricularis,* 1-*S. cohnii*	8-Mcoc, Coryne
5	Nurse Station - Right side	10cm/10cm		1-Mcoc	1-*S. epidermidis*	1-NHS
6	Patient information computer system	Keyboard area	10-*S .haemolyticus,* 11-*S. epidermidis*	29-Baci, Mcoc, Cols	16-*S.epidermidis* , 12-*S. haemolyticus*	48-Mcoc, AHS
7	Drug Trolley - Red (near Bed 3/8)	10cm/10cm	9-*S. haemolyticus*	31-Mcoc, Cols	1-*S. warneri,* 1-*S. hominis*	
8	Treatment Room - Work surface	10cm/10cm	No growth		1-*S. warneri*	2-Mcoc
9	SMART Clock-In Unit	10cm/10cm	9-*S. epidermidis,* 9-*S. auricularis*	36-Cols, NHS	17-*S. haemolyticus* , 9-*S. cohnii*	22-Mcoc, Coryne
10	Rt. Side double door to BMT Push-plate	20cm/10cm	1-*S. capitis,* 2-*S. epidermidis*		1-*S. hominis*	
	BMT					
1	Corridor - Computer	Keyboard area	22-*S. epidermidis*	24-Mcoc, Cols, Baci	11-*S. caprae* , 4-*S. capitis,* 11-*S. haemolyticus*	>100-Mcoc, Coryne
2	Work station surface - Front	10cm/10cm		8-AHS, Baci	No growth	
3	Work station surface - Indoor area rear end	10cm/10cm	No growth		No growth	
4	Treatment Room - Work surface	10cm/10cm	3-*S. hominis*		1-*S.hominis*	
5	Macerator handle	10cm/10cm	2-*S. hominis*	24-Mcoc, Cols	7-S. *haemolyticus*	>100-AHS, Coryne, Mcoc, NHS
6	Bed 5 - Side table	10cm/10cm		2-Coryne	No growth	
7	Door plate - Macerator room	20cm/10cm		1-Mcoc	No growth	
8	Double door plate (next to Bed 4)	20cm/10cm	1-*S. warneri*	1-Mcoc		2-Mcoc
9	Door handle room - Bed 4	Whole area	No growth		No growth	
10	Push plate- Exit door to North	20cm/10cm	No growth			1-Mcoc

Table 2-5: Distribution and number of CoNS species-specific isolates and other bacterial species isolated from the same ward area- 10 points of contact, screened over two times periods, March and May 2011. The other bacterial isolates included: Non-Haemolytic Streptococci (NHS), Alpha Haemolytic Streptococci (AHS), *Micrococcus* (Mcoc), Bacillus (Baci), *Corynebacterium* (Coryne) and Coliforms (Cols).

Overall data analysis of bacterial distribution in March 2011, areas of frequent use or equipment handling by staff such as PICS – Computer system keyboard areas had the highest percentage of CoNS species isolates compared to other areas on both the haematology and the BMT ward areas. From the colony counts, it was observed that the overall number of other skin organisms such as *Micrococcus* species, *Corynebacterium* species, streptococci and other bacterial isolates such as *Bacillus* species and coliform organisms was also high.

Although all health workers commonly accessed both ward areas, the haematology ward area had the highest distribution of different CoNS species-specific isolates including a number of other bacterial organisms. The prevalence of these isolates was noted to be proportionally higher around the computer system keyboard area. The PICS keyboard area in the haematology ward had almost similar distribution of CoNS species, 42% and 58% in March and May 2011, respectively. *S. epidermidis* 22% in March and 21% in May 2011 was the predominant CoNS species in this area followed by *S. haemolyticus* 20 and 15% respectively.

The distribution of CoNS species isolated from the computer keyboard area differed over the two time periods screened in the BMT ward area. In March 2011 *S. epidermidis* (48%) was the only CoNS species isolated of the total bacterial load. In May 2011 however, <20% of the total bacterial load were CoNS species of which *S. caprae* and *S. haemolyticus* (<9%) the predominate species isolated followed by *S. capitis* (<3%).

Figure 2-5 illustrates the overall distribution frequency of the eight main CoNS species isolated from the two ward areas sampled over the two different time periods

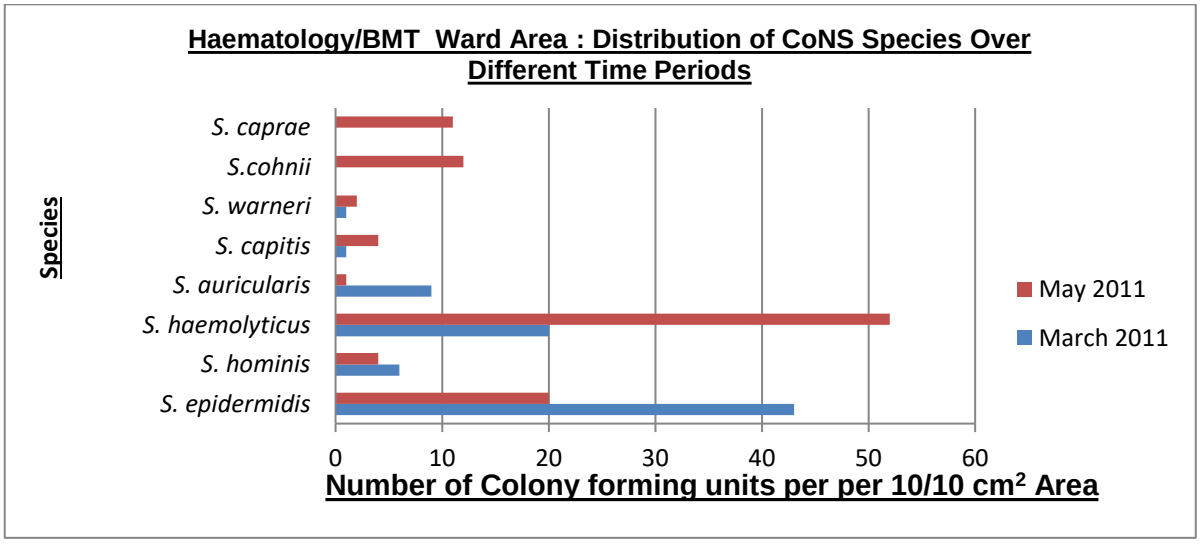


Figure 2-5: Environmental screening distribution of the CoNS species and bacterial load in specified ward areas over the two time period slots of March and May 2011.

The screening of the two geographically linked ward environmental areas showed that the distribution frequency of the different CoNS species-specific isolates varied considerably both between the March and May 2011.

There were 25% more CoNS species types identified in the same areas in May compared to test sampling in March 2011. Considerable variation was also noted in the numbers of colony forming units, per cm^2 of the CoNS species isolated in the different areas sampled over the two time periods. Of the CoNS species, *S. caprae* and *S. cohnii* were the two CoNS species present in May but not in March and *S. caprae* and *S. warneri* were the two species found only in the BMT area. During March, greater than 40/CFU of *S. epidermidis* per 10x10cm^2 area sampled were isolated in comparison to around 20/CFU sampled in May making this the predominant of the CoNS species isolated. Conversely *S. haemolyticus* was the predominant CoNS species isolated in May compared to March 2011 whereby this species was the preceded by *S. epidermidis* as the predominant CoNS species. All CoNS species isolates identified in this environmental study were species that form part of the normal microbiota of human skin.

2.8.1 Comparison of environmental CoNS species relatedness isolated from the two ward areas in March and May 2011

Biochemical profile analytic results using the VITEK®2 displayed as Bionumbers of the eight CoNS species isolated from environmental sampling are detailed in Table 2-6. As the species identification is based on the specificity of the Bionumbers, the results show how well matched these profile results are and hence how close the species clonal relatedness.

Concordance was observed in two isolates of *S. cohnii* recovered from haematology area 1 and 2, from bed 16 side table and the drugs trolley tested in May 2011. The other colonies of *S. cohnii* isolated were not closely related. A relatively high degree of phenotypic relatedness was observed among some isolates of *S. epidermidis, S. haemolyticus* and *S. hominis* based on the closely matching Bionumbers derived from biochemical profile analysis (Table 2-6). Results show a high degree of phenotypic clonal relatedness between some *S. epidermidis* isolated from haematology ward area 6 in March with those isolated in haematology ward area 1 in May 2011.

Ward	Area	Period	CoNS species	Bionumber
Haematology	1	May-11	*S. epidermidis*	000000074620211
Haematology	6	Mar-11	*S. epidermidis*	000000074220211
Haematology	9	Mar-11	*S. epidermidis*	000000074020211
Haematology	10	Mar-11	*S. epidermidis*	020000076221211
BMT	11	Mar-11	*S. epidermidis*	020000074221211
Haematology	4	May-11	*S. epidermidis*	010400010621211
Haematology	5	May-11	*S. epidermidis*	010000032421211
Haematology	6	May-11	*S. epidermidis*	000000074621011
Haematology	2	Mar-11	*S. hominis*	040000010220021
Haematology	2	May-11	*S. hominis*	040000012222021
Haematology	7	May-11	*S. hominis*	040000014260021
Haematology	10	May-11	*S. hominis*	000000016330011
BMT	14	Mar-11	*S. hominis*	010000014220021
BMT	14	May-11	*S. hominis*	040000054320111
BMT	15	Mar-11	*S. hominis*	010002006360231
Haematology	1	May-11	*S. haemolyticus*	010002042620231
Haematology	2	Mar-11	*S. haemolyticus*	010002142660231
Haematology	2	May-11	*S. haemolyticus*	010002442660231
Haematology	6	Mar-11	*S. haemolyticus*	030002044660231
Haematology	6	May-11	*S. haemolyticus*	050002107362271
Haematology	6	May-11	*S. haemolyticus*	070002147362231
Haematology	9	May-11	*S. haemolyticus*	010002002760211
BMT	11	May-11	*S. haemolyticus*	010002002320231
BMT	15	May-11	*S. haemolyticus*	010002040660231
Haematology	9	Mar-11	*S. auricularis*	010000000000201
Haematology	4	May-11	*S. auricularis*	000002000020201
Haematology	10	Mar-11	*S. capitis*	010000000061201
BMT	11	May-11	*S. capitis*	010000002461201
BMT	18	Mar-11	*S. warneri*	050002003262231
Haematology	7	May-11	*S. warneri*	010002002220231
Haematology	8	May-11	*S. warneri*	010000012060231
Haematology	1	May-11	*S. cohnii*	010000002060221
Haematology	2	May-11	*S. cohnii*	010000002060221
Haematology	4	May-11	*S. cohnii*	000000000070221
Haematology	9	May-11	*S. cohnii*	010400002070221
BMT	11	May-11	*S. caprae*	010002012461221

Table 2-6: Environmental Screening in March and May 2011. Distribution of the predominant CoNS species in accordance with the bio-analysed profiles (Bionumber).

Although 75% of the total environmental CoNS species isolates were from the haematology ward area, phenotypic relatedness appeared limited. Isolates of *S. hominis* and *S. haemolyticus* recovered from different areas of the haematology ward over the two time period slots show that they were closely related similar clonal isolates. The difference in the Bionumber obtained through biochemical reaction for identification of each species isolates indicates that these although closely related clonally are different phenotypes. This degree of similarity was not observed in the BMT ward area CoNS species-specific isolates.

Further comparison of biochemical profile Bionumbers with the 100 CoNS isolates each, from the in-patient and control group was carried out to determine phenotypic relatedness. Results of this comparison showed that none of the environmental CoNS species isolates Bionumbers had a 100% match with those of the similar CoNS species-specific isolates from the two patient groups. Overall result comparison indicated a relatively high degree of phenotypic un-relatedness within patient groups and the CoNS species-specific isolates from the patient ward areas.

2.9 Discussion

The Queen Elizabeth Hospitals, NHS Trust as a major referral centre, provides a range of highly specialist services including haematology and allogenic BMT. Such specialist service units have the highest concentration of immunocompromised patients and the highest ratio of ITU to general beds (CDR 2006). These and other factors, such as the high frequency of invasive techniques in routine use, contribute to the high levels of staphylococcal associated bacteraemia episodes within the unit. CoNS are now increasingly recognised as aetiological agents of a range of infections in particular, blood sepsis in this critically ill major patient population group in the Trust.

CoNS as causative agents of nosocomial bloodstream infection are now frequently reported but their role as the major cause of morbidity and mortality has yet to be ascertained. Clinical surveillance laboratory report audit (Permission of Microbiology Specialist Registrar, Dr S. Larkin; June 2012) showed that there was an incidence rate of 252 isolates in 206 blood cultures from 173 patient episodes over a 12 month period. Of these, the highest rate was attributed to the isolation of 106 isolates of CoNS compared to 36 attributed to other staphylococci including both *S. aureus* and MRSA.

Currently, speciation of blood culture isolates of CoNS, contributes little to the management of patients on the Haematology and BMT units, except to occasionally distinguish multiple

episodes of bacteraemia caused by CoNS. These episodes may be considered as culture contaminants attributed to different species of CoNS or from sustained bacteraemia with the same CoNS species-specific isolate is when identification is requested for epidemiological surveillance. However, it is now becoming increasingly important to accurately identify the isolates to the species level in order to determine their clinical significance as repeated isolation of the same species-specific isolate strongly supports its role as an aetiologic agent (von Eiff *et al*., 2002; Otto, 2010).

The emergence of CoNS as human pathogens requires rapid reliable identification of species type in order to predict potential pathogenicity and clarify the clinical significance of each species. An overview of recent medical literature (Piette *et al.,* 2009) focusing on the role of CoNS as the etiological agents of bacteraemia especially in the immunocompromised host indicates the necessity of accurate sub-species identification enabling better understanding of the pathogenic potential of various species-specific CoNS.

The development and use of simple specie-specific methodologies for staphylococcal identification are therefore of great importance, as this has the potential for directly impacting on patient management and clinical outcome. Several studies have shown that in the in immunocompromised patient group in particular, that characteristics of distinctive CoNS species-specific isolates manifest in a difference in pathology and hence clinical outcome (Emori *et al.,*1993; Barenfanger *et al.,* 1999; Dickema *et al.,* 2001; Bjorkqvist *et al.,* 2002).

CoNS are not at present speciated routinely and are reported generically based on morphology and simple tests such as the inability to produce coagulase which delineates them from the pathogenic species *S. aureus.* However, one of the objectives of this part of the study was to carry out speciation of CoNS to determine the prevalence of species-specific isolates from bacteraemic haematology and BMT patients and those skin-colonising isolates obtained from the pre-admission patient control group. Additionally, comparative distribution of CoNS isolates indigenous to the ward environment was carried out to demonstrate the prevalence of CoNS species-specific isolates in each group. Speciation and prevalence of CoNS isolated from the two patient groups and the environment further allowed comparisons between identification methodologies and evaluation of phenotypic relatedness within and between each of these groups.

With the use of the automated VITEK®2 identification system, characterisation of CoNS was carried out by exploring the utility of biochemical phenotypes which are usually reflective of stable, established chromosomal isolate characteristics (Spanu *et al.,* 2009). This methodology is reliant on testing for expressed gene products that are in turn limited by the propensity of the organisms to alter the expression of their genes depending on external

factors such as incubation period and temperature. These phenotypes based on biochemical/enzymatic test reactions are considered to be relatively consistent across all isolates of a given bacterial species, usually reflective of stable established chromosomal strain characteristics. Therefore, the use of this test methodology in this study comes close to biotype “fingerprinting” for CoNS species-specific isolate identification.

VITEK®2 automation criteria results are based on >95% positive or >95% negative discriminatory power application for species identification and are displayed as Bionumbers based on the numerical probability calculations of the biopattern. Published evaluations indicate the GPI-card correctly identifies between 70 and >90% of CoNS species (Pfaller and Herwaldt, 1988; Spanu *et al*., 2003). Greater accuracy is further achieved by combining automated results with off-line coagulase test results as recommended by manufacturer (bioMérieux SA, Marcy-'Etoile, France). The VITEK®2 database contains a large number of Bionumbers inclusive of new species variants, which makes it possible to resolve species into subspecies sufficient for CoNS species characterisation.

Identification of the one hundred blood culture isolates of CoNS, collected over two years in comparison to the 100 predominant CoNS isolated from pre-admission patient skin-colonisers using the VITEK®2 system revealed *S. epidermidis* as the most frequently recovered species (57% and 32% respectively). The second most isolated species in both these patient groups was *S. hominis* (18 and 24%) followed by *S. haemolyticus* (16 and 17%). With respect to the total colony counts per environmental area sampled in the haematology and BMT wards, *S. epidermidis* (53%) was again the predominant species isolated. These findings correlate well with previous studies which have shown that *S. epidermidis* is the most frequent of the CoNS species to be associated with nosocomial BSIs, predominantly as biofilm-associated infections of indwelling medical devices (Worthington *et al*., 2000; Wolf *et al*., 2008; Piette *et al*., 2009). However, the frequency of occurrence can be variable, dependent on the geographical region of isolation (Sader *et al*., 2001). As such environment sampling showed that although the frequency of occurrence varied, *S. epidermidis* was still the predominant species found both in patient ward area and skin-colonisers in the control patient group. The predominance of *S. epidermidis* in the in-patient group could therefore be associated with the propensity of the species to be an opportunistic pathogen sourced from the environment or skin microbiota.

S. hominis was the second most common of the species isolated from both patient groups and is known to be the second most abundant of the skin colonisers. However, clinically it is *S. haemolyticus* that has been cited as the second most common cause of BSIs related to implantation and subsequent manipulation of intravascular medical devices (Kristóf *et al*.,

2011). The pathogenic potential of *S. haemolyticus* second only to *S. epidermidis* especially in the immunocompromised host has been reported by other authors (Sader *et al*., 2001; von Eiff *et al*., 2002). Interestingly, although *S. haemolyticus* was the third most common of the species found in both the blood cultures of bacteraemic haematology patients and skin-colonising isolates of the control group; this species was the least common of the CoNS found in the patient ward area environment. The observation made from this study analysis suggests that *S. haemolyticus* has an apparent niche preference, such as regions endogenous to the patient but not to the patient's environment.

The other minor group of CoNS species isolated from the haematology and BMT ward areas included S*. capitis, S warneri, S. auricularis* and *S. cohnii.* These CoNS species are known to be predominantly native to human skin. However, *S. caprae* a rare isolate of human microbiota was recovered from the computer located in BMT ward area 11 corridor sampled in May 2011(Pfaller and Herwaldt, 1988; Kloos *et al*., 1994). This suggests that the presence of this contaminant CoNS species may have been from a source other than from patients or healthcare staff. Result analysis of the bacterial load from each in-patient ward area sampled (Table 2-6) show that the overall load, including the distribution of CoNS species isolated in the individual bed space areas of the BMT ward was much lower than those isolated from the haematology ward area. A low isolation rate of all CoNS species as well as the complete absence of bacterial isolates was noted in most areas of the BMT ward area in comparison to the haematology ward area. This is reflective of the infection prevention policies and procedures in place protecting the vulnerable state of the neutropenic and immunocompromised patients. The BMT ward area comprising of single isolation rooms are disinfected between each use with accelerated hydrogen peroxide vapour, providing a sterile environment, hence the low bacterial load observed. The air filters and positive air flow in this ward unit further ensures a protective environment by reducing contamination, exposure to and risk of infection with opportunistic pathogens thus the control of infection measures set in place support reduced organism transmission (Syndor *et al*., 2001).

Hence the low numbers of bacteria from environmental sampling observed in this study evidences that infection control procedures were well adhered to by staff common to both ward areas. This was further evident from results showing that most areas sampled in the BMT unit had either had no bacteria isolated or were present in relatively low numbers. Comparison of the isolation rate of common CoNS species from both ward areas revealed that in the BMT ward area CoNS isolates were present in only 9(11%) out of the total 40 area points sampled. Higher numbers of CoNS species, indigenous to skin were isolated in areas of greater access, such as PIC system. This may have been possibly due to healthcare staff

not having to wear essential personal protective equipment such as gloves in areas of no direct patient contact.

Relatively higher bacterial loads were however observed on the BMT macerator handle where health workers dispose patient waste in comparison with the macerator handle of haematology ward which had comparatively few bacterial isolates. These comparatively higher contaminant bacterial loads was observed in both the March and May sampling periods. The difference in contamination may possibly have been due perhaps to a different approach for waste disposable in the BMT area in comparison to the haematology ward area. On the other hand, this could be due to inadequate following of infection control procedures in place for this specified ward area.

Conversely the four bed shared ward units of the haematology ward areas had a higher bacterial load, including the number of CoNS species-specific isolates. Some patient areas had a higher bacterial load per cm^2 of normal skin flora including CoNS species similar to the BMT ward. Likewise, high access sampled areas also revealed greater bacterial loads. The higher bacterial loads observed in the haematology ward area are probably due to the absence in this area of positive air flow system and no air filters, hence contamination is more likely even though control of infection protocols are still applicable in this area. Nonetheless no common pathogenic bacteria isolates such as *S. aureus* were identified in this environmental study.

Although bacterial loads were variable dependent on sample area, the distribution of species-specific isolates was also inconsistent with time of sampling. The numbers and prevalence of species-specific CoNS isolated in May were greater than those isolated in March 2011. *S. epidermidis* was the predominant CoNS species isolated in March compared to dominance of *S. haemolyticus* isolation in May 2011. Comparatively, *S. epidermidis* and *S. hominis* were the most prevalent of the CoNS species isolated in this environmental study. This correlates well with the predominance of *S. epidermidis* in both the in-patient and control patient group isolates investigated. Although the level of predominance of all CoNS species varied dependent on the method of identification used, the level of variation was statistically insignificant ($p > 0.05$). However, when comparing species identification analysis individually for the control patient group, there was a significant difference dependent on the identification system used ($p < 0.05$). This strongly suggests that speciation was dependent on the type of identification system used for control patient group isolates.

In both patient groups, it was apparent that speciation was more robust with MS MALDI-TOF than with VITEK®2. The VITEK®2 system based on miniaturised biochemical reactions may have some limitations as identification is dependent on the variable expression of phenotypic

characteristics that are used as diagnostic parameters. Studies have shown that such definitive identification is dependent on growth of the organism *in-vitro* to ascertain reactivity of the organism with specific biochemical tests (Spanu *et al*., 2003; Layer *et al*., 2006). This methodology is therefore limited by the propensity of the organisms to alter gene expression which is dependent on factors affecting growth such as environmental temperature and incubation period of 18-24 hours or more. These factors affect the biochemical colorimetric or fluorometric changes analysis by VITEK®2, consequently resulting in subjective interpretation hence leading to ambiguity in species identification. Several drawbacks with this methodology also include technical factors which are primarily time dependent such as include time-dependent test set up and correct inoculum size, which can lead to subjective interpretation, rendering the result susceptible to misinterpretation.

Studies by various investigators comparing the performance of VITEK GP-ID card for clinically relevant bacteria in comparison with established molecular reference methods, demonstrate a high degree of agreement with the results obtained in this study (Spanu *et al*., 2003; Ligozzi *et al*., 2002; Layer *et al*., 2006; Funke *et al*., 2005). These studies show an accuracy of between 90 and 93% of CoNS to the species level with the VITEK®2 system. This correlates well with the results obtained in this study particularly for the in-patient CoNS isolates where only 6% of the 100 isolates tested gave a differing identification when compared to results obtained by the proteomic test methodology. Three out of the 18 isolates of *S. hominis* together with one isolate of *S. auricularis* identified as *S. epidermidis,* whereas species of *Kocuria* known to be biochemically inert but morphologically similar to CoNS species were reliably characterised as *S. capitis* and *S. auricularis* based on proteomic analysis. These finding concur with comparative study describing the erroneous reporting by the VITEK®2 system of some CoNS as *Kocuria* species, a group likely to be representative of misidentification (Layer *et al*., 2006). Of the other minor CoNS group of isolates, *S. capitis* was found to be the predominate species in both patient groups and ward environment. *S. capitis*, as a part of the normal skin flora usually resides on the upper half of the human body and has been implicated as the cause of opportunistic infection in the immunocompromised host (Kloos *et al*., 1994). Hence, *S. capitis* nosocomial acquisition and infection in vulnerable hospitalised patients could be associated with its predominance observed in both patient groups and ward areas.

Bannerman and colleagues, (1993) in their study evaluating the VITEK-GPI card showed that 92% of *S. epidermidis* isolates were identified accurately by VITEK®2 however, of these 3% were unidentified and 5% misidentified. Accuracy for identification of *S. haemolyticus* and *S. capitis* was 95% and 88% respectively, whereas *S. hominis* was identified with the least accuracy 63%, of which 43% were misidentified as *S. epidermidis*. These results concur with

the findings of this study, where 17% of the in-patient *S. hominis* isolates identified as *S. epidermidis*, with the use of the VITEK-GPI card. True reproducible differences between distinct strains and inconsistency in test findings are due to a number of factors which affect biochemical reactions of a given species (Bannerman *et al*., 1993). Therefore, environmental or technical factors include inoculum, culture conditions and incubation temperatures may have affected the biological expression of the phenotype. It is therefore possible that misidentification of *S. hominis* isolates as *S. epidermidis* may have been due to test performance variability affecting the phenotypic markers used for strain biotypic differentiation,

This is further evidenced by Spanu and co-workers (2003) in their study using the VITEK®2 system for the rapid identification of staphylococci isolates from BSIs. The authors suggest that there is a need for further improvement in the identification of some CoNS species in particular, *S. hominis* as the degree of misidentification of this species is higher in comparison to other species types. Similarly, for the control patient group CoNS isolates, 7 out of the 24 *S. hominis* identified as *S. epidermidis*. However, the difference in species identification was greater in this group where 22% of the isolates gave differing species-specific identification, compared to identification using VITEK MS MALDI-TOF system. Additionally, higher rates of species identification differences were noted with the speciation of the other minor CoNS group of isolates, where 6 out of the 7 isolates of *S. warneri* identified as either *S. hominis, S. haemolyticus* or *S. epidermidis*. Similarly, 3 out of the total of 5 (60%) isolates of *S. lugdunensis* identified by VITEK®2 were classified as *S. epidermidis* by MS MALDI-TOF. Accurate identification of clinical isolates of *S. lugdunensis* is important as this isolate with closely resembling characteristics *S. aureus* is often reported to be associated with serious infection when found in normally sterile sites (Huebener *et al*., 1999; Hellbacher *et al*., 2005). Conversely four out of five isolates of *S. epidermidis* in the control patient group were identified as the predominant minor group of skin commensals, *S*. *capitis* by MS MALDI-TOF. It is, therefore, highly possible that these isolates of *S. epidermidis* may have been misidentified due to factors affecting their biochemical profiling. Previous published studies looking at different comparative identification methodologies together with their stated limitations, confirm similar differences in misidentification of some CoNS species (Cuna *et al*., 2004; Layer *et al*., 2006). Therefore, accuracy in identification of clinically relevant CoNS species associated with serious infections becomes increasingly important, for optimal patient management (Dettenkofer *et al*., 2003; Goto *et al*., 2013).

S. epidermidis and *S. haemolyticus* are commonly encountered species associated with nosocomial BSIs particularly among immunocompromised patients. As these isolates are increasingly categorised as emerging pathogens the value of differentiating CoNS isolates to

the species level is therefore also becoming clinically relevant. Commonly used biotyping methods although easy to perform in routine laboratories, have a lower discriminatory power showing an over accuracy of 50-70% in comparison to genotypic based (Tan *et al*., 2012). Conventional biotyping systems for CoNS identification appear to be limited, mainly due to the relatively small numbers of recognised phenotypes and homogeneity of human strains and species distribution in clinical setting (Christensen *et al*., 1983; Kleeman *et al*., 1993). Although VITEK®2 automation provides a highly acceptable level of identification accuracy, species-specific isolate delineation is however dependent on both morphological and metabolic activity (Layer et al 2006). Conversely, the MALDI-MS methodology criteria are, however, not dependent on phenotypically expressed characteristics therefore, species differentiation is maximised, and technical limitations are minimised to a large extent. Evaluating the species identification results using two different methodologies in this study shows that proteomic based identification has a higher degree of accuracy in comparison to the bio-analytical method used for isolate sub-species identification.

The mass spectra generated for each test isolate by application of MALDI-TOF MS proteomics are representative of the organism's peptide sequences characteristic of the state of proteins in the biological sample are therefore used to determine organism identity. Subsequent comparisons of these spectra to those in the database identifies microorganisms from different genera and species to the sub-species level (Carbonnelle *et al*., 2010). This relatively new proteomic approach, independent of subjective interpretation, allows for accurate identification of bacteria as spectral "fingerprints" are specific to genus, species and subspecies. The MALDI-TOF MS has a more superior knowledge base to VITEK®2 database as each species and species group is represented in the database by an average of 10 isolates per species with a range of 2 to 475 depending on the organism (Rychert., *et al* 2013). There are comparatively fewer limitations and greater specificity with this technology in comparison to VITEK®2, such as inoculum dependency and requirement for additional off-line tests, thus providing a more specific, highly accurate identification system for CoNS speciation (Loonen *et al*., 2011).

The few limiting factors of the MS MALDI-TOF system affecting identification accuracy are in some instances, include bacterial culture growth conditions. These factors can affect the bacterial RNA sequences and differences in chemical extraction processes in turn affecting spectral peak intensities. The resulting spectra may also differ dependent on the nature and application of the matrix used with the MS for the ionisation process hence impacting on the degree of accuracy for isolate identification (Dubois *et al*., 2012; Patel, 2015). Such limiting factor dependency, in this study was overcome by ensuring that the CoNS test isolates were fresh 24 hour double-passaged cultures before use, thus ensuring stabilisation of CoNS

phenotype. The matrix application standard methodology was adhered to, in accordance with the manufacturers instruction and all CoNS isolates from both patient groups were double tested for enhanced accuracy. From the reproducible speciation results obtained in this study with MS MALDI-TOF it is evident that the identification test sensitivity and specificity was ≥ 99%.

Of the 100 CoNS in-patient isolates tested with MS system, all but one of the CoNS isolates not identified to the species level. However, this isolate was grouped with the other minor group of CoNS in the study as it was identified as *Staphylococcus* species by the reference laboratory by molecular analysis. On the other hand, biochemically inert *Kocuria* species isolates identified as *S. auricularis* and *S. capitis* giving a species level identification value of 99%. This high degree of species-specific identification was also noted with *S. hominis* isolates identifying as *S. epidermidis.* These isolates may have been affected by factors impacting on phenotypic expression, manifested in biochemical reaction variations, thus resulting in misidentification by VITEK®2 methodology. Difference may also be attributable to either metabolic activity and expression that both *S. epidermidis* and *S. hominis* have in common and/or influences of external technical and environmental factors including inoculum preparation and incubation.

As MS MALDI-TOF is based on whole bacterial colony protein structure analysis and independent of external factor effects, specificity and accuracy is greater therefore, resulting in the less likelihood of CoNS species misidentification. Overall result comparisons revealed a minimal error difference (6%) in species identification for the in-patient group isolates, this however made no difference to the overall species-specific CoNS distribution. Conversely, identification difference and species distribution was noted to be greater in the control patient group isolates. Comparatively, in-patient isolates of *S. hominis and S. lugdunensis* also identified as *S. epidermidis* by MS MALDI-TOF methodology. Noticeably 6 out of a total of 7(86%) isolates of *S. warneri* in this group were identified differently among control patient group isolates. In comparison to the in-patient isolates, a higher degree of differences (22%) was observed overall in distinguishing species-specific isolates. These differences could be due to VITEK®2 systems low discriminatory power based on biochemical analysis which could be the limiting factor hence affecting result interpretation. The MS database comparatively, showed scores of ≥95-99.9% considered as high-confidence genus level identification with all the CoNS isolates tested, thus indicating a high probability of correct species level identification. Such species-specific isolate identification result accuracy has been corroborated by other authors, comparing staphylococci identification by replacing the conventional biochemical method with the MS MALDI-TOF system providing better identification to genus and species level for the majority of Gram-positive isolates

(Carbonenelle *et al*., 2012). Likewise, this study also attests to the fact that some CoNS isolates gave inconsistent or low discriminatory power identification by biochemical analysis, whereas correct species level identification was achieved with the MS MALDI-TOF systems. The greater accuracy of MS system comparable to "gold standard" molecular approaches is attributed to the identification of specific protein markers in GP-isolates such as aconitate hydratase; thus, providing clearer differential results for staphylococcal speciation (Patel, 2015).

The overall results obtained comparing the identification of CoNS isolates using the two different methodologies also correlate well with other studies that demonstrate MS MALDI-TOF performs better than biochemical identification systems. Studies demonstrated that 98% of clinical isolates of staphylococci, streptococci and enterococci were correctly identified to the genus level in comparison to the reference methodology (Carbonenelle *et al*., 2010; Patel, 2015). Furthermore, comparisons made with an entire repertoire of biochemical, immunological and genotype tests, the MS MALDI-TOF, proteomic based system displayed a high degree of accuracy in species level identification of CoNS (Rychert *et al*., 2013). *S. epidermidis* in the present study remained the predominate CoNS isolate identified in both patient groups, with the use of the two different identification methodologies. However, difference in CoNS species distribution were more apparent in the control patient group whereby *S. haemolyticus* was the second commonly isolated of the species instead of S. *hominis* when identification was carried out using MS MALDI-TOF.

Further analysis carried out to verify the significance of CoNS speciation result differences obtained using the two different identification systems revealed some dissimilarities. For the in-patient group there was statistically no difference ($p > 0.05$) in CoNS species identification with the use of either the VITEK®2 or MSMALDI-TOF system. However, for the control patient group there was a statistically significant difference ($p < 0.05$) observed in species identification and thus distribution dependent on the identification system used. Further statistical analysis based upon the linear logistic regression model resulting in a salient p value of <0.05, suggests a high significant difference in species identification performance with the use of either the VITEK®2 or the MS MALDI-TOF systems. Moreover, this was also significantly dependent on site of isolation that derived from either in-patient or the control patient group.

This study therefore demonstrated that MS MALDI-TOF was a more reliable platform for identification of clinically important isolates such as those from BSIs. This is especially beneficial for patient management as surveillance programmes have become increasingly important in defining species distribution of potentially pathogenic isolates associated with

BSIs. Accurate CoNS species determination can therefore also aid clinicians in implementing targeted empirical therapeutics. Furthermore, these measures have been shown to influence reduction of morbidity and mortality rates particularly among the critically ill (Wisplinghoff *et al*., 2004; Ortega *et al*., 2005; Worth *et al*., 2009; Ge *et al*., 2016).

This study further investigated clonal relatedness of CoNS species in each of the patient groups, based on phenotypic characterisation. The relationship between CoNS species-specific isolates based on biochemical profiling was explored using Pearsons-Principle component dice coefficient analysis with UPGMA (Figures 2-2 and 2-3). This analysis allowed the determination of relationships between and within each of the CoNS species isolates. The results revealed a high degree of phenotypic relatedness amongst the predominant CoNS species showing five distinctive cluster groups (Figure 2-2). However, significant differences in the biochemical profiles amongst individual isolates of each of the predominant CoNS species was observed amongst the in-patient group. These differences were not observed amongst the control patient group CoNS isolates, where a high degree of diverse clonal types were observed within the predominant CoNS species clusters. These observations were based on significant differences noted in biochemical profiles of individual species-specific CoNS isolates, with very few clusters (<2) of 100% showing matching profiles. Similarly of the 51 profiles of *S. epidermidis* in-patient group isolates only six showed 100% similarity. Conversely, *S. haemolyticus* isolates showed a greater level of similarity with four of the seven different profile cluster groups showing 100% similarity. Furthermore, both patient groups *S. hominis* and the other minor CoNS group of isolates showed no profile matches, suggesting the presence of extremely diverse range of clonal unrelatedness. Overall, no similarity in biochemical profiles was detected amongst majority of species-specific CoNS isolates, suggesting that there were diverse phenotypes within each species what were associated with bacteraemia in the haematology patient group. Comparatively, differences in profiles within and between all species-specific CoNS isolates was much higher in the control patient group. The greater degree of diversity observed within and between each of the CoNS species-specific isolates suggests that these are unlikely to be related to those recovered from the in-patient group. However, these isolates could potentially be variants of the predominant clonal groups present on the skin or the environment. Clonal changes which may have been triggered by adaptive responses for survival thus resulting in aetiological agents associated with nosocomial infections.

The overall species distribution of CoNS isolates identified from both the in-patient and control groups confirm results reported by other researchers of similar studies (Kleeman *et al*., 1993; Piette *et al*., 2009; Argemi *et al*., 2015). A high degree of hyper-variability was noted among and between all species-specific CoNS isolates of both patient groups. As

such, it was possible to distinguish 51 different phenotypes among the 57 in-patient *S. epidermidis* isolates, hence clonal dissimilarity. This phenomenon has been explored in a previous study showing that a single isolate was considered to be "composed of cell populations of common or clonal origin" with the possibility that phenotypic variants could be considered as separate strains or clonal variants from the same parental cell (Kleeman *et al*., 1993). The variability observed in the biochemical patterns within and between isolates of the same CoNS species and the rare presence of phenotypically identical isolates of CoNS in both patient groups could therefore be reflective of this phenomenon.

A further objective of this study was to explore the possibility of environmental CoNS isolates and those representative of skin colonisers from the control patient group as the possible source of in-patient CoNS-associated bacteraemia. The distribution of CoNS species among the environmental isolates identified *S. epidermidis* as the most prevalent species similar to both patient group isolates. Analysis comparisons based on Bionumbers (biochemical based biotypic-analysis) of CoNS species-specific isolates showed extremely limited similarity with no exact matches within and between species-specific CoNS of both patient groups and the ward area sample isolates. One isolate of *S. cohnii* exceptionally, recovered from haematology ward area 1 however demonstrated 100% match with the same species isolates found in haematology ward area 2 during May 2011 sampling. Additionally, further data analysis indicated the presence of unique phenotypes within the ward environment. This relatively high degree of heterogeneity observed within and between CoNS species-specific isolates from the ward environment suggests that these isolates are least likely to be exposed to micro-niches similar to the conditions found in the human host. Therefore, it is possible that these biochemically distinct environmental CoNS species-specific isolates may have been derived from the same clonal linage but possess characteristics that allows them to adapt for survival in the hospital environment. The incredible plasticity of these isolates to survive and adapt in all environments may be evolutionary; such that transient exposure to the human host either as colonisers or potential pathogens is indicated by the number of different phenotypes observed in this study.

Therefore, the difference in unrelatedness observed in CoNS species derived from both patient groups and ward areas is highly suggestive of the fact that both environmental and CoNS skin-colonisers are highly unlikely to be the source of bacteraemia in the patients on the ward. However, it is possible that in-patient CoNS isolates due to their hyper-variable nature may have originated from the same clonal strains in the environment or skin but consequently to their adaptive survival characteristics become the aetiological agents of such nosocomial BSIs. Reported results obtained from other studies evaluating potential sources of CoNS-associated bacteraemia using molecular techniques, suggested that although skin

isolates could not be excluded as a potentially important source of bacteraemia particularly related to CVC infections, there was a significant lack of evidence suggesting it was the primary source of CoNS bacteraemia (Costa *et al*., 2004). Nonetheless, the study reports that among cancer patients, there is a strong correlation between BSI CoNS isolates and those recovered from CVC hub areas and mucosal colonisers. Furthermore, the molecular–relatedness studies demonstrated a molecular match between BSI and skin CoNS isolates in 24 of the 91(26%) of patients with CVC CoNS-related bacteraemia. However, it has been suggested that additional studies are required to access the exact role of skin as primary source of bacteraemia in relation to its clinical significance (Costa *et al*., 2004; Widerström *et al*., 2012). As CoNS isolation from bacteraemic patients remains both subjective and dependent on clinical-specific factors, isolate speciation requiring specialised technologies have yet to be implemented routinely in most laboratories

The clinical microbiology laboratory, nonetheless, plays a critical role in the care of patients by providing rapid identification of the infective organism using definitive diagnostic methods. Species-specific identification for optimal clinical outcomes can therefore be valuable in providing therapeutic guidance for targeted and effective antimicrobial administration. This has been shown to correlate directly with decreased mortality in patients with BSIs (Ge *et al*., 2016). In the present study it could not be conclusively proven that the potential source of CoNS-associated bacteraemia in the haematology patients was derived from either the skin-colonising isolates or the ward environment. However, technologies such as MS MALDI-TOF providing faster, more accurate identification of most clinically important CoNS isolates when compared to the more conventional methodologies, can directly influence patient diagnosis and management. Studies have demonstrated the potential benefit of rapid species-specific level identification correlating with better clinical outcomes in bacteraemic patients diagnosed with CoNS-associated BSIs (Harbath *et al*., 2003; Galar *et al*., 2012)

2.10 Conclusion

S. epidermidis followed by *S. hominis* and *S. haemolyticus* accounted for the dominant CoNS species isolated from both the blood cultures of patients with haematological malignancies and those isolated from among the skin-colonising strains of the control patient group.

Despite the lack of discriminatory power demonstrated by biotyping in comparison to the superiority of MS technology phenotypic characterisation of CoNS isolates at this level revealed no significant difference in the overall distribution of CoNS species-specific isolates in both patient groups. Low levels of phenotypic similarity and a high degree of heterogeneity within and between CoNS species-specific isolates of both patient groups was evident in this study, thus reflecting the diverse and adaptable nature of these ubiquitous species. The findings of this part of the study therefore do not conclusively support the hypothesis that endogenous skin-colonising isolates could be the potential source of CoNS-associated BSIs in the haematology patient group.

Chapter 3 Genotypic Characterisation of CoNS isolates from bacteraemic in-patients in the haematology and BMT unit and those skin-colonisers isolated from the pre-admission patient group to determine CoNS species-specific relatedness.

3.1 Introduction

CoNS species are increasingly recognised as the leading cause of bacteraemia in neutropenic patients with indwelling prosthetic devices and as such results of specific diagnostic assays can be a key factor for medical decision making for antimicrobial choice for patient therapy and epidemiological studies (Galar *et al*., 2012).

Establishing the primary source of clinically significant CoNS associated BSIs in cancer patients in particular, originating from either mucosa, skin or device colonisation is critical in prevention and management of infection (Costa, 2004; Worth *et al*., 2009). Routinely used conventional methodologies for identification of such CoNS species have limited application, as diagnostic parameters are based on the variable expression of phenotypic characteristics which can result in anomalous result interpretation. Although phenotypically expressed traits in species-specific isolates of CoNS may be predictive of therapeutic outcomes, these aspects however, may not be able to distinguish different strains of the same species expressing identical characteristics (Cunha *et al*., 2004). Clonally related strains of CoNS can therefore be distinguished further by their genetic fingerprint thus providing a distinct link between potential sources of clinically defined infections.

Microbial genotyping techniques incorporating molecular biology to reliably distinguish bacterial strains and clones have been shown to be superior to the conventional methodologies (Sloos *et al*., 2005). Studies on the molecular epidemiology of CoNS incorporate genotypic techniques such as DNA banding patterns and DNA sequencing. Other methodologies for the determination of microbial genetic relatedness and diversity include multilocus sequence typing (MLST), ribotyping, PCR ribotyping, DNA sequencing - randomly amplified polymorphic DNA (RAPD), PFGE, repetitive sequence-based polymerase chain reaction (rep-PCR) and whole genome sequencing (Miragaia *et al*., 2002; van Belkum, 2003; Healy *et al*., 2005). These methodologies greatly improve discrimination and characterisation of CoNS species. Of these the rep-PCR technique commercially adapted to a semi-automated format provides a PCR-based genotyping system based on amplification of flanking regions between repetitive DNA sequences found naturally interspersed within the bacterial genome. Therefore, for strain delineation the system utilises automation to include changes in rep-PCR chemistry, thermal cycling parameters, incorporation of microfluidics-based amplicon fractionation followed by detection and result analysis assisted by an integrated internet-based computer application module (Shutt *et al*., 2005). This

epidemiological tool is commercially available as the DiversiLab® System (bioMérieux, Marcy l'Etoile, France) consisting of hardware, software and consumables for genotyping of bacteria. For purpose of CoNS strain delineation, the process includes, amplification of DNA extracted from the CoNS isolates using rep-PCR, separation of amplified fragments leading to the generation of unique fingerprint patterns. These fragments are then compared to each other and to a database of characterised microbial isolates for strain relatedness evaluation (Healy *et al*., 2005).

Although this PCR-based genotyping methodology is less labour intensive in comparison to other methods, it has however been reported to be less discriminatory than PFGE, regarded as the gold standard technique for establishing the true relatedness of bacterial strains (Tenover *et al*., 2009; Widerström *et al*., 2012). As such, despite being relatively costly and time consuming PFGE is a highly discriminatory and reproducible technique often used to fingerprint CoNS species to distinguish characteristics that determine clonal relatedness (Dobbins *et al*., 2002). In this part of the study, rep-PCR and PFGE methodologies were therefore assessed for their discriminatory power to determine strain delineation and clonal relatedness of skin-colonising CoNS species-specific isolates with those from bacteraemic patients in the haematology and BMT unit. Comparisons of isolate and species-specific genotypic characteristics could then be used to elucidate skin colonisers as the potential source of bacteraemia in the haematology patient group.

3.2. Aim of this current study

(a) To compare genotypic characteristics of CoNS isolates recovered from bacteraemic patients in the haematology and BMT unit with those that are predominant skin colonisers recovered from the pre-admission patient group

(b) Molecular characterisation and genotypic relatedness of the CoNS isolates from both patient groups was determined by using two different techniques: microbial DNA typing by automated Repetitive sequence-based PCR (rep-PCR) and PFGE.

(c) To investigate and determine the prevalence of clones and the degree of genetic relatedness of CoNS species within each patient group by PCA analysis.

(d) To determine the possible acquisition of bacteraemic CoNS species associated with those isolates that are skin colonisers.

3.3 Materials and Methods

3.3.1 Bacterial isolates

Previously phenotypically characterised strains of CoNS from both patient groups were investigated in this study as described in section 2.3.3.1 and 2.3.3.5. The isolates stored on Microbank™ beads were recovered by subculture onto blood agar plates incubated at 37°C for 18 hours in ambient atmosphere. Two further subcultures of the CoNS isolates were undertaken prior to further investigation for genotyping.

3.3.2. Selection of isolates for rep-PCR analysis

A total of all the 200 previously phenotypically characterised CoNS isolates from both patient groups were investigated for genotypic characterisation using rep-PCR methodology.

3.3.2.1 Repetitive sequence-based PCR using the DiversiLab® System

All CoNS strain typing kits were supplied by Diversilab™ (bioMérieux SA, France). Bacterial DNA extraction was carried out using the MoBio Ultra Clean microbial DNA isolation kit following the manufacturers protocol (MO BIO Laboratories Inc.).

For each sample isolate tested, a density >5 McFarland standard suspension was prepared. This was achieved by adding two, 10µl loops full of fresh 24 hour CoNS culture to a MicroBead tube consisting of a silicon column, into which 300µl MicroBead solution had been added. Following addition of 50µl of MD1 solution the tubes were vortexed (Vortex-Genie 2 Sigma-Aldrich) for 15 minutes at maximum speed. The vortexed MicroBead tubes were then centrifuged (Sigma1-14) at 10,000*g* for 30 seconds.

The supernatant from each of the MicroBead tubes was then carefully removed avoiding the sandy particles and transferred to a clean 2ml clean collection tubes into each of which 100 µl of MD2 solution had been previously added. The sample tubes were vortexed briefly and incubated at -20°C for 15 minutes, followed by centrifugation for one minute at 10,000*g.* 200 µl of MD2 supernatant was then transferred into pre-prepared clean tubes containing 450 µl of MD3 solution. Following a brief vortex-mix the tubes were centrifuged in short pulses for a few seconds at 10,000*g*, after which 650µl of the MD3 mixture was then transferred into clean spin filter tubes. The spin filter tubes were centrifugation at 10,000*g* for 30 seconds and the flow through solution left in the bottom was discarded. This was followed by addition of 300 µl of MD4 solution to the spin filter, centrifuged for 30 seconds at 10, 000*g* and the flow through solution discarded again. The tubes were then dry spun for 1 minute at 10,000*g*.

Discarding the collection tube, the spin filter was removed and placed in a clean collection tube to which 35µl of MD5 solution was added ensuring that the addition was made at the centre of the spin filter. The tubes were centrifuged at 10,000*g* for 30 seconds and incubated at room temperature for 2 minutes. Each spin filter was then discarded, and the extracted bacterial DNA retained in the collection tubes was quantified using a NanoDrop™ Lite Spectrophotometer (Thermo Scientific, USA) following the manufacturers protocol. Absorbance readings were performed at 260_{nm} and the number generated equates to the estimated yield and purity of DNA. Therefore, the calculated absorbance ratio of $260/280_{nm}$ equivalent to requisite ratio of 1.7-1.8 equated to good quantitative extracted DNA free from protein contamination hence acceptable for further analysis.

3.3.2.2 rep-PCR DNA amplification using DiversiLab species-specific fingerprinting kit

The extracted CoNS DNA was amplified in accordance with manufacturer's protocol using the DiversiLab Staphylococcus DNA fingerprinting kit. This procedure was carried out in a "Clean room" and all equipment was cleaned with 70% ethanol and left under ultra-violet light for 15 minutes before use. The kit containing the reagents for the Master Mix were kept frozen and thawed for 20 minutes prior to use with the exception of AmpliTaq® DNA Polymerase (Thermo Fisher Scientific). Genomic DNA of each sample extracted was diluted using the MD5 solution supplied in the kit to give a final concentration of 35ng/µl for testing.

Preparations for rep-PCR in the clean cabinet were carried out by first preparing the Master Mix in a 1.5 ml tube according to the number of samples tested in each test batch. A positive- and negative-control supplied in the kit was included in each test batch. For a final volume per reaction tube of Master Mix was made up of 18µl rep-PCR MM1, 2.5µl GeneAmp of 10 x PCR buffer, 2µl Primer mix and 0.5µl of AmpliTaq® DNA polymerase. Using reverse pipetting, 23µl of the mixed and briefly spun Master Mix was added to the bottom of each labelled reaction tube placed in a cold block. Addition of 2µl of DNA test sample and controls was then added to the bottom of each of the test tubes and mixed gently by pipetting up and down.

The preheated thermal cycler (PCR cycler 5 Eppendorf Mastercycler Personal) was set at the following thermal cycling parameters: initial denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds and extension at 70°C for 90 seconds and a final extension at 70°C for 3 minutes.

3.3.2.3 Semi-automated rep-PCR DNA fingerprinting

Detection of DNA amplicons and analysis was performed with the DiversiLab system and accompanying software linked to a customer-specific secured web-based application accessible via the internet. The generated PCR products were separated via electrophoresis performed in a microfluids DNA LabChip device.

The protocol for loading the DNA LabChip device in accordance with the manufacturer's instructions, illustrated in Figure 3-1 are as follows; all reagents supplied were brought to room temperature prior to use. An aliquot of 9µl pre-prepared Gel-dye mix was reverse pipetted into the chip priming well. The chip was then pressurised for 30 seconds with the syringe initially set at 1ml. Similarly, 9µl aliquots of Gel-dye mix were added to the remaining two G wells with 5µl of marker added to the rest of the assigned sample wells marked 1 to 13. This was followed by the addition of 1µl of the test sample and molecular ladder agent provided in the kit. The chip was then placed onto the vortex (Sigma-Aldrich) and the contents mixed for one minute. The DNA LapChip was then run on the Agilent 2100 BioAnalyser® which provided Rep-PCR fingerprints through microfluidic capillary electrophoresis (Figure 3-1).

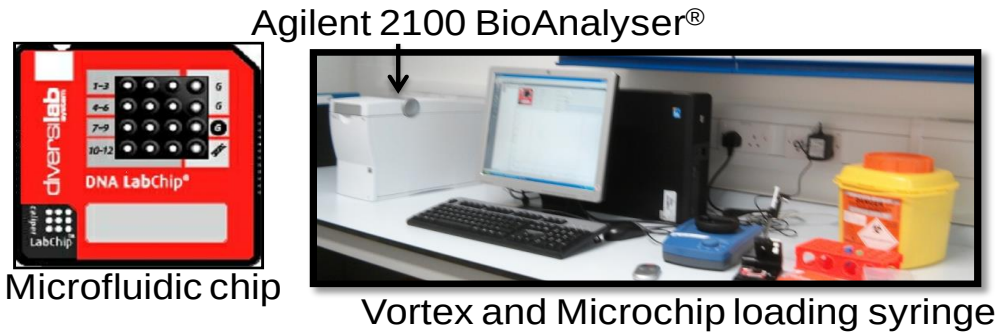


Figure 3-1: DiversiLab system utilising the microfluidics DNA LabChip for the separation of amplified fragments via electrophoresis run on Agilent 2100 BioAnalyser®.

Using the DiversiLab software (version 3.4 bioMérieux, Inc. USA) analytical tools, the raw signals generated for each test sample with the high and low markers showing the beginning and end of a fingerprint curve were processed to normalise the graphs generated as illustrated in figure 3-2. Sample amplifications that yielded low intensity fluorescence or very few non-comparable peaks were repeated for analysis using freshly extracted DNA.

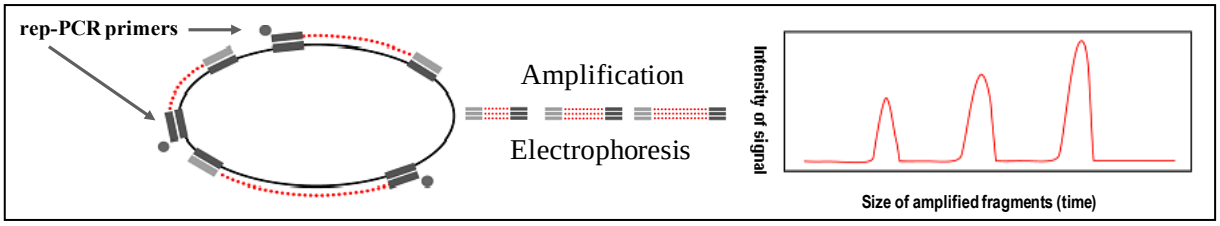


Figure 3-2: rep-PCR technology for CoNS fingerprinting: Multiple fragments of many specific repetitive sequences throughout the genome, amplified and separated, generating unique fingerprint pattern made from the fragments. (Schematic diagram adapted from the DiversiLab® Training Guide 2014).

3.3.2.4 rep-PCR data analysis using the DiversiLab web based Analysis-Tool.

General guidelines for interpretation of DNA patterns for genetic relatedness were followed for rep-PCR in accordance with the manufacturers guidelines using the specified *Staphylococcus* kit and DiversiLab Software Guide v: 3.4. Interpretation of bands to distinguish between strains of CoNS at the species level included the following criteria:

0 band difference = indistinguishable strains (>95% similarity)

1 to 2 band difference = similar strains

More than 2 band difference = different strains (<95% similarity)

The DNA band patterns generated were converted to virtual gel images and further analysis was carried out using the interactive DiversiLab Bacterial Barcodes software program which uses curve-based Pearson correlation coefficient to determine distance matrices for pairwise similarity scores. For data interpretation, the resultant banding patterns were analysed to create dendrograms with a percentage similarity index set at ≥ 95% for cluster analysis in accordance with the manufacturer's instructions. This was carried out using the customer-specific web-based computer application via the internet.

3.3.3 Pulse-field gel electrophoresis analysis of CoNS

This part of the study was kindly carried out courtesy of Professor Angela Kearns at the *Staphylococcus* reference unit laboratory, PHE Colindale London (UK) assisted by Mr. Mark Ganner.

The protocol followed was that of the national *Staphylococcus* reference laboratory which has extensive experience with the technique for genotyping CoNS isolates. The reference laboratory has developed and uses PFGE protocols with standardised parameters

demonstrating a high degree of reproducibility and an established national database for comparison (Kaufmann, 1998; Murchan *et al*., 2003).

3.3.3.1 Selection of isolates for PFGE

Fifty out of 200 (25%) of the predominant species of CoNS isolates were randomly selected from both the in-patient and pre-admission patient control groups for investigation. This included 15 isolates of *S. epidermidis* and 5 isolates each of *S. haemolyticus* and *S. hominis* from each patient group.

3.3.3.2 Preparation of chromosomal DNA

Chromosomal DNA of the 50 CoNS isolates was prepared according to the PHE standard genotyping methodology used for comparing isolates of *Staphylococcus* species (Kaufmann, 1998).

The CoNS test isolates cultured on blood agar incubated at 37°C for 18 hours were prepared by suspending each isolate in 1ml of SE buffer (75mM NACL, 25 mM EDTA pH7.5) to a turbidity of 4 to 4.8 McFarland standard using a densitometer. (DensiCHEK bioMérieux) resulting in a concentration of $1X10^9$ cells/ml. An equal volume (400µl) of bacterial suspension was then mixed with 2% low-melting temperature agarose (MacroSieve LM agarose, Flowgen Bioscience) and dispensed into the assembled well moulds. After cooling and solidification of the molten mixture, each test block was placed in a bijoux and treated with 5mg/L of high concentration lysis buffer (6mM Tris, 0.5M EDTA, NaCl 0.5% w/v Brig 58, 0.25%w/v Sodium deoxyclate, 0.5% N-Lauroyl sarcosine, 1mM $MgCl_2$), 1.5mg/ml, Lysozyme and 3µL Lysostaphin (Sigma-Aldrich, UK). This was followed by incubation at 37°C with gentle shaking at 150rpm (New Brunswick Scientific innOva 2100 Platform shaker) for four hours.

After incubation the first lysis buffer was removed and discarded from each test bijoux. This was followed by the addition of 3ml of Alkaline Lysis Buffer (1% w/v N-Lauroyl sarcosine, 0.5 M EDTA pH 9.5) and 1mg/L Proteinase K (Sigma-Aldrich). The bijoux were incubated overnight at 50°C water bath (Grant SBBS Waterbath) with gentle shaking.

A modified DNA chromosomal preparation was used for CoNS isolates that gave weak or unreadable bands which included treatment and washing of agarose blocks using a higher concentration of Lysostaphin (5mg/ml) and a prolonged incubation period of 20-24 hours.

The blocks were subsequently washed three times with TE buffer (10mM Tris, 10mM EDTA pH 7.5) after removal of the Alkaline Lysis Buffer and Proteinase K solution. Each wash step included addition of fresh TE buffer and incubation at 4°C for 30 minutes. After the final wash step the agarose blocks containing extracted whole CoNS chromosomal DNA were suspended in TE buffer ready for the restriction enzyme digestion step.

The prepared agarose blocks prepared were digested with endonuclease FastDigest *Sma*I (Roche, UK) in buffer incubated at 30°C for 30 minutes prior to loading into the wells of a 1% agarose gel for electrophoresis.

3.3.3.3 Pulse-field gel electrophoresis.

Agarose gel was prepared by dissolving 2.04g agarose (Certified™ Molecular Biology Agarose, Bio-Rad Laboratories Inc.) in 153ml distilled water and 17ml of 5 x TBE buffer commercially prepared solution (Invitrogen Ultrapure 10x TBE Buffer). Agrose plug sections containing the digested DNA were loaded into the wells in the gel together with two molecular weight markers (Lambda Ladder PFG Marker, New England BioLabs.) in the first and last well of each gel. All the wells were sealed with the previously prepared molten agarose kept at 56°C.

Sufficient electrophoresis buffer (2 litres 0.5x TBE per gel) prepared and maintained at 12°C was poured into the tank of the electrophoresis apparatus (Biorad CHEF DRII) to cover the gel. The following electrophoresis parameters on the control module were set for *Staphylococcus* species: Total run time 30 hour; Initial switch time 1 second; Final switch time 80 Seconds; Voltage/cm of 6 volts.

3.3.3.4 Staining, Gel visualisation and interpretation

On completion of electrophoresis the gel was stained with 60µl of 10,000 x Gel Red (Biotium) diluted in 200mL 0.1M NaCl for one hour protected from light at all times. Visualisation of the gel was carried out under ultraviolet transillumination and photographed using the laboratories gel documentation system. Digital images of the gel were saved and stored as electronically as TIFF files and were analysed visually with the GelCompar system (Applied Maths, Belgium). The PFGE generated banding patterns were categorised according to the proposed guidelines described by Tenover and co-workers (1995) as follows: (a) banding patterns from the majority of the related CoNS isolates appearing identical in size and

number of bands were considered to be representative of the same strain type and was designated as pulsotype A; (b) CoNS isolates differing from the main banding pattern by three or less band differences were designated as those of the subtype within the main group (e.g. pulsotype A-1); and (c) banding pattern that differed from the main pulsotype by more than four or more bands were designated as different strain types (e.g. designated as pulsotypes B, C, D and E etc.).

Further data analysis of *Sma*I PFGE patterns was carried out using the UPGMA clustering algorithm method to produce a hierarchical dendrogram. Genetic similarity between CoNS species isolates was calculated using Dice coefficient with a cut-off values set at >90%. This data analysis was performed using the Bionumeric software Statistica version 10 package.

3.4 Results

3.4.1 rep-PCR fingerprint profiles

The requisite quantitative genomic DNA, linear range absorbance target performed at 260_{nm} equivalent to value of >1.7 was achieved for all the one hundred CoNS isolates from each of the two patient groups. Amplification of the rep-PCR or the separation of the amplified fragments for analysis was not achievable for all the isolates in both patient groups. Samples that failed analysis were repeat tested twice. However, 14% and 12% of the CoNS isolates from in-patient and the control group respectively resulted in abnormal rep-PCR DNA fingerprints. The isolates (26%) that presented discrepant results were removed from final analysis due to large difference in either overall intensity resolution or migration differences leading to poor unresolved fingerprints.

In total 234 rep-PCR fingerprint profiles were generated including one ladder standard and analysed for the 174 (87%) out of the 200 CoNS isolates tested for genotyping using the DiversiLab system. Analysis included repeated testing of some CoNS isolates for better resolution or to run a full thirteen slot, LabChip for electrophoresis. CoNS species classification was based on previous phenotypic characterisation of strains (Chapter 2) for rep-PCR analysis. The identities of all CoNS isolates were entered as ‘Alternative ID’ for complete data generation analysis and report presentation.

Figure 3-3 illustrates a typical rep-PCR DNA graphical fingerprint profile with multiple bands of varying intensity representative of most of the *S. epidermidis* isolates in both patient groups. The other CoNS species produced fewer peaks of lower intensity or more peaks of

variable intensities dependent on electrophoretic resolution as observed with *S. haemolyticus* isolates.

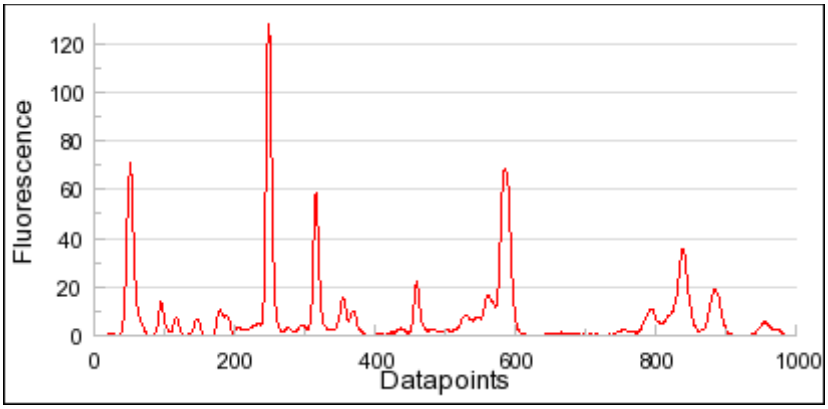


Figure 3-3: rep-PCR fingerprint profile for *S. epidermidis* in-patient isolate A3.

Using the analytic tool application, the relevant graphical data within the specified criteria for all the CoNS isolates was converted to virtual gel profiles for visual assessment of strain relatedness. The gel profiles were visualised as representative of 150-5000 base pairs of the rep-PCR elements.

3.4.1.2 rep-PCR generated fingerprint data analysis results

To ascertain strain relatedness between CoNS isolates and species, the interactive interpretation DiversiLab tool was used to create a dendrogram of rep-PCR fingerprint profiles of all CoNS isolates tested from both patient groups. Automated analysis was based on Pearson's correlation methodology to calculate percentage similarities between CoNS isolates, based on emphasis on rep-PCR electrophoretic peak intensities rather than peak presence or absences. Overall data analysis results using the specified criteria for strain relatedness of all the 233 CoNS rep-PCR fingerprints generated as a complete dendrogram are displayed in Appendix 1 revealing a total of nine clusters.

The observed clusters included CoNS isolates from both patient groups, that showed a high level of similarity but demonstrated a variable degree of relatedness within each of the species-specific isolates. The majority of clusters consisted of groups of closely related isolates of the same CoNS species with a genetic similarity index of >95% as well as a number of isolates demonstrating clonal similarity. This level of isolate similarity was identified in clusters 2, 3, 4, 6, 7 and 9. Conversely cluster 1, 5 and 8 consisted of different CoNS species-specific isolates from both patient groups exhibiting poor genetic similarity (<90%). The dendrogram results of CoNS species-specific isolates from both patient groups that were indistinguishable, i.e. exhibiting 100% strain similarity are summarised in Table 3-1.

CoNS Species	Indistinguishable Strains (100% Similarity)	
	In-patient Isolates	Control Patient Isolates
S. epidermidis	A25	G11, G36
S. epidermidis	A89, A7, A3	
S. epidermidis	A63, A47, A45	G75
S. epidermidis	A26, A48	
S. epidermidis		G100, G96
S. epidermidis	A88, A14, A38	
S. epidermidis	A5, A50, A24	
S. epidermidis	A29, A42, A5	
S. epidermidis	A72, A95, A92, A43	G93, G91
S. epidermidis		G51, G40, G83 - *S. hominis*
S. epidermidis	A77, A60, A36, A98 - *S. hominis*	
S. epidermidis	A9	G23
S. epidermidis	A45, A51, A46	G13
S. haemolyticus	A58, A39, A67	
S. haemolyticus	A99, A86	
S. haemolyticus	A87, A80	
S. haemolyticus	A8	G79
S. haemolyticus	A10	G6
S. haemolyticus	A81	G71
S. capitis		G34, G39, G37
S. capitis	A34, A11	

Table 3-1: Dendrogram (Appendix 1); result summary of in-patient and control patient group CoNS species-specific isolates demonstrating clonal relatedness.

Of a total of 174 CoNS isolates tested from both patient groups, clonal relatedness was observed in 24% and 10% of isolates from the in-patient and control patient groups respectively. Isolate similarity was observed most commonly amongst the *S. epidermidis* species in both patient groups. Conversely, there was low degree of clonal relatedness between species-specific CoNS isolates recovered from the two patient groups. Out of a total of 60 isolates demonstrating 100% strain similarity 12 (20%) control patient group CoNS exhibited clonal relatedness with 18 (30%) of the in-patient isolates. Of the other minor group of CoNS species-specific isolates, clonal relatedness was demonstrated only by isolates of *S. capitis* in both patient groups. Conversely, clonal relatedness was not observed in and between any CoNS species-specific isolates of *S. hominis* in both patient groups.

Of the CoNS isolates 23 (13%) were re-run at least twice to obtain well resolved rep-PCR fingerprint profiles for analysis. Fingerprint profiles for 15 (9%) of these CoNS isolates exhibited 100% similarity proving reproducibility of the rep-PCR assay. Conversely, 4(1%) strains of *S. epidermidis* isolate A88, *S. lugdunensis* isolate G45, *S. hominis* isolate A71 and *Kocuria* species isolate A44 exhibited 95% similarity on repeat re-analysed fingerprint

profiles. The differences of <95% strain similarity was observed in one *S. haemolyticus,* strain A81, tested three times and in two *S. hominis* isolates A71 and A69 tested twice. The disparity in isolate similarity was even more evident in one, *S. capitis*, isolate A34 analysed twice on the same DNA Lab-Chip. The difference in similarity observed with this isolate was at <75% indicating a lack of reproducibility may possibly have been affected by technical and/or environmental factors in this case.

Overall characterisation by rep-PCR revealed similarity at 90 and 95% between most CoNS species-specific isolates from the two patient groups. However, the same degree of similarity was not apparent within and between isolates of the same CoNS species in each of the two patient groups.

Results of further analysis conducted to highlight any potential similarities between each of the CoNS species-specific isolates from both patient groups are summarised in scatterplot Figure 3-4. The 2D PCA rep-PCR fingerprint analysis of all the 233 CoNS isolates tested from both patient groups enabled visualisation of overall pair-wise similarity thus demonstrating relatedness within and between similar CoNS species-specific isolates.

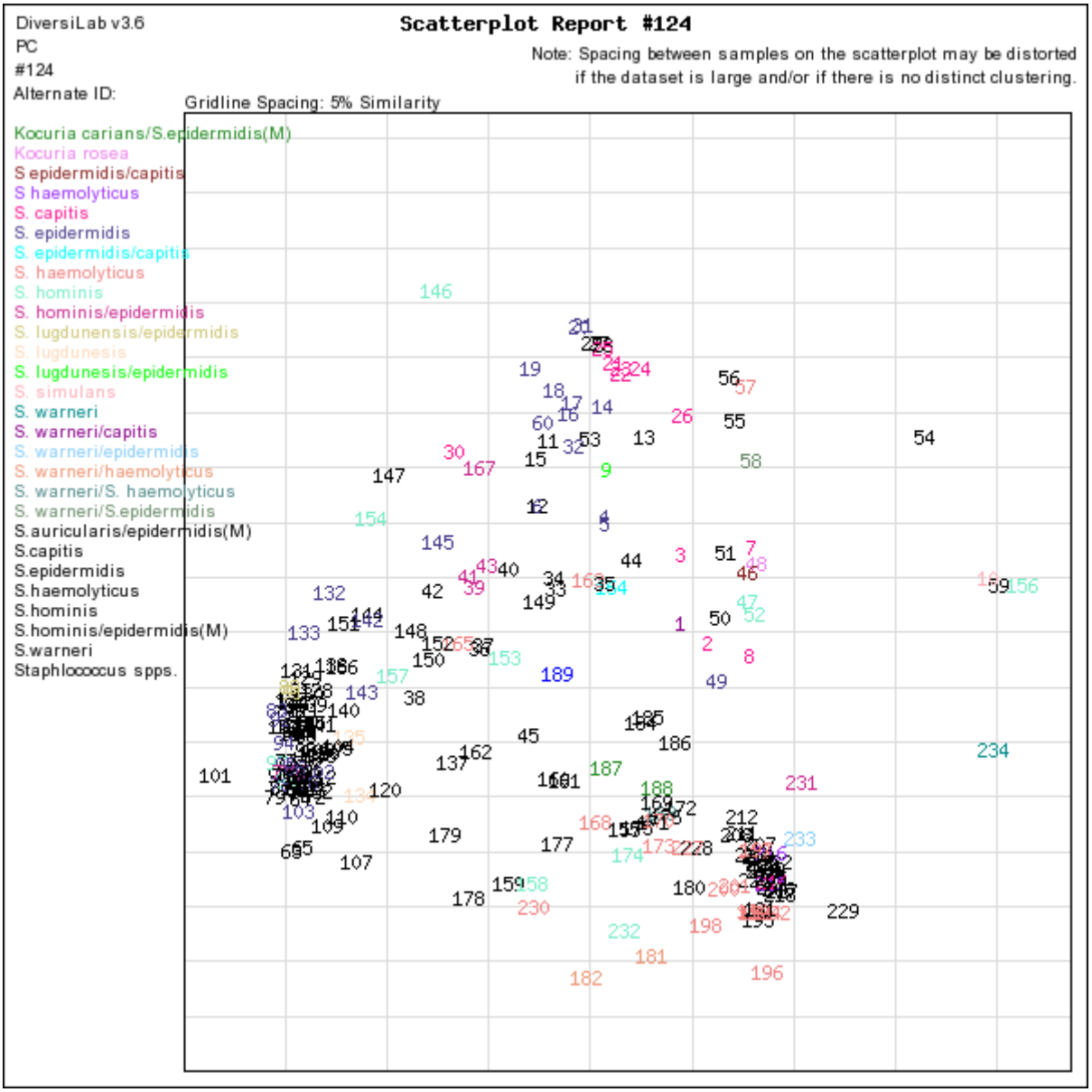


Figure 3-4: Scatterplot summary of 2D PCA rep-PCR fingerprint analysis of the 233 CoNS isolates from both patient groups. Colour-coded data clustering indicates degree of similarity between each of the CoNS species-specific isolates.

The results of the scatterplot demonstrate the direct relationship each CoNS isolate has with each of the other isolates from both patient groups. The 5% similarity spacing points set on the grid of the scatterplot illustrate that the majority of the CoNS isolates are spaced at distances of >5% which is indicative of a relatively low degree of isolate and species-specific similarity.

One hundred and eight (46%) of the 233 CoNS isolates tested were observed to be dissimilar as determined by their distance from each other on the plot. The colour-coding in the scatterplot exhibiting a high level view of clustering also highlights a degree of species-specific similarity within CoNS isolates from both patient groups. The results of 2D PCA analysis of rep-PCR fingerprint of CoNS species-specific isolates from both patient groups

that have a higher direct percentage similarity to each other are represented by the close grouping within three clusters in the plot. This plot also highlights the close relatedness observed in the clusters between and within different CoNS species from both patient groups. The same species-specific isolates in comparison when represented on the dendrogram (Appendix 1) displayed a level of strain similarity of between >70% and >90% within the nine defined clusters.

3.4.1.2.1 Definitive analysis of rep-PCR fingerprint profiles using a randomly selected smaller data set of in-patient CoNS isolates

Further data analysis to explore strain similarity on a more defined scale from the large amount of rep-PCR fingerprint data generated, was carried out on a randomly selected number of CoNS isolates. Analysis was limited to up to 50 isolates at any one time due to data handling constraints of the DiversiLab analytical tool as specified by the manufacturer.

Analysis of the rep-PCR fingerprint data, refined by selecting a random sample of 44 CoNS isolates from the in-patient group, reveal a high degree of species heterogeneity between some isolates of the same species. However, with a few exceptions, rep-PCR profiles also reveal closely relatedness of the same CoNS species-specific isolates grouping well within specified clusters as shown in dendrogram figure 3-5.

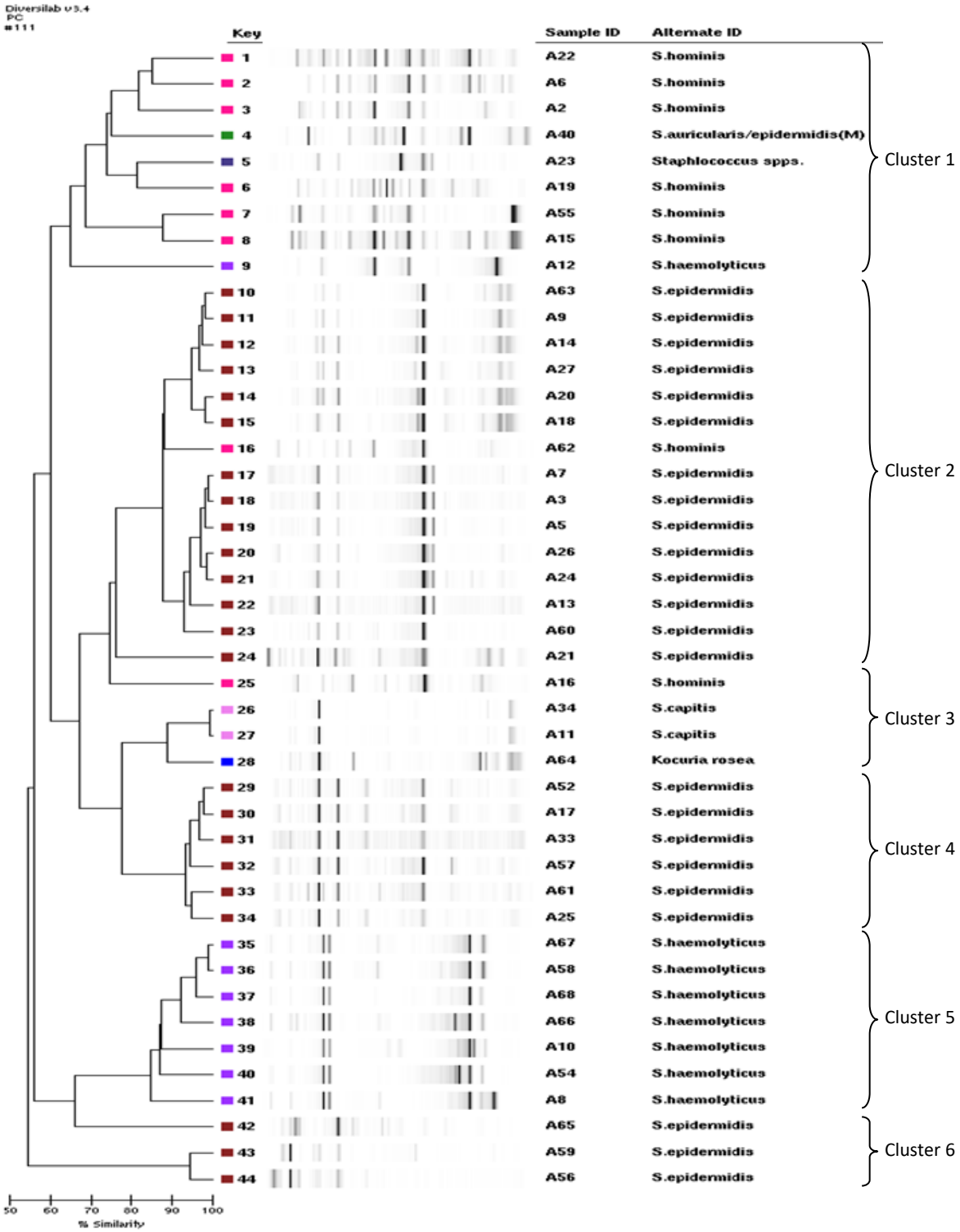


Figure 3-5: Dendrogram illustrates the rep-PCR electrophoresis profiles of a collection of 44 CoNS species-specific isolates from the in-patient group. The scale bar below the dendrogram represents percentage similarity with the cut-off value set at >95%.

Isolates of *S. hominis* represented in cluster 1 all at a similarity index of <80% included two other *Staphylococcus* species strain displaying the same level of similarity. However, one strain of *S. hominis* isolate A62 appeared to be closely related (similarity index <90%) to all the other strains of *S. epidermidis* represented in cluster 2 displaying similarity of >95%. This may have been due to the misclassification of phenotypic identity of the *S. hominis* strain as biochemically expressed traits are remarkably similar to those of *S. epidermidis*.

Indistinguishable strains of *S. epidermidis* isolates A7 and A3 were closely related to isolate A5 (>95% similarity) as were the two other indistinguishable isolates A26 and A24 present in cluster 2. However there was a relatively higher degree of strain diversity displayed between the other strains of *S. epidermidis* isolates in clusters 2, 4 and 6. Conversely, although strains of *S. haemolyticus* grouped (cluster 5) well together, the difference in strain similarity was higher at less than 90% similarity with the exception of the indistinguishable strain isolates A67 and A58. These results were reflective of the overall rep-PCR dendrogram based data analysis.

3.4.1.2.2 rep-PCR analysis using the Similarity Matrix

Further comparisons of rep-PCR fingerprint profiles of the 44 in-patient isolates to assess the percentage similarities between two individual isolates of the same CoNS species instead of averages between clusters are presented in the similarity matrix plot Figure 3-6.

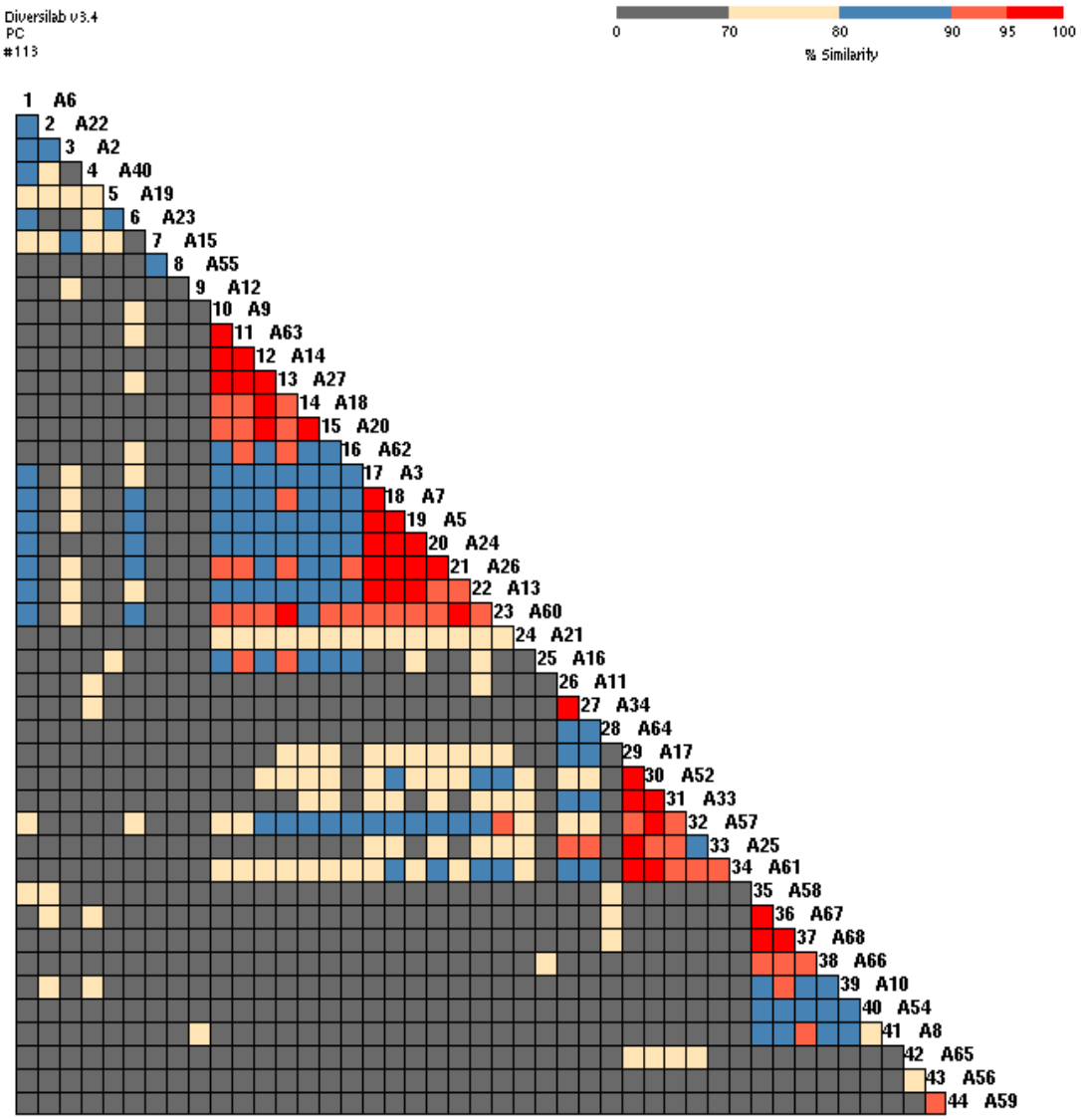


Figure 3-6: Similarity matrix analysis of the 44 in-patient CoNS isolates to determine pair-wise similarities. Level of similarity is reflected by the coloured percentage similarity scale at the top. The triage of red boxes (95-100% similarity) indicates isolate clusters.

For individual CoNS strain comparisons, the similarity matrix of the 44 CoNS isolates represented in the same order as the dendrogram Figure 3-5 show four clusters consisting of isolates demonstrating >95% pair-wise similarity. The lack of significant clusters (red boxes) further demonstrated CoNS species heterogeneity of the 44 isolates analysed indicated by the predominant grey area (<70% similarity) of the similarity matrix.

3.4.1.2.3. Analysis using a randomly selected smaller data set for comparison of CoNS species-specific isolate similarities from both patient groups

Forty eight CoNS isolates from both patient groups, representing well resolved rep-PCR fingerprints were randomly selected for further comparative analysis of similarity. Analysis data included ten isolates each of *S. epidermidis*, *S. hominis, S. haemolyticus* and four isolates each of the other CoNS species from each of the two patient groups.

Dendrogram analysis of rep-PCR profiles comparing the distribution of CoNS isolates from the in-patient and control patient group demonstrate clustering of the same species within each cluster with few exceptions as illustrated in Figure 3-7.

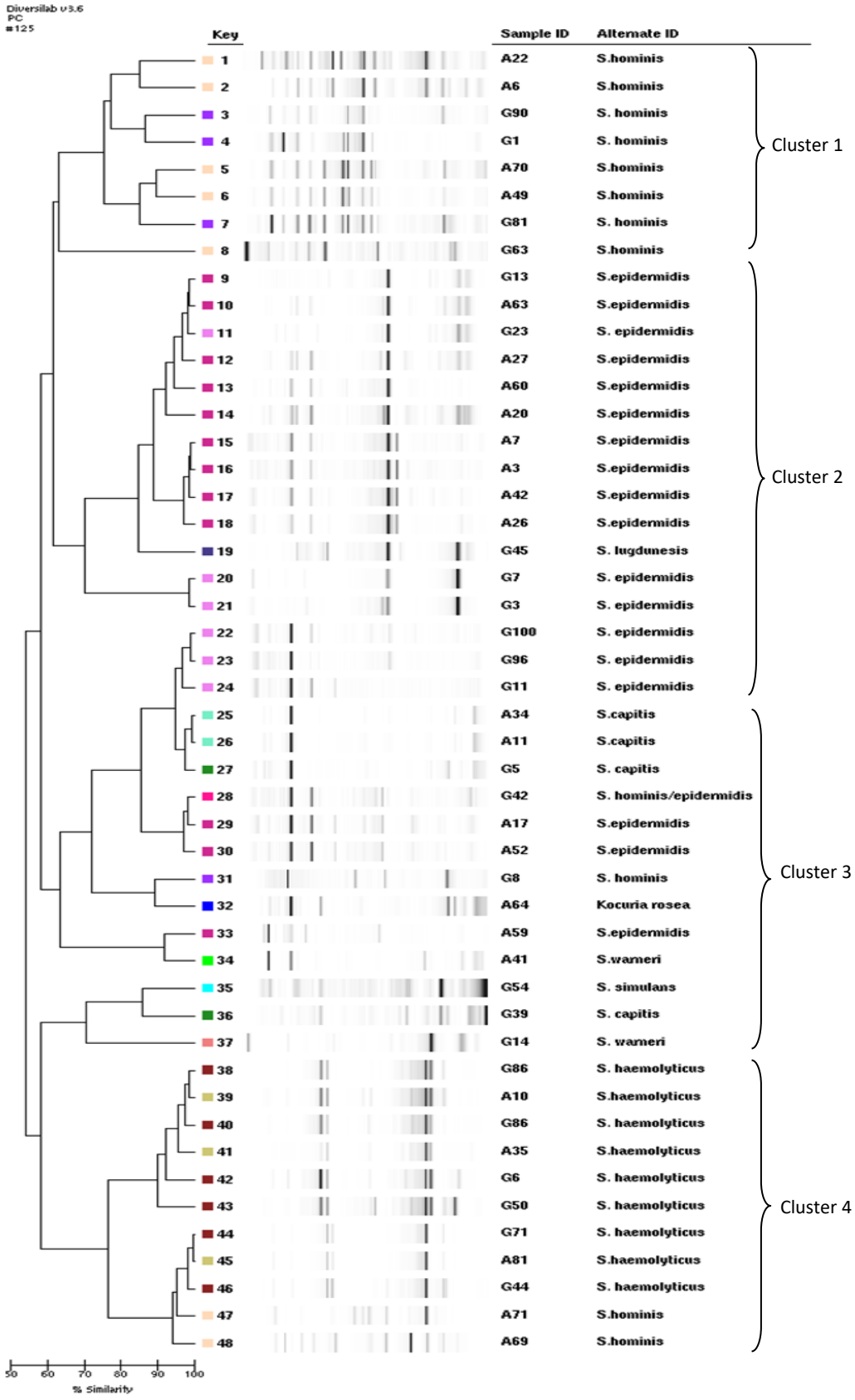


Figure 3-7: Dendrogram illustrates the comparative analysis of rep-PCR profiles of 48 CoNS species-specific isolates recovered from the two patient groups (Similarity: >95%).

With reference to Figure 3-7, comparing isolates from both patient groups, species-specific diversity was most notable between isolates of *S. hominis* at a similarity index of <90% (cluster 1). *S. epidermidis* isolates, A7 and A3 (cluster 2) demonstrated 100% similarity and isolate A42 demonstrated close relatedness to these two isolates at a similarity of > 95%. Similarly, *S. epidermidis* isolates G100 and G96 from the control patient group demonstrated clonal relatedness. Similarity of 100% was also observed between two in-patient isolates of *S. capitis* A34 and A11. These two clonally related isolates however, also demonstrated similarity at >95% with a control patient group isolate G5. Conversely, clonal relatedness between species-specific isolates from the two patient groups was only observed among *S. haemolyticus* isolates G86 and A10 as well as G71 and A81 (cluster 4).

Overall comparative profile analysis of isolates recovered from the two patient groups indicates that clonal relatedness is more common within species-specific CoNS isolates. Conversely, there was poor correlation of clonal relatedness observed between similar CoNS species-specific isolates from the two different patient groups. Clonal similarity at 100% between species-specific isolates was observed in 8% of the total CoNS recovered from the two patient groups. The differences in pair-wise similarity were further demonstrated in the scatterplot of the 48 CoNS isolates recovered from both patient groups (Figure 3-8).

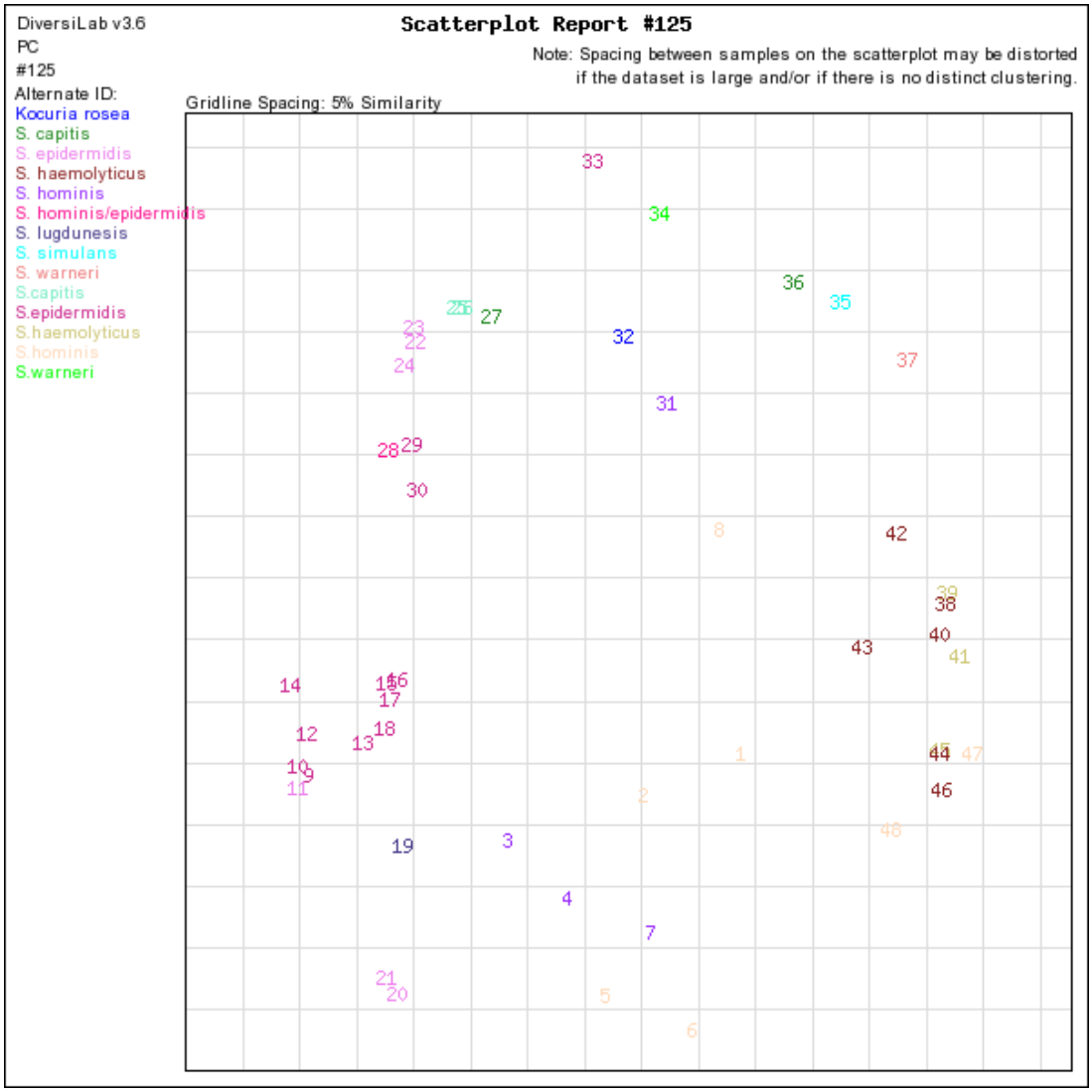


Figure 3-8: Scatterplot summary of 2D PCA rep-PCR fingerprint comparative analysis of the 48 CoNS species-specific isolates from both the patient groups. Colour-coded data clustering indicates degree of similarity between each CoNS species.

The comparative result analysis of the 2D PCA rep-PCR fingerprint plot of isolates from the two patient groups demonstrates a high degree of dissimilarity with no evidence of clustering. The colour-coded data clustering that indicates the degree of isolate similarity between each patient group, species-specific CoNS show very few isolates exhibiting >5% similarity. Less than half of the 48 CoNS isolates (43%) from the two patient groups exhibited similarity between each of the same species-specific isolates.

Overall rep-PCR data analysis results indicate that the degree of strain relatedness was relatively high within the same species-specific CoNS isolates from the two patient groups as observed in the dendrogram analysis (Figure 3-7). Conversely, the degree of similarity exhibited between the same species-specific isolates was comparatively much lower indicating a high level of diversity within and between the same CoNS species-specific isolates (Figure 3-8).

3.4.2 PFGE Data analysis

3.4.2.1 SmaI restriction generated DNA-band profile data analysis

The number and sizes of electrophoretically separated DNA profiles generated by *Sma*I restriction endonuclease digestion of the 50 isolates of the three predominantly isolated CoNS species varied dependent on species-specific type. Isolates of *S. hominis* from both patient groups yielded DNA-band profiles varying from between five to fifteen bands ranging from 50 to 1018 Kb (Figure 3-9). The DNA-band profiles of *S. haemolyticus* isolates from both patient groups varied from between six to ten bands ranging from 50 to 533Kb. *S. haemolyticus* in-patient isolates A53 and A81 together with control patient isolates G4 and G81 that were poorly resolved in the first PFGE gel were re-run giving a better band resolution in the second gel (Figure 3-9 Lanes 2a – 5a). The in-patient *S. hominis* isolate A55 remained unresolved yielding no band profiles due to the indigestible nature of its DNA (Figure 3-9 Lanes 3 and 1a)

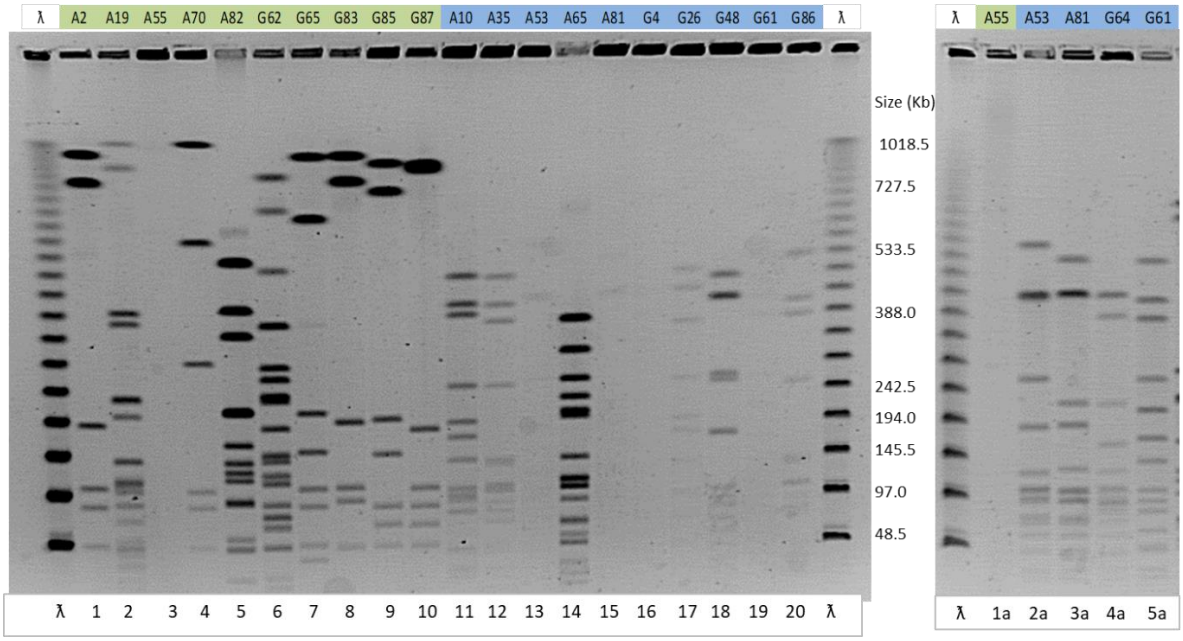


Figure 3-9: PFGE after *Sma*I digestion of *S. hominis* and *S. haemolyticus* isolates from the in-patient and the control patient groups. Lane marked λ= molecular weight ladder. Numbers to the middle indicate molecular size standard (kilobases). Lanes 1-5 represents in-patient *S. hominis* isolates A2, A19, A55, A70 and A82. Lanes 6-10 represent *S. hominis* control patient group isolates G62, G65, G83, G85 and G87. Lanes 11-15 represent in-patient *S. haemolyticus* isolates A10, A35, A53, A65, A81 and Lanes 16-20 represent control patient group *S. haemolyticus* isolates G4, G26, G48, G61, G86. Repeat PFGE gel run: Lane 1a = in-patient *S. hominis* isolates A55 (undigested). In-patient *S. haemolyticus* isolates Lane 2a = A53, Lane 3a = A81, and control patient group *S. haemolyticus* isolates Lane 4a = G4, Lane 5a = G61.

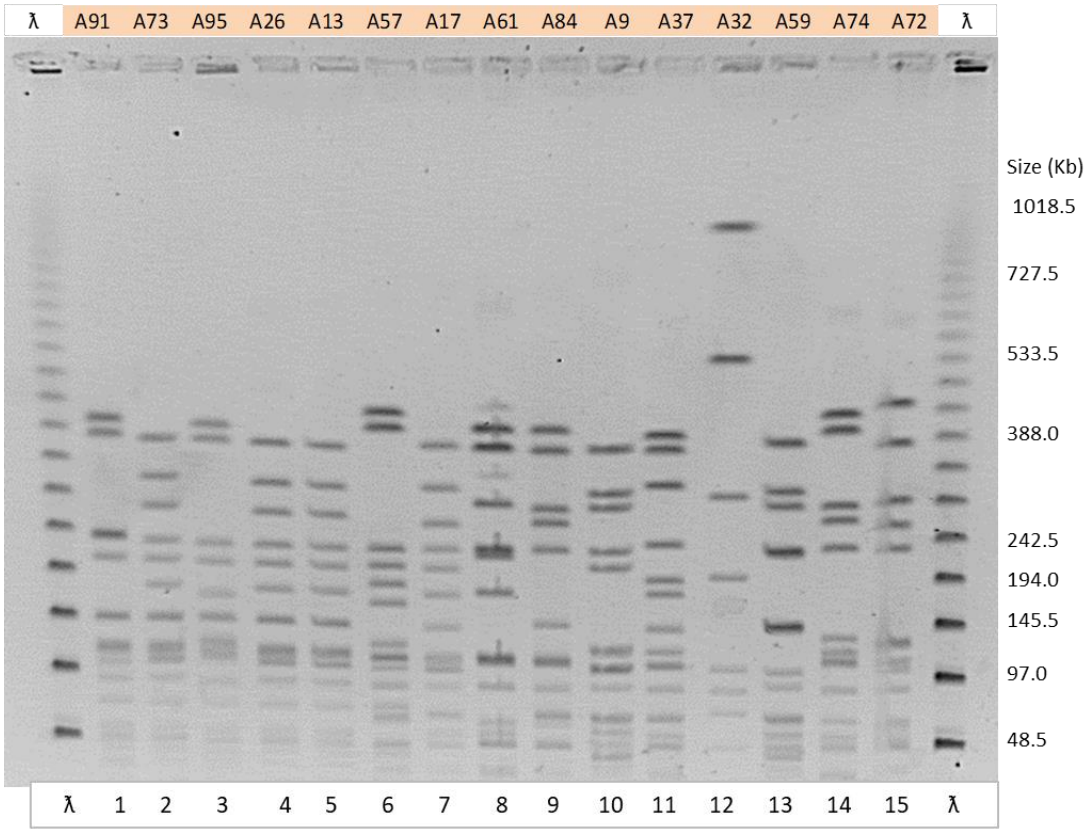


Figure 3-10: PFGE generated banding pattern based DNA fingerprints of *S. epidermidis* isolates from the in-patient and the control patient groups. Lane marked λ= molecular weight ladder. Numbers to the right indicate molecular sizes (kilobases). Lanes 1-10 represent, in-patient isolates A91, A73, A95, A26, A13, A57, A17, A61, and A9. Lanes 11-15 represent control patient isolates G69, G70, G75, G96 and G100.

Similarly, the *Sma*I restriction DNA-profiles generated for the *S. epidermidis* isolates from patient groups yielded banding patterns varying in number and size. The number of bands yielded varied from eight to a maximum of fifteen bands ranging from 50 to 1000kb (Figure 3-10 and 3-11).

The majority of *S. epidermidis* isolates yielded up to fifteen closely positioned DNA-band profiles in the range of 50kb to 485kb. The exceptions to this were the two in-patient and two control patient group isolates A32, A37 and G9, G70 respectively (Figure 3-10, Lane 4,11 12 and Figure 3-11, lane 12). These four isolates of *S. epidermidis* displayed banding patterns varying in number up to a range of 1000kb.

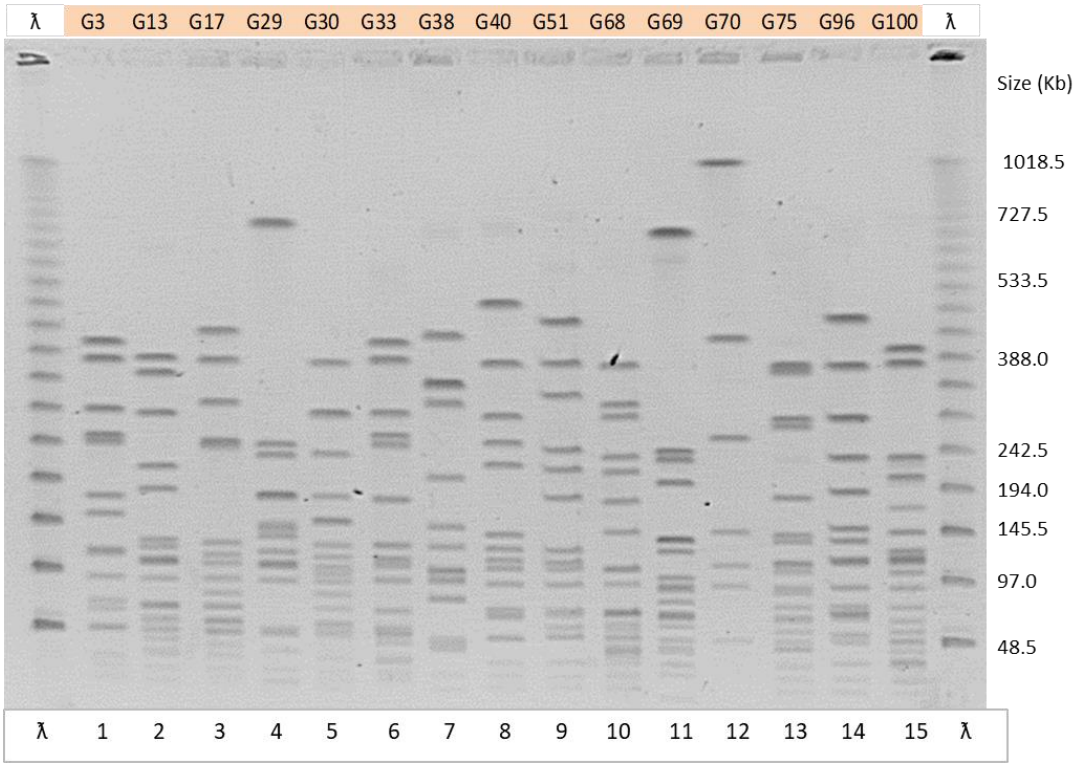


Figure 3-11: PFGE generated banding pattern based DNA fingerprints of *S. epidermidis* isolates from the in-patient and the control patient groups. Lane marked λ= molecular weight ladder. Numbers to the right indicate molecular sizes (kilobases). Lanes 1-10 represent control patient isolates G3, G13, G17, G29, G30, G33, G38 G40, G51 and G68. Lanes 11-15 represent in-patient isolates A37, A32, A59 A74 and A72.

3.4.2.2 Interpretation of DNA restriction patterns produced by PFGE

The banding patterns of the 50 isolates of the predominant CoNS species subjected to PFGE were analysed and categorised in accordance with the criteria established by Tenover and colleagues, (1995) to identify clones and strain similarity.

Each unique electrophoretic DNA- banding pattern fingerprint subjected to visual inspection was defined as a pulsotype for interpretation and comparison. For each of the CoNS species analysed, the resulting band pattern pulsotypes were designated alphabetically with letters A onwards and each closely related band pattern or subtype was given a number in relation to the specified pulsotype (Table 3-2).

In-patient Group	
S. epidermidis	**Pulsotype**
A91	A
A73	B
A95	A-1
A26	B
A13	B
A57	C
A17	B-1
A61	D
A84	E
A9	F
A37	G
A32	H
A59	I
A74	J
A72	A-1

Control Patient Group	
S. epidermidis	**Pulsotype**
G3	K
G13	L
G17	M
G29	N
G30	O
G33	P
G38	Q
G40	R
G51	S
G68	T
G69	U
G70	V
G75	W
G96	X
G100	R-1

In-patient Group	
S. hominis	**Pulsotype**
A2	A
A19	B
A55	Undigested
A70	C
A82	D

Control Patient Group	
S. hominis	**Pulsotype**
G62	E
G65	F
G83	A
G85	G
G87	H

In-patient Group	
S. haemolyticus	**Pulsotype**
A10	A
A35	B
A53	C
A65	D
A81	E

Control Patient Group	
S. haemolyticus	**Pulsotype**
G4	F
G26	G
G48	H
G61	I
G86	B-1

Table 3-2: The number and sizes of electrophoretically separated DNA *Sma*I digested fragments of 50 CoNS species-specific isolates from both patient groups assessed by visual inspection were assigned Pulsotypes in accordance with the Tenover criteria for strain characterisation.

Application of the designated comprehensive criteria for standardised interpretation of PFGE profiles used for clonal similarity of the 50 CoNS isolates recovered from both patient groups revealed multiple species-specific isolate profiles. Out of the 50 isolates tested only four (8%) similar isolate sub-types were identified. The large number of different pulsotypes identified in both patient groups indicates a wide genotypic heterogeneity among all the isolates of the three predominant CoNS species.

Considering *S. epidermidis* isolates from both patient groups 27 different PFGE pulsotypes were assigned among the 30 isolates studied (Table 3-2). Prevalent PFGE clonal pulsotypes (A to W) common to the two patient groups were not identified. However, the numbers of similar and closely related strains were predominantly observed only in the in-patient *S. epidermidis* isolates in comparison to the control patient group isolates. In accordance with the Tenover criteria for strain similarity, the three (10%) *S. epidermidis* in-patient Isolates A73, A26 and A13 assigned as pulsotype B strains were considered indistinguishable as these isolates displayed identical banding patterns. Only one (3%) of the in-patient isolate A17 pulsotype sub-type B-1 was observed to be closely related with less than three band difference to the pulsotype B isolate. Similarly, two (7%) *S. epidermidis* sub-type A95 and A72, in-patient isolates assigned pulsotype A-1 were observed to be closely related to isolates A91 pulsotype A.

Among the control patient group all 15 (100%) *S. epidermidis* isolates tested from this group were assigned different PFGE pulsotypes representative of multiple genetic variants. However, sub-type isolate G40 was closely related to G100 with a three band difference observed. Overall analysis of restriction DNA-band pulsotypes indicates that there was no genetic similarity between in-patient and control group *S. epidermidis* isolates. Conversely one in-patient *S. hominis* isolate A2 (Pulsotype A) exhibited 100% clonal similarity to control patient group isolate G83. Close clonal relatedness was likewise observed in one in-patient *S. haemolyticus* isolate A35 (Pulsotype B) with control patient isolate G86 (Pulsotype B-1).

3.4.2.3 UPGMA dendrogram analysis of PFGE profiles

The level of genetic similarity between each of the three predominant CoNS species isolates from both patient groups was determined by means of UPGMA dendrogram analysis of PFGE profiles. Multiple genotypes were identified within each of the 30 isolates of *S. epidermidis* and the ten isolates each of *S. hominis* and *S. haemolyticus* from the two patient groups.

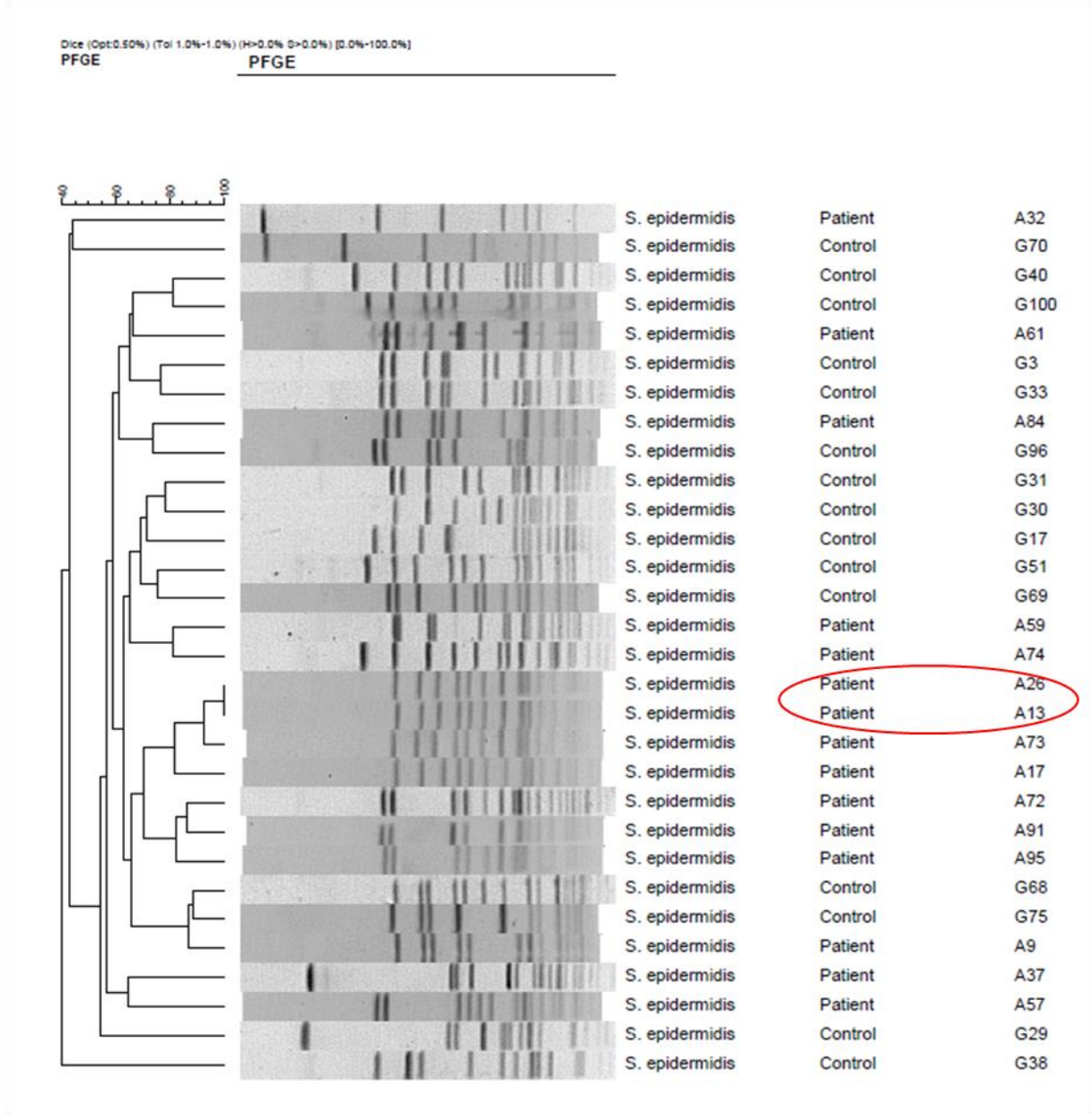


Figure 3-12: Dendrogram (UPGMA cluster analysis based on Dice correlation coefficient) comparison of PFGE DNA-band profiles of the 30 *S. epidermidis* isolates of the predominant CoNS species from both the in-patient and control patient groups. The scale bar at the top represents percentage similarity with the cut-off value set at >90%. Both isolates A26 and A13 show 100% similarity.

Comparative genotypic characterisation based on PCA analysis of PFGE DNA-band profiles of *S. epidermidis* isolates from the two patient groups revealed a significant lack of association between and within isolates (Figure 3-12). The majority of *S. epidermidis* isolates from both patient groups demonstrated similarity of between 60 and 80% with the exception of three in-patient isolates exhibiting >90% similarity. The in-patient, *S. epidermidis* isolates A26 and A13 displayed indistinguishable DNA fingerprints thus

considered to be clonally related (same isolate). The genetic fingerprint of isolate A73 differing by less than a three band difference (>95% similarity) was considered to be closely related to the clonal isolates A26 and A13. Comparing the PFGE profile results by means of UPGMA analysis with those obtained with the visual interpretation of banding patterns (Table 3-2) indicated clonal similarity between all three isolates A26, A13 and A73 (Pulsotype B). Sub-type A17 (Pulsotype B-1) was closely related however UPGMA dendrogram analysis revealed that this isolate was not closely related (<90% similarity) to any of the three Pulsotype B-*S. epidermidis* isolates. Likewise, isolate A91 (Pulsotype A) revealed close relatedness to isolate sub-types A95 and A72 (Pulsotype A-1) however the dendrogram based analysis revealed a genetic similarity of <90% between these three isolates.

Higher variations in genetic similarities and differences were also observed within and between in-patient and control patient group isolates of *S. haemolyticus* (Figure 3-13).

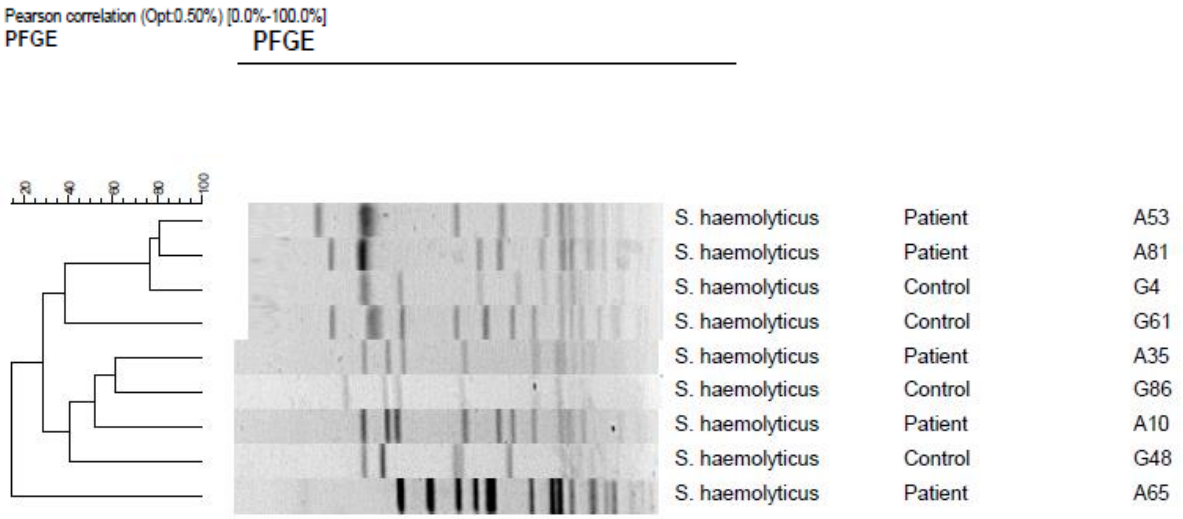


Figure 3-13: UPGMA dendrogram and band-based Dice analysis and comparison of PFGE profiles among *S. haemolyticus* isolates from both in-patient and control patient groups. The scale bar at the top representing percentage similarity with the cut of value set at >90%.

The dendrogram analysis of *S. haemolyticus* isolates from the two patient groups identified two in-patient isolates, A53 and A81 at a genetic similarity of 80% with the rest of the isolates (70%) displaying genotypes at <60% similarity. In comparison, the visual band-pattern analysis results showed control patient groups *S. haemolyticus* G86 (Pulsotype B-1) was closely related to in-patient isolate A35 (Pulsotype B). However, genetic similarity between these two isolates was identified at 60% as determined by means of UPGMA dendrogram analysis.

Multiple genotype strains were also identified amongst strains of *S. hominis* from the two patient groups (Figure 3-14).

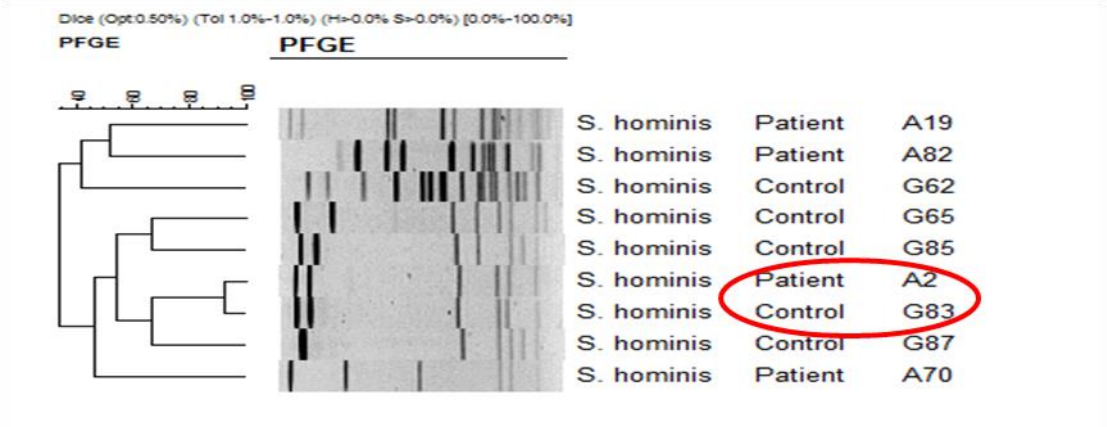


Figure 3-14: UPGMA dendrogram and band-based Dice analysis and comparison of PFGE profiles among *S. hominis* isolates from both in-patient and control patient groups. The scale bar at the top representing percentage similarity with the cut of value set at >90%.

The in-patient *S. hominis* isolate A2 was closely related to control patient isolate G83 at >90% similarity however, visual band analysis results indicated that these two strains were clonally related (Pulsotype A). In comparison distinct genotypes were identified in *S. hominis* isolates from both patient groups at a genetic similarity of <70%.

Overall analysis of PFGE generated fingerprint profiles demonstrated the presence of genotypically divergent isolates within the same CoNS specie-specific isolates recovered from the two patient groups.

3.5 Discussion

Bloodstream infections due to CoNS are the most common cause of bacterial nosocomial infections associated with high rates of morbidity in patients with haematological disorders (Worth *et al*., 2009). Epidemiological studies of infection caused by CoNS species are complicated by the fact that these organisms are also ubiquitous commensals of the healthy human skin and mucosa (Costa *et al*., 2004). However, the predominant skin colonising CoNS species, particularly *S. epidermidis, S. hominis* and *S. haemolyticus* have emerged as opportunist pathogens associated with clinically significant bacteraemia in those patients with intravascular devices and underlying immunocompromised states. These opportunistic infections are usually associated with species-specific strains possessing determinants which facilitate their survival on skin and device surfaces (Sloos *et al*., 2000; Coello *et al*., 2003). Establishing the source of CoNS related bacteraemia in the immunocompromised host is therefore critical in the prevention and management of infection (Coello *et al*., 2003).

Continuing progress in genomic and molecular epidemiology has provided an interesting insight into the flexibility of skin colonising species of CoNS generating novel genotypic variants associated with opportunistic infections in the immunocompromised host (Schoenfelder *et al*., 2010). To establish the link between potential sources and clinical infection, molecular techniques are now routinely employed for definitive genotypic differentiation of species-specific strains of CoNS isolates. The detection of these pathogens usually focuses on single isolate types present in both epidemic and endemic settings as species-specific knowledge is required to assess the impact on transmission and infection from the implicated sources (Carretto *et al*., 2005). Molecular based techniques are highly sensitive and specific and have been shown to perform better than conventional phenotypic methodologies for isolate delineation. The results of these molecular based diagnostic assays can be a key factor in making medical decisions not only for epidemiological studies but more importantly for targeted patient therapy (Galar *et al*., 2012).

The primary objective of this part of the study was to determine the genotypic differences or similarities between bacteraemic in-patient CoNS isolates and those skin colonising strains that could potentially be regarded as inducers of sepsis in the immunocompromised host. In this study rep-PCR and PFGE were the two genotypic methods used to compare the possible genetic relationship between the CoNS isolates from bacteraemic patients with those that are normal skin-colonising strains. The requirement of the molecular methodology employed for isolate typing in this study was based on the ability of the techniques to group genetically related isolates and to differentiate those that were unrelated. Both the genotypic methods provided high discriminatory power, using cut-off values for acceptable levels of similarity whereby indistinguishable isolates could be differentiated by their genetic fingerprints. These two genotypic methods used for typing permitted the delineation of CoNS isolates for characteristic comparison whilst assessing the measure of similarity.

The rep-PCR DiversiLab® system used in this study is an alternative molecular typing technique not routinely used in clinical diagnostic laboratories. The system has however been evaluated for various Gram-negative and Gram-positive associated hospital outbreaks (Tenover *et al*., 2009; Hurmic *et al*., 2014). The system was evaluated for vancomycin resistant *Enterococcus* species, *Pseudomonas aeruginosa* and *Acinetobacter* species outbreaks and transmission between patients at the Queen Elizabeth Hospital and was kindly loaned out for characterisation of CoNS isolates for this part of the study.

The application of the rep-PCR for CoNS strain typing relies on amplification of the non-coding intergenic (highly conserved) repetitive sequences interspersed throughout the genome using specific primers targeting these regions (Healy *et al*., 2005). The amplification

process therefore created multiple DNA fragments varying in size, generating fingerprints reflecting the potential genetic differences or similarity to be studied for each of the CoNS isolates from the two patient groups.

The DiversiLab software also permitted efficient analysis of the large amount of rep-PCR fingerprint data generated for the 174 CoNS species-specific isolates from both patient groups. Of the 233 isolates, 59 were re-evaluated to obtain well resolved rep-PCR fingerprints. These were analysed for similarity comparisons, shown as a dendrogram (Appendix 1) which demonstrated that isolates from both patient groups generally clustered well by species at >95% similarity. The results of this complex data analysis further revealed the genetic diversity among and between isolates of the same CoNS species from the two patient groups. It is evident from the results of this study that the rep-PCR molecular typing method has a relatively high discriminatory power in determining isolate delineation and clonal relatedness. Of these, most species-specific CoNS isolates grouped together in defined clusters. However, results also showed that some CoNS species displayed genotypic fingerprint profiles that were similar to those of a different CoNS species in some cluster groups.

CoNS identification to the species-specific level was based on their biochemical profiles using the VITEK®2 system and the phenotypic identities were additionally used for further genotypic characterisation. Therefore, it is highly likely that the genotypically similar isolates observed in this analysis may have been inaccurately identified as different CoNS species-specific biotypes. These results are concomitant with previous studies highlighting the limitations of routinely used phenotypic methodologies in comparison to molecular methods for species identification and differentiation (Cunha *et al*., 2004; Metha *et al*., 2009). Such differences were mainly observed among isolates of *S. hominis* clustering predominantly within the *S. epidermidis* isolate clusters. Previous studies have also demonstrated the high probability of misclassification of *S. hominis* isolates as *S. epidermidis* because the phenotypic differential between this species is based on a single biochemical reaction difference (Logozzi *et al*., 2004). Such discrepancies with regards to false negative results for acid production from lactose with the absence of phosphatase activity in *S. hominis* have been demonstrated in previous studies evaluating the VITEK®2 GP-card for CoNS species identification (Bannerman *et al*., 1993; Spanu *et al*., 2003). The rep-PCR typing technique, although not used for species identification appears to be more discriminatory based on genotypic profiles among the isolates that are misclassified by their phenotypic profile. These isolates characteristically clustered together at a similarity cut-off value of >95%. In this study, some isolates of *S. hominis* based on VITEK®2 biochemical analysis were identified as *S. epidermidis* using the MALDI-TOF MS system (Chapter 2). Interestingly, it

was noted that if the MALDI-TOF MS species identification results were uploaded for rep-PCR data analysis instead of the biochemical based identification, these strains would cluster together within species-specific group similarity of >95%. These results confirm a comparative study by Loonen and colleagues (2012) using proteomic methodology for identification of CoNS verifying the accuracy of MALDI-TOF MS for CoNS species identification. As such, in the dendrogram analysis (Appendix 1 cluster 4) genotypic rep-PCR profile of *S. hominis* control isolate G42 was closely related to the in-patient *S. epidermidis* isolate A17. Likewise, *S. hominis* control isolates G88 and G22 identified as *S. epidermidis* species by MALDI-TOF MS system would therefore demonstrate clonal relatedness to *S. epidermidis* isolate A46 and A9. It is therefore evident from the genotypic rep-PCR fingerprint profiles comparison with that of MALDI-TOF MS analysis that these isolates are highly likely to be closely related to the same CoNS species-specific isolates. The DiversiLab system could therefore be used not only for CoNS typing but also facilitate species-specific identification. Studies based on molecular assays for *Staphylococcus* species identification have demonstrated greater accuracy in comparison to routinely used standard phenotypic based methodologies (Mehta *et al*., 2009). This correlates well with the degree of reproducibility observed with this genotyping technique as demonstrated with in-patient strains A97, A52, A84, A59, A39, A67, A8 and A83. These strains when re-run more than once displayed 100% similarity as did control patient isolates G35, G44, G45, G48, G83 and G79 (Healy *et al*., 2005).

Importantly, some limitations of this rep-PCR technique for species-specific isolate differentiation were also noted in this study. The rep-PCR fingerprint analysis results for some CoNS isolates that were re-run either due to poor resolution or to complete the 13 wells of the same DNA LabChip did not exhibit 100% similarity. *S. capitis* control patient isolate G37 and the in-patient *S. epidermidis* isolate A85 amongst a few others displayed less than 95% similarity on repeat analysis, indicating that these species-specific isolates were not indistinguishable. More importantly, G45, the same *S. lugdunensis* control patient isolate re-run twice displayed <90% similarity, suggesting that this isolate would have been classified as different species-specific genotype. In the hospital setting this observation would provide a diagnostic dilemma as the isolate typing result would potentially lead to the mistaken assumption that these dissimilar isolates were not part of an outbreak investigation. Such apparent aberrant results impact directly on early management of suspected outbreaks and patient treatment, particularly in situations involving infections with more virulent isolates of CoNS such as *S. lugdunensis* (Hellbacher *et al*., 2006). Elements of caution were also applied when analysing and interpreting the comprehensively large amount of rep-PCR data generated. The DiversiLab system application is a genotypic tool effectively used for

resolving sources of outbreaks where only a small number of isolates are involved for comparisons of genotypic similarity. Nonetheless, the large data set results obtained in this study correlated well with that displayed in the smaller defined analysis of up to 50 CoNS isolates. Overall results reveal that the degree of similarity exhibited between each isolate of the same species was less than the set defined cut-off value of 95% for isolate similarity. These results, therefore, provide confirmation of the high level of diversity existing within and between CoNS species-specific isolates from both patient groups as illustrated in the scatterplot analysis (Figures 3-4 and 3-8). Moreover, comparisons of CoNS species-specific isolates from the two patient groups, revealed a relatively low degree of clonal relatedness between and within the same CoNS species. It is therefore evident from the results of the rep-PCR data analysis that there were no significant correlations between the skin colonising strains and those CoNS isolates obtained from the bacteraemic in-patient group.

The rep-PCR based DiversiLab system in comparison to other molecular typing tools has had a limited application as a standard differential method for genotyping; restricted mainly to identification of small number of specified bacterial species commonly associated with hospital related outbreaks such as MRSA (Fluit *et al*., 2010; Sabat *et al*., 2013). Currently few studies have been published demonstrating the clinical utility of rep-PCR specifically for genotypic characterisation of CoNS isolates. Although molecular techniques are fast progressing toward DNA sequencing of highly variable genes, PFGE however remains the most widespread used tool for molecular typing. Hence, the PFGE technique was utilised to further verify the clonal relatedness of CoNS isolates recovered from the two patient groups in this study.

DNA fingerprinting by PFGE known to provide better strain discrimination compared to other molecular techniques, is however a both time consuming and costly technique requiring specialised technical skill (Widerström *et al*., 2012). Therefore, due to the cost and time constraints, 50 of the 100 isolates of the predominant CoNS species were subjected to PFGE analysis. Overall result analysis identified a limited degree of similarity between in-patient and the skin-colonising isolates regarded as potential inducers of sepsis in the haematology patient group.

Primary visual analysis of genomic DNA fingerprints based on specific band differences facilitated the comparisons between CoNS species-specific isolates from both patient groups. The visual analysis criteria followed was based on detecting differences in the intensities of specific bands. In addition, the Bionumeric software employed for analysis determined the relatedness of isolates by observing the absence or presence of comparative bands. Based on these criteria, visual analysis carried out with the aim of determining the

degree of clonal relatedness between isolates of the two patient groups revealed a high level of genotypic diversity within the three predominant CoNS species.

Based on the Tenover, (1995) criteria for classification of the PFGE fingerprint assessing band patterns revealed that clonal relatedness within the *S. epidermidis* isolates from the in-patient group was greater in comparison to those from the control patient group. Three of the in-patient *S. epidermidis* isolates classified as Pulsotype B (Table 3-2) were observed to be clonally related. The Pulsotype B isolates were observed to be genotypically similar therefore possibly derived from a common parent. However, researchers suggest that such isolate clonality can only be considered to be relative rather than absolute as PFGE fingerprinting does not have the potential for detecting cryptic genetic changes (Tenover *et al*., 2009). The observed results could be representative of either the possibility of cross-infection between patients on the haematology unit or isolation of the same isolate from a single patient's repeated blood cultures. Conversely, such PFGE band pattern similarities were not observed between the *S. epidermidis* isolates from the control patient group. These *S. epidermidis* isolates were seen to be genotypically distinct in comparison to in-patient isolates. The level of clonal diversity observed between and among *S. epidermidis* isolates from the two patient groups suggests that these skin-colonising isolates may not be inducers of bacteraemia in the in-patient group.

Conversely, visual band analysis revealed one identical *S. hominis* in-patient isolate to be another skin-colonising isolate from the control patient group. These two *S. hominis* isolates possibly sharing clonal ancestry, provide potential indication of sharing a common source. Distinct genotypic band patterns were displayed by *S. haemolyticus* isolates from both patient groups with the exception of one sub-type isolate from each patient group, demonstrating close genotypic relatedness.

Further comparison of PFGE band analysis data calculated by Dice coefficient (1% tolerance level) and represented by UPGMA clustering revealed a significant lack of association between isolates of *S. epidermidis*, *S. hominis* and *S. haemolyticus* from the two patient groups. Overall results, therefore, indicated a high degree of genotypic diversity within and between all CoNS species-specific isolates with the exception of two in-patient *S. epidermidis* strains A26 and A13 demonstrating 100% clonal relatedness (Figure 3-12). Interestingly however application of guidelines set by Tenover and colleagues (1995) for visual interpretation of PFGE generated chromosomal DNA restriction profiles, genetic similarity, classified as closely related, was also observed among four of the in-patient *S. epidermidis* isolates, A26, A13 and A73 and isolate A17. The greater discriminatory power of UPGMA in assessing strain relatedness in comparison to visual band profile assessment

could account for disparity in result interpretation and classification. The statistical protocol, widely used for performing cluster analysis for assessing strain relatedness readily identifies bands within complex genotypic profiles in comparison to assessing band profile visually (Caddick *et al*., 2005). Therefore, isolates A2 and G83 of *S. hominis*, each from the two different patient groups classified previously as clonally related, with UPGMA analysis were classified as similar and not indistinguishable. The closely-related classification between these two isolates is more likely due to the three band differences observed with UPGMA cluster analysis. Likewise, both in-patient and control patient group *S. haemolyticus* isolates were therefore categorised as distinct genotypes displaying as values at <60%, well below the acceptable cut-off value for isolate relatedness. Hence, molecular typing by PFGE in this study identified distinct genotypic isolates prevalent within and between all predominant species-specific CoNS from both patient groups. These results reflect observations made in previous studies demonstrating the presence of heterogeneous PFGE profiles between and within the same CoNS species-specific isolates (Lang *et al*., 1999; Casey *et al*., 2005).

Following data analysis, both the rep-PCR and PFGE molecular typing techniques proved to have the required discriminatory power needed to distinguish unrelated CoNS species-specific isolates. The main point of consideration when comparing the two typing methods was the difference in isolate clonal relatedness assessment. Such that, clonal relatedness through rep-PCR is defined as visualisation of 150-5000 base pairs, whereas PFGE thorough the digestion of the entire genome visualises 50,000 to 500,000 base pairs. These typing techniques subjective to different selective pressures therefore effect the defined cut-off values used for interpretative criteria for isolate relatedness (Fluit *et al*., 2010). The rep-PCR web-based DiversiLab software, however, facilitates comparative genotypic analysis of the large data set generated to assess clonal relatedness between all the CoNS isolates. Such generated rep-PCR fingerprints allowed comparative assessment of detailed genetic variability based on the difference in DNA sequences between and within groups of similar species-specific isolates (Healy *et al*., 2005; Shutt *et al*., 2005). Hence, result analysis comparing rep-PCR CoNS fingerprints highlighted the heterogenic nature of the CoNS isolates, as multiple genotypes were observed within CoNS species-specific isolates from both patient groups. These results were concordant with PFGE DNA band analysis but on a larger scale, whereby several different clusters of the CoNS species-species isolates comprised of similar diverse genotypes. Moreover, some clonal relatedness with rep-PCR fingerprint profiling was also observed between some species-specific isolates from each of the patient groups. More importantly, similar levels of clonal relatedness were however not apparent between in-patient and control patient group CoNS isolates with both typing techniques which demonstrated the presence of diverse genotypes.

It is evident from this study that the genotypically dissimilar CoNS isolates from bacteraemic patients and the skin colonisers are unlikely to be derived from common ancestral clones. Nonetheless, it can perhaps also be envisaged that these clones may have undergone random genetic alterations including point mutations and insertion and deletions of DNA during the course of infection hence resulting in distinct clonal types. Hence, investigation facilitated by the two molecular typing tools in this study exploring the clonal relatedness of CoNS isolates present on the skin as the potential source of bacteraemic infection, demonstrated the presence of clonally diverse isolates.

3.6 Conclusion

CoNS normally identified as skin commensals and their potential role as pathogens associated with nosocomial bacteraemia in patients with haematological malignancies are increasingly being recognised. Although endemic transmission of nosocomial pathogens in the hospital environment are often poorly defined, some epidemiological, clinical and molecular relatedness studies have supported the assumption that translocation of isolates from the skin may be associated with BSIs in the immunocompromised host. Moreover, recent literature indicates that genotypic methods providing highly accurate level of clonal delineation can be used to evidence the pathogenic potential of various CoNS species.

The two molecular typing techniques used in this study to distinguish and characterise both in-patient BSI isolates and skin-colonising isolates demonstrated a high degree of genotypic diversity. Prevalent genotypic diversity within and between the same CoNS species-specific isolates was more evident in isolates of *S. epidermidis, S. hominis and S. haemolyticus* recovered from both patient groups. However, a degree of clonal relatedness was evident within some of the same CoNS specie-specific isolates from the in-patient group. Conversely, there was little evidence of clonal relatedness between in-patient and control patient CoNS species-specific isolates which further underscores the heterogenic nature of these diverse variants. The apparent clonal diversity within and between species-specific isolates of CoNS provides some evidence that dissemination of skin colonisers in association with bacteraemia is limited and therefore, skin as the source of infection may be unlikely.

In summary, the lack of association between CoNS skin-colonising strains and those recovered from in-patient group supports the hypothesis, that skin-colonising strains are unlikely to be the primary source of CoNS-associated bacteraemia in the haematology and BMT patient group.

Chapter 4 Comparison of antibiotic resistance profiles of coagulase negative isolates (CoNS) recovered from blood cultures of patients with bacteraemia with skin-colonising strains isolated from the pre-admission patient group.

4.1 Introduction

Patients with haematological malignancies and those, undergoing allogeneic stem cell transplantation are at an increased risk of acquiring infections due to the profound impairment of their immune system. Due to the wide use of prophylactic antibiotics and indwelling venous catheters, CoNS have emerged as the most frequently blood-isolated pathogen associated with primary bacteraemia in many cancer centres (Collin *et al*., 2001; Wisplinghoff *et al*., 2003).

CoNS infections may be attributable to endogenous strains from patient's normal flora, the hospital environment or transmitted among hospitalised patient populations. Reports relating to the incidence of antimicrobial resistance associated with nosocomial CoNS in-patient infections in immunocompromised patients have increased dramatically (Becker *et al*., 2014). These have mainly been linked with CoNS strains possessing multiple antimicrobial resistance mechanisms in particular, resistance to methicillin (Huebner and Goldmann 1999; Henwood *et al*., 2000; Syndor and Perl, 2011). Methicillin, as an antibiotic of first choice against staphylococcal infections, poses a major problem, particularly in nosocomial strains of *S. epidermidis* demonstrating resistance rates of up to 75-90% (Otto, 2009). Therefore, for better prognosis, the recommended empirical therapeutic agents for such serious staphylococcal infections in most healthcare settings are the cyclic glycopeptides (Menchetti, 1992; Yamada *et al*., 2017).

Currently, of the glycopeptides, vancomycin remains the drug of choice for the treatment of BSIs caused by multidrug resistant CoNS (Diekema *et al*., 2001 Natoli *et al*., 2009). Until recently, there were few reported cases of vancomycin resistance in clinically important isolates of CoNS, however events associated with hospitalisation and increased vancomycin use are thought to be associated with emergence of resistance. Of the CoNS, glycopeptide resistance is the most commonly observed in isolates of *S. haemolyticus (*Veach *et al*., 1990; Biavasco *et al*., 2000; Tabe *et al*., 2001). Other species associated with emerging glycopeptide resistance include *S. epidermidis, S. warneri and S. hominis* (Ma *et al*., 2011). Additionally, heterogeneous forms of glycopeptide resistance may also be present in some CoNS isolates; however, due to the presence of specific resistance mechanisms, their detection, clinical and therapeutic relevance still remains challenging (Wong *et al*., 1999; Biavasco *et al*., 2000: D'mello *et al*., 2008).

Although the prevalence of glycopeptide resistance amongst clinical isolates of CoNS has shown an upward trend, their association with clinical outcome has nonetheless been infrequently reported, due mainly to the poor pharmacokinetic properties and dose optimisation of the drug (Tacconelli *et al*., 2001; Natoli *et al*., 2009; Ahlstrad *et al*., 2011). Increasing multidrug resistance among CoNS and the possible emergence of vancomycin resistant strains has led to the need for newer antimicrobials as alternatives agents to improve clinical outcomes and avoid the development and spread of antibiotic resistance (Harbarth *et al*., 2003; Falcone *et al*., 2015). Of the newer antimicrobial agents, daptomycin has been shown to have excellent activity against all species of CoNS, with few cases of reported resistance (Sakoulas *et al*., 2014). The proven clinical efficacy and safety of daptomycin with few adverse effects may therefore prove to be a favourable alternative for the treatment of serious CoNS infections in the immunocompromised host (Steenbergen *et al*., 2009; Chaftari *et al*., 2010; Sader and Jones, 2012).

As acquired bacteraemia in the haematology patient carries a poor prognosis, information regarding the susceptibility patterns of infective agents can therefore be invaluable in aiding selection of appropriate empirical regimens for therapeutic success. The introduction and use of new antibiotic classes also represents an important contribution to limiting resistance development and spread.

4.2 Aims of this study

(a) To assess the *in-vitro* antimicrobial susceptibility patterns of well-characterised CoNS isolates recovered from blood cultures of patients on the haematology and BMT unit in comparison to skin colonising strains from the pre-admission patient (control) group.

(b) To investigate the minimum inhibitory concentrations (MICs) of a panel of 18 antimicrobial agents against CoNS isolates from both the in-patient and control patient groups.

(c) To evaluate and compare the glycopeptide MICs of in-patient CoNS isolate MICs using two validated susceptibility test methodologies. The MICs for vancomycin and teicoplanin obtained with the automated VITEK®2 system were compared to the MICs obtained for the glycopeptides using the standard E-test gradient methodology.

(d) To evaluate the prevalence of reduced glycopeptide susceptibility (Intermediate/heteroresistance) amongst CoNS isolates recovered from bacteraemic patients using two different susceptibility testing techniques.

(e) To determine the MICs of in-patient CoNS isolates to the newer antimicrobial agents, including daptomycin, tigecycline and ceftaroline using standard E-tests.

4.3 Materials and Methods

One hundred bacteraemic isolates of CoNS together with 100 skin colonising CoNS isolates from the pre-admission patient group were tested for susceptibility using current standard laboratory protocols. All the CoNS isolates previously characterised by conventional biochemical tests using the VITEK®2 system (Chapter 2 section 2.4) saved on Microbank™ beads at -20°C were sub-cultured twice onto Columbia blood agar, incubated at 37°C hours in ambient atmosphere to recover viable colonies.

4.3.1 Antimicrobial susceptibility testing using the automated VITEK® 2 system

Automated antimicrobial susceptibility testing (AST) of the CoNS isolates, to a panel of 18 antimicrobials, was undertaken using the Gram-Positive AST P575 and the updated P578 (2012) susceptibility card (bioMérieux, Marcy l'Etoile, France). In accordance with the manufacturer's product literature, the updated AST-578 susceptibility card validated for routine use, includes an improvement in the formulation of two antibiotics, vancomycin and erythromycin. The MIC ranges of these two antibiotics were extended to allow greater accuracy of resistance detection.

A bacterial suspension of 0.5 McFarland standard, was prepared in 3ml sterile saline solution (aqueous 0.45 w/v NaCl, pH 7 bioMérieux, France) and used to load the test cards in accordance with the manufacturer's instructions. The bacterial suspension was further auto-diluted to give a final density of 1.5 x 10^7 CFU/mL after the AST cards were placed into the VITEK® 2 system. The cards were then automatically filled, sealed and inserted into the system incubator-reader module at an ambient temperature of 37°C in accordance with the manufacturers User-Manual instructions (VITEK®2 systems product information 410791). The VITEK®2 automated system susceptibility testing results were based on fluorescence measurements performed every 15 minutes for up to 18 hours and automatically analysed with the installed VITEK®2 software which is also interfaced with the laboratory informatic system. The updated Global European-based phenotypic EUCAST interpretative guidelines

were automatically applied in compliance with the manufacturers installed VITEK®2 software version 25; 5.04 (2011).

The panel of 18 antimicrobials contained in the VITEK®2 - AST P575 card included the β-lactams, aminoglycosides, macrolides, fluroquinolones, glycopeptides, tetracyclines, trimethoprim, fuscidic acid (Fucidin), mupirocin, chloramphenicol, and rifampicin. The concentrations of the tested antibiotics ranged from 0.125 to 32mg/L. The quality control strain *S. aureus* ATCC 29213 was tested concurrently with each batch analysis as recommended by the manufacturer's product protocol.

4.3.1.2 Data analysis of antibiogram patterns

Following analysis of the MICs determined by the VITEK®2 automated system susceptibility results of tested CoNS isolates were categorised and reported as susceptible, intermediate, or resistant based on the installed software EUCAST (2010) interpretative criteria. Primary antibiogram analysis was carried out based on resistance profiles.

Computer assisted analysis of antibiograms was further carried out by converting the antibiotic resistance patterns of CoNS isolates into TIFF file format. These files were imported into the BioNumeric software database as fingerprint types and processed by definition, of fingerprint lanes, optimisation of the densitometric curves and normalisation of resistance patterns (Gelcompar 2 version 5.1: Applied Maths, Belgium). Further data analysis of the antibiotic resistant fingerprint patterns was carried out using dice coefficient and UPGMA. Antimicrobial resistance patterns with a dice coefficient of ≥95% were assigned as similar types (StatSoft Inc, 2011).

4.3.2 Antimicrobial susceptibility testing using standard E-test methodology

For quantitative antimicrobial susceptibility testing the Epsilometer-test (E-test) antibiotic gradient strips (AB Bodisk, Solna, Sweden; AB bioMérieux, Marcy-l'Etoile, France) were used. These E-test strips at a predefined concentration gradient of antibiotic across 15 two-fold dilutions from 0.016 to 256mg/L, impregnated with vancomycin, teicoplanin daptomycin and tigecycline, were used to determine the MICs of the 100 CoNS isolates recovered from bacteraemic patients. Likewise, for ceftaroline MIC evaluation, the M.I.C.E (M.I.C.Evaluator™ Oxoid, UK), test strips at an antibiotic concentration gradient of 0.015 to 256mg/L were used.

An inoculum of 0.5 McFarland density-adjusted suspension, of colonies from pure culture was prepared for each CoNS test isolate in 0.45% w/v sterile saline solution equating to microbial concentration of $1.5x10^8$cfu/mL. In accordance with the recommended BSAC methodology (2012), a sterile swab was dipped into the prepared inoculum and rolled firmly against the side of the inoculum tube to remove excess inoculum. For each test isolate, the entire surface of a 90mm Mueller-Hinton sensitivity test agar (bioMérieux, France) plate was streaked evenly in a three-way direction, each time rotating the plate 60 degrees to achieve even distribution of the inoculum. The inoculum was then allowed to be absorbed into the agar for 10 minutes prior to applying the E-test strips. Two antibiotic E-test strips were used per sensitivity plate following the manufacturers recommended protocol (ETEST® Application Sheet EAS 300 bioMérieux SA Marcy-l'Etoile France 2011/06). The susceptibility plates were incubated at 37°C for 18 hours in ambient atmosphere. Vancomycin, teicoplanin, daptomycin and tigecycline MIC values were read at the interaction point of growth inhibition with the E-test gradient strip.

Ceftaroline sensitivity was tested using Mueller-Hinton plus 5% horse blood susceptibility agar (bioMérieux, France) as recommended by the manufacturer. The MIC values, with the M.I.C.E test strip were determined nearest the concentration value at the point above the growth/inhibition interaction.

Two control strains, *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 were concurrently tested with each test batch. The sensitivity test plates were incubated at 37°C for 18 hours in ambient atmosphere.

During the initial part of this study, BSAC susceptibility interpretive criteria were applied (Reference E-Test Application Sheets EAS 16029 D-2011/06: Staphylococci). Further to this, the updated, recommended EUCAST susceptibility interpretive criteria guidelines were applied for determination of MIC values (www.eucast.org). The VITEK®2 global automated susceptibility result interpretation rule base criteria for detecting and reporting vancomycin resistance for all staphylococcal species was determined at MIC >2mg/L in accordance with the implemented VITEK®2 software. Conversely, for further data analysis the breakpoint MIC value of >4mg/L for vancomycin was applied in accordance with the EUCAST (2010 v. 2.1) clinical breakpoints for all CoNS isolates.

4.3.2.1 Detection of heteroresistance using the modified E-test macro-method

The presence of heterogeneously glycopeptide-intermediate staphylococci (hGIS) among the 100 in-patient CoNS isolates was determined using the modified macro-method as previously described (Wootton *et al*., 2007).

The macro-method E-test was performed using a 2 McFarland standard turbidity suspension prepared in 0.5% w/v sterile saline solution equating to a bacterial concentration of 6 $x10^8$ cfu/mL. For each CoNS test isolate, a 200µL suspension was used to inoculate the surface of BHI agar (Brain Heart Infusion Agar; bioMérieux, France). The inoculum was spread in a three-way direction across the whole surface of the agar plate using a sterile swab. The inoculum was allowed to absorb into the agar for 15 minutes prior to applying the vancomycin and teicoplanin E-test strips in accordance with the manufacturers recommended protocol (ETEST® Application Guide 16273B-EN 2012). The inoculated BHI agar plates were incubated at 37°C for 24 hours in ambient atmosphere and MICs confirmed at 48 hours.

The vancomycin MIC values were read at the interaction of growth with the E-test strip, looking for slower growing sub-populations presenting as hazes of growth including the presence of both micro-colonies and smaller, isolated variant colonies. This procedure for observing presence of such colonies was carried out under oblique light aided by a magnifying glass. Similarly, teicoplanin MIC values were read at the interaction of growth with the E-test strip. EUCAST (2011) interpretative criteria for MIC breakpoints were applied for susceptibility categorisation.

The following control strains were included in each susceptibility batch test; *S. aureus* ATCC 29213 and the two glycopeptide resistant strains Mu50 ATCC 700699 (GISA) and Mu3 ATCC 700698 (hGISA). These control strains were obtained from the Bristol Centre for Antimicrobial Research and Evaluation, Southmead Hospital, Bristol, UK.

4.3.2.2 Detection of glycopeptide resistance and heteroresistance using the GRD E-test

The double sided, Glycopeptide Resistance Detection (GRD) E-test prototype is unavailable for clinical use but is currently available for research purposes only. These GRD E-tests are a potential screening method for the detection of the slow growing hGIS strains that also display a pronounced resistance to teicoplanin. In accordance with the manufacturer's recommendations, the GRD E-test strip with double sided vancomycin (VA) and teicoplanin (TP) with concentration gradients across seven two-fold dilutions of 0.5-32mg/L was tested with an inoculum of 0.5 McFarland standard. For each CoNS test isolate, 20µL of prepared suspension was used to inoculate Mueller-Hinton plus 5% horse blood susceptibility agar plates (bioMérieux, France). The inoculum was streaked evenly across each test plate and allowed to be absorbed for 15 minutes before applying the GRD E-test strip. Plates were

incubated at 37°C for 24 hours in ambient atmosphere and the results were read at 24 hours and confirmed after 48 hours to detect delayed resistance. The *S. aureus* ATCC 29213 control strain was set up concurrently with each test batch GRD E-test VA:TP with specified quality control ranges of 0.5-2mg/L and 0.5-4mg/L respectively (www.eucast.org). The two glycopeptide resistant control test strains Mu50 (GISA) and Mu3 (hGISA) were also included in each test batch.

Agreed interpretive standards are currently unavailable for heterogenous-intermediate resistance CoNS. Therefore, interpretation of the GRD results were used for CoNS in accordance with the manufacturers reading guidelines. The criteria for which are based on the previously described definitions for *S. aureus* as stated in their Research Use Only (RUO), 2012, instruction leaflet (Supplement GRD RUO, bioMérieux, France). The E-test MIC cut-off values were read at the point of complete inhibition of growth. The recommended protocol for the interpretative endpoints defined for the phenotypic detection of glycopeptide resistance with the E-test GRD strips for CoNS read at 24 and 48 hours were applied as follows: (a) E-test GRD plus VAN or TEI cut-off values of ≥16mg/L result would indicate GIS/hGIS; (b) Glycopeptide intermediate resistance result would be indicated with E-test GRD TEI or VAN MIC value of ≥16mg/L and a standard E-test VAN MIC value of ≥8mg/L (c) hGIS resistance result would be indicated with E-test GRD TEI or VAN MIC value of ≥16mg/l and a standard E-test VAN MIC of <8mg/L.

4.4 Statistical Analysis

Descriptive statistical analysis was performed using the existing software packages (STATISTICA version 10 and IBM® SPSS® Statistic version 24). Comparison of categorical variables was carried out using the Chi-square or Fisher exact test. The difference in the prevalence of each CoNS species antimicrobial resistance rates and between patient groups were analysed using the two-tailed Fisher's exact test and the non-parametric comparisons of antimicrobial susceptibilities were performed by Freemans Test. The Friedman test was used to determine the differences between the antibiotics, patient groups and the techniques used for evaluation of MICs. The difference in frequency of resistance distribution and glycopeptide MICs were analysed using the non-parametric two-tailed Mann-Witney U Test. Statistical significance were represented by p value of <0.05.

R version 3.5.3 (2019-03-11) -- "Great Truth" Copyright (C) 2019. The R Foundation for Statistical Computing Platform: i386-w64-mingw32/i386 (32-bit) was used to construct logistic regression models to compare the proportion of CoNS species inhibited as a function

of antibiotic concentration, the glycopeptide type, CoNS species type and the two test methodologies used. (Data analysis: courtesy of Biomedical Statistician - Dr Martyn Wilkinson)

Data analysis of the phenotypic susceptibility antibiograms of CoNS species isolate comparisons were carried out by calculations of dice coefficient and clustering was performed by UPGMA to construct dendrograms. Analysis was performed using the StatSoft, Inc. (2011) STATISTICA version 10 (USA) Software package. Gelcompar 2 version 5.1 (Applied Maths, Belgium).

4.5 Results

4.5.1 VITEK®2 automated susceptibility testing of CoNS isolates to a panel of 18 anti-staphylococcal agents

The VITEK®2 is the AST methodology system currently in-use in routine clinical microbiology. In total, phenotypic antibiotic resistance profiles were evaluated for the 100 CoNS isolates from both the in-patient and the pre-admission control patient group. These isolates were previously identified to the species level by VITEK®2 bio-analysis. In accordance with the EUCAST guidelines for VITEK®2 automated AST system, susceptibility to oxacillin was used to determine methicillin resistance. For the purposes of this study, the prescribed active isoxazole penicillin antimicrobial agent namely, flucloxacillin is the terminology also used to describe susceptibility to oxacillin (methicillin). Assessments based on the quantitative MIC results of each antimicrobial tested were categorised as susceptible, intermediate or resistant in compliance with the computer software installed global EUCAST (2010) susceptibility interpretive criteria guidelines.

Comparative analysis of the overall resistance profiles of the 100 CoNS isolates from both the in-patient and control patient groups to the panel of 18 anti-staphylococcal agents tested are illustrated in Figure 4-1.

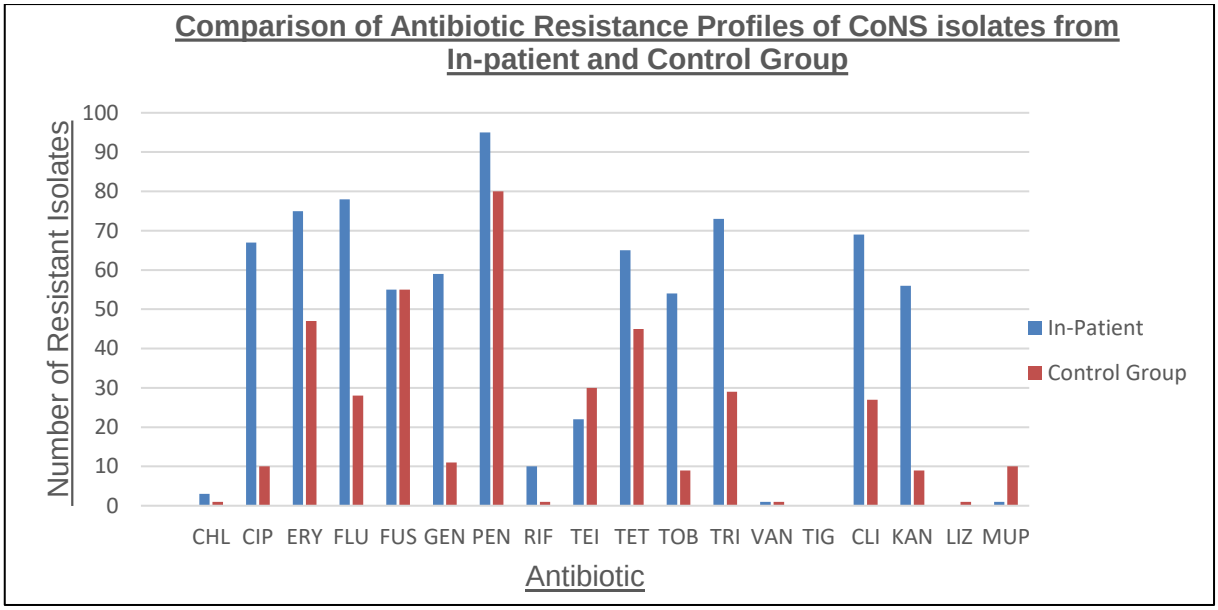


Figure 4-1: Comparison of antibiotic resistant profiles of CoNS isolates from both, the in-patient and control patient groups against a panel of 18 antibiotics using the VITEK® 2 automated micro-dilution methodology. Antibiotic code abbreviations: CHL (Chloramphenicol), CIP (Ciprofloxacin), ERY (Erythromycin), FLU (Flucloxacillin), FUS (Fusidic Acid), GEN (Gentamicin), PEN (Penicillin), RIF (Rifampicin), TEI (Teicoplanin), TET (Tetracycline), TOB (Tobramycin), TRI (Trimethoprim), VAN (Vancomycin), TIG (Tigecycline), CLI (Clindamycin), KAN (Kanamycin), LIZ (Linezolid), MUP (Mupirocin).

Of the 18 antimicrobials tested by VITEK®2, in both patient groups, tigecycline was observed to be the most active compound followed also by linezolid as the most active in the in-patient group only. Overall, resistance was more apparent to the 18 antibiotics tested in the in-patient group in comparison to the resistance profiles displayed by the control patient group isolates. Reviewing resistance rates of all the 100 CoNS isolates from both patient group CoNS isolates, the comparative lowest rates of resistance to specific antibiotic groups differed considerably. In the in-patient group isolates, the lowest rates of resistance were observed to the three antibiotics, mupirocin (1%), vancomycin (1%) and chloramphenicol (3%), with no resistance observed to linezolid. Conversely in the control patient group CoNS isolates, the lowest, 1% resistance was observed to four antibiotics, chloramphenicol, vancomycin, rifampicin and linezolid. Resistance to the aminoglycosides, gentamicin, kanamycin and tobramycin was observed to be just over 55% in the in-patient group compared to 11% resistance in the control patient group. Overall, penicillin resistance was observed to be consistently high (>80%) in both patient group CoNS isolates.

The Friedman test was used to determine the significance of observed difference between the overall distribution of antimicrobial resistance to the panel of 18 antibiotics and CoNS isolates from the in-patient group in comparison to the control patient group. Test analysis showed that there was no significant difference *(*$p = 0.59$ with $1df$*)* between the distribution

frequency of antimicrobial resistance to the 18 agents displayed by CoNS isolates from both the in-patient and control patient groups.

Flucloxacillin or methicillin resistance was noted to be higher in the in-patient CoNS isolates in comparison to the control patient group (78% and 28% respectively). The Mann-Whitney U test was applied to determine the significance of observed difference between CoNS isolates from the in-patient group and the control patient groups and distribution frequency of methicillin resistance as the single variable factor. The results reveal a significant difference in distribution frequency of methicillin resistance between the in-patient and control patient CoNS isolates ($p = 0.0001$).

Following data analysis using similar microbiological criteria, results from the total 100 CoNS species-specific isolates tested for each of the patient groups and the proportional number of resistant isolates to the panel of 18 antimicrobials are summarised in Tables 4-1 and 4-2.

	VITEK®2: Gram-Positive Antibiotic Panel – Number of Resistant Isolates per CoNS species																	
In-patient CoNS Species	CHL	CIP	ERY	FLU	FUS	GEN	PEN	RIF	TEI	TET	TOB	TRI	VAN	TIG	CLI	KAN	LIZ	MUP
S. epidermidis (n=57)	1	45	45	50	39	35	57	7	18	40	34	45	0	0	42	35	0	1
S. hominis (n=18)	0	7	14	12	10	6	17	1	2	10	7	13	0	0	13	7	0	0
S. haemolyticus (n=16)	2	15	14	15	7	14	16	1	3	14	13	14	1	0	12	14	0	0
Other CoNS (n=9)	0	0	2	1	3	0	5	1	0	1	0	1	0	0	2	0	0	0

Table 4-1: Number of in-patient group CoNS species-specific isolates resistant to each antibiotic tested against a panel of 18 antibiotics using the automated micro-dilution VITEK®2 system.

	VITEK®2: Gram-Positive Antibiotic Panel – Number of Resistant Isolates per CoNS species																	
Control Patients CoNS Species	CHL	CIP	ERY	FLU	FUS	GEN	PEN	RIF	TEI	TET	TOB	TRI	VAN	TIG	CLI	KAN	LIZ	MUP
S. epidermidis (n=32)	1	5	18	11	24	2	30	1	19	15	3	9	1	0	10	3	1	5
S. hominis (n=24)	0	1	13	5	17	1	21	0	1	15	1	4	0	0	8	1	0	2
S. haemolyticus (n=17)	0	4	9	9	7	8	12	0	8	9	5	9	0	0	3	5	0	1
Other CoNS (n=27)	0	0	7	3	7	0	17	0	2	6	0	7	0	0	6	0	0	2

Table 4-2: Number of control patient group CoNS species-specific isolates resistant to each antibiotic tested against a panel of 18 antibiotics using the automated micro-dilution VITEK®2 system.

Similar to the overall antimicrobial results obtained for in-patient and the control patient groups, the number of species-specific CoNS isolates resistant to the panel of 18 antimicrobials were observed to be variable dependent on the antibiotic/CoNS species-specific combination. As such, penicillin resistance was more apparent in isolates of *S. epidermidis* in both patient groups in comparison to the other CoNS species. Conversely, the least resistant were the CoNS species comprising the other minor group of CoNS isolates.

The Friedman Test used to determine any significant association between CoNS species-specific isolates and patient group revealed that there was a significant association between the antimicrobial resistant profiles of *S. epidermidis*, *S. hominis* and *S. haemolyticus* to the 18 antibiotics and the patient group from which they were isolated from *(*$p < 0.0001$ with 1*df*). Analysis carried out based on pairwise comparisons of species-specific CoNS isolates for the in-patient group revealed there was a significant difference between the frequency distribution of antimicrobial resistance observed in the other minor group of CoNS and isolates of *S. epidermidis*, *S. hominis* and *S. haemolyticus* ($p \leq 0.001$). Conversely, for the in-patient species-specific isolates there was no significant difference observed between isolates of *S. hominis* and *S. epidermidis* and the frequency distribution of antimicrobial resistance ($p = 0.369$ and 0.35 respectively). Likewise, no significant difference between isolates of *S. epidermidis* or *S. haemolyticus* and the overall frequency of antimicrobial resistance against the panel of 18 antibiotics tested ($p > 0.05$). For the control patient group isolates no statistically significant differences were observed between the frequency distribution of antimicrobial resistance and any of the species-specific CoNS isolates ($p > 0.05$). The notable exception was the resistance frequency observed between other minor group of CoNS isolates and *S. haemolyticus* similar to that also observed for methicillin resistance ($p = 0.003$). There was an overall significant association between the distribution frequency of methicillin resistance and all species-specific CoNS isolates with the exception of the other minor CoNS species isolates from both in-patient and the control patient groups *(*$p < 0.0001$). The other minor group of CoNS isolates in both patient groups demonstrated least resistance to methicillin.

Data analysis was further carried out to assess phenotypic relationship of the CoNS species isolates from each of the patient groups expressing comparable resistance profiles to determine clonal similarity.

Results of the comparative assessment of the CoNS species antibiogram resistant profile analysis from each patient group using PCA with cluster analysis (UPGMA) carried out to

distinguish related CoNS species isolates based on antibiotic resistance profiles are represented in Figures 4-2 and 4-3.

4.5.2 Data Analysis of the antibiotic profiles using Pearsons-Principle Component Analysis (PCA) with Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

Phenotypic antibiotic resistance profiles for the 100 CoNS isolates from each of the patient groups were further analysed using PCA with UPGMA using the Statisca v.10 Bionumeric Software package. Analysis was performed based on levels of similarity or differences of antimicrobial resistance patterns between all the species-specific CoNS isolates. A cut-off value of >95% indicating antibiotic resistance profiles to be identical therefore indicating antibiogram CoNS isolate similarity.

Figures 4-2 and 4-3 represent dendrogram constructs of the antimicrobial resistance profiles of the previously characterised 100 CoNS isolates from the in-patient and control patient group respectively. Computer assisted cluster analysis by UPGMA based on antimicrobial susceptibility similarity matrices did not identify any major phenotypic clusters. For each patient group isolates the results demonstrated a greater number of unique antimicrobial resistance profiles with little similarity (cut-off value set at ≥ 95%).

The UPGMA dendrogram analysis revealed a high degree of antibiotic resistance pattern variability within and between CoNS isolates and among isolates of the same species. A higher degree of diversity was observed to be in and amongst the different species of the in-patient CoNS isolates in comparison to the skin colonising control patient group isolates. In both patient groups, a few isolates of the same CoNS species were indistinguishable. Furthermore, the isolates showing 100% antimicrobial resistance profile similarity, grouped together in small clusters comprising of differing CoNS species-specific isolates.

Figure 4-2: Phenotypic relatedness of 100 in-patient group CoNS: Dendrogram based on Vitek®2 antibiotic analysis using Dice coefficient UPGMA method. The scale bar at the top represents the cut-off value set at >95%.

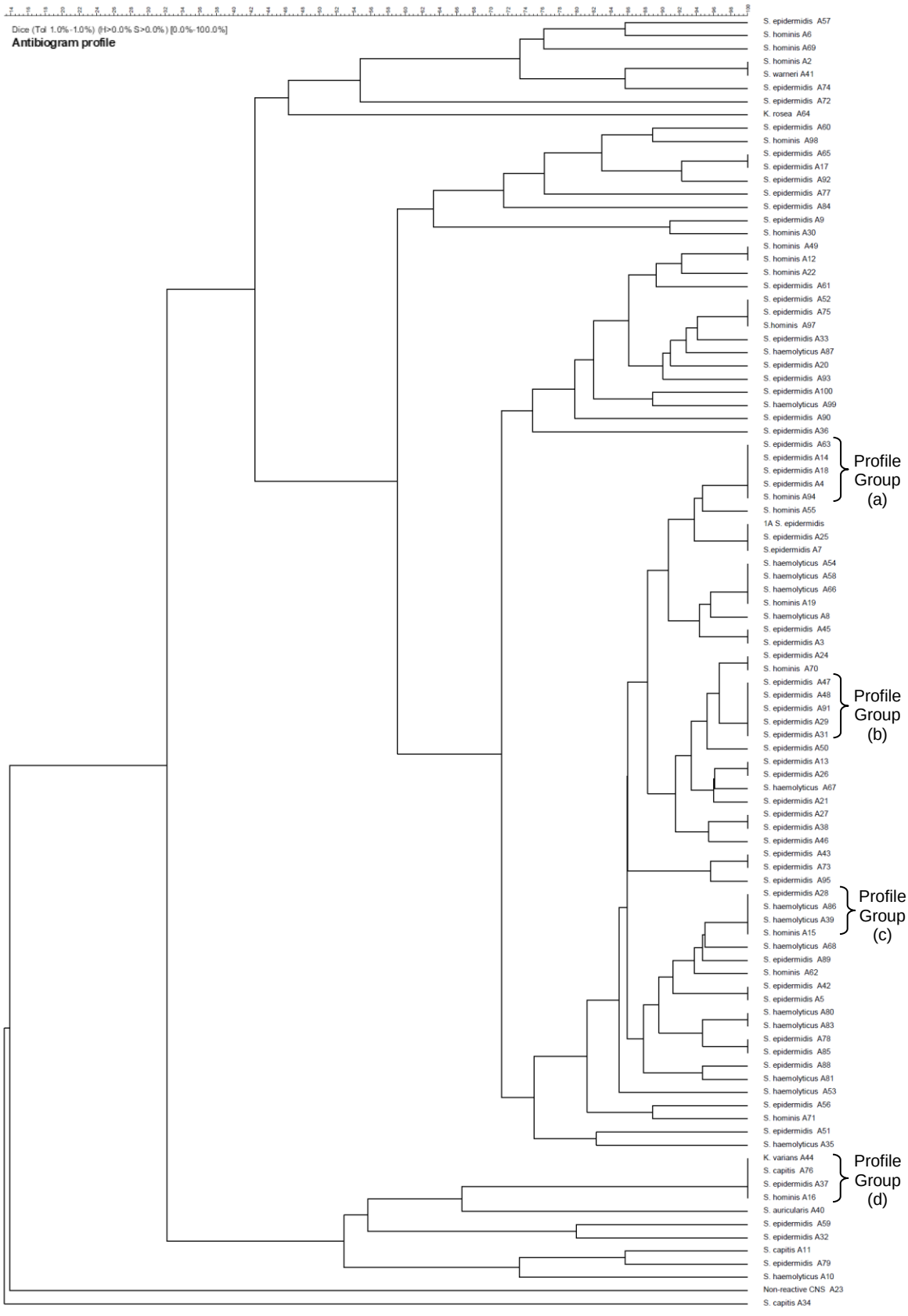
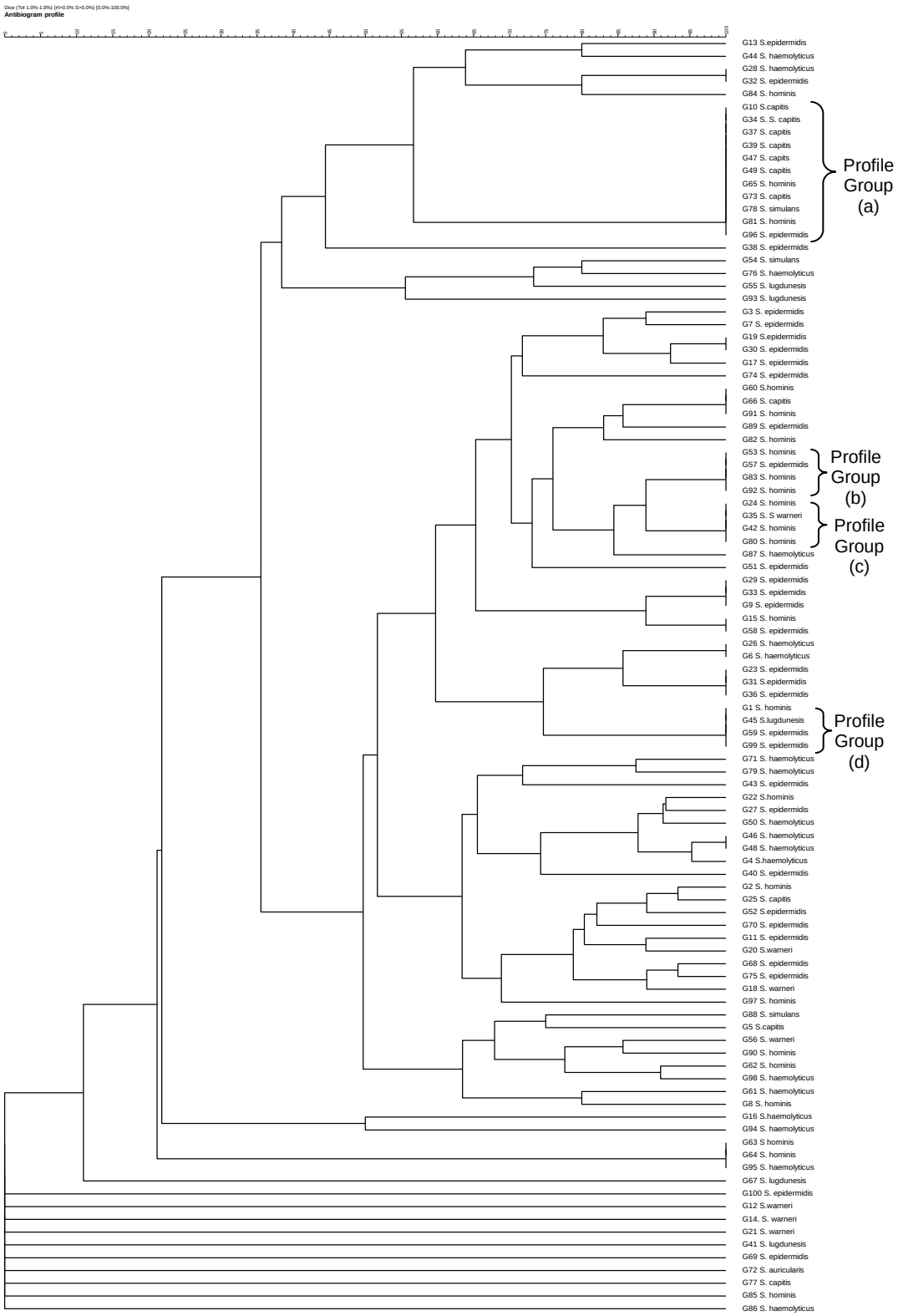


Figure 4-3: Phenotypic relatedness of 100 control patient group CoNS: Dendrogram based on Vitek®2 antibiotic analysis using Dice coefficient UPGMA method. The scale bar at the top represents the cut-off value set at >95%.



4.5.2.1 Interpretation of PCA antibiogram data analysis

For each of the patient groups 100 CoNS isolates dendrograms constructed using UPGMA to investigate species-specific isolates in relation to their antibiotic resistance profiles revealed very few definitive clusters. The levels of similarity of CoNS isolates calculated with Dice coefficient and grouped by UPGMA were predominantly less than the cut-off value set at 95% for similarity antibiogram profile determination. In both patient groups, species-specific CoNS isolates did not group together in large defined clusters in any particular part of the dendrogram axis.

Overall CoNS phenotypic relatedness based on antibiogram resistance profiles, determined by means of UPGMA dendrogram analysis among the 100 in-patient CoNS isolates, revealed 66 different antibiogram profiles as illustrated in Figure 4-2. Overall, 100% similarity correlation was observed in 50% of the isolates independent of the CoNS species type. Of these 50%, identical antibiogram profiles were observed in seventeen small isolate groups. The largest two profile groups (a) and (b), Figure 4-2 consisted of five CoNS isolates whereas the smallest groups showing 100% antibiogram relatedness consisted of two isolates each. In profile group (b) all the five isolates showing 100% similarity were isolates of *S. epidermidis* whereas profile group (a) comprised of four isolates of *S. epidermidis* and one of *S. hominis* with 100% identical resistance antibiograms. Likewise, the other smaller profile groups also consisted of dissimilar CoNS species showing 100% antibiogram relatedness. As such, undistinguishable antibiograms were observed in four isolates in profile group (c) comprising of two strains of *S. haemolyticus* A39 and A86 and one isolate each of *S. hominis* and *S. epidermidis*. Greatest species diversity with indistinguishable antibiograms was observed in profile group (d) consisting of *Kocuria varians*, *S. capitis*, *S. epidermidis* and *S. hominis.* Except for one small group consisting of two different CoNS species with identical antibiograms, which included *S. hominis* (A2) and *S. warneri* (A41). The other small groups were represented predominantly by *S. epidermidis* isolates.

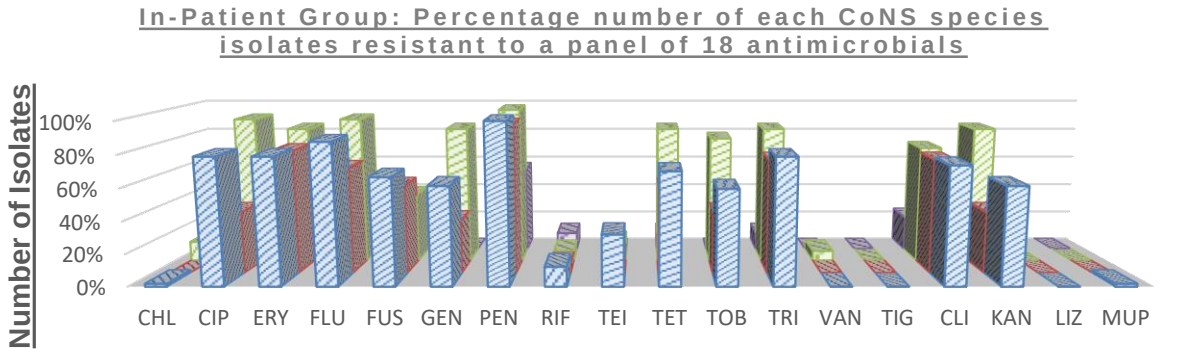
The diversity observed within species-specific CoNS isolates based on antibiogram relatedness was even greater in the control patient group. Results revealed 13 small groups consisting of 45 isolates independent of species-specific CoNS, demonstrating 100% identical antibiogram profiles. Of the 100 CoNS isolates analysed, 68 different antibiogram profiles were observed in the control patient group isolates. The largest of the groups showing 100% similarity was profile group (a) Figure 4-3 consisting of 11 isolates in total, comprising four different species-specific CoNS isolates. This profile group included seven isolates of *S. capitis*, two of *S. hominis* and one each of *S. epidermidis* and *S. simulans*. Profile groups (b) and (c) consisted mainly of *S. hominis* isolates sharing indistinguishable

antibiogram resistant profiles with isolates of *S. epidermidis* and *S. warneri.* However, in profile group (d) only one isolate of *S. hominis* displayed 100% similarity to one isolate each of *S. epidermidis* and *S. lugdunensis.*

The rest of the ten smaller profile groups in the control patient CoNS isolates revealed a high degree of both species and strain diversity within each of the small groups showing 100% antibiogram based strain relatedness. Overall, comparison of UPGMA dendrogram cluster analysis results based on the antibiograms using PCA factor analysis demonstrated a lack of antibiotic susceptibility pattern association or correlation between and among isolates of the same CoNS species in both patient groups.

4.5.2.2 Antibiotic resistance rates among species-specific CoNS isolates

Further to the total number of resistant CoNS species-specific isolates from both patient groups, (Table4-1 and 4-2), the percentage resistance rates exhibited by *S. epidermidis*, *S. hominis, S. haemolyticus* and the other minor group of CoNS species to the 18 antimicrobials are summarised in Figure 4-4 for the in-patient group and Figure 4-5 for the control patient group.



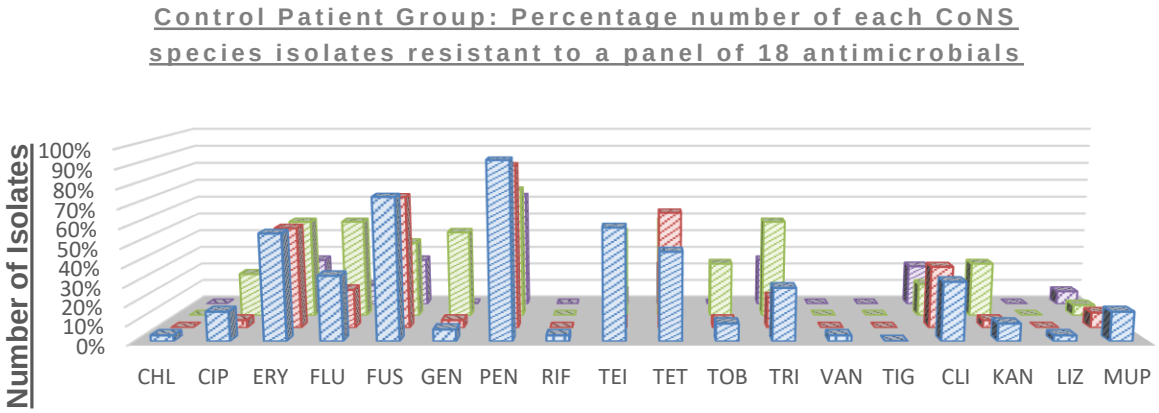
	CHL	CIP	ERY	FLU	FUS	GEN	PEN	RIF	TEI	TET	TOB	TRI	VAN	TIG	CLI	KAN	LIZ	MUP
S.epidermidis (n=57)	2%	79%	79%	88%	67%	61%	100%	12%	32%	70%	60%	79%	0%	0%	74%	61%	0%	2%
S. hominis (n=18)	0%	39%	78%	67%	56%	33%	94%	6%	11%	56%	39%	72%	0%	0%	72%	39%	0%	0%
S. haemolyticus (n=16)	13%	94%	88%	94%	44%	88%	100%	6%	13%	88%	81%	88%	6%	0%	75%	88%	0%	0%
Other CoNS (n=9)	0%	0%	22%	11%	33%	0%	56%	11%	0%	11%	0%	11%	0%	0%	22%	0%	0%	0%

Figure 4-4: Percentage number of *S. epidermidis*, *S. hominis*, *S. haemolyticus* and the other CoNS species in-patient group isolates resistant to each antibiotic tested against a panel of 18 antibiotics using the automated micro-dilution VITEK®2 system.

The proportion of CoNS species-specific isolates resistant to all the classes of antibiotics tested was observed to be highest amongst the in-patient *S. haemolyticus* isolates. In

comparison, the lowest overall resistance rates were observed in the other minor group of CoNS in both patient groups comprising species including, *S. capitis, S. warneri, S. lugdunesis, S. auricularis, S. simulans, Kocuria species and Dermococcus.*

Primary data analysis based on the reported susceptibilities of the in-patient CoNS isolates to the clinicians indicate high rates of penicillin resistance amongst all the three predominant CoNS species. Nonetheless, relatively similar rates of penicillin resistance were also noted amongst the control group skin colonising isolates. Although, proportionally similar rates of penicillin resistance were observed in both patient group isolates, 100% resistance was only exhibited by the in-patient *S. epidermidis* (n=57) and *S. haemolyticus* (n=16) isolates. Conversely, the percentage of isolates demonstrating proportionally higher penicillin resistance rate were the other minor group of CoNS species-specific isolates from the control patient group in comparison those of the in-patient group (27)63% and (9)56% respectively.



	CHL	CIP	ERY	FLU	FUS	GEN	PEN	RIF	TEI	TET	TOB	TRI	VAN	TIG	CLI	KAN	LIZ	MUP
S. epidermidis (n=32)	3%	16%	56%	34%	75%	6%	94%	3%	59%	47%	9%	28%	3%	0%	31%	9%	3%	16%
S. hominis (n=24)	0%	4%	54%	21%	71%	4%	88%	0%	4%	63%	4%	17%	0%	0%	33%	4%	0%	8%
S. haemolyticus (n=17)	0%	24%	53%	53%	41%	47%	71%	0%	47%	53%	29%	53%	0%	0%	18%	29%	0%	6%
Other CoNS (n=27)	0%	0%	26%	11%	26%	0%	63%	0%	7%	22%	0%	26%	0%	0%	22%	0%	0%	7%

Figure 4-5: Percentage number of control patient group isolates of *S. epidermidis*, *S. hominis*, *S. haemolyticus* and the other CoNS species resistant to each antibiotic tested against a panel of 18 antibiotics using the automated micro-dilution VITEK®2 system.

Overall, VITEK®2 system analysis of antimicrobial profiles for the species-specific strains of CoNS show that although the most frequently isolated species was *S. epidermidis* in both patient groups, proportionally, the most resistant were isolates of *S. haemolyticus.* Likewise, variable and lower resistance rates were observed to each of the antibiotic classes amongst isolates of the three predominant CoNS species in the control patient group in comparison to those of the in-patient group. Antimicrobial activity of the individual antibiotic classes within

the panel 18 antibiotics tested, displayed similar activity against all the CoNS isolates in both patient groups. As such, of the aminoglycoside class of antibiotics, resistance to gentamicin, tobramycin and kanamycin was observed to be highest amongst isolates of *S. haemolyticus* followed by *S. epidermidis* and *S. hominis* in both patient groups. Likewise, for both patient groups, the other minor group of CoNS species displayed complete susceptibility to all the aminoglycosides. Similarly, higher resistance activity was observed with the macrolides, erythromycin and clindamycin among isolates of *S. haemolyticus*, *S. epidermidis* and *S. hominis* in the in-patient group. In the control patient group isolates however, for the macrolide class of antibiotics all CoNS isolates demonstrated higher resistance rates to erythromycin *n*=50 (53%) in comparison to clindamycin *n*=18 (33%).

Resistance rates of isolates compromising the other minor group of CoNS were much lower in comparison to the three predominant species in both patient groups. The lowest resistance rates to the panel of 18 antimicrobials tested was observed in one isolate of *S. lugdunensis* (G41) out of the five isolates in the other minor CoNS species in the control patient group demonstrating full susceptibility. The other four isolates of *S. lugdunensis* in this patient group, displayed proportionally, lower resistance rates, exhibiting non-susceptibility to less than two antimicrobials. Similarly, in-patient isolates of *S. warneri* and *S. auricularis* displayed resistance to ≤3 antimicrobials.

Methicillin resistance interpreted in accordance with the 2012 BSAC/EUCAST breakpoints criteria of ≤0.25 to >0.5mg/L, was observed to be much higher amongst all in-patient CoNS species-specific isolates in comparison to those of the control patient group. The highest overall resistance to methicillin was detected in 78 out of the 100 in-patient CoNS isolates in comparison to 28% methicillin resistance observed in the control patient group isolates. Performing CoNS species-specific analysis revealing a degree of methicillin resistance variability between and within isolates of the same species from both patient groups are detailed in Table 4-3. The highest rates of methicillin resistance in both the in-patient and control patient group were observed in isolates of *S. haemolyticus* 15(94%) and 9(53%) respectively. However, among the in-patient CoNS isolates there was a significant association between *S. epidermidis* and its propensity to be methicillin resistant ($p < 0.05$). Of the 57 in-patient group *S. epidermidis* isolates 50 (88%) were classified as methicillin resistant in comparison to 11(34%) resistance in the control patient group. Likewise, although isolates of *S. haemolyticus* 9(53%) of the control patient group displayed higher rates of methicillin resistance no significant correlation ($p > 0.05$) was however revealed between methicillin resistance and any of the other predominant species-specific CoNS isolates.

it is also noteworthy, that resistance to mupirocin, a topical decolonising antimicrobial agent used for the eradication of colonising strains of healthcare associated MRSA, was observed to be more common among the control group CoNS species. Mupirocin resistance in the control patient group was observed to be highest among isolates of *S. epidermidis* (5)16% with the lowest resistance observed in one isolate of the *S. haemolyticus* (6%). Conversely, for the in-patient group, mupirocin resistance was observed in only one *S. epidermidis* isolate (2%).

4.5.2.3 Glycopeptide susceptibility rates among species-specific CoNS isolates

VITEK®2 susceptibility data analysis shows that each of the three predominant species of CoNS in the in-patient group differed in response to the glycopeptides in comparison to those in the control patient group. Resistance to teicoplanin in the control patient group was observed to be greater in CoNS isolates of *S. epidermidis* and *S. haemolyticus,* 19(59%) and 8(47%) respectively. Comparatively, in the in-patient group teicoplanin resistance was observed in 18(32%) of *S. epidermidis* isolates and 2(13%) of the *S. haemolyticus* isolates. Teicoplanin resistance rates displayed by isolates of *S. hominis* were comparatively lower in both patient groups, with nil resistance detected in the in-patient and the other minor group of CoNS isolates. In the control patient group however, 2(7%) isolates of the minor group of CoNS *S. warneri* (G20) and *S. lugdunensis* (G67) exhibited resistance to teicoplanin. The majority of all species-specific CoNS isolates were however, observed to be susceptible to vancomycin in both patient groups with the exception of reported resistance in an isolate of *S. epidermidis* (3%) in the control patient group and in one isolate of *S. haemolyticus* (6%) in the in-patient group.

Computed PCA applied to the in-patient data amongst the different CoNS species tested using the VITEK®2 system to reveal any significant association between species-specific CoNS isolates and susceptibility to the vancomycin and teicoplanin is displayed in Figure 4-6.

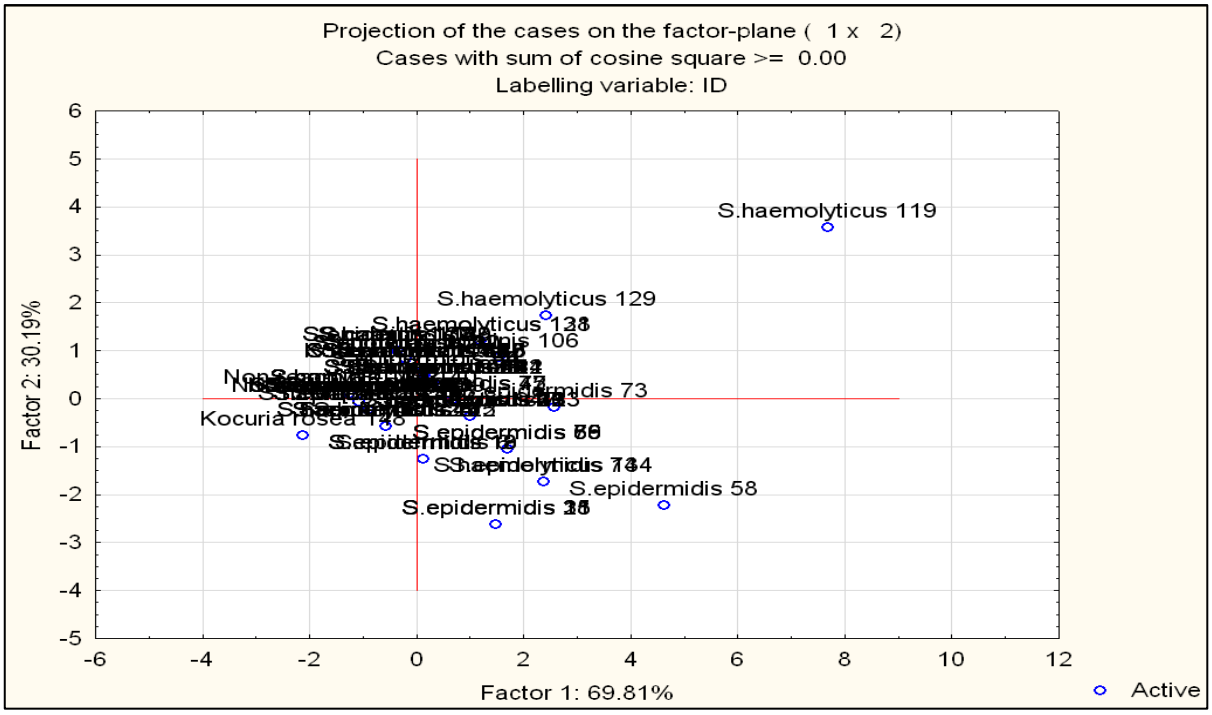


Figure 4-6: Scatter graph of factor loadings calculated by 2D PCA whereby the components are the glycopeptides factor 1 by factor 2 versus the CoNS species-specific identification differences tested using the VITEK®2 system.

The 2D PCA factor plot from the grouping variables which were the speciation identity of CoNS isolates against the two antibiotics, vancomycin and teicoplanin revealed one noticeable in-patient isolate A53, *S. haemolyticus* (plot position 119) away from the main predominating cluster. This suggests that this in-patient *S. haemolyticus* isolate exhibits a significantly distinct susceptibility response to the glycopeptides in comparison to the rest of the species-specific CoNS isolates. The remainder of the CoNS species isolates do not appear to reveal any distinctive clusters with the exception of few outlier isolates of *S. epidermidis* and *S. haemolyticus* near the main cluster. These isolates, although not forming distinct clusters appear individually spaced out on the factorial scale (Factor 2 axis: below zero) due to difference in susceptibility to the glycopeptide antibiotics in relation to species-specific CoNS identification.

Explanatory data analysis was further applied to summarise the vancomycin and teicoplanin inhibitory MIC values obtained with the VITEK®2 system in relation to each of the species-specific CoNS isolates through production of box and whisker plots as shown in Figure 4-7.

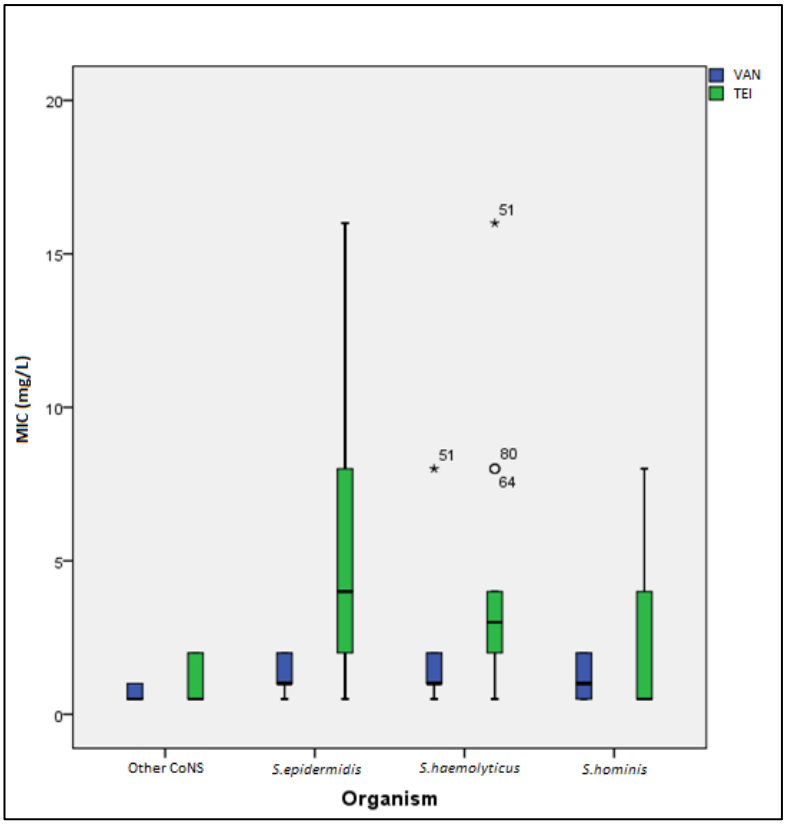


Figure 4-7: Box plot illustrates the overall comparative distribution of vancomycin and teicoplanin susceptibility (MIC mg/L) based on CoNS species-specific isolates from both patient groups tested using the VITEK®2 system.

The box and whisker plot, showing a summary of the overall susceptibility distribution of inhibitory concentrations of the two glycopeptides based on CoNS species-specific isolates, reveals a relatively symmetrical distribution with the exception observed with a few outliers and extreme values observed with isolates of *S. haemolyticus.*

Further application of descriptive statistics, result analysis reveal that there was a significant difference noted between the distribution of both vancomycin and teicoplanin MIC values between the in-patient and control patient group CoNS isolates ($p = 0.0001$ with $3df$).

4.5.2.4 Comparative CoNS species-specific methicillin and multidrug resistance rates

The categorisation of multidrug resistance defined by Magiorakos and colleagues (2012) was applied for the characterisation of multiple antimicrobial resistance. Therefore, following this criteria, multidrug resistance-CoNS (MDR-CoNS) were defined as those isolates demonstrating non-susceptibility to ≥1 agent in ≥3 antimicrobial categories. Hence data analysis of spectrum of overall resistance to multiple antimicrobial classes in the panel of 18 antimicrobials tested, revealed higher rates of MDR in the in-patient CoNS isolates (75%) in comparison to 23% in the control patient group.

The results of data analysis carried out to further assess the correlation between CoNS species-species isolates and their propensity to demonstrate MDR in each of the two patient groups using a generalised linear regression model are displayed in Figure 4-8.

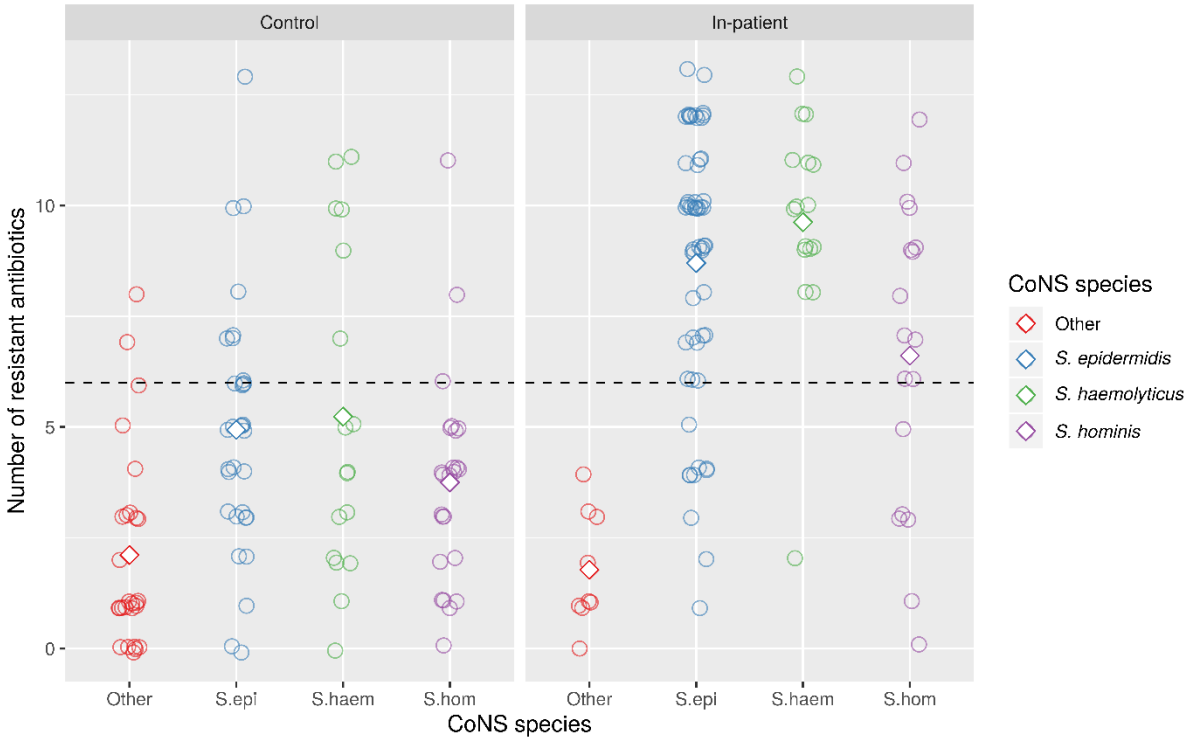


Figure 4-8: Antimicrobial resistance data plot of CoNS species-specific isolates of the in-patient group in comparison to isolates of the control patient group. The hollow circles are representative of each data point. The mean resistance to antimicrobials for each species group is represented by white diamonds and the black dotted line shows the cut-off point for MDR.

The output from the result analysis shows that there is strong evidence that the in-patient CoNS isolates are significantly associated with an increase in the number of antibiotics expressing resistance ($p < 0.0001$). Furthermore, the results show good evidence that isolates of *S. hominis* are associated with expressing resistance to fewer classes of antibiotics in comparison to isolates of *S. epidermidis* and *S. haemolyticus* ($p = 0.0004$ and $p = 0.0001$ respectively). Additionally, the model provided weak evidence that *S. haemolyticus* isolates demonstrated resistance to a greater number of antimicrobials on average than those of *S. epidermidis* ($p = 0.09$).

Based on the defined phenotypic resistance criteria, the *in-vitro* activity of methicillin in comparison to drug resistance to multiple antibiotic groups in the panel of 18 agents tested against CoNS isolates from both patient groups are summarised in Table 4-3.

	Number of Resistant Isolates / Total Number of Isolates Tested (%)	
	Methicillin Resistant	Multi-Drug Resistant
In-patient Group		
S. epidermidis (n=57)	50/88%	47/83%
S. hominis (n=18)	12/67%	13/72%
S. haemolyticus (n=16)	15/94%	15/94%
Other CoNS (*n*=9)	1/11%	Nil
Control Patient Group		
S. epidermidis (n=32)	11/34%	12/38%
S. hominis (n=24)	5/21%	3/13%
S. haemolyticus (n=17)	9/53%	6/35%
Other CoNS (*n*=27)	3/11%	2/7%

Table 4-3: Comparison of methicillin resistance and multi-drug antibiotic resistance patterns of species-specific isolates of CoNS from the in-patient and control patient groups.

Both *S. epidermidis* and *S. haemolyticus* isolates from the in-patient group exhibited high MDR rates (83% and 94% respectively). Additionally, methicillin resistance rates of 15(94%) were observed to correspond equally to MDR rates in, in-patient isolates of *S. haemolyticus* 15(94%). Conversely for the control patient group, almost similar correspondingly high rates of MDR with methicillin resistance were observed in isolates of *S. epidermidis* (38 and 34% respectively) but not in *S. haemolyticus* isolates. The least resistance to all antimicrobial classes was observed mainly amongst isolates of the other minor group of CoNS species in both patient groups. Nonetheless, in isolates of the control patient group two (7%) of the other CoNS, G20 and G25, *S. warneri* and *S. capitis* respectively, expressed resistance to greater than six antimicrobials.

Statistical analysis of overall data reveals that multi-drug resistance is significantly associated with methicillin resistance in both patient groups ($p = 0.0001$ with 1*df*). Analysis further revealed a significant association between MDR, methicillin resistance and in-patient isolates of *S. epidermidis* ($p = 0.0001$). Conversely, there was no significant association observed between methicillin resistance, MDR and isolates of the minor group of CoNS species from both patient groups ($p > 0.05$).

4.5.3 Evaluation of phenotypic susceptibility using the standard E-test method

The MICs of vancomycin and teicoplanin in comparison to tigecycline, daptomycin and ceftaroline were determined by the standard E-test method for 100 in-patient CoNS isolates.

As recommend by the manufacturer the MICs for these antibiotics were evaluated using Mueller-Hinton susceptibility agar (Figure 4-9).

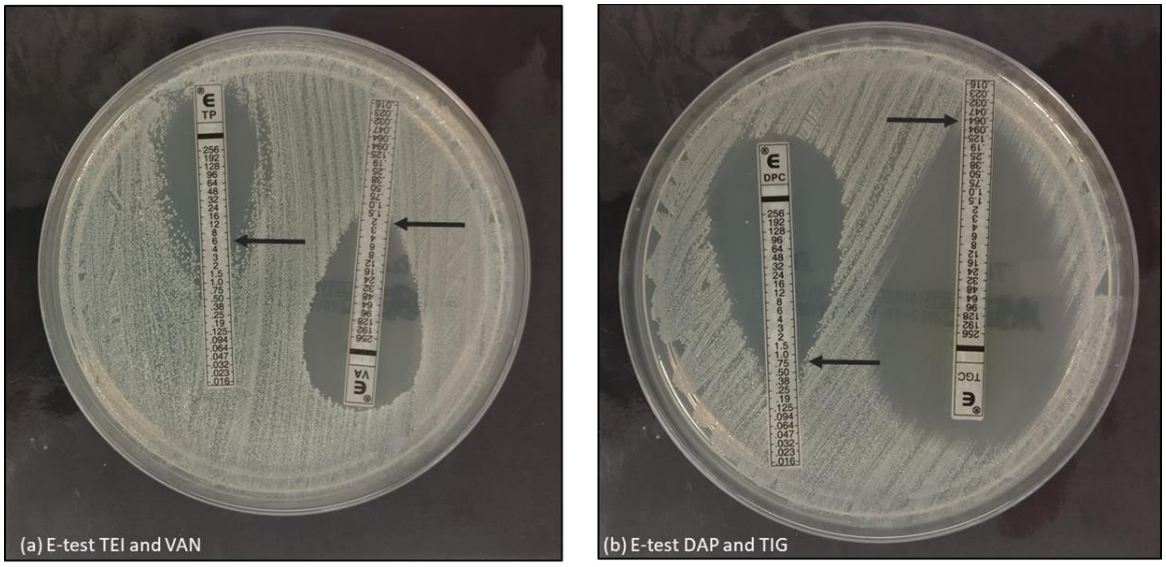


Figure 4-9: A representative in-patient CoNS isolate tested using standard E-test antibiotic gradient strips on Mueller-Hinton agar. (a) TP (teicoplanin) and (VA) vancomycin. (b) DPC (daptomycin) and TG (tigecycline). MIC values read at the point of complete inhibition for each antibiotic are indicated by the arrows.

The standard E-test MIC provided quantitative MIC antimicrobial data generated from the E-test gradient scale ranged from MIC values of 0.016 to 256mg/L for vancomycin, teicoplanin, tigecycline and daptomycin. In accordance with the manufacturers protocol, the MIC for each isolate was determined at the end-point of interaction of the lower part of ellipse shaped growth area with the E-test strip as illustrated in Figure 4-9a and b. The end point of inhibition differed for teicoplanin in comparison to the clear cut endpoints noted for the other test antibiotics. The presence of smaller resistant colonies around the ellipse were also taken into account when evaluating teicoplanin resistance. EUCAST (v.2, 2010/11) susceptibility interpretative criteria was applied as follows: Vancomycin ≤4mg/L, teicoplanin ≤4mg/L, daptomycin ≤1mg/L, tigecycline ≤0.5mg/L and ceftaroline ≤2mg/L. Similar microbiological criteria for *S. aureus* were used to categorise susceptibility to ceftaroline as breakpoint determination by consensus organisations have yet to be established for CoNS and this antimicrobial agent.

MICs for ceftaroline were determined using Mueller-Hinton plus 5% horse-blood with the M.I.C.E standard E-test strips in accordance with the manufacturers recommended protocol. The concentration gradient scale of the ceftaroline standard E-test strip ranged from MIC value of 0.002 to 32mg/L as shown in Figure 4-10. The MIC value for each isolate was determined at the end point of growth inhibition to include small colony variants. As illustrated, the end points of inhibition differed dependent on the CoNS species. Following

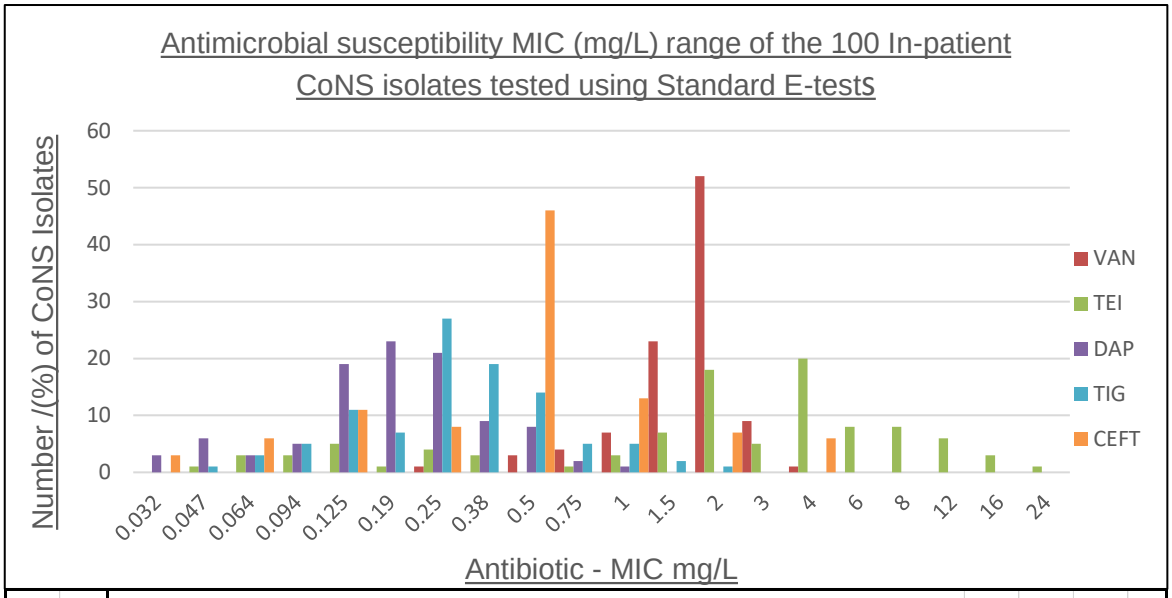
recommendations, for those end-points of growth inhibition falling between the intersection of two MIC value points, the MIC was rounded up to the next two fold dilution value for MIC categorisation as illustrated in Figure 4-10(b).

The MIC measurements obtained for the ATCC reference strains for all the five antimicrobials tested using the standard E-test method fell within the standard published quality control ranges.

Figure 4-10: Two representative in-patient CoNS isolates tested using (CPT) ceftaroline standard E-test antibiotic gradient strip on Mueller-Hinton agar with 5% horse blood. The MIC values were read at the end points as indicated by the arrows (a) *S. epidermidis* MIC value read at the point of inhibition at 0.25mg/L. (b) *S. haemolyticus* inhibition intersection point falling between two MIC values was read at 4mg/L.

Applying the susceptibility interpretative criteria adopted from EUCAST/BSAC (2012) published guidelines, the standard E-test MIC values for vancomycin were all within the susceptibility range of ≤4mg/L with the mean and median MIC value of 2mg/L. On the other hand, the MIC values of teicoplanin were more widely distributed with 26 out of the one hundred in-patient CoNS isolates displaying MICs of >4mg/L. Borderline susceptibility to teicoplanin at breakpoint MIC of 4mg/L was observed in 20(27%) out of the 74 susceptible isolates. The highest MIC of 24mg/L was observed in one out of the 26 CoNS isolates displaying teicoplanin resistance corresponding to a percentage of 0.04% of the non-susceptible isolates.

Figure 4-11 illustrates the overall *in-vitro* activity of the newer antibiotics daptomycin, tigecycline and ceftaroline in comparison to the glycopeptides, vancomycin and teicoplanin against the 100 in-patient CoNS isolates.



	Standard E-test : MIC (mg/L) distribution range of the number of isolates out of the total 100 In-patient CoNS tested																			
	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8	12	16	24
VAN							1		3	4	7	23	52	9	1					
TEI		1	3	3	5	1	4	3		1	3	7	18	5	20	8	8	6	3	1
DAP	3	6	3	5	19	23	21	9	8	2	1									
TIG		1	3	5	11	7	27	19	14	5	5	2	1							
CEFT	3		6		11		8		46		13		7		6					

Figure 4-11: Antimicrobial susceptibility, MIC distribution range of the glycopeptides, vancomycin (VAN) and teicoplanin (TEI) in comparison to daptomycin (DAP), tigecycline (TIG) and ceftaroline (CEFT) for the 100 in-patient CoNS isolates as determined by the standard E-test method.

Based on the standard established susceptibility breakpoints, Figure 4-11 also shows that the MIC susceptibility ranges of the CoNS isolates evaluated by the standard E-test method were variable dependent on the antibiotic tested. Daptomycin (MIC ≤1mg/L) remained the most active compound against all the 100 in-patient CoNS isolates tested. Susceptibility to daptomycin at the upper limit range at MIC 1mg/L was noted in only one *S. capitis* (A7) in the other minor group of CoNS isolates. Of the newer antimicrobials tested similar *in-vitro* potency was not demonstrated by either tigecycline or ceftaroline against all the 100 in-patient CoNS isolates. In total 13 and 6% of the CoNS isolates demonstrated non-susceptibility to tigecycline and ceftaroline respectively. Standard E-test MIC values for teicoplanin were observed to spread across a much wider scale, ranging from 0.047 to 24mg/L in comparison to the MIC ranges displayed by vancomycin, tigecycline and

daptomycin. Similar, MIC values spread across a wider scale were also observed for ceftaroline, ranging from 0.032 to 4 mg/L with a median MIC value of 0.5mg/L.

Higher resistance rates to the newer antibiotics, tigecycline and ceftaroline as determined by standard E-test methodology were noted amongst strains of the predominant CoNS species. In general, tigecycline resistance ranging from MIC 6-24mg/l was observed amongst the13 in-patient CoNS isolates which included 9(16%) strains of *S. epidermidis*, 3(19%) *S. haemolyticus* and 1(6%) of *S. hominis*. Borderline susceptibility to tigecycline was observed in a total of 14 of the hundred CoNS isolates tested at MIC 0.5 mg/L which included 6(11%) *S. epidermidis* and 3 (19%) *S. haemolyticus*. Similarly, all 6(38%) of the ceftaroline resistant isolates were strains of *S. haemolyticus* isolates expressing MIC of 4mg/L. Overall borderline ceftaroline susceptibility at MIC 2mg/L was observed in a total of 7(44%) of the CoNS isolates of these, 6(38%) and 1(2%) were isolates of *S. haemolyticus* and *S. epidermidis* respectively.

The data was further subjected to descriptive statistical analysis to describe the association between the independent variable (antimicrobials) and concentration dependent proportional inhibition of the in-patient CoNS isolates.

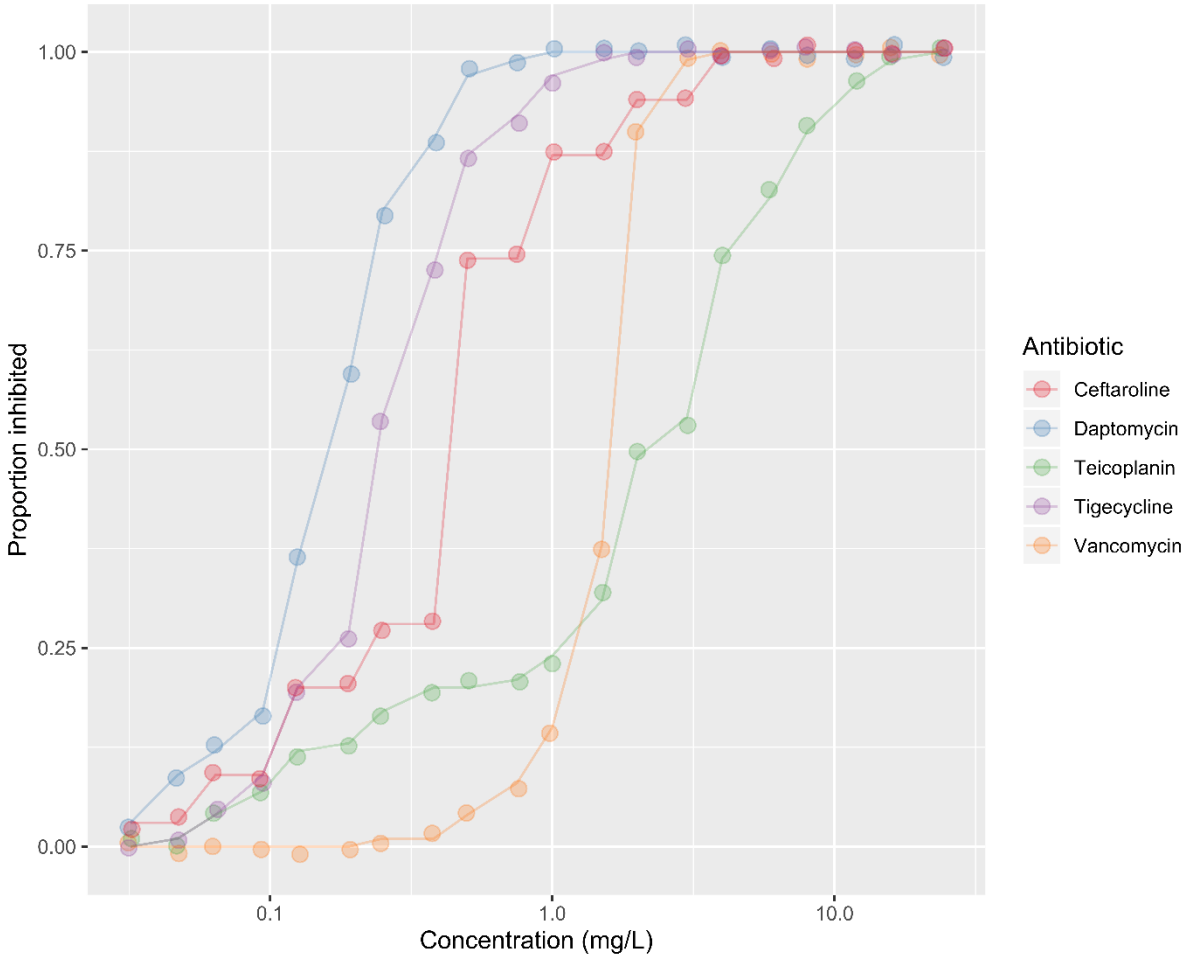


Figure 4-12: Comparative proportion of in-patient CoNS isolates inhibited at specified concentrations (mg/L) (logarithmic scale) by each of the four antibiotics ceftaroline, daptomycin, tigecycline, vancomycin and teicoplanin tested using the standard E-test methodology.

The logistic regression model fitted to the overall susceptibility data obtained for the in-patient CoNS was used to observe the proportion of CoNS isolates inhibited as a function of the specified antibiotic concentration. Figure 4-12 details the proportion of CoNS isolates inhibited at specified MIC concentrations by the newer antibiotics daptomycin, tigecycline and ceftaroline in comparison to the glycopeptides using the standard E-test methodology. The results reveal that there is a significant association between increasing concentration of antibiotic and increase in the proportion of CoNS isolates inhibited. However, the model also demonstrates that in comparison to ceftaroline, the proportion of isolates inhibited by vancomycin at a given concentration is significantly lower ($p < 0.005$). Additionally, the model contains two-way interactions for concentration versus daptomycin, teicoplanin and tigecycline ($p = 0.0014$, $p = 0.0004$ and $p = 0.00048$ respectively), demonstrating that

daptomycin and tigecycline produce significantly greater inhibition at a given concentration and teicoplanin gives a proportionally lower inhibition at the same concentration in comparison to ceftaroline.

Antibiotic	**MIC_{50} (mg/L)**	**MIC_{90} (mg/L)**
Ceftaroline	0.44	1.7
Daptomycin	0.17	0.4
Tigecycline	0.24	0.65
Vancomycin	1.62	2
Teicoplanin	2.2	8

Table 4-4: Comparative MIC_{50} and MIC_{90} (mg/L) values for the newer antibiotics evaluated against vancomycin and teicoplanin by standard E-test.

In-vitro MICs of the newer antibiotics ceftaroline, daptomycin and tigecycline in comparison to the glycopeptides summarised as MIC_{50} and MIC_{90} values for the 100 in-patient CoNS isolates are presented in Table 4-4. The medians MIC_{50} and MIC_{90} for daptomycin are the lowest followed by tigecycline and ceftaroline in comparison to the glycopeptides. The results indicate the daptomycin is deemed to be significantly the most potent ($p = 0.001$) in comparison to the other antimicrobial agents. Conversely, the highest MIC_{50} and MIC_{90} displayed by teicoplanin indicates that significantly higher concentrations ($p = 0.0004$) of this antimicrobial are required to achieve comparatively similar proportional inhibition rates relative to the other four antibiotics tested.

4.5.3.1 Comparison of glycopeptide susceptibilities determined by the two validated AST methodologies

Overall comparative distribution of vancomycin and teicoplanin MICs for the 100 in-patient CoNS species-specific isolates determined by the standard E-tests with those determined by the automated microdilution methodology are illustrated in Figure 4-13. Comparative glycopeptide inhibitory concentrations (MIC mg/L) displayed by the two test methodologies used for detection of susceptibility revealed an overall MIC value similarity of 42% and 52% for vancomycin and teicoplanin respectively.

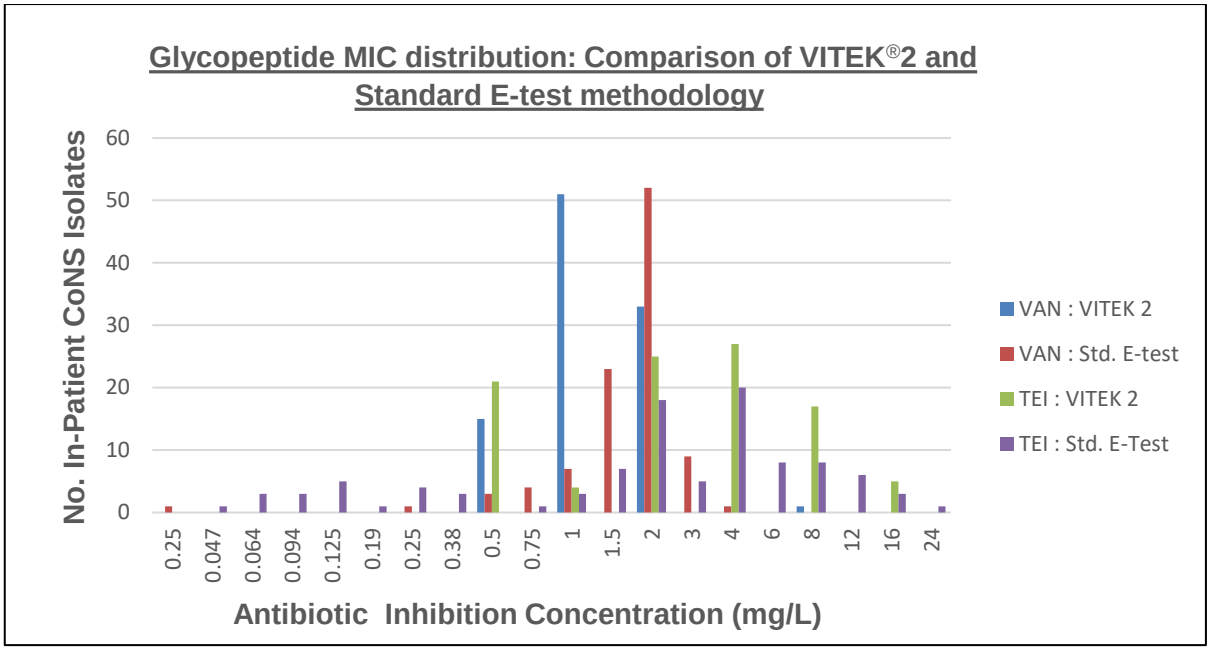


Figure 4-13: Comparison of antimicrobial inhibition concentration MIC (mg/L) distribution range of the glycopeptides, vancomycin (VAN) and teicoplanin (TEI) for the 100 in-patient CoNS isolates as determined by both the standard (Std.) E-test and VITEK®2 automated methodologies.

Vancomycin and teicoplanin MIC distribution by automated VITEK®2 system were relatively different from those evaluated by standard E-test. The VITEK®2 system demonstrated performance within a much narrower MIC susceptibility range in comparison to the standard E-tests. Of these a total of 74 isolates in comparison to 77 CoNS isolates expressed susceptibility to teicoplanin as determined by E-test and VITEK®2 respectively. Twenty (27%) of the isolates analysed by E-test were at the lower end of the teicoplanin susceptibility range, falling below 0.5mg/L, similar to those obtained by the automated method 21(27%), however the susceptibility distribution range at the upper end of the MIC value ranges differed considerably. Nonetheless, the highest number of isolates exhibiting teicoplanin MICs of 2mg/L and 4mg/L were similar with both test methodologies.

Likewise, CoNS isolates by standard E-test method were observed to express teicoplanin inhibitory MICs across a much wider MIC value range in comparison to the automated microdilution method. Therefore, comparatively fewer isolates at each MIC inhibitory value across a much wider scale were observed with the standard E-test method. Additionally, due to the greater sensitivity and specificity of E-test methodology, in total 26 CoNS isolates were classified as resistant to teicoplanin in comparison to resistance observed in 22 isolates with the VITEK®2 method. Similarly, higher teicoplanin susceptibility rates were noted in isolates tested by VITEK®2 with 25(24%) and 27(35%) of the CoNS isolates in comparison to 18(24%) and 20(27%) with E-tests exhibiting MICs of 2mg/L and 4mg/L respectively.

Conversely, the number of CoNS isolates exhibiting teicoplanin resistance at higher MICs, ranging from 6-24mg/L were observed in a total of 26 isolates investigated by E-test. Comparatively a lower resistance rate was observed with the VITEK®2 system, with a total of 22 isolates expressing resistance at MICs between 8 and 16mg/L. However, performing paired sample statistics comparing teicoplanin MIC result distribution revealed no significant difference ($p = 0.624$) between VITEK®2 and the standard E-test susceptibility test methodologies.

Data analysis of the vancomycin susceptible in-patient CoNS isolates on the other hand revealed similar difference in MIC value ranges with the two test methodologies. The majority of in-patient CoNS isolates (n=52) displayed an MIC of 2mg/L as determined by E-test in comparison to the VITEK®2, where the majority of isolates (n=51) were noted to display MIC of 1mg/L with 33 isolates demonstrating MICs of 2mg/L. CoNS isolate A53, on the other hand presented vancomycin MIC of 8mg/L categorised as resistant when determined by VITEK®2 in comparison to an MIC of 4mg/L by E-test method. This one fold dilution difference lower than that obtained by VITEK®2 classifies this isolate as borderline susceptible when tested by E-test. Comparative analysis of vancomycin susceptibilities however, revealed a significant difference ($p = 0.0001$) in MIC result distribution between the two AST methodologies.

VITEK®2 CoNS species-specific MIC inhibitory value range results interpreted according to the 2011 Global European-based phenotypic EUCAST interpretative guidelines for comparative activity of vancomycin and teicoplanin are detailed in Table 4-5. Of note, susceptibility testing analysis for isolate number A64 identified as *Kocuria rosea* in the other minor group of CoNS species could not be performed by VITEK®2 due to the organisms growth condition requirement. Therefore, this isolate was excluded from MIC value range analysis for both the glycopeptides. AST results for this isolate carried out manually on blood Mueller-Hinton agar incubated in 5% Co2 at 37°C for 18 hours were categorised as sensitive or resistance in accordance with the BSAC (2012) disc test guidelines.

VITEK®2 automated microdilution method: Number of Isolates (Cumulative %) Inhibition at MIC in mg/L against 100 in-patient CoNS isolates				
VAN: MIC (mg/L)	*S. epidermidis* (n=57)	*S. hominis* (n=18)	*S. haemolyticus* (n=16)	Other CoNS (n=9*)
<0.5	2(4%)	6(33%)	2(13%)	5(56%)
1	34(60%)	6(33%)	7(39%)	3(33%)
2	21(37%)	6(33%)	6(38%)	
8			1(6%)	
TEI: MIC (mg/L)	*S. epidermidis* (n=57)	*S. hominis* (n=18)	*S. haemolyticus* (n=16)	Other CoNS (n=9*)
<0.5	4(7%)	11(61%)	2 (13%)	4(44%)
1	2(4%)	1(6%)		1(11%)
2	16(28%)		6 (38%)	3(33%)
4	17(30%)	5(28%)	5 (31%)	
8	14(25%)	1(6%)	2 (13%)	
16	4(7%)		1 (6%)	

Table 4-5: MIC value ranges (mg/L) of in-patient CoNS species to vancomycin and teicoplanin investigated with the VITEK®2 automated microdilution system, with application of breakpoint susceptibility criteria of ≤4mg/L for both antibiotics. One isolate of the other CoNS group (n=9*) failed automated AST was excluded from MIC analysis.

All investigated isolates with the exception of one (6%) *S. haemolyticus* isolate, A53 demonstrated susceptibility to vancomycin when tested by VITEK®2. As shown in Table 4-5 species-specific strains of in-patient CoNS expressed differing levels of vancomycin susceptibilities with MIC values ranging from <0.5 to 8mg/L. The lowest inhibitory vancomycin MIC values were found among isolates of the other minor CoNS species 56% and 44% (<0.5 and 1mg/L) respectively.

On the other hand, with the application of the revised interpretative breakpoint criteria used for *S. aureus* to the susceptibility data obtained for the in-patient CoNS isolates, revealed just over 30% of the three predominant species-specific isolates expressed vancomycin MICs of 2mg/L. This current susceptibility breakpoint of 2mg/L, considered to be at the upper limit of the susceptibility range for clinically relevant isolates of *S. aureus* was established to include those isolates expressing low level vancomycin resistance known to be associated with clinical failures (Tenover *et al*., 2007). Data re-analysis following application of the modified interpretive criteria, shows that the highest rates were prevalent in isolates of *S. haemolyticus* at breakpoint of MIC 2mg/L followed by *S. epidermidis* and *S. hominis* (38, 37 and 33% respectively). In comparison to vancomycin, the difference in teicoplanin MIC susceptibility range among in-patient CoNS isolates was more species-specific dependent. Of the 22 teicoplanin non-susceptible species-specific CoNS isolates, *S. epidermidis*, 3(19%) *S. haemolyticus* 18(32%) and one (6%) *S. hominis* demonstrated MIC value ranges between 8 and 16mg/L above the breakpoint value of 4mg/L.

Comparative CoNS species-specific glycopeptide susceptibility data analysis carried out using the standard E-test methodology are detailed in Table 4-6.

Standard E-test: Number of Isolates (Cumulative %) Inhibition at MIC in mg/L against 100 in-patient CoNS Predominant Species Groups				
VAN: MIC (mg/L)	*S. epidermidis* (n=57)	*S. hominis* (n=18)	*S. haemolyticus* (n=16)	Other CoNS (n=9)
<0.5		3 (17%)		1 (11%)
0.75		2 (11%)		2 (13%)
1	1 (2%)	6 (33%)		
1.5	8 (14%)	3 (17%)	5 (31%)	6 (67%)
2	43 (75%)	3 (17%)	8 (50%)	
3	5 (9%)	1 (6%)	2 (13%)	
4			1 (6%)	
TEI: MIC (mg/L)	*S. epidermidis* (n=57)	*S. hominis* (n=18)	*S. haemolyticus* (n=16)	Other CoNS (n=9)
<0.5	1 (2%)	13 (72%)	2 (13%)	5 (56%)
0.75				1 (11%)
1	2 (4%)			1 (11%)
1.5	3 (5%)	1 (6%)		1 (11%)
2	13 (23%)		6 (38%)	1 (11%)
3	5 (9%)			
4	14 (25%)	2 (11%)	5 (31%)	
6	4 (7%)	2 (11%)		
8	7 (12%)		2 (13%)	
12	6 (11%)			
16	1 (2%)		1 (6%)	
24	1 (2%)			

Table 4-6: Comparative distribution of vancomycin and teicoplanin MIC value ranges of species-specific in-patient CoNS isolates investigated using the Standard E-test method.

In total, of the 100 CoNS species-specific isolates from the other minor group of CoNS demonstrated a high degree of susceptibility to both the glycopeptides with MICs ranging from <0.5-2mg/L. With the application of EUCAST (2011/12) susceptibility breakpoint criteria, none of the CoNS species-specific isolates tested exhibited resistance to vancomycin. Only one *S. haemolyticus* isolate A53, expressed an MIC of 4mg/L at the upper end of the susceptibility range. Conversely, application of the revised vancomycin breakpoint of 2mg/L, associated with sub-optimal clinical outcomes in similar *S. aureus* BSIs, a total of 54 CoNS isolates were noted to exhibit susceptibility at the upper end of the susceptibility range with the E-test method. Hence, with application of the modified breakpoint criteria, borderline susceptibility at MIC of 2mg/L was noted to be expressed by *S. epidermidis* and *S. haemolyticus,* 43(75%) and 8(50%) respectively. On the other hand, the lowest rates of teicoplanin susceptibility was observed among 19(33%) in-patient, *S. epidermidis* isolates

followed by 3(19%) and 2(11%) resistance observed in isolates of *S. haemolyticus* and *S. hominis* respectively.

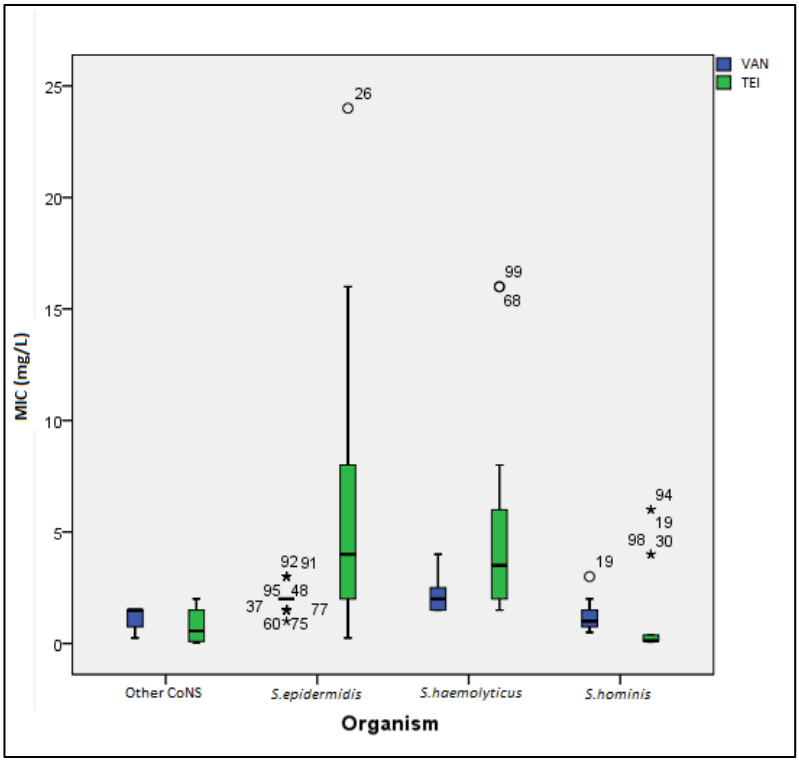


Figure 4-14: Box plot illustrates the overall comparative distribution of vancomycin and teicoplanin susceptibility (MIC mg/L) based on CoNS species-specific isolates tested using the standard E-tests.

The box and whisker plot, Figure 4-14 presents a summary of the overall susceptibility distribution of inhibitory concentrations of the two glycopeptides based on CoNS species-specific isolates using the E-test methodology. With the exception of the other minor CoNS species group, a few extreme values for both vancomycin and teicoplanin were observed in the plot for isolates of *S. epidermidis, S. haemolyticus,* and *S. hominis*. Conversely, a relatively symmetrical MIC distribution with few outliers and extreme values for inhibitory concentrations for teicoplanin alone were observed for all three of the predominant CoNS species isolates.

2D PCA factor analysis (Figure 4-15) was also preformed to further assess the comparative significance of vancomycin and teicoplanin susceptibilities and their association with species-specific isolates of CoNS when tested by VITEK®2 and standard E-test methodologies.

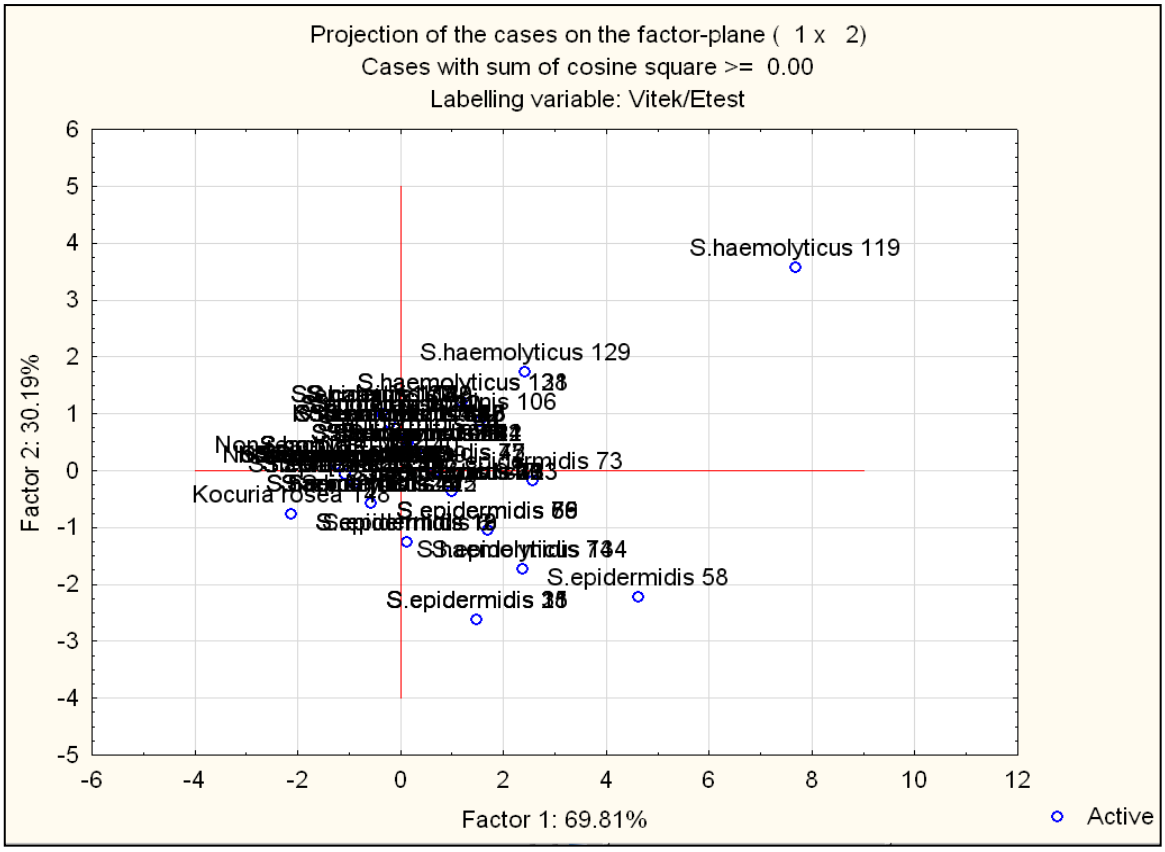


Figure 4-15: Scatter graph of factor loadings calculated by 2D PCA whereby the components are the glycopeptide susceptibilities factor 1 by factor 2 versus the CoNS species-specific differences when tested using the VITEK®2 system or Standard E-test method.

The 2D PCA plot of factor 1 versus factor 2 for the 100 CoNS species isolates shown in Figure 4-15, include the grouping variables which were the test methodology, against species-specific CoNS glycopeptide susceptibility. The PCA of the dataset produced two factors, 1 (VITEK®2) and 2 (standard E-test) accounted for 69.81% and 30.19% respectively in line with the CoNS species related glycopeptide susceptibilities. Based on discriminatory factors, one isolate of *S. haemolyticus* (plot position 119) was pulled out from the main cluster around the factor axis suggesting a significant difference in expressing susceptibility in comparison to the rest of the CoNS species-specific isolates. The overall result analysis suggest that there is no significant difference between the two antimicrobial test methodologies for glycopeptide susceptibility expression in relation to species-specific CoNS isolates.

Linear regression analysis of the three variables, the glycopeptides, VITEK®2 and E-test methodologies and in-patient species-specific CoNS isolates was performed to determine any significant differences with the use of the two validated AST methodologies. The antimicrobial inhibitory concentration dataset was used to construct logistic regression

models, displaying the proportion of CoNS species-specific isolates inhibited by teicoplanin and vancomycin dependent on the AST method used to confirm findings (Figure 4-16).

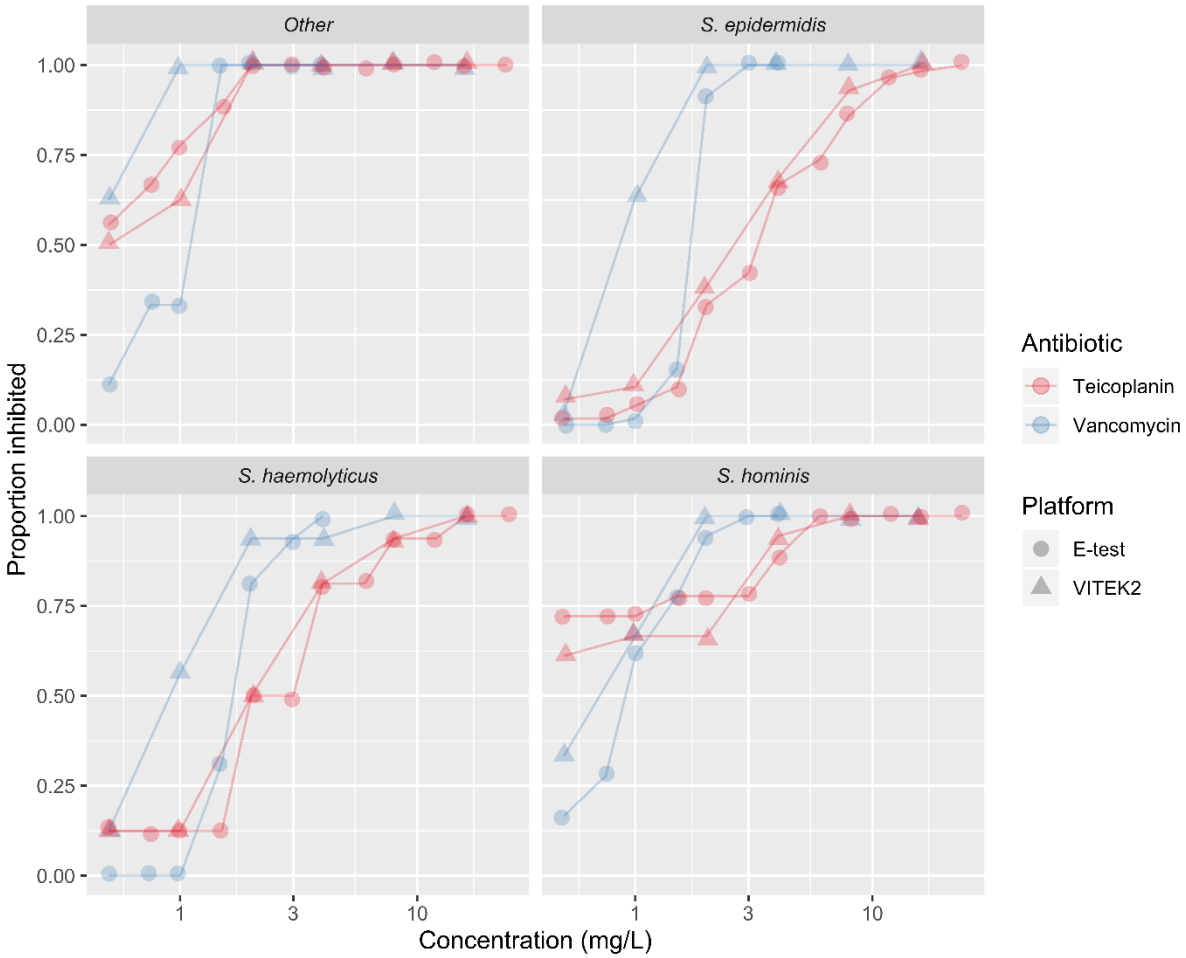


Figure 4-16: Comparative analysis of the proportion of species-specific in-patient CoNS isolates inhibited in relation to glycopeptide antibiotic concentrations (logarithmic-scale) as determined by the VITEK®2 system and the standard E-test method.

The primary model shows the proportion of isolates inhibited as a function of the antibiotic concentrations, the antibiotics namely vancomycin or teicoplanin, the susceptibility test methods either VITEK®2 or standard E-test and the in-patient CoNS species-specific isolates. The results from this analysis demonstrate that there is a significant increase in the proportion of species-specific CoNS isolates inhibited as the concentration of the glycopeptides increased ($p = 0.003$). Conversely, vancomycin was in general, associated with a significantly decreased probability of inhibition ($p = 0.009$). Additionally, there was a two-way interaction observed between vancomycin and VITEK®2 test method, such that, the proportion of isolates inhibited significantly increases when the effect of the antibiotic is

measured on this platform (p = 0.0005) when compared to the E-test method. Significantly, similar two-way interactions were also observed between concentration of the antibiotics and proportional number of species-specific isolates, *S. epidermidis*, *S. haemolyticus* and *S, hominis* inhibited (p values of 0.026, 0.028 and 0.018 respectively).

Moreover, the two-way interaction between vancomycin and *S. epidermidis* further demonstrates that there is a significant tendency for this combination to produce a lower proportion of inhibited isolates (p = 0.0003). Result analysis further reveal that the two-way interactions tends to decrease somewhat the effect that increasing the antibiotic concentration has on the proportion of all CoNS species-specific isolates inhibited, in comparison to those of the other minor group of CoNS species. Overall, the results also show a three-way interaction observed between concentration, vancomycin and only isolates of *S. epidermidis* with the use of E-test. With this methodology an increase in concentration tends to produce a significantly greater increase in the proportion of isolates inhibited when dealing with vancomycin and *S. epidermidis* species (p = 0.0035). Similarly, there was a significantly lower assignment probability (p = 0.008) between concentration, *S. epidermidis,* vancomycin and VITEK®2. Conversely, no significant correlation was observed between concentration, vancomycin and either of the *S. haemolyticus* or *S. hominis* isolates (p = 0.44 and 0.93 respectively) with the VITEK®2 method.

Comparative data analysis was further performed to ascertain the significance of MIC_{50} and MIC_{90} of species-specific CoNS using the two AST test methodologies, the standard E-test and VITEK®2 (Table 4-7) by means of a similar partial liner regression coefficient model. The MIC_{50} and MIC_{90} concentration values were calculated using the following formula:

$$\mathrm{MIC} = \mathrm{LBC} + \frac{\mathrm{TPoI} - \mathrm{LBPoI}}{\mathrm{UBPoI} - \mathrm{LBPoI}} (\mathrm{UBC} - \mathrm{LBC})$$

Whereby, MIC is the minimum inhibitory concentration; LBC is the lower bound concentration which is the highest concentration that gives a proportion of inhibition less than or equal to the target proportion of inhibition (TPoI). LBPoI is the proportion of inhibition at the LBC ad UBPoI is the proportion of inhibition at the upper bound concentration which is the lowest concentration that gives a proportion of inhibition equal to a higher than the target.

		Vancomycin		Teicoplanin	
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
	Isolate				
VITEK®2	*S. epidermidis*	0.89	1.73	2.76	7.51
	S. hominis	0.75	1.7	<0.5	3.68
	S. haemolyticus	0.93	1.9	2	6.8
	Other CoNS	<0.5	0.87	0.5	1.73
Std. E-test	*S. epidermidis*	1.73	1.99	3.32	9.53
	S. hominis	0.92	1.87	<0.5	4.2
	S. haemolyticus	1.69	2.7	2	7.4
	Other CoNS	1.13	1.43	<0.5	1.55

Table 4-7: Comparative MIC_{50} and MIC_{90} value ranges (mg/L) of in-patient species-specific CoNS isolates to vancomycin and teicoplanin investigated with the VITEK®2 automated microdilution system and standard E-tests

The computed MIC results that this model produces shown in Table 4-7 reflects apparent differences in MICs exist dependent on the AST technique used for species-specific MIC determination. Among the 100 in-patient species-specific CoNS isolates the MIC_{50} and MIC_{90} for vancomycin were on average approximately one doubling dilution higher by standard E-test in comparison to those obtained using the VITEK®2 automated system. These differences were also observed with teicoplanin for all three predominant species of CoNS with the exception of the other minor group of CoNS isolates. The single exception was results obtained for teicoplanin in the other minor group of CoNS species, where almost no significant difference was observed for MIC_{50} and MIC_{90} values ($p = 0.9$). The overall assignment probability for vancomycin and teicoplanin susceptibility with both AST methods was determined to be high ($p > 0.05$), demonstrating good correlation between standard E-test and VITEK®2 AST methodologies for the MIC evaluation.

4.5.4 Macro-method E-test for heteroresistance detection

The prevalence of heteroresistance amongst the 100 in-patient CoNS isolates was evaluated using the previously described modified screening method using BHI agar (van Hal *et al*., 2011; Satola *et al*., 2011).

All batch test results that included the ATCC positive controls Mu3 (hGISA), Mu50 (GISA) and the negative control *S. aureus* (ATCC 29213) preformed within the expected quality control ranges.

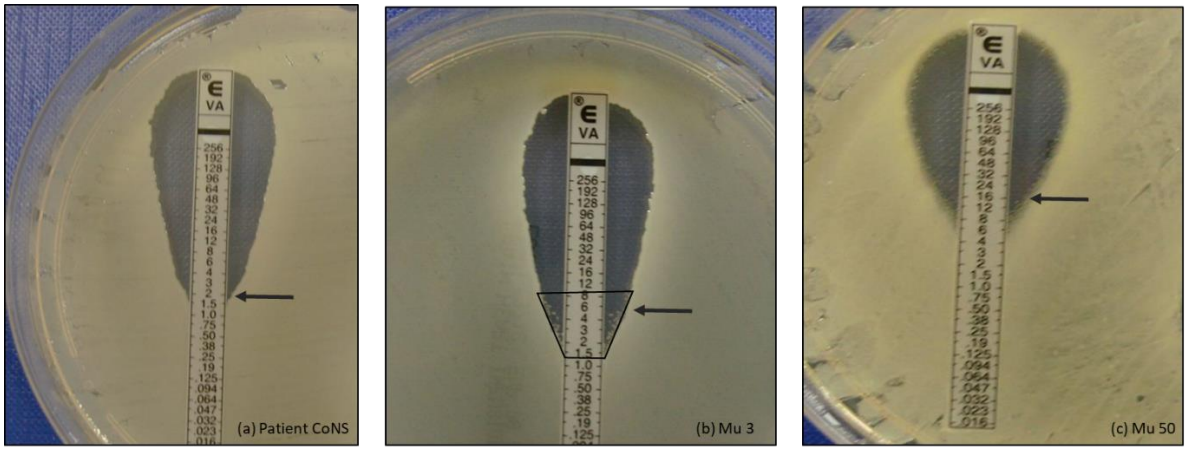


Figure 4-17: Macro-method using BHI agar and E-test antibiotic gradient strips on Brain-Heart Infusion agar. (a) Representative in-patient CoNS isolate displaying vancomycin MIC of 2 mg/L, b) Mu3 shows lower VA value MIC of 4mg/L with discernible small-colony variants visible within the inhibition ellipses area. c) Mu50, the GISA strain shows a higher MIC value of 16mg/L.

Due to the lack of defined breakpoints for the interpretation of glycopeptide susceptibility using the macro-method the interpretative criteria as previously proposed by other researchers was applied (Tenover *et al*., 2001; Walsh et al 2001; Wooton *et al*., 2007; Satola *et al*., 2011). The following E-test MIC interpretative criteria for heteroresistance detection for CoNS strains exhibiting MIC of ≥8mg/L for both vancomycin and teicoplanin or MIC of >12mg/L for teicoplanin regardless of vancomycin MICs were applied for evaluation of resistance. These are not true MIC values, but interpretative MICs based on the heavier inoculum of 2 McFarland standard on enriched BHI medium incubated over a longer period of time. The higher inoculum and media combination supports the growth and hence detection of small subpopulations of resistant cells as shown in Figure 4-17.

Based on these criteria the MIC values for the glycopeptide heteroresistant control strain Mu3 was observed to be typically between 4-6mg/L and for intermediate resistance control strain Mu50 was 12-16mg/L for each test batch. The overall comparative MIC distribution ranges of vancomycin and teicoplanin read at 48 hours using the E-test macro-method performed on the 100 in-patient CoNS isolates are illustrated in Figure 4-18. Applying the proposed evaluation criteria for determination of heteroresistance prevalence in CoNS in-patient isolates, 3 and 28 out of the 100 isolates tested exhibited MICs ≥8mg/L for vancomycin and teicoplanin respectively.

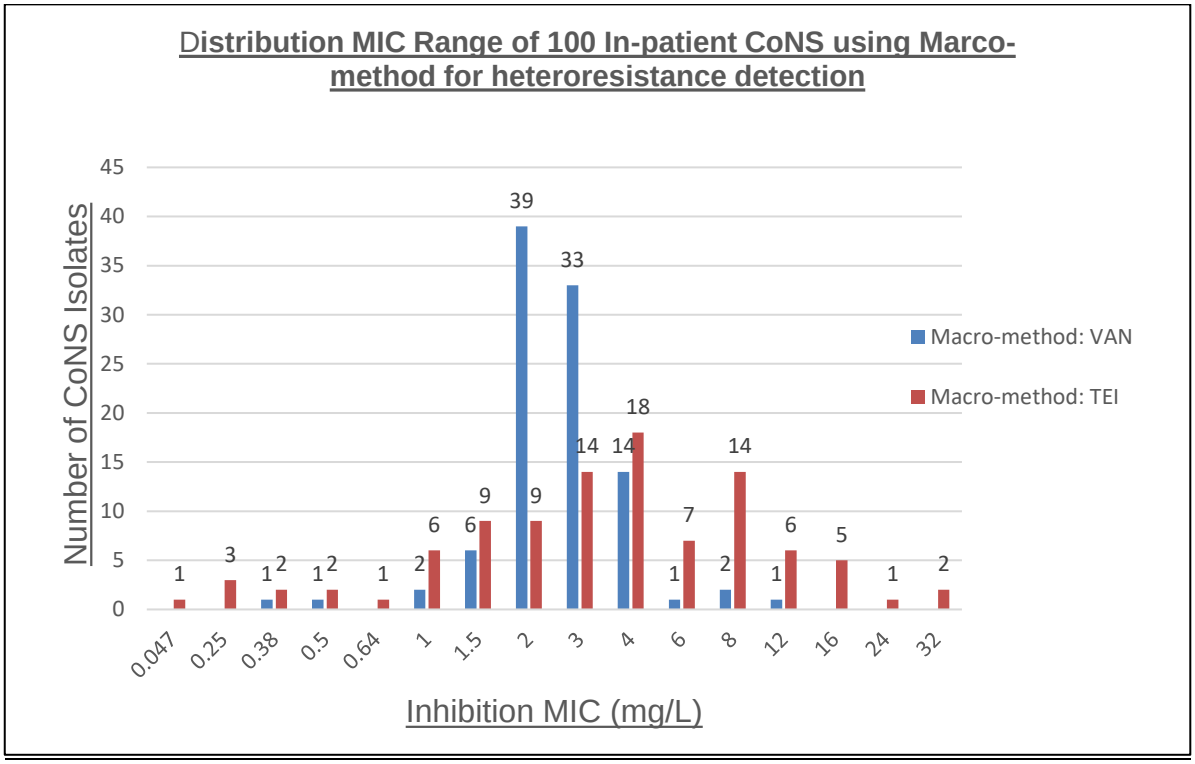


Figure 4-18: MIC (mg/L) distribution range of the glycopeptides, vancomycin (VAN) and teicoplanin (TEI) for the 100 in-patient CoNS isolates as determined by the macro-method for detection of heteroresistance.

Further evaluations carried out based on the criteria for isolates expressing teicoplanin MICs ≥12mg/L regardless of the vancomycin susceptibilities 14 of the 100 CoNS isolates tested with MICs ranging from ≥12-24mg/L met the criteria for hVIS. In total these 14 isolates would be considered for the presence of hVIS, as the number of isolates expressing teicoplanin MICs of 8 and >12mg/L, were within the defined criteria inclusive of the 3 isolates demonstrating vancomycin MICs of ≥8mg/L. Therefore, the overall result evaluations reveal a probable total prevalence of 14% heteroresistance amongst the 100 in-patient CoNS isolates tested using the BHI macro-method for detection glycopeptide resistance.

Further analysis of the *in-vitro* activity of vancomycin and teicoplanin against species-specific CoNS isolates tested using the BHI macro-method are detailed in Table 4-6.

BHI Macro-method: Number of Isolates (Cumulative %) Inhibition at MIC in mg/L against 100 in-patient CoNS Predominant Species Groups				
BHI Macro-method VAN: MIC mg/L	*S. epidermidis* (n=57)	*S. hominis* (n=18)	*S. haemolyticus* (n=16)	Other CoNS (n=9)
<0.5				2 (22%)
1		1 (6%)		1 (11%)
1.5	3 (5%)	2 (11%)	1 (6%)	6 (67%)
2	18 (32%)	8 (44%)	7 (44%)	
3	24 (42%)	3 (17%)	6 (38%)	
4	9 (16%)	4 (22%)	1 (6%)	
6	1 (2%)			
8	1 (2%)		1 (6%)	
12	1 (2%)			
BHI Macro-method TEI: MIC mg/L	*S. epidermidis* (n=57)	*S. hominis* (n=18)	*S. haemolyticus* (n=16)	Other CoNS (n=9)
<0.5		6 (33%)		2 (22%)
0.64				1 (11%)
1	1 (2%)	3 (17%)		1 (11%)
1.5	3 (5%)	3 (17%)		3 (33%)
2	9 (16%)			
3	12 (21%)	1 (6%)	1 (6%)	1 (11%)
4	11 (19%)	1 (6%)	5 (31%)	1 (11%)
6	5 (9%)	1 (6%)	1 (6%)	
8	7 (12%)	3 (17%)	4 (25%)	
12	5 (9%)		1 (6%)	
16	4 (7%)		1 (6%)	
24			1 (6%)	
≥32			2 (13%)	

Table 4-8: MIC value ranges (mg/L) of in-patient species-specific CoNS to vancomycin and teicoplanin investigated using the E-test BHI Macro-method.

The other minor group of CoNS isolates did not demonstrate vancomycin and teicoplanin MICs above 1.5 and 4mg/L respectively therefore demonstrating nil resistance to the glycopeptides. Similarly, Isolates of *S. hominis* did not exhibit heteroresistance as vancomycin and teicoplanin MICs were <8 and 12 mg/L respectively which were below the criteria range for presence of heteroresistance determination. Conversely, heteroresistance prevalence was apparent in a total of 9(16%) *S. epidermidis* isolates of which all nine exhibited teicoplanin MICs of ≥12mg/L including 2(4%) which exhibited vancomycin MICs ≥8mg/L. Similarly, hVIS resistance was apparent in a total of five isolates of *S. haemolyticus* with 5(31%) exhibiting teicoplanin MICs ≥12mg/L including the isolate A53 that also expressed vancomycin, MIC of 8mg/L. Analysis carried out based on pairwise comparisons between standard E-test method and BHI macro-method for the glycopeptides, revealed no

significant differences between the frequency distribution of either vancomycin or teicoplanin MICs for the in-patient CoNS species-specific isolates tested ($p > 0.05$).

4.5.4.1 GRD E-test method for glycopeptide intermediate/heteroresistance detection

The presence of GIS or hGIS strains was determined for all the 100 in-patient CoNS using the new prototype double sided gradient E-test strip in accordance with the manufacturer's instructions. Results were read after an incubation period of 48 hours to allow detection of the slower growing resistant colonies within the susceptible population. Defined heteroresistance/glycopeptide intermediate resistance consensus for CoNS has however yet to be established. As specified, the endpoints read from the GRD E-tests were used for determination of interpretative classification similar to those defined for isolates of *S. aureus*.

Result analysis was primarily carried out following the manufacturers application of interpretative criteria for glycopeptide resistance indication. For heterogenous resistance, interpretative criteria was defined as E-test GRD TEI or VAN cut-off MIC value of ≥16 mg/L and a standard E-test VAN MIC of <8 mg/L. Accordingly, the results for the resistant control strains Mu3 and Mu50 VA:TP values were 4:16 mg/L and 16:>32 mg/L respectively. GRD MIC values for the glycopeptide sensitive *S. aureus* ATCC 29213 VA:TP varied typically between 0.5-1.5 mg/L and 1-2 mg/L for each test batch which was within the specified quality control range.

Figure 4-19 shows example of results obtained for two in-patient CoNS isolates using the GRD E-test strips in comparison to those obtained with the Mu3 control strain.

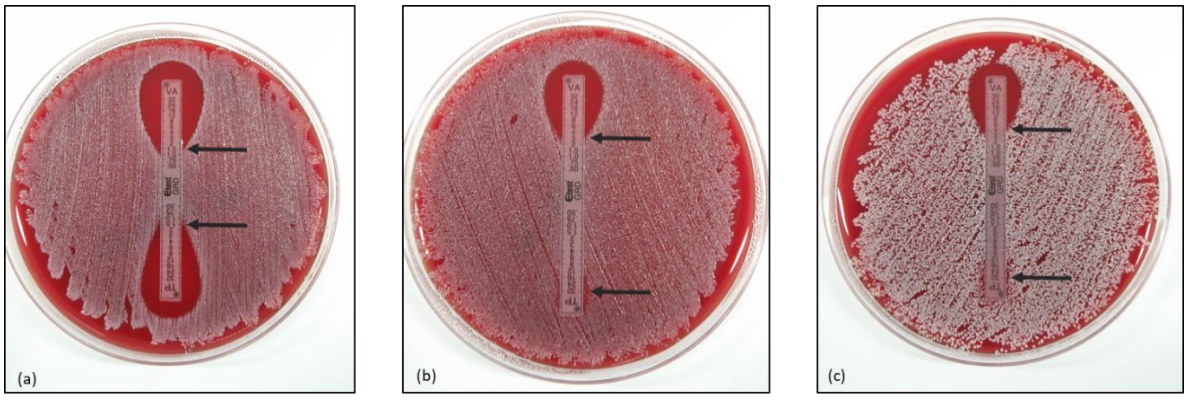


Figure 4-19: Susceptibility results obtained with the prototype GRD (VA)/(TP) vancomycin/ teicoplanin, E-test strips for two in-patient representative CoNS isolates and the hGIS control isolate. a) Susceptible *S. epidermidis* isolate displaying VA/TP MICs of 1.5/1.5 mg/L b) *S. epidermidis* isolate displaying VA/TP MICs of 2/32mg/L c) Mu3, isolate displaying VA/TP MICs of 4/32mg/L.

The overall comparative MIC distribution ranges of vancomycin and teicoplanin read at 48 hours using the GRD E-test method performed on the 100 in-patient CoNS isolates are illustrated in Figure 4-20.

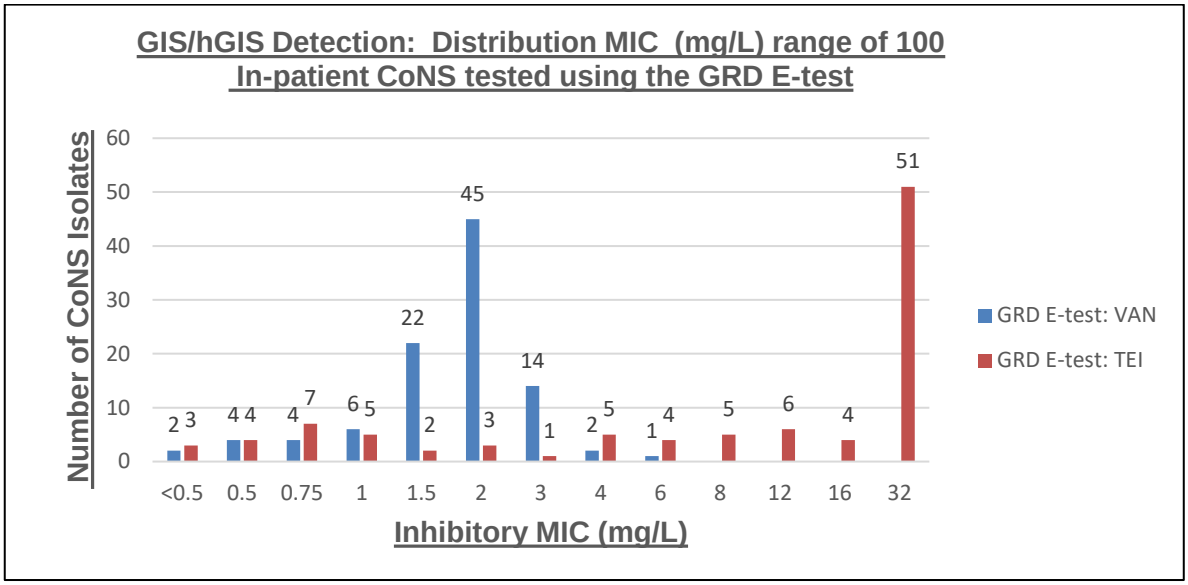


Figure 4-20: MIC (mg/L) distribution range of the glycopeptides, vancomycin (VAN) and teicoplanin (TEI) for the 100 in-patient CoNS isolates as determined by the GRD E-test method.

Overall, the use of GRD E-test methodology indicates that although in total 70% of the CoNS in-patient isolates displayed high teicoplanin resistance ranging from 6 to >32mg/L, with the exception of one isolate, all others remained vancomycin susceptible. Likewise, neither of the two CoNS isolates expressing vancomycin MICs of 4 mg/L were observed to display typical growth of small colony variants after 48h incubation similar to those observed with the Mu3 (hVISA) control strain. Mu3 displays sub-populations (micro-colonies) of cell growing up to vancomycin concentration level of ≥4mg/L as shown in Figure 4-19c.

Comparing results for vancomycin and teicoplanin MIC values, although a total of 55 in-patient CoNS exhibited GRD E-test teicoplanin resistance at MIC values of ≥16mg/L, none of these isolates expressed vancomycin MIC value of ≥8mg/L tested by standard E-test methodology. Therefore, based on the application of manufacturers interpretive guidelines, none of the in-patient CoNS isolates met the criteria for the presence of heteroresistance detection. Likewise, none of the 100 in-patient CoNS isolates demonstrated the presence of GIS with the GRD E-test, based on the manufacturers interpretative criteria of TEI or VAN MIC value of ≥16 mg/L and a standard E-test VAN MIC value of ≥8 mg/L.

Conversely, results based on the application of modified interpretive cut-offs for the GRD E-test strips evaluated by other researchers, demonstrates a likelihood of heteroresistance

among the 100 in-patient CoNS strains tested (Yusof *et al*., 2008). Data for the 100 CoNS in-patient isolates was re-evaluated based on interpretive cut-off criteria proposed by the researches of TP or VA MIC ≥8mg/L and vancomycin standard E-test MIC value of ≥6mg/L for GIS and TP or VA MIC ≥8mg/L and standard E-test VA MIC ≤4mg/L for hGIS detection. Data analysis based on these interpretive cut-offs values for GIS detection of TP:VA MIC of ≥8mg/L and standard E-test vancomycin MIC ≥6mg/L, results reveal an absence of glycopeptide intermediate resistance among the 100 CoNS in-patient isolates. However, re-evaluated results based interpretive cut-offs values for hGIS detection reveal the possible presence of heteroresistance in a total of 66 CoNS isolates. This includes all isolates exhibiting GRD E-test TP MICs of ≥8mg/L and the one isolate exhibiting breakpoint VA MIC of 4mg by standard E-test.

GRD E-Test (Double-Sided) Method: Number of Isolates (Cumulative %) Inhibition at MIC in mg/L against 100 in-patient CoNS isolates				
GRD: VAN MIC mg/L	*S. epidermidis* (n=57)	*S. hominis* (n=18)	*S. haemolyticus* (n=16)	Other CoNS (n=9)
<0.5		3 (17%)		3 (33%)
0.75		4 (22%)		
1	1 (2%)	4 (22%)		1 (11%)
1.5	10 (18%)	2 (11%)	8 (50%)	2 (22%)
2	34 (60%)	4 (22%)	4 (25%)	3 (33%)
3	11(19%)		3 (19%)	
4	1 (2%)	1 (6%)		
6			1 (6%)	
GRD: TEI MIC mg/L	*S. epidermidis* (n=57)	*S. hominis* (n=18)	*S. haemolyticus* (n=16)	Other CoNS (n=9)
<0.5		4 (22%)		3 (33%)
0.75	1 (2%)	5 (28%)		1 (11%)
1	2 (4%)	3 (17%)		
1.5	1 (2%)			1 (11%)
2	1 (2%)	1 (6%)		1 (11%)
3				
4	3 (5%)		2 (13%)	1 (11%)
6	3 (5%)		1 (6%)	
8	2 (4%)		3 (19%)	1 (11%)
12	3 (5%)		2 (13%)	
16	2 (4%)		2 (13%)	
≥32	39 (68%)	5 (28%)	6 (38%)	1 (11%)

Table 4-9: MIC value ranges (mg/L) of in-patient species-specific CoNS to vancomycin and teicoplanin investigated with the Double-Sided GRD E-Test.

Data analysis carried out using of the GRD E-test to detect the prevalence of heteroresistance based on the distribution of MICs measured for vancomycin and teicoplanin for each of the species-specific in-patient CoNS isolates are presented in Table 4-9.

With the exception of one, all CoNS species-specific isolates tested by double-sided GRD E-Test, expressed vancomycin MIC values ranging from <0.5 to ≤4mg/L. Accordingly, all species-specific CoNS were classified as vancomycin susceptible with the exception of 1(6%) *S. haemolyticus* isolate displaying MIC of 6mg/L. Conversely the highest GRD E-test teicoplanin resistance rates were observed in 14(88%) *S. haemolyticus* followed by *S. epidermidis* 49(86%). Comparatively lower TP: GRD resistance rates were observed in isolates of *S. hominis* and the other minor group of CoNS 5(28%) and 2(22%) respectively ranging from MIC 6 to >32mg/L. Based on the manufacturers interpretative guidelines for GRD E-test overall results therefore reveal an absence of CoNS species-specific glycopeptide intermediate/heterogenous resistance.

In comparison, using the interpretive MIC cut-offs for hGIS evaluations with GRD E-test as proposed by Yusof and colleagues (2008), one (16%) isolate of *S. haemolyticus* in particular would be defined as expressing heterogenous glycopeptide intermediate resistance. Therefore, *S. haemolyticus* isolate, A53 expressing vancomycin standard E-test MIC of 4mg/L and GRD E-test, VA:TP 6:>32mg/L would fit the hGIS classification criteria. Moreover, using these MIC cut-offs interpretive criteria, heterogenous resistance expression could also be demonstrated in a total of 66 species-specific in-patient CoNS isolates. These include, 46(81%) *S. epidermidis*, 13(81%) *S. haemolyticus,* 5(28%) *S. hominis* and in 2(22%) of the other minor CoNS species displaying TP MICs ≥8mg/L, GRD E-test values therefore meeting the defined criteria for heteroresistance detection.

GRD E-test glycopeptide MIC data further subjected to pairwise sample statistics revealed that there was a significant difference observed between CoNS species-specific, distribution of vancomycin and teicoplanin MICs and the two test methodologies BHI macro-method and GRD E-test method ($p = 0.0001$ with 99 *df*). Similarly, a significant difference was observed between MIC frequency distribution of teicoplanin in comparison to use of the standard E-test method and GRD-E-test methodology for detection of glycopeptide resistance ($p = 0.0001$ with 99 *df).* Conversely, no significant difference was however demonstrated between vancomycin MIC susceptibility distribution with use of GRD-E-test and the standard E-test method ($p = 0.075$ with 99 *df*).

4.6 Discussion

Colonisation with potentially pathogenic isolates of staphylococci which may become invasive are common amongst the haematology patient population, where immunity defects are related to underlying malignancy or therapy. The clinical significance of CoNS disease in the healthcare setting continues to increase as strategies in advancing medical practice lead to more invasive procedures. CoNS infections have been noted to compose a serious problem among immunocompromised patients, not only as a result of widespread use of vascular catheters but also the extensive use of antimicrobial prophylaxis (Feld, 2008). CoNS in-patient infections in this vulnerable patient group have become a major concern because of their extraordinary ability to rapidly adapt to antibiotic stress due to the extended usage of broad-spectrum antibiotics (Livermore, 2000). Antibiotic resistance may therefore also contribute to the poor rates of bacteraemia-related patient outcomes following treatment through insufficient clearance of infection (Harbarth *et al*., 2003). Hence, effective antimicrobial therapy is critical in controlling commonly encountered CoNS bacteraemia in the haematology and BMT patient group. Clinically significant CoNS in this patient group are reported to have become increasingly resistant to commonly used antimicrobials and hence remain a significant challenge to the provision of successful therapeutic outcomes (Deikema *et al*., 2001; Yadegarynia *et al*., 2003; Falcone *et al*., 2015).

Appropriate antimicrobial therapy is continually challenged not only by the emergence of antibiotic resistance but also the underlying disease state such as developing febrile neutropenia complicating the *in-vivo* response to implemented therapeutics (Collin *et al*., 2001; Bal and Gould 2007). Effective empirical antibiotic therapy is therefore dependent on the microbiology laboratory providing accurate information of existing spectrum of resistance among local strains of CoNS causing BSIs in these patients. AST is therefore necessary, not only to confirm susceptibility to the chosen empirical antimicrobial agent but more essentially, for the detection of acquired resistance in species-specific isolates of CoNS. This is because anti-staphylococcal agents from several different antibiotic classes demonstrate differing resistance mechanisms hence affecting drug efficacy.

May and colleagues (2014) in their study demonstrated the significant increase in antibiotic resistance in CoNS pathogens over a 13 year period. The importance of these results further indicates the value of monitoring resistance trends within specialist healthcare units as has it has become apparent that CoNS can serve as sentinels of resistance. Additionally, study results revealed that CoNS colonisers played a role in potential pathogenesis, acting as reservoirs for resistance genes responsible for nosocomial transmission to other pathogens. The link between inappropriate broad-spectrum antibiotic prescribing and

resistance levels suggests the critical importance of curbing the reservoir of resistance in these potentially pathogenic CoNS colonisers (Piette and Verscharegen, 2009). Hence awareness of the predominant BSI isolates and their antibiograms that depict *in-vitro* susceptibility patterns of the prevalent potential pathogens are of importance for clinical selection of effective initial empirical therapy. In most healthcare institutions the provision of quantitative MIC antimicrobial data is a prerequisite for the management of in-patient infections among the critically ill. Antimicrobial susceptibility testing is, therefore, important not only to confirm susceptibility to the chosen empirical antimicrobial agent but also detection of acquired resistance mechanisms especially important in species-specific *Staphylococcus* isolates. Hence, provision of accurate MICs ensures correct dosage guidance for clinicians to ensure targeted patient therapies, which limits antimicrobial resistance development thus leading to optimal clinical outcomes (Raney *et al*., 2002; Nonhoff *et al*., 2005).

In routine diagnostic laboratories, antibiotic susceptibility profiles are the "first-line" phenotypic typing scheme, often used towards identification of clinically significant strains of CoNS and their emerging spectrum of resistance. Therefore, the main purpose of this part of the study was to determine the prevalence of resistance among the 100 in-patient CoNS isolates recovered from haematology patient group to the commonly used anti-staphylococcal drugs. Performance of comparative antibiotic profile analysis of CoNS isolates from the pre-admission control patient group in this study also aimed to provide an insight into the incidence of resistance amongst the skin colonisers to the same panel of antimicrobials. This was based on the postulation that these skin colonising CoNS isolates could be the potential source of nosocomial acquisition and transmission in the haematology/BMT patient group.

The study results using the VITEK®2 automated AST system for all one hundred CoNS isolates from both patient groups, demonstrated variable resistance to all classes of antibiotics to the panel of 18 agents tested with the exception of tigecycline. The lowest resistance rates were observed to vancomycin, chloramphenicol, linezolid, rifampicin and mupirocin in both patient groups. These results are consistent with other study reports, showing that there has been minimal to no resistance to linezolid noted among isolates of CoNS with the exception of a few recovered from blood cultures of patients who had undergone previous extensive treatment with linezolid (Kelly *et al*., 2006). Although the use of linezolid is known to be limited in the haematology hospitalised patient group, nonetheless, 1(3%) skin colonising *S. epidermidis* isolate (G70) was found to exhibit linezolid resistance. However, none of the in-patient CoNS isolates expressed resistance to linezolid. These finding suggest that the absence of resistance to linezolid noted in the in-patient isolates may

be due to lack of antibiotic pressure. Linezolid is exceptionally limited for use in the haematology patient group because of its low bioavailability associated with immunosuppression and haematopoietic toxicity (Kuter, 2001).

With the exception of penicillin, the other minor group of CoNS isolates in both patient groups, presented the lowest percentage resistance to all the other classes of antibiotics in the panel of 18 antimicrobials tested. The overall antibiogram resistant profiles obtained from both patient groups show that less than 10% of the CoNS isolates remained susceptible to a few of the commonly used anti-staphylococcal agents, including antimicrobials such as rifampicin. Although infrequently used, rifampicin usually forms part of combination therapeutic regimen with either vancomycin or the aminoglycosides for treatment of infections that are difficult to eradicate (Rose and Poppens, 2009). Similarly, of the aminoglycosides, gentamicin is a drug frequently used in combination with vancomycin or rifampicin particularly for the treatment for MRSA BSIs in healthcare facilities for optimal clinical outcomes in high-risk patient groups (LaPlante and Woodmansee, 2007; Rehm *et al*., 2008). While resistance to rifampicin was observed to be low in both patient groups comparatively high resistance to all three aminoglycosides, gentamicin tobramycin and kanamycin was observed in the in-patient isolates. Overall, 50% of the in-patient CoNS isolates in comparison to around 10% of control group skin colonising isolates demonstrated resistance to the aminoglycosides. It is possible that the higher rates of aminoglycoside resistance in the in-patient isolates may have disseminated from the reservoirs of resistance present in skin colonising CoNS isolates. These findings are supported by evidence from other studies, reporting that the predominant aminoglycoside resistant phenotype is commonly found among CoNS isolates colonising the skin of hospitalised patients. Hence, the prevalence of aminoglycoside resistance observed among nosocomial isolates causing serious infection in high-risk patient populations may be due to dissemination from skin colonising resistant isolates of CoNS (Klingenberg *et al.,* 2004; Koksal *et al.,* 2009). The present study results therefore suggest that 10% of the control group skin colonising isolates may be representative of the resistant aminoglycoside phenotype involved in the spread of nosocomial infections.

The overall percentage resistance rates to all the other classes of antibiotic including the β-lactams in particular to methicillin was higher amongst the in-patient CoNS isolates in comparison to skin colonising isolates of the control patient group. However, there was no significant difference noted between the overall distribution of resistance to all antimicrobial classes in both patient group CoNS isolates ($p > 0.05$), with the exception of resistance to methicillin. Through *in-vitro* studies authors have previously, demonstrated that the majority of staphylococcal species are resistant to the penicillins, due to production of beta-lactamase

with methicillin resistance among clinical isolates of CoNS having a worldwide prevalence of 75% (Brown, 2001; Miragaia *et al*., 2002; Stefani and Varaldo, 2003). Similar levels of methicillin resistance were observed in the in-patient CoNS BSI isolates in comparison to much lower rates displayed by the control group isolates.

Results obtained with the VITEK®2 system analysis has been shown to have excellent accuracy for the detection of methicillin resistance in CoNS isolates with a sensitivity/specificity of 99/96% accuracy with the use of the anti-staphylococcal antibiotic AST-P507 test card (Raney *et al*., 2002; Ligozzi *et al*., 2002; Nonhoff *et al*., 2005). Based on the BSAC/EUCAST breakpoint of 0.25mg/L for oxacillin (a proxy antibiotic drug used for the detection of resistance to methicillin) the highest resistance to methicillin was detected in 78% of the in-patient CoNS isolates in comparison to 28% resistance in the control patient group. The results of this study are also concordant with a previously published study of susceptibilities of blood stream isolates from BMT patients reviewed over a seven year period; showing a trend of increasing resistance among staphylococcal species to semisynthetic penicillins, ciprofloxacin and gentamicin. The authors demonstrated that some staphylococcal species remained uniformly susceptible with the exception of others, including isolates of CoNS (80%) demonstrating increasing resistant to methicillin over this period (Collin *et al*., 2001). Likewise, the present study results indicated high levels of resistance among isolates of the in-patient group, with less than five percent of CoNS strain remaining susceptible to agents such as linezolid, vancomycin, tigecycline and linezolid. Additionally, these in-patient CoNS isolates also demonstrate a significantly higher percentage resistance to methicillin in comparison to the skin colonising strains ($p < 0.05$). The overall resistance rates observed in haematology patient group in-patient CoNS isolates concur with those of a UK based surveillance study of 25 hospitals, where 68.9% of the CoNS isolates from blood or line infections were reported to be resistant to methicillin (Henwood *et al*., 2001). Furthermore, MR-CoNS rates in this study investigation were also consistent with data collated from worldwide surveillance programmes, showing that the rate of methicillin resistance in CoNS varied from between 70-80% (Fluit *et al*., 2001). The variation in resistant rates are reported to be dependent on prevalence of MR-strains concomitant with the different geographical regions of Europe, USA, Canada and the Latin American countries (Falcone *et al*., 2004; Vincent *et al*., 2006).

Observational studies indicate that hospitalisation and antimicrobial treatment tend to influence colonisation patterns which impacts on both the risk of cross-transmission between patients and probability of acquiring nosocomial infections. The lower rates of MR (28%) observed among control patient group skin colonising isolates were also consistent with studies demonstrating that CoNS isolated from the skin of healthy persons were considerably

less resistant in comparison to clinical isolates and those belonging to indigenous nosocomial populations (Agvald-Öhman *et al*., 2003). Furthermore, Cherif and colleagues, (2013) in their study comparing skin *S. epidermidis* isolates from healthy volunteers with those causing CR-BSIs in hospitalised patients also demonstrated that resistance to methicillin and other β-lactams was found to be much higher amongst CR-BSI isolates than those that were CoNS skin colonisers. Similarly, the observed, overall distribution of MR in CoNS isolates recovered from blood cultures of patients in the haematology unit were comparatively higher than those of the pre-admission skin-colonisers, results which are corroborated by other studies (Yamada *et al*., 2017). The authors demonstrated that infections attributed to MR-CoNS (60%) were more common in febrile neutropenic patients with haematological malignancy in comparison to other high-risk patient group isolates. The observed inefficacy of methicillin therefore, limits treatment options for CoNS associated BSI in the haematology patient group related to unfavourable clinical outcomes.

It was indeed interesting to find that most species-specific CoNS isolates of the control group were resistant to mupirocin a topical decolonisation antimicrobial agent frequently used for the eradication of healthcare associated methicillin resistant *S.aureus* colonisers. Mupirocin resistance was observed to be present in up to 16% of all the CoNS skin colonising species isolates however, only one, in-patient *S. epidermidis* isolate (2%) displayed resistance to mupirocin. Predisposition to mupirocin resistance is a factor known to be significantly associated with persistent carriage of MR-strains in particular MRSA with failure of decolonisation strategies increasing the risk of adverse clinical outcomes (Poovelikunnel *et al*., 2015). Therefore, study results demonstrate that the therapeutic indication for mupirocin would perhaps be limited in up to 16% of the ward population if these patients were colonised with mupirocin resistant CoNS isolates. This level of mupirocin resistance displayed by skin colonising strains raises cause for concern, because of the potential risk of selection and spread of methicillin resistance among susceptible CoNS associated with nosocomial BSIs in high-risk patient groups. MR-CoNS responsible for healthcare associated BSIs and their associated multidrug resistance potential also adds to challenge of patient management for optimal clinical outcomes. These factors, particularly among vulnerable patient groups are known to play as important drivers for the emergence and dissemination of highly resistant epidemic CoNS strains which may also essentially be related to species-specific traits (Rossolini *et al*., 2010). Observations made in the present study revealed some further important points relating to the antimicrobial spectrum of species-specific in-patient CoNS in comparison the skin colonising isolates.

Documented evidence suggests that resistance may be elicited via transfer of resistance elements from nosocomial CoNS strains, acting as reservoirs for resistant genes and are

postulated to be the main source of horizontal transfer between species-specific isolates (May *et al.,* 2014). Therefore, of the CoNS species-specific isolates phenotypically expressed resistance traits to the 18 principle anti-staphylococcal drugs explored further to determine clonal similarity highlighted some important differences. Comparative analysis of species-specific CoNS antibiogram resistance profiles facilitated by use of multivariant statistical PCA methodology revealed a high degree of species-specific isolate unrelatedness in both patient groups. Additionally, dendrograms of percent similarity calculated with Dice coefficients from the antimicrobial resistant data from both the in-patient and control patient groups, using a cut-off set at 95% revealed no major clusters among species-specific CoNS resistant isolates. Sixty-six and 68 antibiograms were distinguished in both in-patient and control group respectively. This phenotypic antibiotic typing scheme revealing a large number of unique resistance patterns indicates the presence of highly heterogenous populations among CoNS isolates from both patient groups. PCA-UPMGA analysis further revealed a significant lack of correlation between antimicrobial resistance profiles among and between species-specific CoNS, particularly noticeable amongst isolates of the control patient group. Such significant differences were more apparent among the other minor group of CoNS species-specific isolates of the control patient group in comparison to the in-patient isolates. Of these, delineation levels revealed 30% of the isolates exhibiting distinct antibiograms comprising predominantly isolates of *S. capitis*, *S. lugdunensis* and *S. warneri.* Although antibiogram typing is a relatively simple method with a low discriminatory power compared to genotypic methodologies for determination of species-specific isolate delineation; results of this study confirm a high degree of unrelatedness among and between species-specific CoNS isolates of both patient groups. Quantitative antibiogram determination combined with cluster analysis can therefore be valuable as a simple screening method to determine species-specific isolate and clonal relatedness in order to elucidate the source of infection.

The occurrence of highly diverse range of antimicrobial resistance profiles observed between and among species-specific in-patient CoNS isolates in comparison to those of the control group skin colonisers may also be due a number of different factors that influence resistance acquisition. As such, it could be postulated that the hospital environment may be involved in evoking various adaptive responses in colonising strains for survival. Conversely, prevalence of diverse phenotypes among the in-patient CoNS population may be because of evolving adaptive responses due to selective pressure and propagation of resistance as a consequence of antibiotic overuse. Such adaptive and coincidental mutational events in the context of antimicrobial resistance are well documented, notably amongst clinical isolates of staphylococci demonstrating high frequency of resistance to various classes of antimicrobials

in particular to the β-lactams (Klingenberg *et al.,* 2004; Arslan and Özkardes, 2007; Koksal *et al.,* 2009). Antibiogram analysis for the determination of phenotypic species-specific isolate delineation, although of a lower discriminatory power in comparison to genotypic characterisation, could therefore also be used for the clear purpose of confirming and elucidating the source and transmission of infection due to species-specific CoNS in the hospitalised patient group. In this study a major diversity in antibiotic susceptibility profiles found between and among the different species-specific CoNS isolates in both patient groups suggests the in-patient CoNS may belong to hypervirulent clones as opposed to being drawn from the hospital environment of patient endogenous skin colonising isolates.

All antibiotics are known to exert selective pressure favouring resistant variants, such that some have a stronger effect in comparison to others as a consequence of their pharmacodynamics and mode of action demonstrated by the development and spread of MR-staphylococci associated with MDR in the hospital environment. Such effects of antimicrobial resistance development have been noted to be more frequent in isolates of *S. epidermidis* and *S. haemolyticus* in comparison to all other species of CoNS (Archer and Climo, 1994; Diekema *et al*., 2001). A pattern of high variability of resistance profiles to more than one antibiotic in the panel of 18 antimicrobials tested was found for both in-patient and control patient CoNS isolates. Evaluation of species-specific resistance patterns in this study showed that isolates of *S. epidermidis S. haemolyticus* as well as those of *S. hominis* in both patient groups remained uniformly resistant to penicillin but expressed differing rates of resistance to all the other antimicrobial classes. MDR was significantly more apparent in the in-patient isolates of *S. haemolyticus* ($p < 0.05$), in comparison to the lowest resistance observed in isolates of the other minor group of CoNS species. Of note, one these isolates also expressed resistance to vancomycin. Nonetheless, of the control patient group isolates of *S*. *epidermidis* demonstrated higher rates of MDR with one isolate also expressing vancomycin resistance. By comparing antibiotic resistance patterns results show that of the control patient group isolates there was no significant correlation between *S*. *epidermidis* isolates and the predisposition for MDR expression ($p > 0.05$). These findings are supported by data from other studies of species-specific resistance patterns of clinical CoNS isolates (Biavasco *et al*., 2000; Tabe *et al*., 2001; Kristóf *et al*., 2011). Conversely in both patient groups, the other minor group of CoNS species-specific isolates including those of *S. capitis S. lugdunensis*, *S. auricularis* and *S. warneri* generally demonstrated least resistance not only to the β-lactams but also exhibited higher levels of susceptibility to the other antimicrobial classes as well. The CoNS resistance patterns noted in this study illustrate the role of some species-specific traits in response to particular anti-staphylococcal agents. These species-specific differences of non-susceptibility to the various antimicrobial agents,

particularly among in-patient isolates suggests that such resistance exhibited by these clinical isolates may have emerged under definitive antibiotic pressures.

Becker and colleagues (2014) in their review publication of clinical and other relevant CoNS, report that over the past few decades CoNS isolated from clinical specimens have become significantly more resistant to the commonly used antimicrobials. Previous reports comparing antibiotic resistance patterns demonstrate that methicillin resistant species-specific isolates usually also display MDR, defined as resistance to three or more antibiotic classes (Diekema *et al*., 2001). Moreover, with the continuously increasing percentage of MDR-CoNS noted in patients with haematological malignancies with Gram-positive BSIs, MDR raises particular concerns because of limited treatment options and the associated consequence of higher mortality rates (Marin *et al*., 2014). MR-CoNS in-patient isolates in this study were generally observed to demonstrate a higher degree of multi-drug resistance in comparison to those that were susceptible. The overall frequency of MDR and MR among the 100 CoNS in-patient isolates was high (75% and 78% respectively). These results are concordant with results of a study which demonstrated that MR-CoNS blood culture isolates (67.5%) from septic patients also displayed resistance to more than three antibiotic classes in comparison to isolates expressing methicillin susceptibility (Koksal *et al*., 2009). Comparatively, lower rates of MDR and MR were observed to be exhibited by the skin colonising control group CoNS isolates (23% and 28% respectively). Overall analysis of both patient group CoNS antimicrobial resistance data indicated that there was a significant correlation between prevalence of MDR and corresponding high rates of methicillin resistance ($p = 0.0001$). Although *S. haemolyticus* exhibited higher resistance rates to all antimicrobial classes in comparison to all other species isolates; a significant association between MDR and the propensity to express methicillin resistance was observed only in the in-patient *S. epidermidis* isolate ($p = 0.0001$). These findings are supported by data from other studies showing resistance to other classes of antimicrobials are much lower in methicillin sensitive CoNS in comparison to those that express methicillin resistance (Sader and Jones 2012).

VITEK®2 analysis of species-specific antibiotic resistance profiles in this study revealed that of the three predominant CoNS species from the in-patient group the highest rates of methicillin and MDR was observed among 15 out of the 16(94%) isolates of *S. haemolyticus.* Of these one (6%) in-patient *S. haemolyticus* isolate notably also demonstrated resistance to vancomycin, results which are concordant with observations made by authors of previous studies. Falcone and colleagues (2006) study of patients with haematological malignancies over a seven month period, reported that *S. haemolyticus* exhibiting high rates of methicillin and teicoplanin resistance were second only to *S. epidermidis* as causative agents of

bacteraemia in post-transplantation period, further concurring with the results of the present investigation. Furthermore, clinical isolates of *S. haemolyticus* have been consistently documented as emerging nosocomial pathogens, particularly characterised by their tendency to develop MDR with a higher predisposition to glycopeptide resistance (Schwalbe *et al*., 1987, 2007; Stefani and Varaldo, 2003). Exploration of inter and intraspecies relationships isolate relatedness utilising PCA dataset analysis producing two factors variables, antimicrobial resistance and species-specific characteristic (Figure 4-6 and 4-14), demonstrated that one of the in-patient isolates of *S. haemolyticus* was significantly unrelated to the others in the sample group based on the Euclidean distance between the other species and this isolate. In comparison to all the other species-specific CoNS, this particular in-patient *S. haemolyticus* isolate appears to demonstrate a significantly distinct response not only to vancomycin but also to all the other 18 antimicrobials in the test panel. Although the majority of *S. haemolyticus* isolates demonstrated higher levels of MDR and MR in comparison to the other species-specific CoNS, isolates of *S. epidermidis* from both the in-patient and the control patient group demonstrated a higher prevalence of MDR significantly associated with methicillin resistance 88/83% and 34/38% respectively ($p < 0.05$). Interestingly, in the control patient group although not statistically significant, the prevalence of MR associated with MDR was observed to be higher in isolates of *S. epidermidis* in comparison to *S. haemolyticus* (34%/38% and 21%/13% respectively). Additionally, the observed resistance to vancomycin in one (3%) *S. epidermidis* skin colonising isolate would be of concern as this could potentially be the causative agent of nosocomial BSI in the haematology patient group. The prevalence of such MDR-CoNS isolates including resistance to vancomycin limit therapeutic options in high-risk patient groups. In recent years however, concerns have also been raised regarding the efficacy of vancomycin in the treatment of serious BSIs caused by MR and MDR-staphylococci. There is increasing evidence of high rate of vancomycin therapeutic failures of BSI caused by such organisms. This has been noted particularly amongst MRSA isolates displaying vancomycin MICs of 1.5mg/L and at the breakpoint of 2mg/l, although considered susceptible in accordance with the BSAC, CLSI and EUCAST guidelines, such isolates have failed *in-vivo* eradication (Sakoulas *et al*., 2004; Steinkraus *et al*., 2007). Although, acquired resistance to the glycopeptides has also been recognised among MR-CoNS, teicoplanin resistance has been reported to occur more commonly than vancomycin resistance particularly among isolates of *S. haemolyticus* followed by *S. epidermidis* in comparison to vancomycin resistance (Biavasco *et al*., 2000). Unlike such previously published data, teicoplanin resistance in this study was noted to be much higher amongst in-patient *S. epidermidis* isolates in comparison to *S. haemolyticus* (34% and 19% respectively); with even higher rates of resistance noted in those of the control patient group (49 and 47% respectively). Additionally, a significant

association between isolates expressing MR with those demonstrating resistance to teicoplanin was noted only amongst *S. epidermidis* in-patient isolates (p >0.05). Of note, such resistance rates are also known to correlate with treatment failures in high risk patient groups. As such, D'Antonio and colleagues (2004), in their study of teicoplanin or vancomycin for the treatment of Gram-positive cocci infections in neutropenic patients with haematological malignancies, documented there were there were more therapeutic failures associated with use of teicoplanin than with vancomycin treatment. Furthermore, these therapeutic failures rates were noted to be greater in episodes caused by *S. epidermidis* and to a lesser extent by *S. haemolyticus* with use of teicoplanin than with vancomycin. The authors demonstrated that the response rate to both teicoplanin and vancomycin was higher in *S. hominis* isolates, further corroborating the findings of the present study. Conversely, a case controlled retrospective comparative analysis of MR-bacteraemia in patients with haematological malignancies, demonstrated an overall reduced susceptibility to teicoplanin noted to be higher among MR-*S. haemolyticus* in comparison to bacteraemia caused by MR-*S. epidermidis* isolates (47.5% and10% respectively), (Falcone *et al*., 2004). Multi-drug resistance and the increased use of vancomycin necessitates the availability of reliable methods for detection of resistance especially in cases of decreased susceptibility to the glycopeptides

Clinical isolates are known to have the ability to accumulate antibiotic resistance determinant and therefore, with the substantial increase in the incidence of MR-CoNS noted over the last few decades has resulted in the frequent use of glycopeptides for the treatment of infections with such isolates(Tacconelli *et al.,* 2001; Stefani and Varaldo, 2003). The glycopeptide, vancomycin has long been established as the foundation of chemotherapy for the treatment of CoNS BSIs in the haematology patient group. Progressive antibiotic resistance caused by unwarranted vancomycin therapy is however, thought to be a possible risk factor for limiting susceptibility and therefore continues to be an important determinant of clinical outcomes in the high-risk haematology patient group (Biavasco *et al*., 2000; Boisson *et al*., 2001; Natoli *et al*., 2009; Chen *et al.,* 2010). Currently few studies exist demonstrating the link between reduced vancomycin susceptibility and the potential clinical significance associated with MDR-CoNS BSIs, in comparison to the numerous published reports linking vancomycin treatment failure associated with MRSA infections (Tenover *et al*., 2001; Howden, 2005; Howden *et al*., 2004; 2010). However, with the extensive use of vancomycin due to widespread MR for the treatment of Gram-positive cocci in neutropenic patients with haematological malignancies more therapeutic failures have been documented associated with infections particularly caused by *S. epidermidis* and *S. haemolyticus* isolates (D'Antonio *et al*., 2004; Nunes *et al*., 2007; Ahlstrand *et al*., 2011). Such isolates have however been

noted to demonstrate a much lower response to teicoplanin in comparison to vancomycin. It has therefore become imperative to reliably detect developing resistance to the glycopeptides which may be associated with species-specific CoNS isolates concomitant with adverse clinical outcomes. With the potential of emerging resistance possibly due also to the presence of isolates exhibiting heterogenous resistance to vancomycin, it becomes increasingly important for clinical laboratories to distinguish such isolates using more sensitive phenotypic detection methodologies. This study therefore further focused on investigating the prevalence of reduced glycopeptide susceptibilities among the in-patient CoNS isolates based on MIC evaluations using AST techniques which had greater specificity than those currently available in routine laboratories.

Both the VITEK®2 and the standard E-test susceptibility testing methods provide quantitative results (MICs) and categorical qualitative assessments of susceptibilities which fulfil the requirements for earlier microbiological diagnosis and appropriate directed therapy based on evaluated *in-vitro* antimicrobial efficacy (Reller *et al*., 2009). MIC testing of the antibiotics for the causative organisms remains the 'gold-standard' methodology. The VITEK®2, AST system utilising micro-broth *in-vitro* diagnostics, calculates MIC values by extrapolating growth curves and application of dedicated algorithms. However, the standard E-test method which incorporates both intermediate and traditional two-fold dilutions provides a more sensitive susceptibility marker for MIC evaluations in comparison to the VITEK®2 system. Therefore, comparative analysis to ascertain the true prevalence of glycopeptide resistance amongst the 100 in-patient isolates revealed differences in MIC values and hence susceptibility classification with the use of E-tests. The generation of MICs across a gradient of 15 dilutions with the E-test technique providing a greater degree of sensitivity and specificity allows for easier detection of small changes that occur in MICs over time due to antimicrobial pressure (Balouiri *et al*., 2016). Thus, this technique provides a useful means for tracking trends in resistance development over time due to the prevalent use of vancomycin in most healthcare institutions.

In-patient CoNS isolates in this study exhibited overall susceptibility to vancomycin, however, a number of isolates expressed variable susceptibility to teicoplanin with the use of the two test methodologies. There was an overall 22% and 26% disparity observed between teicoplanin susceptibility as determined by VITEK®2 and E-test method respectively. However, comparing overall teicoplanin MIC result distribution revealed no significant difference ($p = 0.624$) between VITEK®2 and the standard E-test susceptibility test methodologies. Conversely, however the highest E-test MIC_{50} and MIC_{90} displayed by teicoplanin indicates that significantly higher concentrations ($p < 0.0004$) of this antimicrobial are required to achieve comparatively similar proportional inhibition rates relative to

vancomycin. Interestingly, although the majority of CoNS isolates displayed susceptibility to vancomycin, there were considerable difference in MIC distribution rates with this antimicrobial and the two test methodologies. The majority (52%) of the in-patient CoNS exhibited vancomycin MICs at 2mg/L when tested by E-tests, whereas when tested by VITEK®2, the majority (51%) displayed MIC of 1 mg/L with one isolate classified as resistant, exhibiting an MIC of 8mg/L. These observed significant difference with vancomycin MIC distribution was dependent on the AST methodology used ($p = 0.0001$). These findings are evidenced in a similar study comparing vancomycin MICs obtained by E-test with broth microdilution methodology (Paiva *et al*., 2010). The authors of the study demonstrated that the E-test method provided MICs of vancomycin 1-2 fold dilutions which were significantly higher ($p < 0.0001$) than those determined by broth microdilution. Additionally, the majority of *S. epidermidis* isolates in their study presented MIC of 1mg/L by broth microdilution and despite *S. haemolyticus* isolates being more resistant to the glycopeptides, the isolates presented MICs between 1-2 mg/L. Similarly, comparisons of the species-specific CoNS glycopeptide MIC evaluations performed on the in-patient isolates in this study reflected similar differences in vancomycin MICs as determined by E-tests. The *S. epidermidis* in-patient isolates demonstrated comparable difference in MICs evaluation using the two test methodologies. The majority of isolates (75%) demonstrated MICs of 2mg/L by E-test and 60% at MIC of 1mg/L by VITEK®2. Interestingly, comparative analysis of the performance of the E-test revealed that significantly higher proportions of *S. epidermidis* isolates ($p = 0.0035$) were inhibited with increasing antibiotic concentrations in comparison to VITEK®2. On the other hand, no such correlation ($p > 0.05$) was noted between isolates of *S. haemolyticus*, *S. hominis* and the other minor group of CoNS species-specific isolates with VITEK®2 (Figure 4-15). On the whole, among the 100 species-specific in-patient CoNS isolates the MIC_{50} and MIC_{90} for vancomycin were on average approximately one doubling dilution higher by standard E-test in comparison to those obtained using the VITEK®2 automated system.

The disparity in MIC evaluations using the two AST methodologies was however, observed to be greater among isolates of *S. haemolyticus*. The majority of in-patient *S. haemolyticus* (n=16) 50%, presented MICs of 2mg/L by E-test and 19% MICs >2mg/L with none of the isolates expressing MIC at 1mg/L. On the other hand, with the VITEK®2, the majority (76%) of MDR-isolates of *S. haemolyticus* presented MICs between 1 and 2mg/L with one isolate expressing resistance at 8mg/L. However, this resistant *S. haemolyticus* isolate A53, demonstrated borderline susceptibility to vancomycin at MIC 4mg/L, one doubling dilution lower than that observed with VITEK®2 and therefore classified as susceptible when tested by E-test. Similar differences were also observed with teicoplanin for all three predominant species-specific CoNS with the exception of the other minor group of CoNS isolates. In total,

twenty two and 24 CoNS isolates demonstrated resistance to teicoplanin when tested by VITEK®2 and E-tests respectively. Resistance to teicoplanin was observed predominantly in *S. epidermidis* in just over 30% of the isolates in comparison to (19%) of the *S. haemolyticus* with MICs ranging between 6-24mg/L. The level of teicoplanin resistance expressed was however, significantly different with E-tests, demonstrating teicoplanin activity across a much wider MIC distribution range in comparison to VITEK®2 ($p < 0.05$). The higher E-test MIC_{50} and MIC_{90} displayed by teicoplanin and those obtained by VITEK®2, in comparison to vancomycin indicates that significantly higher concentrations of the antibiotic ($p = 0.0004$) are required to achieve relatively similar proportional inhibition rates. The single exception was results obtained for teicoplanin with the other minor group of CoNS species, where almost no significant difference was observed for MIC_{50} and MIC_{90} values ($p = 0.9$).

These significant difference in CoNS species-specific MIC results observed with the use of the two different AST methods are concordant with results of other studies reporting that the determination of vancomycin MIC, in particular is method specific (Sader *et al*., 2009; Paiva *et al*., 2010). However, in the present study, with the exception of *S. haemolyticus,* overall comparative MIC dataset analysis by PCA, suggest that there is no significant difference between the two antimicrobial test methodologies for glycopeptide susceptibility expression in relation to species-specific CoNS isolates. Nonetheless, variability in susceptibility categorisation based on evaluated MICs by different test methods, would clearly influence clinical decisions for targeted therapies and impact on resistances selection.

Successful treatment of complicated staphylococcal infections is particularly challenging especially if the isolates are MDR. Selective pressure increasingly leads to antibiotic resistance thus limiting therapeutic options and increased use of vancomycin (Kirst *et al*., 1998). The high use of vancomycin in the haematology unit may be an important factor that has influenced the higher levels of glycopeptide MICs noted particularly amongst in-patient isolates of *S. epidermidis* and *S. haemolyticus.* This would be a genuine cause for concern in the haematology patient group as the higher observed MICs may be indicative of the possible rise in resistance development due to antibiotic pressure. More importantly, nosocomial CoNS, known for their innate adaptive capability have the potential to acquire and spread such resistance among and between other susceptible species-specific isolates which would, therefore, significantly impact on patient clinical outcomes. The, use of reliable susceptibility methods are therefore fundamental for the adequate prediction of clinical response. The findings in study support the fact that E-tests in comparison to the current VITEK®2 laboratory technology provide more definitive MIC results. This is evidenced by reports of data of similar analysis from other studies demonstrating that vancomycin MIC evaluations by E-tests were comparatively more reliable for detecting antimicrobial

resistance and are therefore also more reliable for the predication of treatment responses (Sakoulas *et al*., 2004; Sader *et al*., 2009). Moore and colleagues (2011) in their study, comparing clinical outcomes of patients on vancomycin treatment for BSIs due to MRSA also used E-test methodology to demonstrate clinical failures; reporting that this was due to isolates exhibiting higher vancomycin MICs. On the other hand, although E-tests are observed to overestimate MICs generally by 0.5-1.5 log_2 dilution difference, authors of other studies have found an agreement between vancomycin MICs by E-test and other comparative test methods (Hope *et al*., 2013). The log_2 dilution differences observed with E-tests have been attributed to the different calibration standards of the test methodologies which are thought not to impact significantly on overall susceptibility results (Raney *et al*., 2002). Other studies however have shown the importance of evaluating vancomycin MICs using reliable test methodologies whereby the reported results impact significantly on clinical guidance particularly for the management of MRSA or MR-CoNS BSIs (Sakoulas *et al*., 2004; Moore *et al*., 2011). Therefore, antibiotic resistance detection using appropriate test techniques is an issue of importance for CoNS-associated BSIs in the haematology patient group. Results of the overall assignment probability for vancomycin and teicoplanin susceptibility with both AST methods in the present study was however, determined to be high ($p > 0.05$), thus demonstrating good correlation between standard E-test and VITEK®2 AST methodologies for the MIC evaluation. The retrospective result analysis of these comparisons suggest that the laboratory reported VITEK®2 based vancomycin MICs for CoNS-associated BSIs are potentially reliable for predicting treatment response to vancomycin in the haematology patient group.

Although glycopeptides, in particular vancomycin as initial empirical therapy, until recently have been effectively used for the treatment of MR-staphylococci, the number of isolates demonstrating reduced susceptibility or complete resistance to the glycopeptides however, have been increasingly reported (Tabe *et al*., 1998; Baivasco *et al*., 2000). However, low-level glycopeptide resistance associated with therapeutic failure has been more commonly described in *S. aureus* infections (Howden, 2005; Howden *et al*., 2010; Chen *et al*., 2011; Satola *et al*., 2011). On the other hand, glycopeptide heteroresistance among invasive CoNS infection has had limited characterisation to date (Van Der Zwet *et al*., 2002). CoNS isolates with decreased susceptibility to the glycopeptides are difficult to detect, particularly, where heteroresistance sub-populations are present. Standard methodologies are limited when attempting to detect resistance amongst a heterogenous population consisting of cells expressing susceptibility and a sub-population expressing low-level susceptibility to vancomycin. These limitations have been noted with even the latest version of the VITEK®2 software, which may not report vancomycin MICs above 4mg/L for most species-specific

CoNS except for isolates of *S. haemolyticus*. The VITEK®2 displays low sensitivity for the detection of heteroresistant subpopulations as it tends to categorise VIS strains as susceptible. VITEK®2 therefore remains unreliable for the detection of heteroresistant strains because the standard AST inoculum used is below the requisite threshold for the detection of the resistant subpopulations (Wootton *et al*., 2007). The clinical relevance of low-level glycopeptide resistance in potential pathogenic isolates of CoNS remains difficult to ascertain however, similar to infections with hVISA and VRSA these may be associated with treatment failures (Jones, 2006). In the haematology patient group, BSI-associated CoNS expressing decreased susceptibility to the glycopeptides not only limits treatment options but more importantly requires the implementation of control of infection measures to restrict nosocomial spread of such resistant isolates. The lack of standardised protocols for the phenotypic detection of slower growing cells expressing resistance to the glycopeptides within a predominantly susceptible population does not allow elucidation of such resistance in the routine laboratory (Del'Alamo *et al*., 1999; Howden *et al*., 2010).

The standard method for optimal detection of hVIS strains is the PAP-AUC, an impractical method, currently confined to reference laboratories and does not provide results in a clinically relevant time frame (Howe et al 2000; Wooton et al 2001). However, alternative methods purposed for evaluating staphylococci expressing heterogenous or intermediate resistance to the glycopeptides were employed in this study to describe the prevalence of heteroresistance amongst in-patient CoNS from the haematology patient group. Although no standardised diagnostic methodology exists for these tests either, both the E-test macro-method and GRD E-test methodology used in this study to assess susceptibility to both vancomycin and teicoplanin revealed differing levels of activity against the hundred CoNS in-patient isolates. In general, these variable difference in test results are thought to be mainly due the influence of various technical factors associated with these protocols (Wooten *et al*., 2007; Howden *et al*., 2010; Satola *et al*., 2011). Furthermore, as the criteria used to classify heterogenous of intermediate resistance has yet to be defined for CoNS; application of the CLSI/EUCAST MIC breakpoints categorisation for hVISA and VISA used for the evaluation of heterogenous resistance in the in-patient CoNS isolates may not be a definitive of the actual prevalence of heterogenous isolates in the haematology patient group.

Significant differences defining the presence of reduced susceptibility to the glycopeptides were observed with the two test methodologies. Using the manufacturers interpretative guidelines, the double sided GRD E-test evaluations revealed an absence of glycopeptide intermediate or heterogenous resistance in all 100 of the in-patient CoNS isolates. Conversely, based on the method specific interpretative criteria, heteroresistance was defined in a total of fourteen CoNS isolates using the E-test macro-method. Of these,

heteroresistance was expressed predominantly by 5(31%) and *S. haemolyticus* and 9(16%) isolates of *S. epidermidis.* This method optimises detection of hVIS isolates because the enriched BHI media used, supports the growth of slower growing subpopulations and the predefined stable E-test gradient is also largely inoculum tolerant (Walsh et al 2001). On the other hand, reclassification, using defined interpretive MIC cut-off values purposed by Yusof and colleagues (2008) with the GRD E-tests, demonstrated heterogenous resistance in the majority (81%) of both *S. epidermidis* and *S. haemolyticus* isolates. Five (28%) *S. hominis* isolates and 2(22%) of the minor group of CoNS species-specific isolates would also be defined as expressing heteroresistance. The apparent variability in result evaluation observed with the two test methods is not only dependent on the technicality of these test protocols but also on the non-standardised criteria used to define hVIS.

As stated by authors of previous studies, evaluation of heteroresistance is method dependent and technical factors have an inevitable variability inherent within the test protocol which affects differentiation of staphylococcal isolates with MICs around the susceptibility breakpoint (Walsh *et al*., 2001: Wootton *et al*., 2007; Vaudaux *et al*., 2010). With the use of these defined characteristics, the main differences in the degree of glycopeptide antimicrobial resistance noted with use of E-test macro-method in comparison to GRD E-tests was primarily the test media. The main critical variable in the macro-method is the use of BHI agar media for AST together with the use of higher inoculum density. This non-susceptibility test media is basically an enriched culture medium which allows growth of slower growing resistant isolates requiring prolonged incubation period of up to 48 hours. Therefore, established interpretive guidelines are not applicable for definitive heteroresistance identification. However, researchers have ascertained that these methodologies could be used as initial screening methods for evaluating presence of heteroresistant isolates, as these methods incorporate media supplements that improve their detection (Wootton *et al*., 2007; Howden *et al*., 2010; Satola *et al*., 2011). On the other hand, the GRD E-test method utilises horse blood enriched Mueller-Hinton, an AST medium with a defined composition and a standard inoculum which however also requires prolonged incubation for isolation of slow growing resistant sub-populations. The use of a standard inoculum with this methodology, however, increases the risk of reduced sensitivity for the detection of slow-growing or low-frequency heteroresistance subpopulations thus increasing the risk of underestimating resistance. Nonetheless despite the limitations of the two screening methodologies used in this study for heteroresistance detection, it is interesting to note, that definitive low level vancomycin resistance was potentially expressed by the one MDR-*S. haemolyticus* (A53) that expressed teicoplanin resistance but borderline vancomycin resistance when tested by standard E-test methods. This isolate exhibited high level

vancomycin resistance at MIC 8mg/L by both BHI macro-method and VITEK®2, however confirmation of heteroresistance would be determined by PAP-AUC method. Overall result analysis revealed not only significant differences between the two test methodologies, but also significant differences in MIC distribution of teicoplanin ($p = 0.0001$) but not with vancomycin.

Previous studies have established that increasing resistance to teicoplanin is frequently accompanied by an increase in vancomycin resistance expressed at a relatively much lower level. Furthermore, all identified VISA strains to date have also been shown to express reduced susceptibility to teicoplanin (Hiramatsu *et al*., 1997; Hiramatsu, 2001). Other studies using quantitative MIC tests have also demonstrated that isolates with higher MICs within the susceptible category range for vancomycin are likely to be associated with treatment failure rather than success (Sakoulas *et al*., 2004). *In-vitro* glycopeptide resistance in staphylococci evaluation studies have demonstrated that teicoplanin resistance is a previous stage for development of resistance to vancomycin, thus making this glycopeptide a useful maker for isolates demonstrating intermediate and potentially heterogenous resistance to vancomycin (Sieradzki *et al*., 1998; Sader *et al*., 2001, Liu and Chambers, 2003). Indeed, investigation of the prevalence of intermediate or heterogenous resistance to the glycopeptides therefore provides crucial data for clinical relevance and therapeutic choices for better patient outcomes (Tevell *et al*., 2014).

Prevalence of teicoplanin resistance among the in-patient CoNS isolates were observed to significantly higher in isolates of both *S. epidermidis* and *S. haemolyticus* particularly with evaluations using the GRD-E-tests ($p = 0.0001$). Interestingly, no significant differences were observed with MIC susceptibility distributions with the BHI macro-method, GRD-E-tests and with the standard E-tests. The high rates of observed teicoplanin resistance among CoNS isolates suggests that this may be due to extensive use of vancomycin for the treatment of CoNS-associated BSIs in the haematology patient group. Similarly, although the clinical significance of decreased susceptibility to teicoplanin at MICs >4mg/L, still remain somewhat unclear, the disparity in teicoplanin susceptibility as noted in this study versus that of vancomycin has been well documented in other studies (Del'Alamo *et al*., 1999; Liu and Chambers 2003; Nunes *et al*., 2007). Furthermore, Bertin and colleagues (2004) in their study evidenced that the high incidence of CoNS isolates with decreased susceptibility to teicoplanin correlated significantly with high vancomycin use in haematology patients in their institution. The findings of this study are further corroborated by authors of other studies correlating the high incidence of teicoplanin resistance with an increase in vancomycin use, resulting in a lower clinical response in comparison to isolates that were susceptible (Falcone *et al*., 2004; 2006). Additionally, increasing vancomycin pressure favours the selection of

rare clones that demonstrate resistance to teicoplanin but under continued antibiotic pressure favour development of heterogeneous resistance. In such cases isolates expressing teicoplanin resistance is a strong indicator of developing resistance to vancomycin and hence suboptimal clinical responses (Liu and Chambers 2008). The present study, because of its retrospective nature however limits correlation of evaluated teicoplanin MICs with therapeutic outcomes. It would have been of interest to note if the differences in higher teicoplanin MICs observed predominately amongst both *S. epidermidis* and *S. haemolyticus,* in-patient isolates were associated with therapeutic failures in the haematology patient group, where empirical use of vancomycin is high for the treatment of staphylococcal BSIs.

It is possible that CoNS bacteraemia caused by antibiotic-resistant species-specific isolates may be related to features that are intrinsic to resistant isolates under antibiotic pressure or are characteristics that are also unique to the immunocompromised patient infected with these CoNS. Widespread antimicrobial resistance is therefore an important problem challenging the treatment of CoNS-associated infections in the immunocompromised host thus leading to the frequent use of vancomycin. However, excessive long term vancomycin therapy with minimal changes detected by conventional MIC determination methods can be seen to severely compromise the therapeutic efficacy of severe infections with MR-CoNS with a tendency to also express MDR. This is evidenced in a prospective surveillance study regarding the incidence and the risk factors of glycopeptide resistance among CoNS-associated BSIs, demonstrating a significant correlation between high glycopeptide use in individual wards and the development of glycopeptide resistance (Tacconelli *et al*., 2001). Vancomycin inefficacy facilitated by the immunosuppressive state of haematological patient therefore adds to the risk of serious infections due to MDR-CoNS becoming notoriously difficult to treat with current drugs of choice.

Overcoming the limitations of currently in use anti-staphylococcal drugs, the implementation of newer antimicrobial agents may have a potential role particularly for the treatment of recalcitrant BSIs. These new antimicrobials may be less susceptible to mechanisms of resistances that render them ineffective and therefore their use has the potential to improve patient outcomes. In view of the resistance observed in majority of CoNS isolates and the lack of effective of antibiotics available for the treatment of infections caused by MDR-CoNS isolates; it was therefore of interest to investigate the *in-vitro* efficacy of newer antibiotics against the in-patient isolates in this study, utilising the standard E-test AST methodology. Of these, daptomycin, tigecycline and ceftaroline were the agents investigated as these agents have been recently introduced into clinical practice for the treatment of serious infection due to MR-staphylococci and MDR-CoNS. Daptomycin, a cyclic lipopeptide,

tigecycline a glycylcycline and ceftaroline a fifth generation cephalosporin have all been shown to have good *in-vitro* activity against most Gram-positive isolates (Kratzer *et al*., 2007; Guay, 2011). Daptomycin has been described to have superior activity to vancomycin in patients with bacteraemia caused by MR-CoNS. Additionally, greater resolution of clinical infection has been associated with patients receiving daptomycin in comparison to vancomycin therapy (Falcone *et al*., 2012).

In the present study, in comparison to tigecycline and ceftaroline the highest *in-vitro* activity against all 100 species-specific CoNS in-patient isolates was demonstrated by daptomycin. Additionally, in comparison to both the glycopeptides, tigecycline and ceftaroline MIC_{50} and MIC_{90} for daptomycin observed in this study was significantly lower ($p = 0.001$) demonstrating the greater potency of the drug at comparatively lower concentrations. Both daptomycin and tigecycline in comparison to ceftaroline produced significantly greater inhibition for a given antibiotic concentration ($p = 0.001$). These results are concomitant with results of a previous study demonstrating the superior efficacy of daptomycin used for the treatment of primary bacteraemia associated with CoNS isolates expressing low level vancomycin in severely ill patients (Kelesidis, 2011). Similarly, Moore and colleagues, (2011) also demonstrated that daptomycin was more effective and associated with better clinical outcomes compared to vancomycin use for treatment of MRSA bacteraemia expressing high vancomycin MICs (≥1 mg/L) or in patients that were unresponsive to vancomycin therapy. Daptomycin use, as an alternative to vancomycin could therefore be deemed to be more efficacious with negligible toxicity for the treatment of MDR-CoNS associated bacteraemia in the haematology patient group.

Tigecycline a broad spectrum antimicrobial, known to be efficacious against both Gram-negative isolates and MR-staphylococci on the other hand, demonstrated comprehensive susceptibility against all in-patient CoNS isolates when tested by VITEK®2. However, tigecycline resistance was noted in 13% of the isolates when tested by the more sensitive standard E-test methodology. Tigecycline working within a narrow susceptibility spectrum when compared to the other antimicrobials was noted to demonstrate resistance predominantly in isolates of *S. haemolyticus* followed by *S. epidermidis* and *S. hominis* (19%, 16% and 6% respectively). Similar results were reported by Natoli and colleagues (2009) in their study, of MDR-CoNS in haematology patients with reduced susceptibility to the glycopeptides. The authors reported results concordant with those of this study where isolates of *S. epidermidis* and *S. haemolyticus* responsible for BSIs demonstrated susceptibility to daptomycin, linezolid and tigecycline with a few however, also expressing resistance to tigecycline.

The development of broad spectrum bactericidal fourth and fifth generation cephalosporins such as ceftaroline that demonstrate efficacy have been shown to have time-dependent killing activity against Gram-positive organisms that are clinically resistant to the β-lactams (Guay, 2011; Laudano, 2011). Primarily, ceftaroline as an alternative therapeutic agent has been documented to have good activity against MRSA bacteraemia and in cases of reported failures in patients on vancomycin therapy demonstrating elevated vancomycin MICs (Polenakovik and Pleiman, 2013). Other studies have also documented similar activity against both methicillin susceptible and MR-CoNS isolates of *S. epidermidis*, *S. haemolyticus*, *S. hominis* and *S. lugdunensis* (Mushtaq *et al*., 2007; Saravolatz *et al*., 2011; Sader *et al*., 2013). In the absence of defined EUCAST/CLSI for CoNS and using the susceptibility criteria for *S. aureus* for ceftaroline susceptibility evaluation, the present study results showed that ceftaroline exhibited high *in-vitro* activity against the majority of all methicillin sensitive and resistant in-patient CoNS isolates. The results obtained in this study are similar to those obtained in a study by Ge and colleagues (2008). The authors demonstrated that ceftaroline exhibited potent activity against both methicillin sensitive and methicillin resistant isolates with MIC_{90} of 0.12mg/L and 0.5mg/L respectively. Likewise, in the present study with the exception of resistance noted in 6(38%) MDR-*S. haemolyticus* expressing MIC of 4mg/L the majority displayed MIC_{50} and MIC_{90} of 0.09 and 1.7mg/L respectively demonstrating good activity against both methicillin sensitive and resistant in-patient CoNS isolates. Thus, the greater activity of ceftaroline suggests it could be a better alternative for the treatment of CoNS associated BSIs particularly against those that also express reduced activity to teicoplanin with limited susceptibilities to other commonly used antimicrobials. Moreover, the *in-vitro* profile of daptomycin in comparison to both ceftaroline and tigecycline in particular, suggests that this drug could potentially be an important addition to the diminishing arsenal of currently available antimicrobial therapies for the treatment of nosocomial-acquired CoNS BSIs.

4.7 Conclusion

Overall data analysis of this study provides affirmation that there are high levels of resistance to antimicrobials, in particular to methicillin amongst haematology patient group CoNS isolates. These infective isolates exhibited resistance to the majority of antimicrobial classes widely used in this setting, including resistance to β-lactams, aminoglycosides, macrolides quinolones and the glycopeptides in particular to teicoplanin. Although in-patient *S. haemolyticus* isolates demonstrated high levels of MDR, the prevalence of both MR and MDR was significantly associated with *S. epidermidis,* the predominantly isolated CoNS

species. It was, however, alarming to note that similar but relatively lower levels of antimicrobial resistance were also expressed by the pre-admission patient group skin-colonising isolates. Monitoring of antimicrobial resistance in these isolates which act as reservoirs of resistance determinants, is of importance as these isolates are known to be commonly associated with both cross-transmission and acquisition of nosocomial infection in high-risk patient groups.

With respect to the criteria studied, phenotypic antimicrobial resistance profile typing assessing the correlation with species-specific CoNS isolates from both the in-patient and control patient group revealed great diversity between and among the same CoNS species-specific isolates. PCA cluster analysis further highlighted species-specific isolate and clonal unrelatedness, which therefore suggests that such skin colonising isolates are highly unlikely to be the common source of CoNS-associated bacteraemia in the haematology patient group.

Although vancomycin continues to be the mainstay of therapy for CoNS-associated BSIs in the haematology patient group, virtually all isolates expressed *in-vitro* susceptibility to vancomycin (MIC_{50} and MIC_{90} of between 1-2mg/L), with the exception of one in-patient *S. haemolyticus* isolate. This isolate however, expressed borderline susceptibility (MIC=4mg/L) when tested by the more sensitive standard E-test methodology, signifying that the observed MIC distribution ranges for the glycopeptides are test-method dependent. However, the excessive levels of teicoplanin resistance identified predominantly, amongst in-patient isolates of both *S. epidermidis* and *S. haemolyticus* may potentially be predictive of unfavourable response to vancomycin therapy. As such *in-vivo* vancomycin efficacy may be compromised because resistance expressed to teicoplanin is thought to be a precursor stage to developing vancomycin resistance, based on their comparable mode of resistance. In the haematology patient group isolation of BSI CoNS expressing high levels of teicoplanin resistance and a low clinical response to vancomycin therapy could therefore be linked to potential therapeutic failures particularly in bacteraemic episodes caused by *S. epidermidis* and *S. haemolyticus*.

Comparative testing of both vancomycin and teicoplanin by conventional routinely used AST automated system with standard E-tests and screening protocols for heteroresistance prevalence, revealed some major assay-dependent differences in MIC distribution of each glycopeptide. Distinguishing heteroresistance phenotypes from isolates demonstrating overall vancomycin susceptibility was problematic because of the protocol lacking sensitivity for accurate evaluation of low-level resistance to the glycopeptides, a critical factor for the detection of hGIS. Despite these indications, following the criteria allowing discrimination of

hGIS isolates from those that were vancomycin susceptible, there is a possibility that a small percentage of in-patient isolates may potentially express heteroresistance. The data results further correlate with findings of other studies showing that although some isolates primarily characterised as vancomycin susceptible, express significantly elevated teicoplanin MICs. As teicoplanin serves a good marker for developing resistance, indication are that both in-patient isolates of *S. epidermidis* and *S. haemolyticus* observed to express significantly high levels of teicoplanin resistance are also likely to also express heteroresistance. Further studies using established protocols for hGIS/GIS are however required to confirm the prevalence of low-level resistance in the in-patient CoNS isolates and correlation with clinical outcomes.

Current evidence demonstrating the possible inefficacy of vancomycin commonly used to provide coverage against CoNS-associated BSIs in the haematology patient group has necessitated the use of alternative therapeutics. The findings in this study demonstrated that daptomycin's excellent spectrum of activity distinguishes it not only from the glycopeptides but also from the other new competitors, tigecycline and ceftaroline. The significantly high *in-vitro* susceptibility demonstrated by daptomycin against all species-specific CoNS isolates therefore indicates its greater potential as a suitable alternative therapeutic agent to vancomycin for the treatment of CoNS BSIs. Therefore, for optimal clinical outcomes, in the high-risk haematology patient group the use of daptomycin would be particularly suitable, especially in situations where MDR-CoNS expressing reduced susceptibility to teicoplanin are suspected. Hence, improved therapeutic options using reliable diagnostic AST methodologies will optimise treatment for better clinical outcomes whilst also reducing selection of antibiotic resistance.

Chapter 5 Assessment of the potential virulence factors present in the in-patient CoNS isolates in comparison to the pre-admission patient group skin colonisers and their association with antibiotic resistance.

5.1 Introduction

CoNS demonstrate a narrow spectrum of virulence factors in comparison to *S. aureus*, nonetheless their pathogenic potential is greatly enhanced due to their increasing antibiotic resistance. Of the virulence factors produced by various species-specific CoNS isolates, apart from biofilm formation or slime production as the major known factor, others include the exoenzymes, lipases and proteases which are involved in host tissue damage (Otto, 2004). As a result, post-chemotherapy and post-transplant patients with malignant diseases are at a greater risk of such CoNS incited infections.

S. epidermidis is the most frequently isolated aetiological agent associated with a spectrum of infections linked to biofilm formation particularly in connection with intravascular devices (O'gara and Humphreys, 2001). The ability to form biofilm, in *S. epidermidis* has been postulated to be representative of a virulence factor that promotes phenotypic resistance to antimicrobial therapy (Holby *et al.,* 2009). Antibiotic efficacy is primarily dependent on the antimicrobial agents ability to reach the target site and accumulation within planktonic bacterial cells at a sufficient concentration to achieve eradication. In patients with haematological malignancies with an already compromised immune response, the biofilm mode of growth particularly on surfaces of intravascular catheters renders bacteria embedded in biofilms more resistant to antibiotics. The polymeric matrix (slime) causes reduction of antimicrobial efficacy by decreasing infiltration, neutralisation and binding, essentially reducing the antimicrobial to sub-lethal concentration levels prior to contact with the infective agent (Hogan and Kolter 2002). Clinical and *in-vitro* research both support the theory that antimicrobial inefficacy associated with biofilm producing bacterial strains also negatively affects treatment outcomes (Stewart, 2002; Stewart and Franklin 2008).

Clinical efficacy *in-vivo* is predicted by evaluating the MIC and MBCs of antimicrobials *in-vitro* essentially against planktonic organisms in the exponential growth phase. These evaluations are therefore an assessment of antibiotics effectiveness against the aetiological agent in acute infections. However, in chronic and device-related bacteraemic infections, clinical failures have been noted despite antibiograms of the causative organisms displaying susceptibility to standard antimicrobials at appropriate attainable concentrations. In such cases, clinical failures are usually attributed to refractory infections related to surface-adherent biofilms, where standard susceptibility tests fail to predict *in-vivo* susceptibility. Furthermore, of deep concern is the limited effectiveness of vancomycin therapy used for treatment of nosocomial acquired CoNS BSIs due to antibiotic tolerance demonstrated by

biofilm producing CoNS isolates (Claessens *et al*., 2015). In such cases, eradication of biofilm-associated CoNS infections has been shown to be highly effective with the use of combination therapeutics instead of vancomycin monotherapy associated with persistent bacteraemia (El-Azizi *et al*., 2005). Recommended synergistic combination therapies usually include the use of gentamicin or rifampicin in addition to vancomycin. These combinations may however prove problematic in the immunocompromised host, as gentamicin increases the risk of nephrotoxicity and rifampicin increases the potential of adverse drug interactions and resistance development (Stewart, 2000; Fey, 2010). Greater achievement of bactericidal activity has nonetheless been documented against more than 90% of biofilm producing isolates of *S. epidermidis*, by the addition of rifampicin to gentamicin or vancomycin for treatment of chronic staphylococcal infections (John and Harvin, 2007).

On the other hand, there is little information at present to support the use of newer therapeutics such as daptomycin in combination with commonly used synergetic agents for the treatment of BSIs caused by biofilm producing Gram-positive organisms. (LaPlante and Mermel 2007, LaPlante *et al*., 2007;2009; Kaplan 2009; Kaplan *et al*., 2011). The use of combination antimicrobial regimens have nevertheless been associated with higher survival rates of patients with MDR Gram-positive infections (Dall *et al*., 2018). Further investigations are therefore merited to assess the *in-vitro* activities of daptomycin and vancomycin alone and in combination with other commonly used anti-staphylococcal agents against clinically important isolates of CoNS. Additionally, *in-vitro* activities of these antimicrobials against biofilm producing CoNS associated with BSI also necessitates evaluation due to increasing concerns over the decreasing efficacy of vancomycin associated with sub-optimal patient outcomes.

5.2 Aim of the current study

a) To investigate the association of antimicrobial resistance with the virulence factors amongst blood culture isolates of well-characterised CoNS recovered from in-patients in the haematology unit. These characteristics were compared with skin colonising isolates from the pre-admission control patient group.

b) To evaluate four well established virulence traits expressed by CoNS including exopolysaccharide (slime), haemolysin, protease and lipase. These were further assessed and correlated with the resistant phenotypes.

c) To investigate the *in-vitro* activity of vancomycin, daptomycin, gentamicin and rifampicin combination for synergy or antagonisms against three *S. epidermidis* BSI isolates from the in-patient group, representing a range of susceptibility patterns using the E-test methodology.

d) To evaluate the antimicrobial efficacy of the four antimicrobials (vancomycin, daptomycin, gentamicin and rifampicin) individually and in binary combinations against the panel of three BSI isolates of *S. epidermidis* to determine antibiotic synergy or antagonistic effect using time-kill assays.

e) To evaluate the activity of vancomycin, daptomycin and rifampicin individually and in combination using an *in-vitro* model of the well characterised biofilm producing *S. epidermidis* reference strain RP62A/ATCC 35894. The minimum antibiotic concentration required to inhibit biofilm growth (biofilm MIC) and the minimum bactericidal concentration (biofilm MBC) required to inhibit biofilm load was also determined.

5.3 Materials and Methods

5.3.1 Isolates

The four most common virulence factors produced by CoNS isolates were studied in all the one hundred of the in-patient CoNS isolates as well as in the 100 skin colonising CoNS isolates from the pre-admission patient group.

Three well characterised BSI isolates of *S. epidermidis*, representative of three differing susceptibility categories were used for assessment of antimicrobial synergy. The isolates from the haematology patient group used in the assay included, *S. epidermidis* isolates, A37 exhibiting susceptibility to all antimicrobial classes with the exception of penicillin; A57 exhibiting intermediate resistance (resistant to ≥4 but <6 antimicrobials) and the MDR isolate A91 (resistance to 12 antimicrobials). Isolates A37 and A91 were also positive for slime production, whilst A57 was slime negative. Fresh 24 hour stock cultures were prepared of each isolate maintained at minus 20°C on Microbank beads by defrosting and sub-culturing twice on nutrient agar at 37°C for 18 hours in ambient atmosphere. *S. aureus* quality control strain NCTC 6571/ATCC 9144 expressing high level susceptibility to all antimicrobial classes was used as the control for both E-test synergy studies.

For biofilm formation in 96-well microtitre plates and subsequent susceptibility assays; several single colonies of a fresh 24 hour culture of *S. epidermidis* RP62A biofilm producing

strain grown initially on nutrient agar and then in tryptic soya broth media was used for further investigations.

5.3.1.2 Antimicrobial agents

Vancomycin, rifampicin and gentamicin (250mg) in powder form were sourced from Sigma-Aldrich, UK. Stocks of each antibiotic were freshly prepared for use in accordance with manufacturers recommendations. The antibiotics were dissolved in sterile distilled water to give a working stock concentration of 50mg/L and filter-sterilised using 0.2µm syringe filter (Sartorius™ Minisart®, SigmaAldrich, UK). The prepared antibiotic stock solutions were stored at 4°C and brought to room temperature prior to use.

Similarly, for daptomycin lyophilised powder (Cubicin®) was reconstituted as recommend with the addition of 10% (v/v) dimethyl sulfoxide (co-solvent) to an equal volume of sterile distilled water supplemented with 50mg/L calcium and 12.5mg/L magnesium chloride (Sigma-Aldrich, UK). Addition of Ca^{2+} was added as recommended to effectively improve the bactericidal activity of daptomycin *in-vitro* and the filter sterilised stock concentration of 50mg/L stored at -20°C was defrosted and brought to room temperature prior to use. However, daptomycin solution in a ready to use formulation (Cubicin®; Cubist Pharmaceuticals, UK) kindly supplied by the Queen Elizabeth Hospital pharmacy through the microbiology pharmacist, was used for all subsequent biofilm assays.

Interpretation criteria for susceptibility testing was based on BSAC and EUCAST (2013) published guidelines.

5.3.2 Virulence factors

All media and reagents used were sourced from Oxoid (Basingstoke, UK) unless where stated otherwise.

5.3.2.1 Lipase

For the detection of lipase production, previously described method (Lang, PhD Thesis: 2000) was used to produce olive oil agar using, 1% (w/v) Tryptone, 1.3% (w/v) agar No.1, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl, mixed with distilled water and sterilized following the standard parameters of 121˚C for 15 minutes. The media was then allowed to

cool to 60˚C, mixing constantly prior to the addition of the olive oil 2.5% (w/v) (Thornton & Ross Ltd, Huddersfield) and 0.001% (w/v) aqueous rhodamine B, which were filter sterilised using a 0.5 µm pore size cellulose nitrate filter (Sartorius Stedium, GMBH). The agar poured to a depth of 4mm in 90mm petri dishes was then left to set at ambient room temperature for 18 hours before use.

CoNS colonies from a 24 hour sub-cultured blood agar plate were used to prepare a bacterial suspension in 0.5% saline equivalent to McFarland standard 0.5. A sterile swab dipped into the prepared bacterial suspension was pressed lightly against the side of the tube to remove excess fluid prior to inoculating the olive oil agar plates. *Pseudomonas aeruginosa* ATCC15442 as the positive control and *Staphylococcus epidermidis* RP62A/ATCC 35894 as the negative control strain were included in each test batch. Plates were incubated at 35˚C for 18 hours in ambient atmosphere. Positive lipase activity was detected by production of fluorescence by isolates when subjected to ultraviolet light (UV Light Box DaRO UV Systems II).

5.3.2.2 Proteases

For the detection of protease production by CoNS, a previously described method was used to produce skimmed milk agar (Lang, PhD Thesis: 2000). Agar No.1, 1% (w/v) and 1% (w/v) skimmed milk powder were dissolved in 1000ml distilled water and autoclave sterilized at 120˚C for 15 minutes. The prepared media was dispensed to a depth of 4mm in sterile petri dishes and left to set at room temperature prior to inoculation. Four, equally spaced 5mm diameter wells were cut into each plate using a sterile glass pipette end. Aliquots of 50µL bacterial suspension prepared equivalent to McFarland standard 0.5, was then added to each well. The suspensions were allowed to absorb into the agar before incubating the plates at 37°C for 18 hours in ambient atmosphere. An equivalent volume per well of protease positive control strain, *Pseudomonas aeruginosa* ATCC15442 and sterile distilled water as the negative control were included in each test batch. Hydrolysis signified a positive result indicated by zone of clearance around the well area of protease producing isolates.

5.3.2.3 Slime

The modified, Congo red agar test was utilised to Investigate bacterial isolate slime production using previously described phenotypic methods (Freeman *et al*., 1989; Kaiser *et*

al., 2013). Brain heart infusion broth (37g/L) was prepared with 1L of distilled water. A volume of 50mL of the well mixed BHI broth was removed into a glass universal, to which an aqueous solution of Congo red dye (0.8g/L) was added and autoclaved separately. To the rest of the 950mL BHI broth, 1% (w/v) agar No.1 and 5% (w/v) sucrose was then added, and autoclave sterilised. The sterilised solutions were allowed to cool to 55˚C. The Congo red in BHI broth solution was then aseptically added to the rest of the BHI sucrose broth and mixed thoroughly. The agar plates were then poured and allowed to set at ambient temperature prior to inoculation.

A sterile swab dipped into McFarland standard 0.5 of each test bacterial suspension, pressed lightly against the side of the tube to remove excess fluid was used to evenly streak half the Congo red agar plate. The inoculated plates were initially incubated for 24 hours at 37˚C followed by incubation at ambient temperature for a further 24 hours prior to reading results for slime production. A positive slime producing control strain of *S. epidermidis* RP62A/ ATCC 35894 and the negative control, *S. hominis* ATCC 35982 a non-biofilm producing strain were included in each batch test.

Positive slime production was determined by the appearance of black crystalline colonies with areas of transparency surrounding the colonies, where diffusion of slime production into the agar causes clearance of the Congo red pigment. Slime negative colonies were determined by their appearance remaining dark and/or opaque with no surrounding zones of transparency.

5.3.2.4. Haemolysin

The expression of haemolysin was determined on pre-poured plates containing Columbia blood agar base and 5% sheep blood (COS agar bioMérieux, France). A sterile 10µL loop of CoNS bacterial suspension prepared in 0.5% (w/v) saline equivalent to McFarland standard 0.5 was used to inoculate each half of a COS agar plate.

A haemolysin negative control strain, *S. epidermidis* ATCC 12228 and a haemolysin positive, *S. haemolyticus (*clinical isolate) was included in each test batch. The inoculated plates were incubated at 35˚C for 18 hours in ambient atmosphere. A positive result was indicated by the formation of zones of haemolysis around the colonies.

5.3.3 In-vitro antimicrobial combination activity

All test assays were performed independently in duplicate using the E-test technique to determine combination antimicrobial interactions against three *S. epidermidis* BSI isolates. The three *S. epidermidis* isolates were randomly selected based on their susceptibility category, including susceptibility to all but one of the antimicrobials in the panel of 18 test antibiotic, moderate susceptibility and a MDR isolate.

5.3.3.1 Combination antimicrobial susceptibility testing using E-tests

Determination of vancomycin, daptomycin, rifampicin and gentamicin MICs against each of the three in-patient BSI *S. epidermidis* isolates were performed individually, in duplicate, using the E-test methodology. Combinations of each of the four antibiotics, were subsequently tested against each of the three *S. epidermidis* isolates A37, A57 and A91. Cultures of each isolate including the control strain were grown in nutrient broth, incubated at 37°C for 18 hours in ambient atmosphere to ensure isolates were in the stationary growth phase prior to each batch test assay.

The E-test method as previously described (Chapter 4) was used to determine MICs of the antibiotic individually. Using a sterile swab, a 0.5 McFarland standard inoculum of each bacterial strain was used to inoculate Iso-Sensitest Agar (ISA) plates in a three way direction to create a lawn of semi-confluent growth. For synergy testing combinations of each the four antimicrobials, the E-test strips were placed on the inoculated ISA plates in a cross formation at a 90° angle. The E-test strips were placed such that the MIC value of the first antibiotic was positioned at the base intersection of the respective MIC value of the second antibiotic for the test isolate (Figure 5-5). After incubation at 37°C for 18 hours in ambient atmosphere, the MIC of each antimicrobial was evaluated. The combined effect of the two agents against each test isolate was calculated using the following previously defined algorithms: MIC of antibiotic A (MIC_A), MIC of antibiotic B (MIC_B), MIC of antibiotic A in the presence of antibiotic B(MIC_{AB}) and MIC of antibiotic B in the presence of antibiotic A (MIC_{BA}). Fractional Inhibitory Concentration (FIC) index calculations were performed using the formula FIC index = $MIC_{AB}/MIC_A + MIC_{BA}/MIC_B$. Results of antimicrobial combinations were accordingly classified as, synergy if FIC ≤ 0.5, additive FIC > 0.5 and ≤ 1, indifference if FIC > 1 and ≤ 4 and antagonism FIC > 4 (White *et al*., 1996; EUCAST, 2010).

5.3.4 Antimicrobial efficacy determination against S. epidermidis RP62A biofilm

Qualitative and quantitative evaluation of vancomycin, daptomycin and rifampicin singly and in combination was ascertained against the *S. epidermidis* RP62A biofilm producing reference strain, using previously described *in-vitro* chequerboard techniques for determination of antibiotic susceptibilities of bacterial biofilms (Christensen *et al*., 1983,1985; Steenbergen *et al*., 2009). For antimicrobial efficacy evaluation against *S. epidermidis* RP62A biofilm the test assays were performed in triplicate and repeated twice on independent occasions.

Figure 5-1 illustrates the checkerboard method used for testing the *in-vitro* efficacy of the three antibiotics either individually or in combinations against either *S. epidermidis* RP62A planktonic cells or developed biofilm. The three antibiotics tested, included vancomycin, daptomycin and rifampicin individually and in binary combinations.

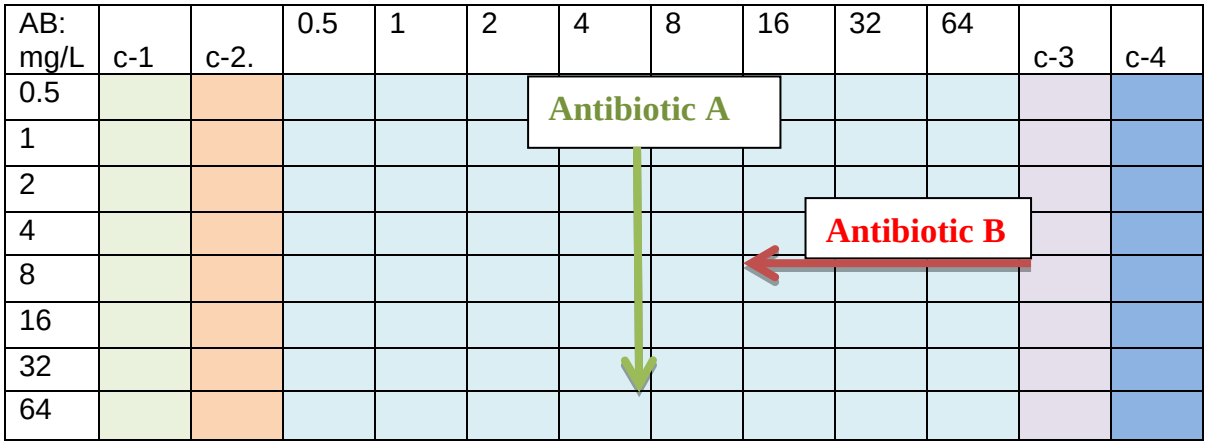


Figure 5-1: Schematic of checkboard method layout for the testing of two-antimicrobial combinations, concentration range 0.5-64mg/L. Antibiotic A (vancomycin) interaction activity in combination with Antibiotic B (daptomycin or rifampicin against *S. epidermidis* RP62A test strain. Control wells c-1 and c-2 contain *S. epidermidis* planktonic culture in MHB, c-3 and c-4 MHB only. For tests against biofilm: control wells, c-1 consists of biofilm, c-2 biofilm and MHB, c-3 empty and c-4 MHB only.

5.3.4.1 Planktonic MIC and MBC determination

Cultures of *S. epidermidis* RP62A in nutrient broth incubated at 37°C for 18 hours in ambient atmosphere were obtained at stationary phase growth for test assay. *S. epidermidis* RP62A culture inoculum were standardised to give a one McFarland turbidity equating to approximately $1x10^9$ CFU/mL and further adjusted by diluting with sterile nutrient broth solution to obtain a final concentration of $1x10^5$ CFU/mL. Using aseptic techniques under the laminar flow cabinet, 150µL of the prepared bacterial culture was added to each well of a

round bottomed 96 well polystyrene microtitre plate (Sterlin™ Clear Microtiter™ plates, Thermo-Fisher Scientific, UK) with the exception of the first two wells and last well of each row serving as controls. The first two control wells contained a 300µL aliquot bacterial culture, Figure 5-1 wells c-1 and c-2 (absence and presence of MHB respectively). The last control well of each row contained 300µL aliquot of sterile MHB only (Figure 5-1, well c-4). With the exception of the control wells, to each well a 150µL aliquot of previously prepared series of log_2 antibiotic dilutions prepared in MHB was added to give final serial antibiotic concentrations of 0.5 to 64mg/L in a total volume of 300µL per well.

To allow the antibiotics to mix well with the bacterial culture, the prepared microtitre plates were then transferred onto an incubator shaking platform and agitated at the lowest speed (25 rpm) for 30 seconds. The microtitre plates were sealed with clear adhesive plate seals (VWR™, UK) prior to incubation statically at 37°C for 18 hours in ambient atmosphere. Each test assay was performed in triplicate.

Visual observations were made after incubation for MIC determination, defined at the point of lowest concentration of antibiotic in which there was no visible growth. For quantitative MBC evaluations,100µL from the contents of three wells at the point of MIC were removed and serially diluted and sub-cultured in duplicate on freshly poured MH-agar. The plates were incubated overnight at 37°C for colony counts and MBC was determined as the lowest antibiotic concentration showing ≥99.9% kill after incubation.

5.3.4.2 Biofilm formation for susceptibility testing

Tryptone soya broth (TSB) supplemented with 1% glucose was prepared in accordance with manufacturers instruction, autoclave sterilised at 121°C for 15 minutes and stock solution stored at 4°C for use as the growth suspension medium for biofilm production. Cultures of biofilm forming reference strain *S. epidermidis* RP62A in nutrient broth incubated at 37°C for 18 hours in ambient atmosphere were obtained at stationary phase growth for biofilm test assays using a modified version of previously described techniques for testing of antibiotics against biofilm producing strains (Christensen *et al*., 1985; Stepanović *et al*., 2000; Oliveira *et al*., 2010). *S. epidermidis* RP62A growth culture suspension inoculum standardised to give a one McFarland turbidity equating to approximately $1x10^9$ CFU/mL was then prepared in TSB growth media and diluted further to obtain a final concentration of $1 x10^5$ CFU/mL. With the exception of last two rows of wells per assay plate, 200µL aliquots of the prepared bacterial suspension equating to $1 x10^5$ CFU/mL was then added to each well of a 96 well round bottomed polystyrene microtiter plate (Sterlin™ Clear Microtiter™ plates, Thermo-

Fisher Scientific, UK). A 200µL aliquot of sterile TSB was then added to each but one well of the last row of the microtiter plate serving as negative controls (Figure 5-1 wells c-3 empty and c-4 TSB only). The plates were incubated for 18 hours at 37°C in ambient atmosphere to allow the *S. epidermidis* RP62A bacterial cells to form biofilm. After incubation, using aseptic techniques, the contents of each well were gently aspirated using a sterile pipette and discarded and replaced with 200µL aliquots of fresh growth medium. This process was carried out over a three day period to allow development of an adequate biofilm layer in each well.

Prior to susceptibility assays, using aseptic techniques the biofilm formed in each well was washed three times with phosphate-buffered saline (PBS) (Sigma Aldrich, UK), to eliminate the presence of free-floating planktonic cells. To ascertain biofilm formation, 200µL volumes of crystal violet stain were added to each well initially and left for 10 minutes before washing off with distilled water. The plates were left to air dry prior to undertaking absorbance readings. Using the automated plate reader (Pharmacia Biotech, Ultrospec 2000) the extent of the fixed air dried adherent bacterial cells in biofilm layer were quantified spectrophotometrically by determining the optical density at 570nm (OD_{570nm}). Experimental test readings were taken in triplicate with negative blank control well readings obtained subtracted from the calculated average test values (Figure 5-1 empty well c-3, background reading). OD_{570nm} readings for *S. epidermidis* RP62A formed biofilms were furthermore considered positive when the average reading for these were significantly higher than those obtained for the negative wells. The resulting optical density data from the plate reader software system were imported into Microsoft Excel application for further analysis.

5.3.4.3 Evaluation of S. epidermidis RP62A biofilm MIC and MBCs

The biofilm forming reference strain *S. epidermidis* RP62A was challenged with vancomycin, rifampicin, and daptomycin alone and in combinations to evaluate biofilm MIC and MBC to assess antimicrobial efficacy. Biofilm MIC and MBC determination was performed following an adaption of previously described 96-well microtitre plate biofilm susceptibility testing method (Oliveira *et al*., 2010; Leite *et al*., 2011).

MIC determination of the tested antimicrobials was performed, starting with a stock concentration of 128mg/L, two-fold serial dilutions in MHB of each filter-sterilised test antibiotic prepared in a separate microtitre plate for subsequent testing. For daptomycin test assays the serial two-fold antimicrobial dilutions were prepared using MHB supplemented with 50mg/L of calcium and magnesium chloride. Subsequently 200µL aliquots of each of

the antibiotic concentrations at two-fold dilution ranging from 0.5-64mg/L in MHB were then added to each well of the microtitre plates comprising formed biofilms. The first and last two control wells per row of the 96-well microtitre plate included in the assays consisted of control wells as detailed in Figure 5-1. The first two control wells, c-1 consisted of *S. epidermidis* RP62A biofilm with the absence of MHB and well c-2 consisted of addition of MHB 200µL aliquot. The last but one well was left empty with one each of the last negative control wells per row contained only MHB without the presence of bacterial biofilm. Wells containing uninoculated MHB were used as sterility controls and also served as spectrophotometric blanks. The antibiotic challenged biofilm microtitre plates were incubated for 24 hours at 37°C in ambient atmosphere. After antibiotic exposure, using the plate reader, the extent of adherent bacterial cells in the biofilm layer were quantified spectrophotometrically by determining the OD_{570nm}. The results obtained were derived from the two separate experiments run in triplicate and average biofilm cell density OD_{570nm} values were expressed as ± standard deviations. Additionally, calibration curves quantifying biofilm *S. epidermidis* isolates previously produced by other researchers were subsequently used to convert spectrophotometric values to conventional CFU/mL to assess overall antimicrobial biofilm efficacy. Biofilm MICs were determined as the lowest concentration of antibiotic required to inhibit biofilm embedded bacterial growth (Adams *et al*., 2005; Leite *et al*., 2011).

After exposure, the antibiotic solution was discarded, and the wells washed again three times with PBS ensuring neutralisation of residual antibiotic. 200µL aliquots of fresh MHB were then added to each well of the washed biofilms and microtitre plates re-incubated for 24 hours at 37C in ambient atmosphere. The degree of cell regrowth within antibiotic neutralised biofilms after re-incubation was further assessed by spectrophotometric determination of biofilm cell density at OD_{570nm}.

Similar to the previously described methodology, combinations of daptomycin and rifampicin with vancomycin were studied against *S. epidermidis* RP62A produced biofilm in order to determine synergy. According to the previously described checkerboard method, with the exception of the control wells c-1 and c-2, the first test antibiotic was added across each row giving a final serial concentration ranging from of 0.5 to 64mg/L (Figure 5-1). Serial concentrations of the second antibiotic was then added, such that the bottom row containing the highest concentration (64mg/L) with the lowest final serial dilution across the top row. Experiments were again performed in triplicate and analysis of results were based on taking spectrophotometric absorbance reading at 570nm. The overall results of combination antibiotic efficacy against *S. epidermidis* RP62A produced biofilm were again expressed as ± standard deviations.

Calculated FIC index values were further used to interpret the biofilm checkerboard test as previously described. The FIC index calculation of the two combinative antibiotics against biofilm embedded cells was based on both direct visual observation and the spectrophotometric absorbance values obtained at the interaction point of wells showing turbidity and non-turbidity/decreased turbidity. Average values of the experiments preformed in triplicate were taken to determine the mean or cumulative FIC index value and results of antibiotic interactions were categorised in accordance with previously described classification of synergy, antagonism, additive or indifference (Bonapace *et al*., 2000).

To determine biofilm MBC, after the 24 hour incubation period with antibiotics, the biofilm cells were dislodged from each of the test wells around the interaction points following a simple scrape method previously described by Adams and colleagues, 2005. Suspension of cells from each of the specified wells were then serially diluted in sterile saline. An aliquot of 100µL of each dilution was then inoculated and spread on MH-agar plates and incubated for 24 hours at 37°C in ambient atmosphere. Viable colony counts were performed after incubation and cell density accordingly calculated as $\log_{10}$ CFU/mL. MBCs were determined as the lowest concentration to show a highly significant or 99.9% reduction in biofilm CFU/mL, in comparison to the control wells containing no antimicrobials.

5.4 Statistical analysis

Data were analysed using the SPSS statistical software for Windows. Descriptive statistics were computed for all variables in this study. Difference testing between the CoNS isolates obtained from the two patient groups, was performed using the Chi-squared test, and the Students t-test was utilised to compare antimicrobial combination pair variables with statistical significance accepted at a confidence level of >95%.

Data evaluations of virulence factors produced by both in-patient and control patient skin colonising isolates in relation to their previously characterised phenotypic antibiotic resistance profiles, were analysed using Pearsons-Principle component analysis (PCA) StatSoft, Inc. (2011) STATISTICA version 10 (USA) Software package. Gelcompar 2 version (Applied Maths, Belgium). The linear-regression model constructed using the Holm-Bonferroni method was used to explore the association of CoNS species-specific isolates from each of the patient groups with the slime production, the predominant virulence factor.

Reduction in biofilm density after 24 hour antimicrobial challenge were compared for single and antimicrobial combinations with assays performed in triplicate, run twice independently

and data presented as means of optical densities ± standard deviation. The R Foundation Statistical Computing Platform R version 3.6.2 (2019) was used to perform the Kruskal-Wallis *H* test to assess the efficacy of vancomycin, daptomycin and rifampicin individually and in binary combinations against biofilm producing *S. epidermidis* reference strain RP62A. The Conover-Iman test, the non-parametric multiple-comparison method was further applied to the analysed data to determine significant differences between individual and combination antibiotic efficacy against biofilm embedded cells. For all data analysis comparisons, a p-value of ≤0.05 were considered to donate statistical significance.

5.5 Results

5.5.1 Virulence

Of the one hundred previously well characterised CoNS isolates tested, slime and haemolysin were the most commonly expressed virulence factors amongst both the in-patient and control patient groups (58% and 46% respectively). Haemolysin positivity was 70% in the in-patient group in comparison to 52% in the control patient CoNS colonising isolates. Lipase expression on the other hand was absent in the control patient group isolates and poorly expressed among CoNS isolates of the in-patient group. Similarly, protease production was also low, expressed by only six and 1% of the in-patient and control group isolates respectively. Figure 5-2 shows examples of the positive and negative results obtained using the selective test agars for the detection of the four commonly expressed virulence factors.

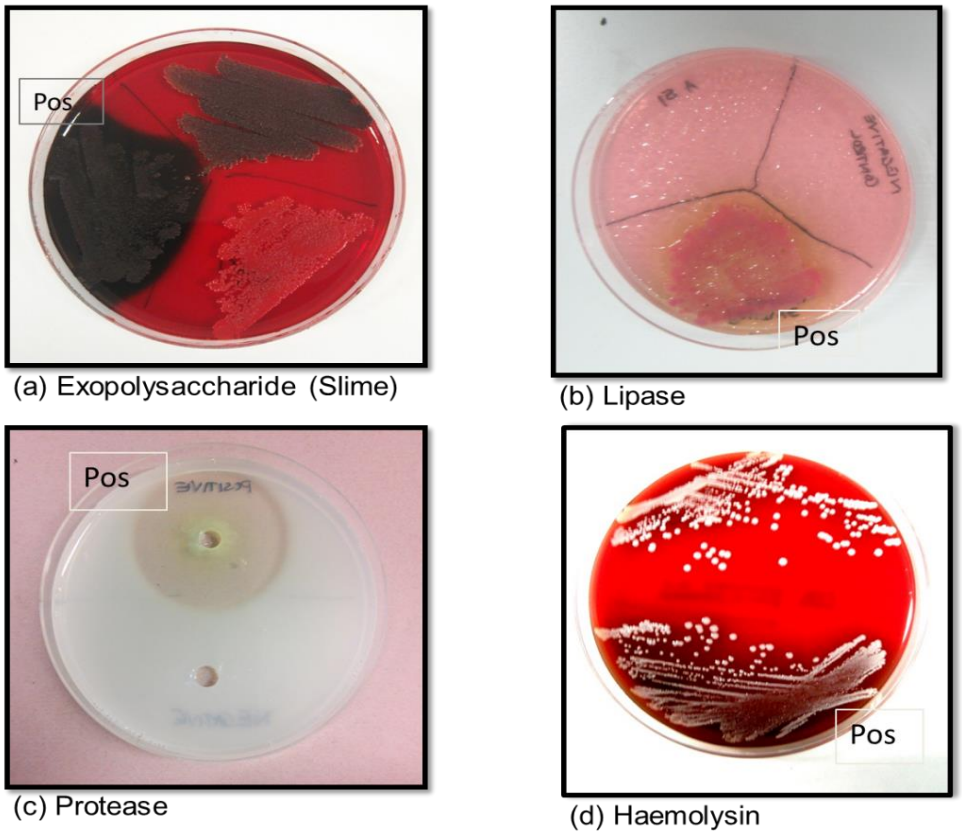


Figure 5-2: Detection of four commonly expressed virulence factors by CoNS isolates. a) Slime positive is demonstrated by zones of transparency around black crystalline colonies b) Lipase positive isolates produce fluorescence under UV light c) Protease positive isolates demonstrate zones of clearance d) Haemolysin production is observed as zones of complete haemolysis around colonies.

Comparative results detailing the detection of virulence factors produced by both the in-patient and control group CoNS species-specific isolates are presented in Table 5-1. The results reveal clear differences in positivity rates for the production of the four different virulence factors in each of the patient group isolates.

	Number of isolates expressing virulence factors/ Total number (%) of isolates tested for virulence factors			
	Slime	**Protease**	**Lipase**	**Haemolysin**
In-patient Group				
S. epidermidis (n=57)	41/72%	5/9%	1/2%	36/63%
S. hominis (n=18)	8/44%	Nil	Nil	13/72%
S. haemolyticus (n=16)	5/31%	Nil	Nil	15/94%
Other CoNS (*n*=9)	4/44%	1/11%	1/11%	6/67%
Control Group				
S. epidermidis (n=32)	16/50%	Nil	Nil	14/44%
S. hominis (n=24)	10/43%	Nil	Nil	13/54%
S. haemolyticus (n=17)	6/36%	1/6%	Nil	15/88%
Other CoNS (*n*=27)	14/52%	Nil	Nil	15/56%

Table 5-1: The distribution of species-specific isolates of CoNS from the in-patient and control patient groups according to the production of the four virulence factors.

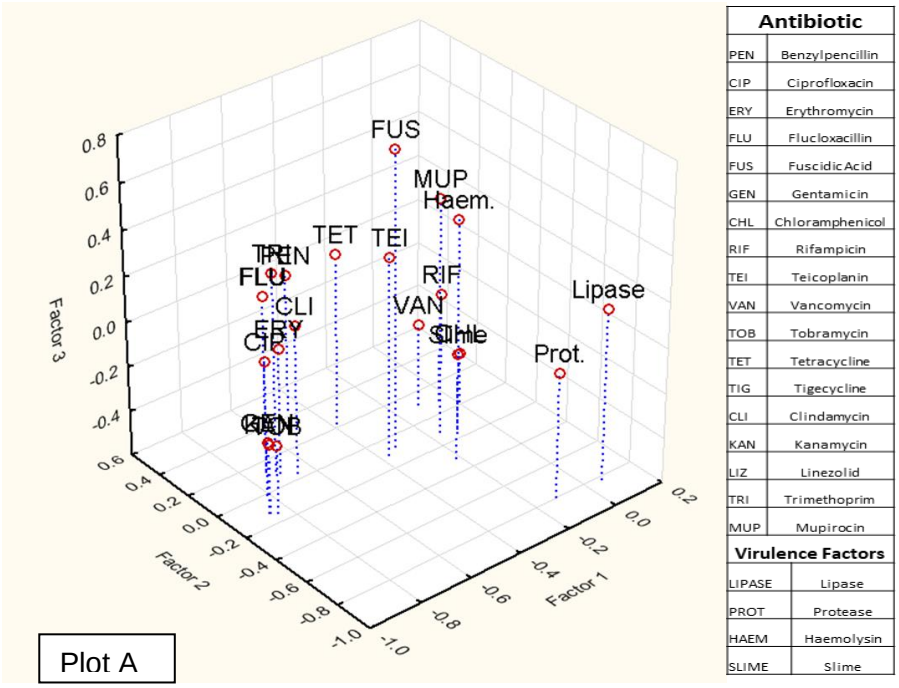
Slime production was noted predominantly amongst isolates of *S. epidermidis* in both the in-patient and control patient group (72% and 50% respectively), followed by isolates of *S. hominis* and *S. haemolyticus*. However, the other minor group of CoNS species of the control patient group also expressed slime at a similar frequency (52%); predominantly expressed by *S. warneri* and *S. captis*. On the other hand, such predominance of slime production was not noted amongst the in-patient minor group of CoNS isolates.

Similar to the haemolysin produced by *S. aureus* the δ-type haemolysins produced by CoNS species demonstrating haemolysis on blood agar containing sheep erythrocytes was observed predominantly amongst *S. haemolyticus* isolates in both the in-patient and control patient groups (94% and 88% respectively). However, the frequency of haemolysin production was notably greater among in-patient and control patient group *S. hominis* isolates and equally in the other minor CoNS species of the control group (72, 54 and 56% respectively). Conversely, with the exception of one protease positive isolate of *S. haemolyticus* (6%), none of the control group isolates were noted to produce either lipase or protease. Similarly, all of the in-patient group *S. haemolyticus* and *S. hominis* isolates were also negative for protease and lipase production. Conversely, low frequency production of both protease and lipase was noted amongst in-patient isolates of both *S. epidermidis* and one, *S. capitis* isolate of the minor group of other CoNS species.

Data analysis subjected to the Holm-Bonferroni method was used to construct a generalised linear regression model to assess whether the proportion of CoNS isolates that produce the predominant virulence factor, slime was associated with CoNS species-specific isolates.

Application was based on the assumption that slime production (response variable) follows a binomial distribution and the in-patient and control groups respectively as explanatory variables. The constructed linear regression model demonstrates there was no evidence of a difference in the proportion of slime production in isolates of *S. epidermidis* and *S. haemolyticus* when compared to the other minor group of CoNS species ($p = 0.293$, $p = 0.121$ and $p = 0.441$ respectively). Similarly, there was no significant difference observed between the in-patient and control group of species-specific CoNS isolates and the proportion of isolates associated with slime production ($p = 0.230$). However, application of partial regression coefficients for the different species-specific CoNS isolates using the Holm-Bonferroni method to control the family-wise error rate, resulted in analysis showing strong evidence that isolates of *S. epidermidis* contain a significantly higher proportion of slime producers in comparison to *S. haemolyticus* isolates in both patient groups ($p = 0.0284$).

Additionally, the relationship between the production of the four virulence factors and antimicrobial resistance expressed to the panel of 18 anti-staphylococcal agents previously evaluated for the 100 CoNS isolates of both patient groups was explored using PCA factor analysis. The results of three dimensional PCA analysis performed on the data evaluated for each of the patient group CoNS isolates to determine the relationship between the two principle variable components, antibiotic resistance and the production of the four main virulence factors are show Figures 5-3 and 5-4. Computed analysis of both data sets for each of the patient group CoNS isolates and the antimicrobial resistance to the panel of 18 antibiotics, with respect to the production of the four virulence factors to create the 3D PCA scatter plot A; demonstrated no correlation between any of virulence factors and resistance to the antimicrobials. This was further represented in the linear regression plot B, demonstrating the relationship between the two principle component variables, resistance to each of the 18 antibiotics and the virulence factors expressed by the 100 CoNS test isolates.



Antibiotic	
PEN	Benzylpencillin
CIP	Ciprofloxacin
ERY	Erythromycin
FLU	Flucloxacillin
FUS	Fuscidic Acid
GEN	Gentamicin
CHL	Chloramphenicol
RIF	Rifampicin
TEI	Teicoplanin
VAN	Vancomycin
TOB	Tobramycin
TET	Tetracycline
TIG	Tigecycline
CLI	Clindamycin
KAN	Kanamycin
LIZ	Linezolid
TRI	Trimethoprim
MUP	Mupirocin
Virulence Factors	
LIPASE	Lipase
PROT	Protease
HAEM	Haemolysin
SLIME	Slime

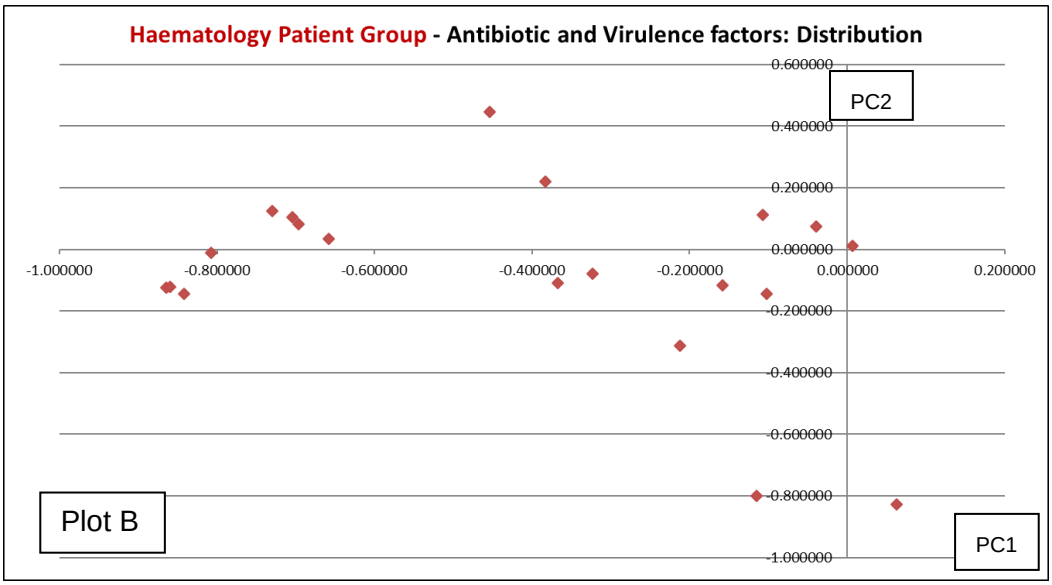
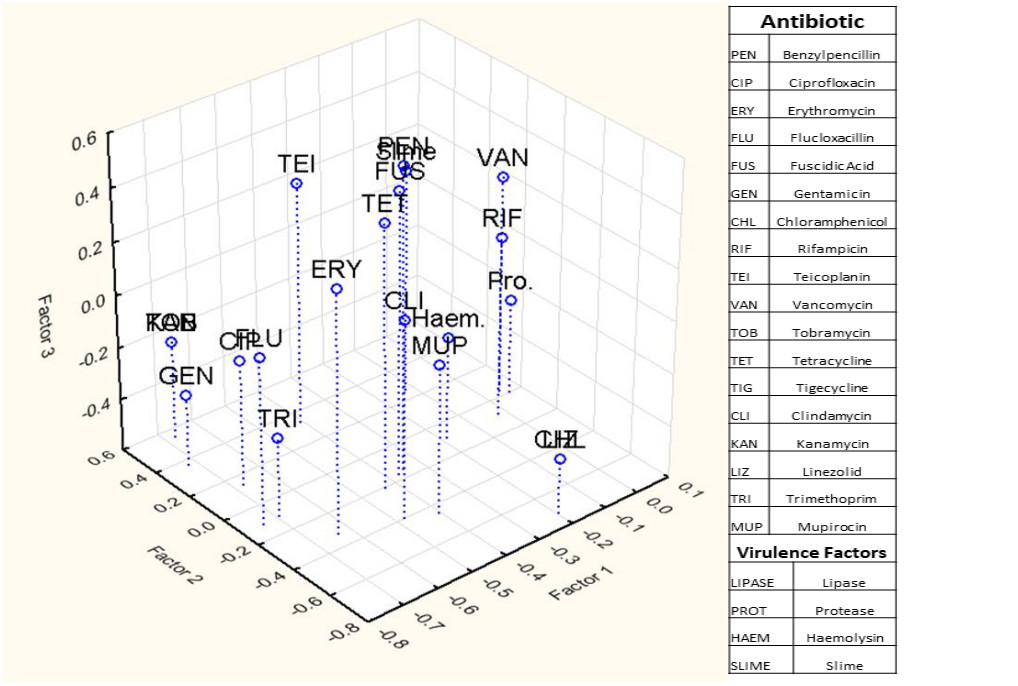


Figure 5-3: Plot A shows 3D Extraction Principle Components Analysis scatter plot of resistance to a panel of 18 antimicrobials and their association with the four virulence factors produced by CoNS isolates from the haematology patient group. Plot B represents PC1(x-axis) vs. PC2(y-axis) plot of data variables to identify correlation of antibiotic resistance profiles with the four virulence factors.



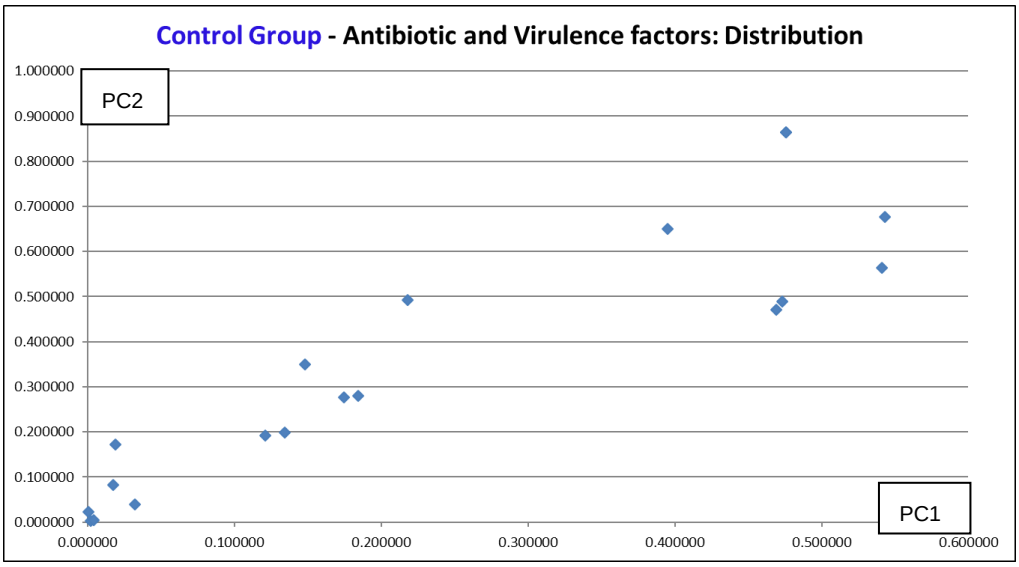


Figure 5-4: Plot A shows 3D Extraction Principle Components Analysis scatter plot of resistance to a panel of 18 antimicrobials and their association with the four virulence factors produced by CoNS isolates of the control patient group. Plot B represents PC1(x-axis) vs. PC2(y-axis) plot of data variables to identify correlation of antibiotic resistance profiles with the four virulence factors.

PCA result analysis for both patient group CoNS isolates represented by the variance in data plots demonstrate a lack of correlation between the two principle variables. The distribution of data plots do not demonstrate any significant clustering, therefore indicating there is no association between antimicrobials resistance to the 18 agents and the expressed virulence factors. Similarly, comparative scatter plot data analysis showed no association between the two principle variants, antimicrobial resistance and virulence factors with respect to the 100 CoNS isolates investigated from each of the two patient groups (Figure 5-5).

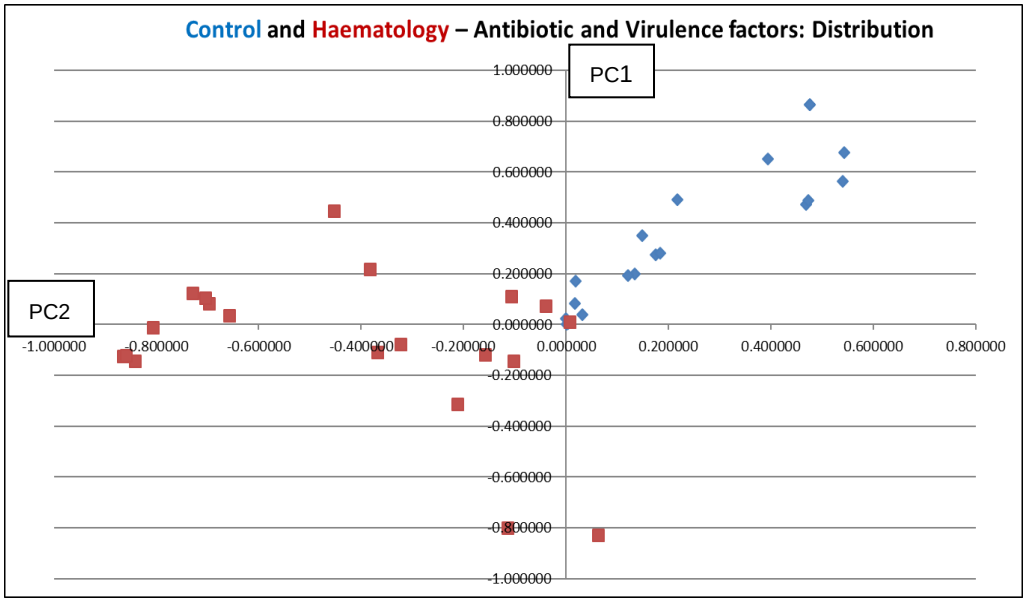


Figure 5-5: Overall comparison of PCA scatter plots obtained for the in-patient haematology patient group (red squares) with the control patient group (blue diamonds) to investigate association of the two variables, virulence factor production with the antibiotic resistance profiles of CoNS isolates from the two patient groups.

The resultant data points for each of the patient groups associated with the two variable vectors were observed to be projected on different sides of the plot axis with little overlap or clustering. Therefore, the overall PCA result analysis of the multivariant data indicates a significant lack of correlation between antimicrobial resistance and the four virulence traits expressed by both patient group CoNS isolates.

Comparisons specifying the percentage distribution of isolates expressing MDR in relation to the major virulence trait, slime production or absence by species-specific CoNS isolates in both patient groups are detailed in Table 5-2. The results reveal a difference in the rate of distribution of species-specific isolates that demonstrate MDR not associated with slime production. Isolates of *S. epidermidis*, *S. hominis* and *S. haemolyticus* in comparison to the other minor group of species-specific CoNS isolates had the greatest number of slime-producers but did not expressing MDR (44% and 41% in the in-patient and control group respectively). Conversely, the greater proportion of non-slime producing isolates exhibiting MDR were predominantly *S. haemolyticus* in both the in-patient and control patient group (69% and 18% respectively).

	Number of Isolates Slime Positive/ MDR Negative	Number of isolates Slime Negative/ MDR Positive
In-patient Group		
S. epidermidis (n=57)	5/ 9%	12/ 21%
S. hominis (n=18)	6/ 33%	2/ 11%
S. haemolyticus (*n*= 16)	1/ 6%	11/ 69%
Other CoNS (*n*=9)	4/ 44%	Nil
Control Group		
S. epidermidis (n=32)	10/ 31%	4/ 13%
S. hominis (n=24)	8/ 33%	1/ 4%
S. haemolyticus (n=17)	3/ 18%	3/ 18%
Other CoNS (*n*=27)	11/ 41%	1/ 4%

Table 5-2: The distribution of none-MDR species-specific CoNS isolates but slime-positive in comparison to slime-negative isolates expressing MDR from both the in-patient and control patient groups.

A generalised linear regression model was further constructed to statistically assess the differences between the proportion of isolates that express MDR and variation dependent on CoNS species. Result analysis based on the proportion of isolates that expressed MDR as the response variable and the CoNS species-specific isolates, slime production and the two patient groups as the explanatory variables are summarised in Table 5-3.

	Mean Number Resistant	Standard Error - Resistance	Proportional Number of MDR isolates	Proportional Number of Slime-positive isolates
In-patient Group				
S. epidermidis (n=57)	8.7	0.4	0.825	0.719
S. hominis (n=18)	6.61	0.825	0.667	0.444
S. haemolyticus (*n*= 16)	9.62	0.625	0.938	0.312
Other CoNS (*n*=9)	1.78	0.434	0	0.444
Control Group				
S. epidermidis (n=32)	4.94	0.5	0.375	0.5
S. hominis (n=24)	3.75	0.494	0.125	0.417
S. haemolyticus (n=17)	5.24	0.897	0.353	0.353
Other CoNS (*n*=27)	2.11	0.428	0.111	0.519

Table 5-3: Summary data of each of the patient groups dependent on the proportion of species-specific CoNS isolates expressing MDR in comparison to the proportional number of slime producers.

From the model used to assess the significance of association between MDR, species-specific CoNS isolates, slime production and patient groups, it was observed that *S. epidermidis* and *S. haemolyticus* were both associated with an increase in the proportion of isolates expressing MDR in comparison those isolates comprising the minor group of CoNS ($p < 0.0001$). Overall result analysis presented strong evidence that CoNS isolates belonging to the in-patient group were significantly associated with an increase in the proportion expressing MDR ($p < 0.0001$). Furthermore, there was good evidence showing that isolates expressing increasing resistance to the tested antimicrobials were significantly associated with the production of slime ($p < 0.0098$). No such correlation between slime production and expression of MDR was observed among species-specific CoNS isolates of the control patient group ($p = 0.222$).

5.5.2 Antimicrobial synergy testing using E-tests

The in-patient *S. epidermidis* isolates A37, A57 and A91 tested against combinations of vancomycin, daptomycin, rifampicin and gentamicin were evaluated for demonstration of synergy using the quantitative E-test synergy method. The E-test strips were placed on the susceptibility agar in a cross formation at the interaction point corresponding to the previously determined individual MICs of the respective test antibiotics. Figure 5-6 shows an example of the E-test results evaluating combination of daptomycin with rifampicin for synergy testing.

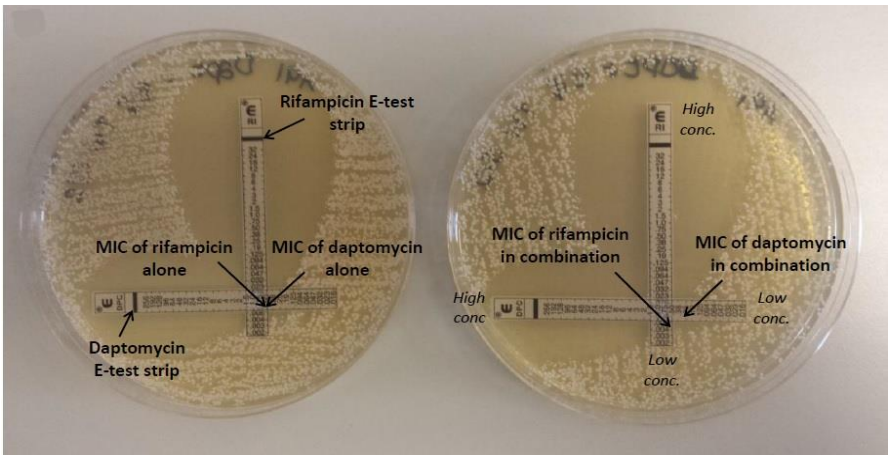


Figure 5-6: E-test synergy test method showing the placement of daptomycin and rifampicin E-test strips in cross formation with a 90° angle at the interaction points of their respective MIC values tested against *S. epidermidis* isolate A91.

The combination antimicrobial MIC was interpreted as the value at zone of inhibition at the point of interaction of the scale on each respective antimicrobial E-test strip. The MIC for the

combined effect of the two antibiotics calculated using the previously defined algorithms were used to calculate the FIC index to evaluate the efficacy of the combination. The *in-vitro* activities of the test antimicrobials in combination and the interpretive classification criteria based on the FIC index calculation are summarised in Table 5-4 for the control strain, *S. aureus* NCTC 6571 and in Table 5-5 for the representative in-patient *S. epidermidis* isolates A37, A57 and A91.

Control Isolate	Drug Combination	MIC Value mg/L	FIC Index value	Classification
S. aureus	RIF and GEN	0.002		
NCTC 6571	GEN and RIF	0.047		
			0.68	Additive
S. aureus	RIF and DAP	0.001		
NCTC 6571	DAP and RIF	0.047		
			0.71	Additive
S. aureus	DAP and GEN	0.064		
NCTC 6571	GEN and DAP	0.047		
			0.56	Additive
S. aureus	DAP and VAN	0.125		
NCTC 6571	VAN and DAP	1		
			2	Indifference
S. aureus	RIF and VAN	0.002		
NCTC 6571	VAN and RIF	0.5		
			1.17	Indifference
S. aureus	VAN and GEN	0.5		
NCTC 6571	GEN and VAN	0.78		
			1.28	Indifference

Table 5-4: E-test synergy test method results for combinations of rifampicin (RIF), gentamicin (GEN), daptomycin (DAP) and vancomycin (VAN) measured against the control strain *S. aureus* NCTC 6571.

The *S. aureus* control strain classified as susceptible to all classes of anti-staphylococcal drugs including the β-lactams, showed high level susceptibility to all the four antimicrobials tested individually. Although no synergy was defined, an additive effect was however noted between combination of rifampicin with gentamicin and daptomycin and between daptomycin-gentamicin. Indifference was however, observed between vancomycin combination with daptomycin, rifampicin or gentamicin against the control *S. aureus* strain.

Similar differences were also observed between the different antimicrobial interactions and the representative in-patient *S. epidermidis* isolates. Nonetheless, no antagonistic effects were observed with any of the binary drug interactions. The results displayed in Table 5-5 show that the majority of the interactions were predominantly additive, defined as the total sum effect equal to single effects of the two antimicrobials.

S. epidermidis Isolate	Drug Combination	MIC Value mg/L	FIC Index value	Classification
A37	RIF and GEN	0.003		
	GEN and RIF	0.032		
			1.09	Indifference
A37	RIF and DAP	0.002		
	DAP and RIF	0.25		
			1.17	Indifference
A37	DAP and GEN	0.064		
	GEN and DAP	0.032		
			0.63	Additive
A37	DAP and VAN	0.19		
	VAN and DAP	0.5		
			0.71	Additive
A37	RIF and VAN	0.002		
	VAN and RIF	0.5		
			1	Additive
A37	VAN and GEN	0.75		
	GEN and VAN	0.015		
			0.73	Additive
A57	RIF and GEN	0.004		
	GEN and RIF	0.047		
			0.87	Additive
A57	RIF and DAP	0.002		
	DAP and RIF	0.095		
			0.58	Additive
A57	DAP and GEN	0.38		
	GEN and DAP	0.125		
			1.8	Indifference
A57	DAP and VAN	0.172		
	VAN and DAP	0.5		
			0.78	Additive
A57	RIF and VAN	0.002		
	VAN and RIF	0.5		
			0.83	Additive
A57	VAN and GEN	0.75		
	GEN and VAN	0.095		
			1.1	Indifference
A91	RIF and GEN	0.004		
	GEN and RIF	8		
			0.75	Additive
A91	RIF and DAP	0.004		
	DAP and RIF	0.32		
			1.31	Indifference
A91	DAP and GEN	0.5		
	GEN and DAP	32		
			2	Indifference
A91	DAP and VAN	0.188		
	VAN and DAP	0.75		
			0.88	Additive
A91	RIF and VAN	0.002		
	VAN and RIF	0.5		
			0.83	Additive
A91	VAN and GEN	0.38		
	GEN and VAN	6		
			0.44	Synergy

Table 5-5: E-test synergy test method results for combinations of rifampicin (RIF), gentamicin (GEN), daptomycin (DAP) and vancomycin (VAN) measured against the three *S. epidermidis* strains A37, A57 and A91 and the calculated FIC index, defining combination activity.

The highly susceptible isolate A37 (penicillin resistance only), moderately resistant isolate, A57 and the MDR isolate A91, all demonstrated additive interaction between daptomycin-vancomycin and rifampicin-vancomycin (FIC index ≤1). In comparison to the fully susceptible *S. aureus* control strain, all three in-patient *S. epidermidis* isolates with one exception, demonstrated either indifference or additive interaction effect between combinations of rifampicin with vancomycin, daptomycin or gentamicin. Although the control strain showed indifference between combinations of daptomycin-vancomycin and rifampicin-vancomycin, A37, the susceptible isolate and the resistant isolates A57 and A91 all demonstrated an additive effect with these combinations. Moreover, combination of vancomycin-gentamicin was observed to be most effective, as synergy was exceptionally noted against MDR isolate A91. In contrast however, isolate A91 demonstrated vancomycin susceptibility at MIC 1.5mg/L but high-level resistance at MIC >32mg/L to gentamicin individually. Nonetheless, the calculated FIC index result of 0.44 relating to synergy was further evidenced by the defined observation of a greater than fourfold decrease in the MIC produced by combination of gentamicin-vancomycin against isolate A91. More importantly however, none of the binary drug combination evaluations, demonstrated *in-vitro* inefficacy. Combination antimicrobial efficacy assessment using the E-test assay in accordance with the standard definitions for the methodology against the three *S. epidermidis* BSI isolates and the *S. aureus* control strain are summarised in Table 5-6.

	Classification of Combination Antibiotic Interaction based on E-test Assay			
	S. epidermidis A37	*S. epidermidis* A57	*S. epidermidis* A91	*S.aureus* Control
Antibiotic Combination				
VAN and DAP	Additive	Additive	Additive	Indifference
VAN and GEN	Additive	Indifference	Synergy	Indifference
VAN and RIF	Additive	Additive	Additive	Indifference
RIF and DAP	Indifference	Additive	Indifference	Additive
RIF and GEN	Indifference	Indifference	Indifference	Additive
DAP and GEN	Additive	Indifference	Indifference	Additive

Table 5-6: Comparison of the E-test assay results for classification of combination antimicrobial interactions of rifampicin (RIF), gentamicin (GEN), daptomycin (DAP) and vancomycin (VAN) against the three *S. epidermidis* isolates A37, A57, A91 and the control strain *S. aureus*.

Of all the six antimicrobial combinations assessed an overall additive effect was observed for combination of vancomycin-daptomycin and vancomycin-rifampicin against all of the three in-patient *S. epidermidis* isolates tested for synergy. Likewise, all three test isolates demonstrated an indifference effect with rifampicin-gentamicin combination whereas the other antibiotic combination exhibited differing interaction effects.

5.5.4 Antimicrobial activity assessed against S. epidermidis RP62A adherent biofilm

5.5.4.1 Effect of vancomycin, rifampicin and daptomycin challenge on biofilm

Vancomycin, daptomycin and rifampicin showed good activity against the biofilm forming reference strain *S. epidermidis* RP62A in the planktonic mode of growth. For all the tests run in triplicate the average MICs evaluated by the microtitre broth microdilution method for vancomycin and rifampicin were within the susceptibility range of 1 and 0.5mg/L respectively. Conversely, the MIC of 2mg/L for daptomycin was one doubling dilution above the susceptibility MIC breakpoint in accordance with the EUCAST susceptibility breakpoint of 1mg/L. MBC values obtained for rifampicin, vancomycin, and daptomycin were 0.5, 2 and 8mg/L respectively. Overall results indicated the superior activity of rifampicin against planktonic state *S. epidermidis* RP62A isolate in comparison to the other test antimicrobials.

The effect of daptomycin, vancomycin and rifampicin was further assessed for biofilm killing across a range of concentrations from 0.5 to 64mg/L after 24 hour incubation. The biofilms developed in the 96-well microtitre plates were characterised in terms of the total killing of adherent cells and the calculated logarithmic cell density reduction with the selected antimicrobial agents (Figure 5-7). The overall comparative *in-vitro* activity of vancomycin, rifampicin and daptomycin assessed against the *S. epidermidis* RP62A adherent biofilm, are presented in Figure 5-7A. These were quantified spectrophotometrically by determining the OD at 570_{nm} and the spectrophotometric values obtained converted to conventional CFU/mL to evaluate and assess average biofilm cell density reduction for all tests run in triplicate.

This isolate demonstrated sufficient biofilm formation after three days incubation with an OD_{570nm} of 0.875 ± 0.01 equating to biofilm cell density of 1.457 x 10^8 CFU/mL on average. This first negative control wells after 24 hour incubation demonstrated OD_{570nm} of 0.99 ± 0.21 equating to an average biofilm cell density of 1.92 x 10^8 CFU/mL. In comparison, the negative control well with no bacterial cell growth remained constant, resulting in an average background reading at OD_{570nm} of O.085. The second sterility check, negative control well containing MHB only, presented an OD_{570nm} value of 0.096 ± 0.005. The sterility check performed on the contents of the control wells subsequently plated out after incubation on MH-agar showed all assay plates were culture negative therefore, contaminant free.

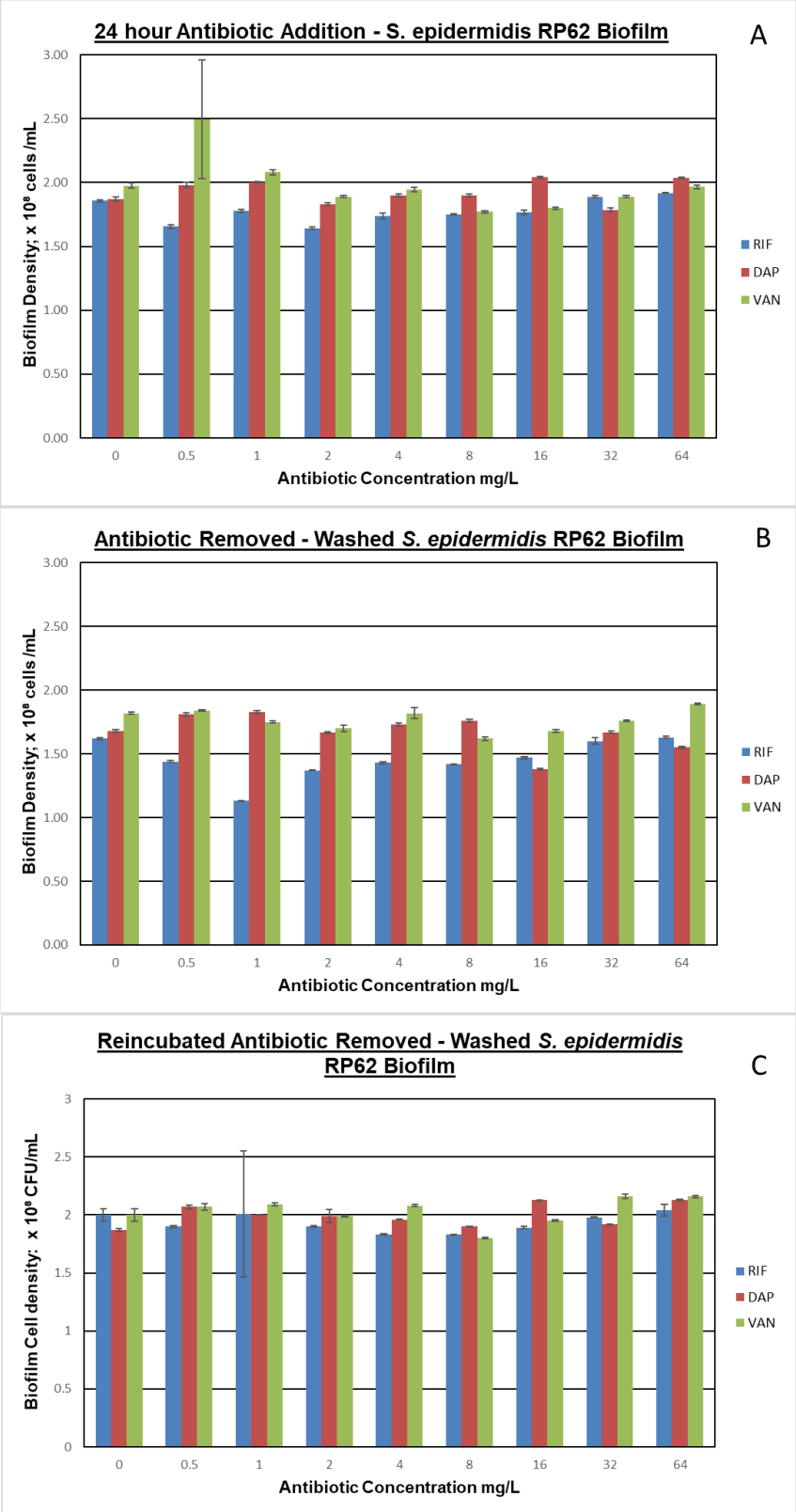


Figure 5-7: Activity of rifampicin, daptomycin and vancomycin individually against biofilm encased *S. epidermidis* RP62A cells. A) Antimicrobial activity after 24 hour incubation with antibiotics B) Biofilm cell density after washing antibiotic affected biofilm cells with the addition of MHB C) Antibiotic neutralised, washed biofilm cell density after further 24 hour re-incubation.

The MIC of vancomycin was determined at 8mg/L for *S. epidermidis* RP62A adherent biofilm, which was four fold higher than the MIC observed for the isolate in planktonic state. For all the tests run in triplicate the total average calculated biofilm cell density reduction was equivalent to 0.54 x 10^8 CFU/mL after 24 hour exposure to vancomycin. Furthermore, an exponential decrease in biofilm cell density was not observed with exposure to increasing concentrations of vancomycin from 16-64mg/L. Similarly, the biofilm MIC value for rifampicin and daptomycin visually observed at concentration of 1 and 2mg/L resulted in an average calculated biofilm cell density reduction of 0.22 and 0.04 x 10^8 CFU/mL respectively at these concentrations. Similar to the activity of vancomycin, both daptomycin and rifampicin showed no further decrease in cell density with increasing antimicrobial concentrations. The overall calculated average logarithmic reduction value for all three antimicrobials tested was <1 $\log_{10}$ CFU/mL.

Base-line assessment of biofilm cell density was carried out prior to re-incubation of the antibiotic neutralised biofilm to assess the presentation of residual antimicrobial activity within the biofilm layers. Figure 5-7B details the antibiotic neutralised washed biofilm cell densities with the addition of MHB. The average calculated biofilm cell density of the control wells and those that had been antibiotic challenged previously were, 1.773 ± 0.15 x 10^8 CFU/mL and 1.716 ± 0.26 x 10^8 CFU/mL respectively. After 24 hour re-incubation the control well biofilm cell density increased on average by 0.196 ± 0.02 x 10^8 CFU/mL. Similarly, vancomycin, daptomycin and rifampicin neutralised biofilm cell densities on average increased by 0.267 ± 0.06x 10^8 CFU/mL as detailed in Figure 5-7C. This indicates a comparative difference of 0.071x10^8 CFU/mL, equivalent to <1 $\log_{10}$ CFU/mL increase in calculated cell densities of re-incubated biofilm.

Furthermore, the average calculated cell densities for biofilm MBC determination for vancomycin at concentration of 8, 16 and 32mg/L ranged from 2.44, 4.95 and 6.96 x10^8 CFU/mL and for daptomycin 3.65, 1.33 and 1.11 x10^8 CFU/mL respectively. Similarly, for rifampicin the average calculated MBC was 5.12 x10^8 CFU/mL at the upper concentration level of 32mg/L. The overall data result analysis indicates, that defined MBCs determined as the lowest concentration to show a highly significant or 99.9% reduction in biofilm CFU/mL was not achieved at the upper limits of the test concentration for all three tested antibiotics.

Spectrophotometrically quantified results for similar comparisons of antimicrobial efficacies of vancomycin, rifampicin and daptomycin against *S. epidermidis* RP62A biofilm embedded cells individually and in combination obtained at OD 570_{nm} are detailed in Figures 5-8 and 5-9 respectively. The error bars reflect ± standard error of the mean. Figure 5-8 1a, 2a and 3a represent individual activity of vancomycin, rifampicin and daptomycin respectively against biofilm embedded cells.

There were no significant difference observed in base-line OD readings taken of all test assay plates with the addition of MHB plus the antibiotics prior to incubation ($p = 1.000$). Similar results were obtained for average OD readings taken after the addition of fresh MHB to the washed and antibiotic neutralised biofilms prior to re-incubation for a further 24 hours ($p = 1.000$).

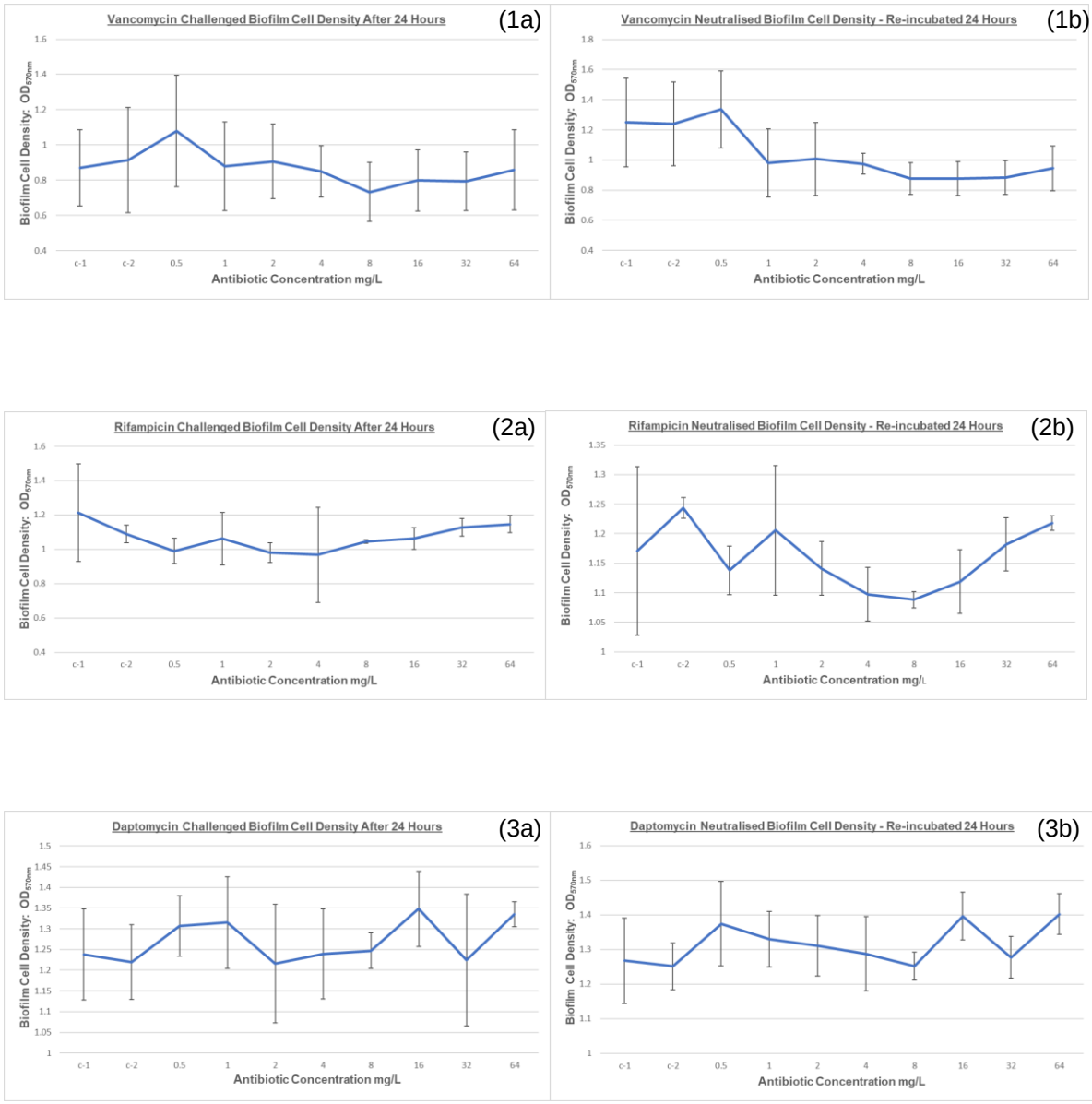


Figure 5-8: Effect of vancomycin (1a), rifampicin (2a) and daptomycin (3a) challenge on *S. epidermidis* RP62A biofilm cell density. Spectrophotometric measurements at OD_{570nm} of cell-density across antimicrobial concentration range of 0.5-64mg/L were determined and compared to effect on biofilm cell density after vancomycin (1b), rifampicin (2b) and daptomycin (3b) neutralisation and re-incubation. Controls c-1 and c-2 (biofilm and biofilm with MHB respectively).

The overall measured OD_{570nm} result values of the experimental assays for all the three single antibiotic challenged biofilm after 24 hour incubation, indicate that none of these tested antibiotics were capable of appreciably reducing biofilm cell densities across antibiotic concentration range of 0.5 to 64mg/L.

The presence of viable *S. epidermidis* cells was further assessed within the antimicrobial neutralised biofilm by removing unbound sessile cells and residual antibiotics by washing the biofilm with PBS. Spectrophotometric measurements at OD_{570nm} applied to quantify viable bacteria within each antibiotic neutralised biofilm microtitre assay after 24 hour incubation at 37°C are detailed in Figure 5-8 1b, 2b and 3b for vancomycin, rifampicin and daptomycin respectively. In comparison to the antibiotic treated biofilm test assay the mean OD_{570nm} values of untreated biofilm samples (controls c-1 and c-2) remained constant at 1.16 ± 0.98 on average. This indicates that substantive increase in biofilm cell density in the control well biofilm samples was not observed on further incubation with the addition of fresh MHB.

The mean OD_{570nm} values of vancomycin and rifampicin neutralised biofilm similarly demonstrate a small rate of increase in OD_{570nm} values across the entire concentration test range. The small difference in biofilm cell density measured by OD_{570nm} values was however, observed to be comparatively higher in the daptomycin neutralised biofilm assay. Likewise, the mean difference in the increase of OD_{570nm} values for daptomycin were higher in comparison to those obtained for vancomycin and rifampicin after re-incubation. An average overall increase of biofilm cell density (OD_{570nm} value 0.25 ± 0.2) for daptomycin indicates the possible presence of potentially viable persister cells embedded within the biofilm. This may however also be associated with the lower biofilm killing effect of daptomycin in comparison to the activity of vancomycin and rifampicin against the isolate embedded in biofilm.

Significant differences in activity among the three antibiotics, vancomycin, rifampicin and daptomycin against *S. epidermidis* RP6A biofilms after both 24 hour challenge and post neutralisation and re-incubation were evaluated with the application of the Kruskal-Wallis sum test. Overall data analysis results comparing ranks of median distribution of OD values indicate that there was significant differences observed among the three antibiotics tested after 24 hours antibiotic challenge, post neutralisation and re-incubation (Kruskal-Wallis chi squared = 896.8833, *df* =13, $p < 0.00005$).

Comparisons between vancomycin, rifampicin and daptomycin were further made using similar application of the Kruskal-Wallis sum test to determine significant reduction in biofilm cell density through quantitative OD measurements. Therefore, statistical analysis of the data shows that the mean OD values for vancomycin and rifampicin ranked significantly

higher post-neutralisation and re-incubation in comparison to the 24 hour challenged biofilm OD values (Kruskal-Wallis chi-squared = 70.7995 *df* =15, p <0.00005 and Kruskal-Wallis chi-squared = 115.1363, *df* =15 p = 0.0214 respectively). This suggests that there were a discernible number of viable bacteria present within the vancomycin and rifampicin post-neutralised biofilms to allow for the significant increase in biofilm cell density observed after re-incubation (p <0.05). Conversely, no statistically significant difference was observed when comparing the median OD values for daptomycin after 24 hour antibiotic challenge with the OD values obtained after post-neutralisation and re-incubation (p = 1.0000).

Similarly, the biofilm control well with addition of MHB showed mean OD values ranking significantly higher after further incubation of washed biofilm and with addition of fresh MHB (p <0.00005). Conversely, there was strong evidence of no statistical difference observed when comparing the median OD values for the biofilm control well incubated without the addition of fresh MHB after 24 hour incubation and post-incubation after washing of biofilms (p = 0.3198).

Statistically significant differences in OD values were noted when comparing data results of individual antibiotic activity of vancomycin with rifampicin and also with daptomycin against *S. epidermidis* RP62A biofilms pre and post re-incubation (p <0.00005). Similar significant differences were observed when comparing 24 hour challenge of biofilm, individually with rifampicin and daptomycin on biofilm cell density after antimicrobial neutralisation and re-incubation (p <0.00005 and p = 0.0081 respectively). The overall results reveal a significant difference in the overall effect each antibiotic has on OD, as a measure of biofilm cell density after incubation with antimicrobial challenge across the entire antibiotic concentration range of 0.5-64mg/L. Furthermore, there was good evidence showing that the overall effect on biofilm viability after antibiotic neutralisation was similar for each antibiotic neutralised biofilm test assay on re-incubation (p <0.00005). This suggests that the biofilm washing process reduced the concentration of each of the test antibiotic sufficiently to below active levels to allow detectable growth of the viable cells encased within the biofilm layers after further 24 hour re-incubation.

5.5.4.2 Effect of vancomycin combinations with rifampicin and daptomycin challenge on biofilm

The results of mean killing difference of vancomycin-rifampicin, vancomycin-daptomycin and rifampicin-daptomycin combinations against *S. epidermidis* RP62A biofilm assessed spectrophotometrically across concentration range 0.5-64mg/L are presented graphically in Figure 5-9 1a, 2a and 2c.

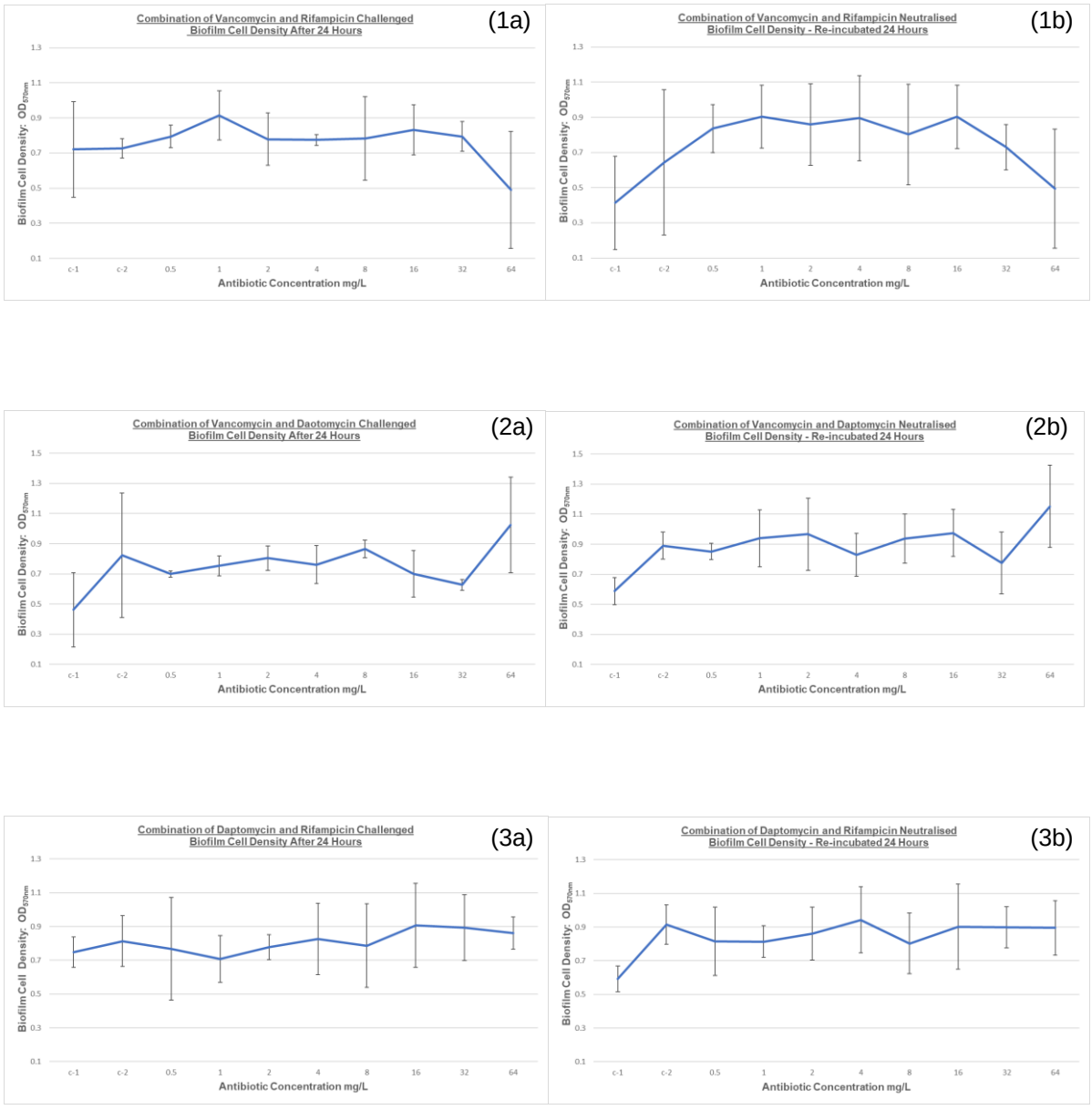


Figure 5-9: Effect of combination challenge of vancomycin with rifampicin (1a) and with daptomycin (2a) and daptomycin with rifampicin (3a) on biofilm cell density. Biofilms formed by reference strain *S. epidermidis* RP62A were determined spectrophotometrically at OD_{570nm} across antimicrobial concentration range of 0.5-64mg/L. Comparison of antimicrobial neutralisation and re-incubation effect on biofilm cell density (1b), (2b) and (3b) respectively. Negative controls c-1 and c-2 (biofilm and biofilm with MHB respectively).

In comparison to the untreated biofilm controls, the killing effect of combinations of vancomycin-rifampicin, vancomycin-daptomycin and daptomycin-rifampicin on *S. epidermidis* RP62A biofilms determined by reduction in OD values of the biofilm cell densities was discernibly low. Additionally, the overall low killing effect for all three antimicrobial combination interactions did not appear to vary considerably with increasing antimicrobial concentrations.

S. epidermidis RP62A biofilms challenged with a combination of vancomycin-rifampicin showed no difference in OD values equating to any noticeable reduction of biofilm cell density up to interaction concentrations of 32mg/L. Conversely the biofilm cell densities after showing a small increase, remained in a steady state with a comparatively small reduction in biofilm cell density, noticeable at the maximum concentration level of 64mg/L. However, the observed OD value at this maximum concentration point did not exceed the value noteworthy of logarithmic reduction in comparison to the values obtained for the untreated biofilm controls. Additionally, combinations of vancomycin-daptomycin and daptomycin-rifampicin demonstrated no visible killing effect on *S. epidermidis* RP62A challenged biofilms across all antimicrobial concentration interactions. Furthermore, the OD values of biofilm cell densities with these two antimicrobial combinations challenged biofilms also appeared to remain in a steady state (Figure 5-9 2a and 3a). The inefficacy of combinations of vancomycin-daptomycin and rifampicin-daptomycin interactions against *S. epidermidis* RP62A biofilms was further noted with no apparent changes in the OD values of antibiotic neutralised biofilms (Figure 5-9 2b and 3b respectively). Similarly, no variation in OD values for vancomycin-rifampicin combination challenged biofilms were observed in comparison to the antimicrobial combination neutralised biofilms (Figure 5-9 1a and 1b respectively).

To further assess the generalised low or indifferent killing effect of three binary antimicrobial combinations against the *S. epidermidis* RP62A biofilm, the mean FIC Index was calculated, based on previously described method for the interpretation of biofilm checkerboard synergy testing (Bonapace *et al*., 2002). This method of assessment was utilised for result interpretation as the requirement for entire rows or columns to show complete growth inhibition is not essential. This standardised biofilm checkerboard assay utilising inhibition of growth as the endpoint, with comparisons made to the positive growth control well without antibiotic addition was used to evaluate the degree of reduced turbidity both visually and by measure of OD. Hence, overall results of the antimicrobial interaction were based on evaluating the point of interface for each of the test antimicrobial combination interactions and assessed visually and by spectrophotometric difference in OD values of selected wells in specified rows with comparison to results obtained for untreated biofilm control wells.

The biofilm FIC index calculations were therefore based on using the concentration value of the first well showing the lowest amount of turbidity compared to the positive control well in each row and column along the interface of high-turbidity/lowest or non-turbidity. Analysis of the data obtained for all the experiments run in triplicate, resulted in the calculated cumulative mean ΣFIC index values of 4.03, 4.72, and 4.345 obtained for combinations of vancomycin-rifampicin, vancomycin-daptomycin and daptomycin-rifampicin respectively against *S. epidermidis* RP62A biofilm. These results interpreted in accordance with the previously defined categorical interpretation for synergy, such that ΣFIC ≤0.5 categorised as indifference, additive as ΣFIC >0.5-4 and ΣFIC >4 as antagonism (Bonapace *et al*., 2002). Accordingly, for the resultant ΣFIC index values of >4 obtained for all three antimicrobial combinations the interactions were all categorised as demonstrating an antagonism effect against *S. epidermidis* RP62A biofilm.

Data results evaluating the activity of individual antibiotics in comparison to the binary combinations both indicate poor activity against biofilm-embedded isolates. Overall, no discernible logarithmic decrease in biofilm cell density, measured through OD_{570} across the test concentration range of 0.5-64mg/L was demonstrated. Furthermore, biofilm bactericidal activity was not observed with any of the three binary antimicrobial combinations at the upper limit of the test concentration range. Single and combination antimicrobial activity therefore appears to be significantly ineffective at reducing biofilm cell density, additionally demonstrating a poor response with increasing antimicrobial concentrations ($p > 0.5$).

The Kruskal-Wallis sum test was further applied to test the significant association of the effect of each binary combination antimicrobial challenge on *S. epidermidis* RP62A biofilm cell density after 24 hour incubation. Overall data analysis comparing ranks of median distribution of OD values for combination of vancomycin-rifampicin did not reveal any evidence of statistically significant difference when tested against 24 hour challenged biofilm across the interaction concentration range of 0.5-64mg/L (Kruskal-Wallis chi-squared = 116.98 *df* =127, p = 0.7271). Similarly, there was no evidence of statistically significant reduction of biofilm cell densities observed with 24 hour challenge of *S. epidermidis* RP62A biofilm with rifampicin-daptomycin combination across the antimicrobial interaction concentration range of 0.5-64mg/L (Kruskal-Wallis chi-squared = 118.68 *df* =127, p = 0.6886). Conversely, data analysis of median OD value distribution for combination antimicrobial effect of vancomycin-daptomycin, demonstrates good evidence of a statistically significant difference to *S. epidermidis* RP62A biofilm cell density after 24 hour challenge with combination (Kruskal-Wallis chi-squared = 299.33 *df* =127, p <0.00001). The statistical analyses results further indicate the greater effect of vancomycin-daptomycin combination against biofilm encased *S. epidermidis* RP62A cells across all the test concentration range in

comparison to the efficacy of combinations of vancomycin-rifampicin and rifampicin-daptomycin.

Evaluating the association between binary combinations on the other hand revealed there no significant differences noted between the activity of vancomycin-rifampicin in comparison to vancomycin-daptomycin and rifampicin-daptomycin efficacy against 24 hour *S. epidermidis* RP62A biofilms ($p = 0.934$ and $p = 0.8967$ respectively). However, comparison of the binary combination activity of vancomycin-daptomycin with that of rifampicin-daptomycin revealed a significant difference in effectiveness against 24 hour challenged biofilm ($p = 0.0092$).

Comparing the OD value distribution of the control wells for combination test assays there was no significant difference observed in biofilm cell densities for the biofilm without addition of MHB pre and post re-incubation ($p = 0.0945$). However, there was a significant difference in observed OD values for the control wells consisting of biofilm with the addition of MHB ($p = <0.00001$). The increase in cell density post-washing with the addition of fresh MHB is likely due to the provision of fresh nutrients required to stimulate growth of viable cells within the biofilm thus showing a significant difference in OD values post re-incubation. Comparatively, similar statistically significant differences in biofilm cell densities were observed with combinations of vancomycin-daptomycin as well as with rifampicin-daptomycin post-neutralised biofilms ($p = <0.00001$ and $p = 0.0005$ respectively). Conversely no significant difference in OD values were observed after 24 hour vancomycin-rifampicin combination challenged biofilms, compared to the OD values obtained post-neutralisation and re-incubation ($p = 0.7271$ and $p = 0.1940$ respectively). Combination of vancomycin- rifampicin interaction in this case suggests growth suppression activity rather than reduction in biofilm density due to eradication of biofilm encased cells.

Further data analysis to determine whether single antibiotics were more effective than in binary combination against *S. epidermidis* RP62A biofilms was carried out by comparing ranks of median distribution of OD values. These values demonstrated that single antibiotics were overall significantly more effective than in binary combination against *S. epidermidis* RP62A biofilms ($p <0.00001$). As such, biofilm antimicrobial challenge individually with vancomycin or rifampicin was significantly more effective than in combination after 24 hour incubation ($p = 0.0422$ and $p <0.00001$ respectively). Likewise, there was a significant difference noted with vancomycin-daptomycin in compassion to the combination of vancomycin-rifampicin interactions against 24 hour challenged biofilms ($p = 0.0002$ and $p <0.00001$ respectively). Rifampicin and daptomycin were also significantly more effective individually, in comparison to rifampicin-daptomycin combination against *S. epidermidis* RP62A biofilms after 24 hour challenge ($p <0.00001$). The overall data analysis suggests

that the kill effect of the three antibiotics individually on biofilm encased cells is significantly greater in comparison to the effectiveness of the antibiotics in binary combination ($p < 0.00001$). Thus, vancomycin, daptomycin and rifampicin individually demonstrate significantly greater efficacy in comparison to their activity in combination against biofilm embedded *S. epidermidis* RP62A cells.

5.6 Discussion

Virulent isolates of CoNS most frequently involved in nosocomial infections particularly among immunocompromised patients are due to their inherent ability to form biofilms on any abiotic surfaces (de Allori *et al*., 2006; Ninin *et al*., 2006; Fey, 2010). Such infections, in particular BSIs are frequently associated with the extensive use of CVCs and other medical devices becoming colonised with biofilm-producing CoNS strains. *S. epidermidis* strains in particular, contribute to the significant pathogenesis of medical device related infection particularly among immunocompromised patients. This is due to their inherent ability to form biofilms on abiotic surfaces hence affording the isolates protection against the antibiotics administered for treatment of infection (O'gara and Humphreys, 2001; Otto, 2004).

There is increasing evidence that virulence factors produced by CoNS significantly contribute to the pathogenesis of serious infection in the immune-incompetent host. These virulence factors are surface expressed or secreted components that include enzymes or toxins that allows the bacterium to invade and cause disease when host defences are compromised (Otto, 2004; de Lourdes *et al*., 2006). Among the CoNS, *S. epidermidis* represents the species most commonly associated with the production of virulence factors, in particular, the formation of multi-layered biofilms surrounded by slime an extracellular polymeric matrix conferring increased tolerance to antimicrobial therapeutics (Holby *et al.,* 2009). The present study therefore aimed to explore the prevalence of some of the few common virulence factors produced by both the skin-colonising and associated potential pathogenic isolates of CoNS causing BSIs concomitant with phenotypic antimicrobial resistance. Based on these considerations, in this study, both the in-patient BSI CoNS and the skin colonising isolates from the pre-admission control patient group were screened for their ability to produce four clinically relevant virulence factors common to CoNS. In addition to the production of protease, lipase and haemolysin, the CoNS species-specific isolates from both patient groups were evaluated for their ability to produce slime, the main virulence factor often associated with resistance to systemic antimicrobial therapy.

Of the in-patient CoNS species-specific isolates, *S. epidermidis* the most frequently isolated species was found to be associated with the production of all four virulence factors evaluated. Slime production, the major virulence factor associated with CoNS pathogenicity in this study was found to be prevalent among both the in-patient and control patient group *S. epidermidis* isolates (72% and 50% respectively). Similar results of higher rates of slime production in pathogenic strains of CoNS compared to normal skin-colonising isolates have been previously reported (Koksal *et al*., 2009). Slime production was also expressed at a similar frequency (52%) among the other minor group of CoNS isolates of the control patient group, predominantly isolates of *S. warneri* and *S. capitis*. Although results show no significant difference in the proportion of slime producing isolates of *S. epidermidis* and *S. haemolyticus* when compared to the other minor group of CoNS species ($p > 0.05$); there was however, strong evidence that *S. epidermidis* isolates, comprise of a significantly higher proportion of slime producers in comparison to *S. haemolyticus* isolates in both patient groups ($p = 0.0284$). These findings are consistent with those presented in other studies showing that the more virulent strains of *S. epidermidis* and *S. haemolyticus*, the predominant species isolated in clinical infections are concomitant with slime production (Voung and Otto, 2002; Arciola *et al*., 2004; Arslan and Özkardes, 2007). Likewise, the comparatively higher frequency of slime producing isolates of *S. epidermidis* associated with bacteraemia in the in-patient group could be due to seeding from colonised intravascular devices that are extensively used in this in-patient group for treatment access. Similar device-related infections with virulent CoNS species-specific isolates have been reported by several other investigators (Ahlstrand *et al.,* 2012; Martiínez-Morel *et al.,* 2014; Yamada *et al.,* 2017). Additionally, the greater capacity for virulence resulting from triggering of exoenzyme expression during the course of infection is likely to be associated with adaptive survival responses of these clinical isolates.

Furthermore, in addition to haemolysin, the expression of hydrolytic exoenzymes, protease and lipase were mainly observed among the BSI CoNS in comparison to the skin-colonising isolates. Lipase and protease were predominantly expressed in six (11%) *S. epidermidis* and by one (11%) *S. capitis* BSI isolates. Conversely, only one (6%) control patient group isolate of the *S. haemolyticus* expressed protease. The role for lipase in virulence although predominantly reported in *S. epidermidis* has also been described in some clinical isolates of *S. haemolyticus* and *S. warneri.* In such cases the associated exoenzymatic activities of these isolates is concomitant with their role as potential pathogens because of their collagen binding ability and survival in fatty sections of infected hosts (Otto, 2004). Similarly, the hydrolytic activity of protease expressed by some *S. epidermidis* strains, trigging degradation of fibrinogen and other proteins associated with the immune defence system causing tissue

damage correlates with potential for virulence. Similar homologues of protease identified in some minor group of CoNS species-specific isolates interacting with other enzymes confer these isolates with the capacity to adhere to host cells. These enzymatic activities expressed by some virulent strains of CoNS enhance their role in biofilm formation, hence significantly contributing to their pathogenic potential (Otto, 2004). Haemolysin, on the other hand, one of the major virulence factors expressed was prevalent among both the in-patient and control patient group CoNS isolates (94% and 88% respectively). Although the positive expression of haemolysin was also observed among all other CoNS, it was notably higher among in-patient species-specific CoNS isolates. These findings were not surprising as haemolysin expressed by pathogenic strains of *S. haemolyticus* is associated with host erythrocytes lysis releasing free iron, the uptake of which promotes growth and subsequent spread of the organism during course of infection when host immunity is compromised (Hellbacher *et al.,* 2006). Hence, the potential pathogenicity of CoNS associated with expression of disease-conferring determinants in immunocompromised patients described by other researchers, further corroborate the findings of this study (Cunha *et al.,* 2006; de Lourdes *et al.,* 2006).

Further evidence indicates, that of the few virulence factors expressed by CoNS contributing to pathogenicity of infection, slime production, is the indicator that tends to be predominantly associated with antimicrobial resistance (Baker *et al.,*2018). The data obtained in this study subjected to PCA analysis to facilitate determination of association between virulence expression and resistance pattern characteristics of both patient group isolates revealed a distinct lack of association between these two variants which are concomitant with potential pathogenesis. The scatter plots generated by PCA indicate that CoNS species-specific isolates from both patient groups exhibiting resistance to the 18 antimicrobials indicate that this variable factor did not influence the expression of either lipase, protease, haemolysin or slime. Additionally, the three-way PCA plot analysis and the generated scatter plots, revealed no evidence of sample grouping and separation based on patterns in the highest significant detected features despite the observed predominance of methicillin-resistant, slime-positive *S. epidermidis* and *S. haemolyticus* isolates, in both patient groups. Other researchers have however demonstrated that slime-positive *S. epidermidis* were significantly associated with MR and other widely used antimicrobials (Koksal *et al.,* 2007). This may be to do with the tiggering of adaptive responses of slime-producing infective strains for both survival and spread, dependent on the physiological condition of the immunocompromised host. Similar assessment of association between slime-positive expression and MDR resulted in both a significant portion of in-patient *S. epidermidis* and *S. haemolyticus* isolates demonstrating MDR correlation with slime production ($p < 0.0001$), a response likely to be

determined in the presence of antimicrobial pressure. Conversely, no such correlation was observed between slime production and expression of MDR among the skin-colonising isolates of the control patient group (p = 0.222). However, studies have provided strong evidence that slime-positive MDR-CoNS skin flora isolates, serving as reservoirs of antimicrobial resistance, colonise hospital environments and are therefore the major determinants of nosocomial acquired infections (Koksal *et al.,*2009).

Resistance to multiple therapeutic agents commonly used for the treatment of CoNS-associated bacteraemia limits clinical efficacy, therefore the use of antimicrobial combination are recommended for eradication of infection and optimal outcomes particularly among the immunocompromised patient group (Islas-Muñoz *et al*., 2018). Effective combination therapy is, however, dependent on evaluation and provision of accurate assessment of synergy between antibiotic interactions as a guide to targeted patient therapy (Raad *et al*., 2007). Consequently, evaluating the efficacy of drug combinations is another clinically relevant aspect associated with predicting *in-vivo* resistance of recalcitrant infections.

Although numerous *in-vitro* methods have been described for gauging synergy between antimicrobial combinations, there are currently no standardised simple to perform methods available routinely in clinical laboratories for synergy detection. For the purposes of this study, the E-test agar diffusion method for synergy testing was used to assess *in-vitro* antimicrobial interaction and activity of. four antimicrobial combinations commonly for the treatment of CoNS-associated bacteraemia. The E-test agar diffusion method for synergy testing (White *et al*., 1996; Doern, 2014). Investigations therefore carried out in order to ascertain the efficiency of vancomycin, daptomycin, gentamicin and rifampicin individually and in binary combinations against three representative in-patient *S. epidermidis* isolates expressed a range of antimicrobial susceptibilities All the three in-patient *S. epidermidis* isolates A37, A57 and the MDR isolate A91 exhibited susceptibility to vancomycin daptomycin and rifampicin. Susceptibility to gentamicin was also expressed by isolates A37 and A57, well within the susceptibility range of ≤1mg/L whereas isolate A91 expressed high level resistance at MIC >32mg/L. Although gentamicin demonstrates a concentration-dependent killing effect *in-vivo*, it is likely to induce nephrotoxicity in patients exposed to drug concentrations above a certain MIC threshold (BMJ-Best Practice, 2017). However, gentamicin is often used in combination with other agents such as vancomycin for broad-spectrum cover for GP- and GN-associated bacteraemia results in better clinical outcomes with few adverse effects (Cohen and Drage, 2011). The efficacy of gentamicin-vancomycin combination was evident in this study with antibiotic synergy demonstrated against the MDR isolate A91 expressing a fourfold decrease in MIC. Although there is no defined relationship

between gentamicin and vancomycin expressed individual and combination susceptibilities, it is likely that synergism occurrence is due to the differing modes of actions against staphylococcal isolates. Hence combined action of the two antibiotics involving different cellular targets, results in synergistic activity as vancomycin inhibiting cell wall synthesis, and gentamicin targeting bacterial ribosomes inhibits growth thus leading to cell death (Rehm *et al*., 2008).

Comparatively, all three S .epidermidis isolates A37, A57 and A91 vancomycin combination with both rifampicin and daptomycin demonstrated an additive effect. These results are concomitant with previous reports of enhanced activity of rifampicin in combination with vancomycin due to its potent bactericidal activity and ability to penetrate cells more effectively is associated with improved clinical outcomes (Rose *et al*., 2009). Concomitant overall additive effects of such combination interactions noted in this study vancomycin-daptomycin and rifampicin would prove to be advantageous in clinical practice as rifampicin is rarely used as an effective mono-therapeutic agent due to resistance development in early stages of infection (Zheng and Stewart, 2002). Synergy testing for all six drug combinations assessed either resulted in an indifference or additive effect with no defined antagonism noted. The ease of use for the *in-vitro* E-test method for testing of combination antimicrobial susceptibilities offers the possibility of synergy testing in routine laboratories for determination of drug combination efficacy. However, further *in-vivo* studies are potentially required to validate the *in-vitro* findings of this study to include patients physiological factors such as immune status and drug-drug interactions encountered during the course of infection which are critical for accurate predication of combination therapeutic outcomes (Doern, 2014).

There are, however, several other limiting factors that influence antimicrobial efficacy which include the interaction between different antimicrobial class combinations, concentration levels, species-specificity and essentially the presence of biofilms. Biofilm-embedded isolates in comparison to planktonic cells consist of phenotypes that exhibit reduced susceptibility to most antimicrobials resulting in persistent infections despite adequate therapy (Kaplan *et al*., 2011; He *et al*., 2017). Several published studies however, report that the antimicrobials limited efficacy is attributable to several exogenous factors produced by infective agents, characterising traits such as tolerance, poor tissue penetration and resistance (Mah and O'toole 2001; Otto, 2004, 2008, 2009; Sakoulas *et al.,* 2004; Casey *et al.,* 2007). It therefore becomes imperative to determine the response of biofilm producing species-specific CoNS to commonly used therapeutics. One of the objectives of the current study was to investigate and compare the effect of three common anti-staphylococcal

agents, individually and combinations utilised for the treatment of potentially biofilm-producing CoNS-associated bacteraemia in the haematology patient group.

Biofilm assays were therefore used to determine the concentration dependent killing effect of the commonly used agents vancomycin, and rifampicin including the newer agent, daptomycin individually and in combinations against *S. epidermidis* RP62A, a well characterised reference strain with a high capacity to form biofilm. Differences between the planktonic MIC and MBC values obtained using the standard microdilution assay, did not however relate to the values obtained with the biofilm plate assay on primary evaluation. Nonetheless, comparative MBC values demonstrate rapid kill of planktonic state *S. epidermidis* RP62A at the lowest concentration of 0.5mg/L for rifampicin in comparison to vancomycin and daptomycin (2 and 8mg/L respectively). Conversely, biofilm culture of *S. epidermidis* RP62A were found to be much more resistant to the inhibitory effects of vancomycin, daptomycin and rifampicin determined both spectrophotometrically at OD_{570nm} and enumeration of viable cells reported as log reduction. Study results revealed no significant bactericidal effect on the *S. epidermidis* biofilm-embedded cells after 24 hour exposure to a range of antimicrobial concentrations. Data analysis indicates that defined MBCs determined for the concentration to show a discernible viable cell reduction in biofilm was not achieved at the upper limit of 64mg/L determined both by calculated log reduction of viable biofilm-embedded cell counts and spectrophotometrically for all for all three test antibiotics (Figures 5-8 and 5-9 respectively). These results are concordant with findings of other studies which describe no killing effect on biofilm embedded bacterial cells at antimicrobial concentrations of $>10^4$ times the MIC (de Allori *et al*., 2006, Høiby *et al*., 2010, 2014; Dall *et al*., 2018).

Furthermore, relative to the effect on cell density, increasing concentrations of rifampicin and daptomycin also appeared to have little effect with regrowth levels noted equivalent to untreated biofilm cell densities, post-neutralisation and incubation (Figure 5-9, 2b and 3c respectively). Overall data obtained for antibiotic challenged *S. epidermidis* biofilms, subjected to statistical analysis using Kruskal-Wallis sum test revealed no significant differences in concentration-dependent cell density reduction for all three antibiotics individually ($p = 0.7$). These finding are concomitant with other reports indicating that biofilm-embedded isolates are inherently resistant to antimicrobial agent concentrations of more than 1000 times higher than their planktonic state MICs (Mah and O'toole, 2001). Although study results confirm the increased resistance of antimicrobials against *S. epidermidis* embedded in biofilm, rifampicin, individually was the most active agent against the planktonic form of *S. epidermidis* RP62, but as a constituent of antimicrobial combinations was distinctly less active against the biofilm embedded cells. However, these finding do not correlate with

studies showing the superior bactericidal activity of rifampicin singly and in combination with vancomycin against *S. epidermidis* associated biofilms (Saginur *et al*., 2006). Other, studies also demonstrate the superior biofilm-associated activity of rifampicin, particularly in combination with other antimicrobials, as it is shown to retain its potency in the presence of slime (John and Harvin, 2007).

Vancomycin due to its potent bactericidal activity is commonly used for the eradication of staphylococci associated with recalcitrant infection. Surprising, in this study vancomycin demonstrated a significant lack of bactericidal activity against biofilm-embedded *S. epidermidis* cells, as biofilm production appeared to be elevated in the presence of higher antimicrobial concentrations of 32-64mg/L ($p = 0.7271$). Additionally, increase in biofilm cell density post-antibiotic neutralisation and re-incubation suggests that there is a likelihood of either reduced penetration of vancomycin through the biofilm, tolerance or most sub-populations within the deeper layers of the biofilm may have persisted in a dormant state refractory to antimicrobial inhibition (Figure 5-9,1b). The lack of penetration through the biofilm layer may also relate to the large molecular weight of vancomycin molecules (molecular weight of 1450) which could become entrapped in the extracellular mucopolysaccharide slime layer thus reducing effective penetration (Souli and Giamarellou, 1998). Similar to these findings the lack of response or tolerance to vancomycin in *S. epidermidis* biofilm associated infection have been previously reported. Investigators have reported diminished antimicrobial response against biofilm-embedded *S. epidermidis* strains despite high concentration achieved in the biofilm environment are linked to microbial growth suppression and inadequate kill-activity (Claessens *et al*., 2015; Dall *et al*., 2018). Additionally, physio-chemical interactions with slime composites may also decreases permeability of some antibiotics through the biofilm (Jefferson *et al*., 2005.). Diminished responses to vancomycin, due to factors other than penetration, associated with slime production, include alteration of charge and free energy on bacterial surface and antimicrobial-tolerant sessile sub-populations found deeply embedded in the biofilm (Raad *et al*., 2007; Otto, 2009; Cerca *et al*., 2014).

The addition of rifampicin is however reported to enhance the activity of vancomycin against staphylococcal biofilms because of its ability to penetrate cells and activity against stationary-phase cells (Rose and Poppens, 2009; Saginur *et al*., 2006). However, vancomycin-rifampicin combinations recommendations are limited related to adverse effects due to drug interaction and possibility of rapid developing resistance in patient groups with underlying comorbidities. Despite limited data availability, daptomycin-vancomycin/rifampicin combinations have been shown to exhibit better *in-vitro* anti-biofilm activity in comparison to other regimens (LaPlante and Mermel, 2007). Unlike the finding of this study, comparing

combinations of vancomycin-daptomycin rifampicin and rifampicin-daptomycin did not show any significant difference in anti-biofilm efficacy ($p = 0.9$). Conversely, vancomycin-daptomycin revealed a significant difference in activity compared to the other evaluated combinations ($p = 0.0092$). Additionally, with no significant reduction in biofilm cell density after antimicrobial exposure the overall response of the combinations demonstrated antagonism based on the calculated cumulative mean ΣFIC index values of ≥0.4, for synergy assessment. Further result analysis however, revealed vancomycin, rifampicin and daptomycin demonstrated significantly greater efficacy individually in comparison to the effectiveness in combinations against biofilm-embedded *S. epidermidis* cells ($p < 0.00001$). Likewise, other investigations have demonstrated that daptomycin individually and in combination against biofilm isolates had better activity compared to vancomycin and rifampicin but did not significantly improve activity when combined with rifampicin and vancomycin (LaPlante and Woodmansee, 2009). Conversely, other studies also report the superior *in-vitro* susceptibility response of daptomycin-rifampicin against *S. epidermidis* biofilm cells whereby approximately 3-4 $\log_{10}$ CFU/mL reduction post-antimicrobial combination exposure was observed (Leite *et al*., 2011). These difference in reported anti-biofilm activities may have been due to the methods used to evaluate antimicrobial efficacies against reference or clinical biofilm-producing isolates.

In accordance with previous investigative techniques, the quantitative microtitre plate assay eliminates subjectivity in reading of results and provides a greater predictive clinical relevance. Hence, the model experiment of OD-measurements applied in this study include taking into account test adherent bacteria both, on the sides of the walls and the bottom of the microtitre plate wells thus increasing the accuracy of evaluated reduction in biofilm cell density due to antimicrobial activity (Stepanović *et al*., 2000). Therefore, overall results analysis demonstrating significantly reduced *in-vitro* activity of vancomycin, rifampicin and daptomycin individually and in combination against the reference biofilm-producing *S. epidermidis* strain, suggests that these antibiotics are not likely to demonstrate reduced antibactericidal activity against biofilm-associated infections *in-vivo*. Moreover, study findings also demonstrated that a significant 99.9% reduction in biofilm cell densities at the highest antimicrobial test concentrations of 64mg/L was unachievable, which suggests that higher concentration would be required to acquire complete eradication of biofilm-embedded cells. Therefore, further test assays would be required to include a series of much higher doubling concentrations of 128, 256 and 512mg/L using similar viable count methodology pre and post antibiotic exposure to assess significant reduction in biofilm cell densities and hence antimicrobial efficacy. Clinically**,** the requirement for much higher antimicrobial concentrations for eradication of biofilm-embedded cells implies serious limitation for

therapeutic application. This is because such high serum dose-levels of >64mg/L are not achievable *in-vivo* as increasing clinical toxicity linked to higher dosing would adversely affect patient management.

The present study provides good evidence that the presence of biofilm appears to have a negative effect on vancomycin, daptomycin and rifampicin activity. These antibiotics may have some activity against the biofilm-embedded staphylococcal cells but none on overall biofilm cell density reduction as significant eradication at antimicrobial concentrations well above the expected MICs. As both biofilm formation and density are known to influence the efficacy of antimicrobials used against biofilm-mediated infections, it would be of interest to study the *in-vitro* response of the biofilm-producing strains of both patient group species-specific CoNS isolates. The bioassay model employed in this study represents a valuable method for assessing antimicrobial efficacy on staphylococcal biofilms, given the frequency of biofilm-associated infection related to bacteraemia in haematology patients. The evaluation carried out with the reference staphylococcal strain and representative antibiotics however requires further studies involving the previously characterised slime-positive in-patient CoNS isolates. Investigative studies including evaluation of species-specific CoNS isolates associated with the production of high density biofilms using previously described methods for quantification of biofilm mass and correlation with rate of antibiotic resistance would contribute significantly towards understanding the mechanisms governing biofilm-associated resistance (Smith *et al*., 2008). Furthermore, evaluation of anti-biofilm synergy interaction would be useful in supporting clinical therapeutic decision involving chronic staphylococcal biofilm-mediated infections and more importantly avoid antimicrobial resistance development

5.7 Conclusion

Virulent skin colonising isolates of CoNS have become the one of the most important pathogens in clinical infections among patients with haematological malignancies, a group compromising highly immunocompromised patients with colonised medical devices as the common source of BSIs. Among the species-specific CoNS isolates slime, the main virulent factor involved in pathogenesis was prevalent among isolates of *S. epidermidis* in both patient groups. The findings in this study therefore support the potential role of skin-colonising *S. epidermidis* slime-producing isolates and their possible role as inducers of CoNS-associated bacteraemia.

The E-test method employed in this study to assess the *in-vitro* efficacies of vancomycin, gentamicin, daptomycin and rifampicin combinations against three representative BSI isolates of *S. epidermidis* demonstrated additive or indifferent effects overall. Synergy was however, only confirmed with combination of vancomycin-gentamicin against the MDR-*S. epidermidis* test isolate Most importantly no *in-vitro* antagonism of vancomycin and the other binary agent combination was confirmed in this study. Conversely vancomycin, daptomycin and rifampicin, individually and in combinations demonstrated antagonistic interactions against the biofilm-embedded cells of *S. epidermidis* RP62A. Hence, these antimicrobials individually and in combination, although demonstrating susceptibility against isolates in the planktonic mode could not be considered of clinical relevance; for the treatment of CoNS-associated bacteraemia in haematology patients who are likely to have CVC-lines colonised with biofilm producing isolates in-situ.

Chapter 6 Final Discussion

This retrospective study aimed to evaluate the incidence of antimicrobial resistance among CoNS-associated BSI isolates from patients in the haematology and BMT unit of the Queen Elizabeth Hospital, Birmingham UK between April 2010 and March 2012. The recovered CoNS (in-patient) isolates were characterised by both microbiological and molecular methods, evaluating both CoNS species-specific isolate prevalence and their antimicrobial resistance profiles. These were compared to the pre-admission patient (control group) CoNS skin-colonising isolate characteristics, proposed to be the potential source of BSIs, for determination of both phenotypic and genotypic clonal similarity. The primary purpose of this study was to ascertain whether the incidence of bacteraemia was attributable to the species-specific isolates of CoNS comprising normal skin flora.

BSIs due to CoNS are the most common cause of nosocomial infection associated with high rates of morbidity in patients with haematological disorders (Worth and Salvin, 2009). These opportunistic infections are usually associated with MDR-CoNS isolates possessing determinants facilitating their survival on skin and medical devices. Therefore, establishing the source of these potentially pathogenic strains is critical in prevention and management strategies (Coello *et al*., 2003). For treatment purposes, the use of bactericidal antimicrobial agents are essential clinically, for the rapid eradication of severe life-threatening BSIs for optimal outcomes in haematology patients (LaPlante *et al*., 2007; Kratzer *et al*., 2007). As such, all febrile patients on the unit, displaying clinical signs and symptoms of infection receive broad-spectrum empirical antimicrobial therapy prior to receiving results of positive blood cultures from the microbiology laboratory. Vancomycin remains the most widely used first line therapeutic agent in combination with other antibiotics for the treatment of serious infections in the haematology patient group (Garcia-Vidal *et al*., 2018). However, vancomycin has several limitations, including its slower bactericidal activity and associated toxicity requiring close monitoring of serum levels and renal function (Kitzis and Goldstein 2004). Moreover, most worrying are reports of emerging resistance due its extensive use and association with suboptimal treatment outcomes in patients with haematological malignancies undergoing BMT (D'Antonio *et al*., 2004; Safdar and Rolston, 2006). Therefore, in addition to the evaluation of resistance to commonly used anti-staphylococcal agents, this study also aimed to investigate the prevalence of reduced susceptibility to the glycopeptides, in particular to vancomycin, related to CoNS-associated bacteraemia, which remains a frequent complication in patients with haematological malignancies. Furthermore,

the efficacy of newer alternative antimicrobials including daptomycin, tigecycline and ceftaroline were also evaluated against these BSI CoNS isolates.

Despite medical and therapeutic advances, nosocomial infections with potentially pathogenic CoNS pose a major threat to patients admitted to hospital with haematological malignancies (Diekema *et al.,* 2001; Wisplinghoff *et al.,* 2003). Because of the presumed assumption that this group of isolates are of low virulence, clinical debates continue regarding the clinical or therapeutic value of speciating BSI-associated CoNS isolates. Nevertheless, evidence from studies have emerged suggesting that the source and the proceeding consequence of infection may differ considerably dependent on species-specific strains of infective CoNS (Piette and Verschraegen, 2009; Chen *et al.,* 2010). Hence, with the increasing incidence of CoNS-associated bacteraemia, speciation may become a necessity because of the link between species-specific strains and serious infection syndromes (von Eiff *et al*., 2002; Ahlstrand *et al*., 2012). In such cases, isolates of *S. epidermidis* have been described to be most frequent aetiological agent associated with nosocomial BSIs (Feld, 2008). Furthermore, CoNS species-specific differentiation provides valuable information relating to antimicrobial resistance pattern associated with expression of virulence factors for clinical correlation targeting therapeutic interventions for optimal outcomes (Heikens *et al.,* 2005; Otto, 2010). Hence, a strong case exists for the identification of potential pathogenic strains of CoNS to the species level that are isolated from normally clinically sterile sites such as the bloodstream, thus supporting their role as aetiological agents of infection.

In the clinical setting, several different simple phenotyping systems such as biotyping and antimicrobial susceptibility profiling are used to identify identical strains of CoNS continuously isolated from the same patient in an attempt to distinguish potentially pathogenic strains. Bio-analysis and antibiogram profiling of these isolates to the sub-species level has therefore become increasingly important in order to ascertain their role not only in colonisation and dissemination but also determine the source of infection in order to prevent nosocomial cross-transmission and infection relapse. Furthermore, isolate identification to the genus/sub-species level is essential for interpretation of antimicrobial resistance patterns which are predictive of therapeutic outcomes (Stuart *et al*., 2011). Consequently, the study findings using the commercial automated VITEK®2 system utilising computer software analysis of unique biochemical test profiles for species differentiation identified *S. epidermidis* as the predominant species among both the in-patient and control patient group isolates (57 and 32% respectively). These results were further corroborated with use of the

advanced proteomic based MS-MALDI TOF application. Proteomic profiling by MS, because of its greater sensitivity and specificity in detecting the spectrum of proteins expressed by organisms, facilitates highly accurate species-level identification (Clark *et al*., 2013). Comparative speciation result analysis evidenced a statistically significant difference in the performance VITEK®2 and the MS^{MS} MALDI-TOF system for isolate identification, predominantly among species-species CoNS isolates from the control patient group ($p = 0.0058$). Nonetheless, although accuracy level of species-specific identification was greater with MS^{MS} MALDI-TOF, the overall species distribution frequency among both patient groups was not affected.

As a prevalent member of the skin flora *S. epidermidis* is also the most frequently encountered aetiological agent associated with device-related BSIs in critically ill patients (Huebener and Goldmann 1999; Marin *et al.,* 2014). The distribution frequency of *S. epidermidis*, recovered from both bacteraemic patient and the control group skin-colonising isolates in this study was in concordance with results of other studies describing CoNS species-specific frequency and distribution. The rising incidence of device-related CoNS-associated bacteraemia has been attributed to *S. epidermidis* the predominant skin flora isolate as well as the principal isolate colonising long-term indwelling medical devices (Diekema *et al*., 2001; Longauerova, 2006; Koksal *et al*., 2009). Similarly, *S. hominis* and *S. haemolyticus* were the second and third most abundant species isolated from both patient groups. These findings are concomitant with the make-up of the human skin microbiome, as both *S. epidermidis* and *S. hominis* are the most abundant species found on the skin and mucosa membranes accounting for 46% and 22% of the total CoNS species recovered from individuals (Kloos *et al*., 1994; Costa *et al*., 2006; Cogen *et al*., 2008). Furthermore, other research studies cite the predominance of *S. haemolyticus* as the second most common isolate of BSIs (D'Mello *et al.,* 1999; Kristóf *et al*., 2011). In clinical settings, this unique specificity is associated with critically ill patients having a greater number of invasive therapeutic interventions thus putting them at a higher risk of colonisation with *S. haemolyticus* strains (Fluit *et al*., 2001; Longauerova, 2006). This study's results however, revealed *S. haemolyticus* as the third most prevalent of the CoNS species, accounting for 16 and 17% of the in-patient and control group isolates respectively. These results, nonetheless, harmonise with those of another study, where *S. haemolyticus* accounted for 5-38% of all CoNS clinical isolates investigated (Kristóf *et al*., 2011). The overall existence of *S. epidermidis*, *S. hominis* and *S. haemolyticus,* representative of the three predominant CoNS species in both patient groups, is also consistent with findings of similar studies reported elsewhere (Raslinksi *et al.,* 2018).

The presence of predominant CoNS species-specific isolates in the healthcare facility and hospitalised patients colonised with acquired microflora may be representative of clonal cross-transmission and hence the source of nosocomial infections (Ziebuhr *et al*., 2006). Colonisation with potentially pathogenic CoNS species is common amongst high-risk patient groups with underlying comorbidities, where these isolates survive on medical devices and in areas of skin surrounding insertion sites. Therefore, the source of infection may be endogenous or exogenous, derived either from the hospital environment of from healthcare staff. Interestingly, environmental sample analysis of highly accessed points by all healthcare personnel around the haematology and BMT wards revealed a difference in bacterial loads and prevalence of species-specific CoNS isolates dependent on contact areas. The frequency and distribution of dominant normal skin-colonising strains comprising of CoNS and other bacterial species such as *Micrococcus*, *Corynebacterium, Bacillus* and streptococci species also differed dependent on sampling period. As such, overall distribution frequency of the eight predominant CoNS species recovered from sampling the high-access contact points in the ward, undertaken during March 2011 revealed *S. epidermidis* as the most frequently isolated species whereas in May 2011, *S. haemolyticus* predominated at a greater frequency across both ward areas. Although all the CoNS species-specific isolates were confirmed as those comprising normal human skin microbiota their predominance and frequency appeared to be transient in the ward environment. Analysis and comparison of biotypic profiles of environmental species-specific CoNS with those of both the in-patient and control group isolates revealed distinctly diverse phenotypes. These findings therefore suggest that the transient presence of different predominant species-specific CoNS isolates in the ward areas are highly unlikely to be associated with dissemination and cause of bacteraemia in the haematology patient group.

From the perspective of phenotypic identification with limited attention given to speciation in general, may have served as a barrier in understanding the epidemiology of CoNS-related bacteraemia in the haematology patient group. Distinguishing potential pathogenic strains of CoNS as aetiological agents from contaminating skin flora however remains challenging, not only due to their extensive presence on the human body but also the spectrum of disease associated with species-specific strains (Otto, 2010). On the other hand, phenotypically, expressed traits can be exploited for the purpose of elucidating the source and transmission of nosocomial infections due to CoNS species-specific isolates. Moreover, CoNS species-specificity has the potential for clinical correlation and together with appropriate therapeutic intervention, a necessity for patient survival (Ahlstrand *et al*., 2012). Hence, both conventional methodologies and molecular techniques with increased sensitivity and

specificity were utilised in this study to determine the phenotypic and genotypic differences or similarities within and between in-patient blood culture isolates of CoNS and those skin colonisers which could be regarded as inducers of bacteraemia.

For the purpose of species identification and demonstration of species-specific differences, biotyping based on biochemical profile analysis using the VITEK®2 system for CoNS isolate delineation was found have a relatively low discriminatory capacity. Nonetheless, biotyping when combined with PCA and cluster analysis increased the discriminatory power confirming overall study findings, that CoNS isolates of both patient groups exhibited high levels of dissimilarity and hence diverse phenotypes. Data analysis performed based on similarity or differences of CoNS biotype profiles (n=100) and dendrograms created, representative of percentage similarity revealed five main clusters comprising of very few isolates that demonstrated clonal relatedness in each of the patient groups. *S. haemolyticus* isolates of the in-patient group demonstrated the greatest degree of biotype similarity followed by those of *S. epidermidis*. Conversely in the control patient group, the same level of similarity was only apparent in isolates of *S. epidermidis.* In both patient groups, however, the number of isolates within each cluster demonstrated a discernibly high level of un-relatedness within and between isolates of the same CoNS species. Conversely, for comparative purposes, antibiogram profiles based on evaluating the prevalence of resistance to the 18 commonly used anti-staphylococcal agents using the VITEK®2 system were further subjected to PCA and cluster analysis for species-specific isolate delineation. Among both patient groups the phenotypic antibiogram profiles of CoNS isolates revealed a distinct of lack of correlation between the antibiograms and isolates of the similar CoNS species

As independent factors that are predictive of true bacteraemia rather than contaminants, antimicrobial resistance patterns in association with biotype although lacking in discriminatory power can be useful for differentiating clinically significant CoNS isolates (Raad *et al*., 1998; 2007). Based on this predictive value, comparative data analysis of biotype in association with antimicrobial resistance profiles, revealed limited biotype similarity. Conversely, amongst each of patient group CoNS isolates a distinct lack of correlation was noted between antimicrobial resistance profiles and isolates of similar species-specific CoNS. These study findings therefore confirm that both patient group CoNS isolates exhibiting high levels of phenotypic dissimilarity are highly unlikely to have been derived from common clonal sources.

Strain-specific characteristics can, however, only be distinguished effectively by genotypic methods rather than by phenotypic appearance or by antibiogram (Miragaia *et al*., 2002; Costa *et al*., 2006). Molecular relatedness techniques allow evaluation determination of skin-colonisers playing a significant role as the source of CoNS bacteraemia in patients with haematological malignancies. Strain relatedness using molecular typing tools such as PFGE and rep-PCR utilised in this study to evaluate skin isolates as the potential source of CoNS BSIs showed highly diverse species-specific CoNS isolates present amongst both patient groups. The results of this retrospective single centre study are concomitant with results obtained by Costa and colleagues (2006) where skin site colonisers were considered to be the unlikely source of bacteraemia. The researchers study results were based on prospective evaluation of the colonising skin microflora from CVC exit skin-site samples post-immunosuppressive therapy with patients receiving vancomycin therapy at time of sampling. These factors could be important in influencing not only colonisation sites but also the sequence of colonisation in relation to CoNS BSI pathogenesis. Hence, investigations revealed translocation of genetically similar mucosal CoNS-colonising strains (nasal and rectal) and not skin-colonisers as the cause of bacteraemia. Nonetheless, other authors in their investigations have evidenced skin-colonisers as the potential primary source of CoNS-BSIs primarily in association with foreign-body or catheter-related infections (Livesley *et al*., 1998; Becker *et al*., 2014).

Advances in molecular-related epidemiological studies provide another interesting insight into the flexibility of CoNS skin-colonising strains generating both phenotypic and genotypic variants associated with life-threatening infections in the immunocompromised patient group (Cuna *et al*., 2004). Although phenotypically expressed traits can differentiate some similarities between potentially pathogenic CoNS isolates, genotyping on the other hand, distinguishes clonally related similar species-specific isolates expressing the same characteristics during the course of infection. These definitive genotypic traits are not only predictive of therapeutic outcomes, but potentially ascertain the source of clinical infections (Sloos *et al*., 2000).

Accordingly, comparative analysis of biochemical and antibiogram profiling using standard phenotypic fingerprint typing protocols revealed a high degree of diversity observed within and between all CoNS species-specific isolates recovered from both patient groups. Conversely, techniques used to assess genomic relatedness among CoNS isolates (n=100)

employing rep-PCR technology based on the amplification of non-coding, repetitive sequences, interspersed throughout the bacterial genome revealed diverse PCR patterns among both patient group isolates. rep-PCR analysis of both patient group CoNS species-specific isolates identified a few distinct clones of similar species-specific isolates clustering in nine closely related rep-PCR genotypes differing by single DNA fragments. Likewise, fifty of the 200(25%) predominant CoNS species-specific isolates randomly selected equally from both patient groups subjected to PFGE analysis, revealed two distinct clones of *S. epidermidis* in the in-patient group. The other isolates discriminated into diverse macrorestriction patterns, characteristics which reveal high level genotypic diversity between and among both patient group isolates. Conversely, rep-PCR data analysis identified indistinguishable species-specific CoNS in 24% and 10% in-patient and control patient group isolates respectively, of which *S. epidermidis* predominated followed by *S. haemolyticus*. Direct comparisons of genotypes was not possible as species-specific isolate relatedness through rep-PCR was defined by assessing amplification of non-coding DNA repetitive sequences, whereas with PFGE this occurs thorough the digestion of the entire genome and resolution of macrorestriction fragments for strain delineation. Nonetheless these two highly discriminatory molecular techniques utilised in this study demonstrated the presence of diverse species-specific genotypes in both patient groups. These findings reflect observations made in previous studies demonstrating the heterogenic nature of CoNS species concomitant with infections of immunocompromised patient groups (Cuna *et al*., 2004; Casey *et al*., 2005).

Furthermore, comparative data analysis of the 50 predominant CoNS species-specific isolates randomly selected equally from both patient groups, investigated for phenotypic and genotypic characteristic comparisons subjected to PCA analysis, revealed no major groups or clusters. The predominant species-specific CoNS isolates analysed demonstrated a significant lack of both genotypic and phenotypic relatedness between and among isolates of the in-patient and control patient group. However, result analysis identified 2(4%) indistinguishable *S. epidermidis* in-patient genotypes and 2(4%) *S. hominis* isolates from each of the patient groups demonstrating a high degree of genetic similarity (Chapter 3 Figures 3-12 and 3-14). Likewise, although some phenotypic clusters were identified by biotypes (Chapter 2 Figures 2-2 and 2-3), no major clusters were distinguished among and between the two patient group CoNS isolates based on antibiogram typing (Chapter 4 Figures 4-2 and 4-3). This indicates high levels of heterogenicity among both the bacteraemic patient and control group skin-colonising CoNS, comprising of few isolates that were indistinguishable or closely related. Due to the retrospective nature of this study, it was

not possible to ascertain whether the two clonally indistinguishable *S. epidermidis* isolates identified by PFGE analysis were recovered from the same patient, presenting multiple episodes of bacteraemia or were isolated from different patients, acquired through nosocomial transmission. Nonetheless, overall results for phenotypic isolate differentiation and genotypic characterisation based on single restriction fragment differences revealed no dominating pulsotypes but diverse heterogenicity between and among isolates of the same CoNS species.

The lack of genotypic similarities or relatedness suggest that as the skin-colonising isolates were no more homogenous than the in-patient BSI-associated CoNS species-specific isolates and are therefore unlikely to be the possible source of bacteraemia as postulated. Similarly, other epidemiological studies using molecular techniques have provided an interesting insight into the flexibility of skin-colonising CoNS isolates generating novel phenotypic and genotypic variant associated with opportunistic nosocomial infection (Cuna *et al*., 2004; Costa *et al*., 2004). The phenotypic and genotypic diversity noted among and between both the in-patient and control patient group CoNS species-specific isolates therefore underscores the fact that the hospital environment, antimicrobial pressure and the nature of the patients disease state may be factors that play a crucial role in promoting the adaptive nature of CoNS isolates and hence their importance as potential pathogens.

Interestingly, the phenotypic antibiogram profiles of both patient group CoNS isolates revealed distinct clonal dissimilarity, with the majority of isolates demonstrating a wide spectrum of antimicrobial resistance to commonly used anti-staphylococcal agents. Administrating antibiotics disrupts the normal skin microbiota which is made up of a complex community of colonising microorganisms with the capability of acquiring resistance, hence acting as reservoirs of resistance genes and therefore the potential for pathogenicity. Hospitalised patients often acquire healthcare-associated colonising strains which partially and progressively replace normal microbiota, dependent on factors such as type of infection underlying co-morbidities, antibiotic pressure and prevailing epidemiology (Agvald-Öhman *et al*., 2003 (Cogen *et al*., 2008). Similarly, of the 18 antimicrobial agents assayed, varied resistance responses were produced among both patient group CoNS isolates in a species-specific dependent manner, with the majority demonstrating resistance to penicillin and others to more than three antimicrobial classes. Understanding the spectrum of commonly used agents against CoNS species-specific isolates is essential for appropriate diagnosis and provision of targeted therapeutics guidance for optimal clinical outcomes. Clinically

significant bacteraemia associated with MDR-CoNS isolates possess determinants that facilitate their survival on skin and device surfaces particularly in patients with underlying immunocompromised states (Coello *et al*., 2003). In the clinical setting prevention and management strategies are critically dependent on ascertaining the source of recalcitrant infections associated with such potentially pathogenic isolates (Costa *et al*., 2004; 2006).

An important characteristic of CoNS isolates is their multi-drug resistance to the antimicrobials commonly used for the treatment of staphylococcal infections. The presence of MDR-CoNS isolates have been shown to present the most critical of compromises to favourable clinical outcomes in immunocompromised patients (Marin *et al*., 2014). In agreement with previously published studies, the primary finding of this study indicated that of the species-specific CoNS isolated from bacteraemic patients, *S. epidermidis* and *S. haemolyticus* (88% and 94% respectively) indeed demonstrated significantly higher levels of MDR associated with high levels of methicillin resistance in comparison to other species-specific isolates. Conversely, species-specific MDR was most restricted among the minor CoNS species-specific isolates in both patient groups. In total 78% of the in-patient isolates demonstrated significant levels of methicillin resistance ($p = 0.0001$), results which are concomitant with several other studies undertaken across Europe and American countries, citing MR rates of 70-80% (Collin *et al*., 2001; Collin *et al*., 2001; Vincent *et al*., 2006). Other study results also concurred with observed rates of MR expressed by skin-colonising isolates (28%) which although lower than those exhibited by blood culture isolates are thought to contribute significantly to nosocomial acquisition (Yamada *et al*., 2017). Therefore, the emergence of MR and MDR-CoNS isolates are an important healthcare problem and a particular concern among patients with malignancies, in whom, BSIs are the most common manifestation because of severe immunosuppression. The risks are further compounded by a number of complex factors which include length of hospitalisation and selective antimicrobial pressure promoting emergence of resistance, predisposing the patient to colonisation with resistant strains (Montassier *et al*., 2013). Reports relating to the incidence of antimicrobial resistance in nosocomial-acquired CoNS are rising however, their association with clinical outcomes especially among immunocompromised patients remain limited (Natoli *et al*., 2009).

Vancomycin however remains the standard of care for these problematic potential pathogenic strains in haematology patients with increasing reported case of suboptimal treatments resulting in clinical failures (D'Antonio *et al*., 2004). The most worrisome issue

with prolonged vancomycin treatment has been the emergence of associated reduced susceptibility to the glycopeptides in CoNS isolates. whereby the precursors of glycopeptide intermediate and/or sub-populations of resistant strains display MICs in excess of 2mg/L. *In-vivo* this would relate to achieving serum drug concentrations of >30mg/L for efficacy. However, maintaining such high serum levels would compound toxicity leading to worse clinical outcomes (Cremniter *et al*., 2010). Furthermore, there is increasing evidence of a significant relationship between elevated vancomycin MICs and treatment failures associated with the emergence and spread of nosocomial MR-staphylococci (Falcone *et al*., 2015).

Associated therapeutic failures are thought to be multifactorial in the immunocompromised patient group including, insufficient drug serum levels achievement, attributable either to poor pharmacokinetic properties of the antimicrobial or heteroresistance expression. Moreover, high protein binding properties of the glycopeptides in particular, teicoplanin leads to reduction of drug optimisation associated with the concentration-related inhibitory properties of the drug *in-vivo* (Howden *et al*., 2004; 2010). The application of techniques to investigate the presence of reduced susceptibility due to the presence of heteroresistant strains remains confined to the research field at present. However, the proposed simple screening techniques although not currently in-use as a diagnostic tool in clinical microbiology despite their limitation can be utilised to determine the potential presence of low-level resistant strains in patients on long-term vancomycin therapy exhibiting signs of clinical failure The results of this study demonstrate that the prevalent MDR-CoNS BSI phenotypes although exhibiting *in-vitro* susceptibility to vancomycin may not show clinical success as a fair proportion demonstrated resistance to the glycopeptide, teicoplanin. *In-vitro* studies to monitor emerging resistance requires observing the presence of high levels of teicoplanin resistance proposed to be a precursor stage to developing resistance to vancomycin. Due to the similarity of the mode of resistance demonstrated by both glycopeptides, the frequency of teicoplanin resistance can therefore act as a marker for emerging vancomycin resistance i(Hiramatsu, 2001).

Staphylococci responding to sub-therapeutic glycopeptide drug levels are known to subsequently select for both heterogenous and variable resistance which are difficult to detect by conventional AST techniques. The VITEK®2 automated AST system is deemed to be an unreliable platform for the detection of heteroresistance because the incubation period together with the standard inoculum used, is below the required threshold for detection of the slower growing heterogenous subpopulations (Wootton *et al*., 2007; Howden *et al*., 2010).

The accurate assessment of vancomycin susceptibility is therefore crucial with increasing numbers of CoNS isolates exhibiting MDR to commonly used anti-staphylococcal agents and reduced susceptibility to the glycopeptides. Distinguishing heteroresistance phenotypes from isolates demonstrating overall vancomycin susceptibility was problematic because of the lack of both simple screening techniques and criteria for defining heteroresistance in CoNS. However, determination of low-level resistance, a critical factor for the detection of heteroresistance was carried out using by application of the macromethod E-tests and GRD E-tests. Using these simple screening techniques, study results reveal that some in-patient isolates of *S. epidermidis* and *S. haemolyticus* may have the potential to express low-level glycopeptide resistance. Due to the limitations of the techniques although heteroresistance in these isolates was not definitively confirmed; the significantly high levels of teicoplanin resistance expressed by both *S. epidermidis* and *S. haemolyticus* in-patient isolates indicates that these isolates are also highly likely to express heteroresistance.

The increasing prevalence of glycopeptide inefficacy against CoNS-associated bacteraemia correlating with potential therapeutic failures therefore merited the evaluation of newer antimicrobials. Of these, daptomycin demonstrated significantly higher levels of *in-vitro* activity in comparison to tigecycline and ceftaroline, against all the in-patient CoNS species-specific isolates ($p = 0.001$). Similar to the findings of other studies daptomycin demonstrated greater potency against all CoNS isolates expressing vancomycin MICs at the upper breakpoint level of 2mg/L; indicating its greater potential as a suitable alternative therapeutic agent to vancomycin for the treatment of CoNS-associated bacteraemia (Kelesidis, 2011; Moore *et al*., 2011). More importantly, this study highlights the difficulties encountered with provision of accurately evaluated results to the clinician using standard AST protocols for therapeutic guidance. Moreover, these *in-vitro* AST results do not take into account the complex relationship interaction between environmental factors, previous antimicrobial therapies and interaction of potential pathogenic strains of CoNS associated with the immune-depressed status of the host. Optimal patient outcomes are therefore reliant on the use of reliable AST *in-vitro* diagnostic techniques for determination of accurate antimicrobial MICs thus ensuring correct dosage guidance and resistance development limitation. Nonetheless, antimicrobial resistance due to aggressive use of broad-spectrum therapeutics for treatment of bacteraemic infections in the high-risk haematology patient group continues to be an important determinant of clinical outcomes (Chen *et al.,* 2010).

Resistance to multiple classes of antimicrobials is estimated to be expressed by about 60-80% of potentially pathogenic CoNS hence limiting therapeutic options for the treatment of severe infections due to such virulent strains in high-risk patient groups (Ma *et al*., 2011). Despite the use of appropriate antimicrobial therapies, MDR CoNS-associated bacteraemia often results in poor clinical outcomes. Serious difficulties encountered with the excessive use of vancomycin and its reduced efficacy against drug tolerant CoNS species-specific isolates and the lack of effectiveness of other commonly used antimicrobials as mono-therapeutic agents for the treatment of CoNS-associated bacteraemia; the use of two antimicrobials in combination are usually recommended for attaining optimal clinical efficacy. Additionally, combination treatment regimens prevent both relapse and difficulties related to antimicrobial-associated adverse effects (Climo *et al*., 1999; LaPlante and Woodmansee 2009). The selection of combination therapeutics are however deemed to be dependent on the process of effective pharmacokinetic antibiotic delivery at serum concentrations that are pharmacodynamically efficient without causing toxicity for improved clinical outcomes (Ahlstrand *et al*., 2012; Martins *et al*., 2013; Islas-Muñoz *et al*., 2018). Therefore, the aim of employing antibiotic combination therapeutics are not only to produce synergistic bacterial killings *in-vivo*; but also, to decrease the risk of resistance development, by providing a predictable extended antimicrobial spectrum in comparison to that achievable by nanotherapeutics. In clinical practice, vancomycin is frequently used in combination with other antibiotics including the aminoglycosides or cephalosporins for the treatment of serious MR-staphylococcal associated infections. However, no clinical data is yet available to support the definitive use of some combination therapies, in particular to those related to specific factors which include potential association with drug toxicities in the immunocompromised patients (Deresinski, 2009).

This study therefore served to characterise the *in-vitro* effectiveness of combinations of vancomycin, rifampicin, daptomycin and gentamicin against three in-patient *S. epidermidis* isolates representative of susceptible, intermediate and MDR susceptibility profiles. The E-test method employing specified conditions evaluated inhibitory data at a single time end-point of antimicrobial combination interactions for synergy assessment. Evidence of antimicrobial interactions between binary combinations of vancomycin rifampicin, gentamicin and daptomycin against the three representative in-patient *S. epidermidis* strains, predominantly demonstrated either an additive or indifference effect, with the exception of synergy demonstrated by the combination of vancomycin-gentamicin against the MDR-*S. epidermidis* isolate A91. More importantly however, antagonism was not observed between any of the four antibiotic combination interactions against all three *S. epidermidis* test

isolates. In clinical practice, optimal antimicrobial therapy is achieved by administration of vancomycin-gentamicin combinations for infections caused by MR-staphylococci (Dompeling *et al*., 1996; Rehm *et al*., 2008). Although combining vancomycin with gentamicin may increase risk of nephrotoxicity however it has been shown that with additional serum level monitoring and appropriate dosing regimens the risk probability is lowered hence enhancing bactericidal activity (Rybak *et al*., 2009).

The increased bactericidal activity of vancomycin when combined with other antimicrobial agents has been noted to provide an extended spectrum of activity against difficult to treat infections and prevent emergence of drug-resistant subpopulations (LaPlante and Woodmansee, 2009). Although vancomycin-daptomycin and vancomycin-rifampicin combinations did not demonstrate the same high level drug potency as combinations of vancomycin-gentamicin an overall additive effect was noted against all the three BSI *S. epidermidis* was in this study. Similar to vancomycin-gentamicin combination, rifampicin, characterised by its potent bactericidal activity and ability to penetrate tissue cells has been reported to enhance vancomycin activity *in-vivo* (Rose *et al*., 2009). Hence, the increased observed activity of vancomycin-rifampicin combination is in concordance with its use in clinical practice. Overall results obtained in this study demonstrate that combinations of vancomycin-gentamicin or rifampicin and the newer antimicrobial, daptomycin in particular can be considered as effective alternatives for improved clinical outcomes in haematology patients, particularly in cases of clinical failures with commonly used monotherapies.

Biofilm producing staphylococcal isolates also referred to as “slime producers” are known to be responsible for many recalcitrant infections associated with antimicrobial resistance (de Allori *et al*., 2006). The pathogenesis of these isolates in particular, *S. epidermidis* biofilm producers are dependent on their ability to produce adherent exopolymers or slime. These exopolymers are an integral component of biofilm, the most important virulence trait of CoNS species, an adaptive survival response particularly associated with CR-BSIs (Otto, 2009; Claessens *et al*., 2015). Of major concern among patients with haematological malignancies is the extensive colonisation of intravascular devices with such slime producing CoNS which also form part of the normal microbiome associated with the patients’ skin and mucosa. Difference in the immunological status of haematology patients and variation in expressed virulence factors among species-specific isolates determine the outcome of colonisation and disease development (Cervera *et al*., 2009 Ahlstrand *et al.,* 2012). Various phenotypic methods have been proposed for the investigation of biofilm producers in relation to skin-colonising strains particularly for diagnosis of line-associated bacteraemia.

In this study, criteria based on visual analysis of colour changes due to the production of slime using the Congo red agar method identified in-patient isolates of *S. epidermidis* (72%) as the predominant CoNS species associated with slime production. Conversely, in the control patient group the other minor group of CoNS isolates, predominantly *S. warneri* and *S. capitis* demonstrated slime production marginally higher than those *S. epidermidis* isolates (52% and 50% respectively). The findings of this study corroborate those of other studies showing that dominant isolates exhibiting MDR, recovered from hospital patients demonstrate increased virulence in comparison to skin-colonising strains (Arslan and Özkardes, 2007; Baker *et al.,*2018). However, data obtained for the virulence study subjected to statistical analysis revealed that there was no significant difference with respect to the proportion of CoNS species-specific isolates association with slime production and patient groups ($p = 0.230$). Nonetheless, a significant proportion of in-patient *S. epidermidis* isolates were associated with slime production in comparison to those of *S. haemolyticus* ($p = 0.0284$).

Furthermore, overall PCA multivariant data analysis used to explore the relationship between species-specific CoNS, production of four main virulence traits and association with antimicrobial resistance; revealed a distinct lack of correlation between all variant factors, including patient groups and isolates. Study findings also showed that despite the predominance of slime producing *S. epidermidis* isolates in both patient groups, there was little evidence of clonal clustering of MDR-isolates related to the production of the four virulence factors (Chapter 5, Figure 5-5). However, further analysis revealed a difference in the rate of distribution of isolates that demonstrating MDR not associated with slime production, which is a trait normally postulated to be associated with a higher degree of antimicrobial resistance (Arslan and Özkardes, 2007). Hence, data subjected to further statistical analysis using the linear regression model to assess the expression of MDR in relation to species-specific isolates producing slime, revealed a significantly high proportion of in-patient MDR-*S. epidermidis* isolates associated with slime production ($p < 0.0098$). These findings are concomitant with numerous published studies which evidence the predominance of highly resistant, virulent, slime-producing *S. epidermidis* and *S. haemolyticus* isolates associated with causing recalcitrant infections in immunocompromised patients (Arciola *et al*., 2004; Martiínez-Morel *et al.,* 2014; Yamada *et al.,* 2017). In comparison, the colonising CoNS isolates may have an inherent ability to produce slime as the virulence factor, however this response is likely to be expressed in the presence of antimicrobial pressure.

Accumulated data analysis in this study suggests that slime is one of the important factors related to virulence in association with either methicillin or MDR predominantly amongst in-patient group *S. epidermidis* isolates. Both clinical and *in-vitro* research findings support the theory that slime, an integral component of biofilm-producing staphylococci negatively affects treatment outcomes due to expression of increased resistance (Klingenberg *et al.,* 2007). Studies have also shown that such isolates involved in biofilm formation are frequently associated with medical implants and devices serving as a common source of bacteraemia associated with suboptimal clinical outcomes (Casey *et al.,* 2006, 2007; Longauerova, 2006; Martiínez-Morel *et al.,* 2014). The direct relationship between the virulence factors assessed and the source (patient group) did not however emerge in the present investigations. Furthermore, the phenotypic variation in slime production observed within and between CoNS species of the hospitalised patient group may also introduce a degree of complexity associated with pathogenicity. Hence, the potential variations linked with the degree of slime production by different species-specific CoNS isolates associated with virulence related to antimicrobial resistance would require addressing in future studies. Investigations would involve quantification of biofilm mass of slime-positive species-specific isolates of CoNS and correlation with rate of antibiotic resistance which contribute significantly towards biofilm-associated resistance.

Furthermore, colonisation of surfaces through the development of biofilms is known to increase the likelihood of resistance and reduces anti-bactericidal activity of commonly used antimicrobial agents leading to increased rates of treatment failures (LaPlante and Woodmansee 2009). Therapeutics for biofilm-associated infections are usually selected according to conventional susceptibility testing. However, this type of susceptibility data is largely inadequate for therapeutic decisions, as biofilm-embedded isolates display diverse mechanisms of antimicrobial resistance. The interaction with antimicrobial agents and the mechanism of biofilm-mediated resistance is dependent on a number of specific factors that influence detection of susceptibility. These include factors such as hydrophobicity and diffusion rates, dependent on the composite nature of the biofilm that influences antimicrobial efficacy *in-vivo* (Patel, 2005). Therefore, *in-vitro* methods that address biofilm-associated susceptibility, need to take such factors into account to ascertain biofilm-mediated response to commonly used therapeutics. Standardised *in-vitro* biofilm susceptibility test methods reflecting *in-vivo* antimicrobial efficacy against adherent staphylococcal isolates are yet to be elucidated. Some well-established *in-vitro* study models although lacking in several components of *in-vivo* biofilm-associated infections, have been utilised to effectively predict the activity of various antimicrobials against adherent cells (Saginur *et al*., 2006). With the

aim of determining antimicrobial efficacy of three commonly used anti-staphylococcal drugs against biofilm encased *S. epidermidis*, this study further explored the concentration-dependent activity of vancomycin, rifampicin and daptomycin individually and in combination utilising the commonly employed bioassay model of *S. epidermidis* biofilm developed *in-vitro*.

Investigation revealed an unexpected outcome in this study with respect to the bactericidal activity of all three antimicrobials assessed individually and in combinations for anti-biofilm activity. Vancomycin, rifampicin and daptomycin appeared to have a negative effect on the reference strain, *S. epidermidis* RP62A biofilm embedded cells. Biofilm-mediated non-susceptibility was demonstrated by insufficient reduction in biofilm cell density measured spectrophotometrically and quantitatively by all three antibiotics individually. Minimum biofilm inhibitory and eradication concentration combinations were well above the normally exhibited MICs. The elevated values obtained were also significantly higher than those expected of peak serum values in patients during therapy. This suggests that the significantly reduced anti-biofilm activity of these commonly used antimicrobials limits therapeutic options for biofilm-associated infections particularly for patients with malignancies and a blunted immune response. These findings are concomitant with clinically relevant features of biofilm-embedded isolates, demonstrating significantly higher antibiotic resistance expression which may be up to 1000-fold higher, relative to planktonic-state isolates (Mah and O'toole, 2001).

The observed biofilm-mediated resistance to individual and combination antimicrobial in clinical practice for the treatment CoNS-associated bacteraemia, indicates the presence of multiple biofilm-specific mechanisms which may have contributed to the high levels of observed *in-vitro* antibiotics resistance. These biofilm-specific resistance characteristics may include the age of biofilm, specificity of antimicrobials such as penetration ability linked with molecule size and concentration-dependent time of antimicrobial exposure. Furthermore, researchers have shown that high antibiotic concentrations failing to achieve eradication and biofilm-mediated infection are due to the presence of persister cells, a dormant viable sub-population found within biofilms. These persister cells have be linked to induction of biofilm-associated re-infections related to unresolving bacteraemia as persister cells disseminating from within the biofilm in the planktonic mode retain their phenotypic resistant characteristics hence causing relapse (Dall *et al*., 2018).

Therefore, reduced susceptibilities observed against biofilms commonly produced by clinical *S. epidermidis* isolates, would generate serious consequences for therapy of biofilm-associated infections. This would subsequently result in serious clinical implications for haematology patients with inserted lines essential for continuous intravascular access that are colonised with CoNS biofilm-producers resistant to standard therapeutics. Additionally, *in-vitro* antagonistic antimicrobial interactions against *S. epidermidis* biofilm embedded cells suggests that vancomycin-rifampicin, -daptomycin, combinations would also be restricted for treatment of device-related bacteraemia caused by CoNS-associated biofilm producers.

Previous investigators have shown that *in-vitro* MIC assays for antimicrobial susceptibility investigations are poor predicators of treatment outcomes due to the extrinsic factors in the host environment that also influence antibiotic efficacy (Raad *et al*., 2007). As such, factors that influence accurate prediction of efficacy include the pathogen's metabolic state during the course of infection. These include, stationary-growth phase biofilm-associated sessile cells or when circulating as 'persister' cells demonstrate increased antibiotic tolerance, hence exacerbating infection progression leading to suboptimal clinical outcomes (Radlinski and Conlon, 2018). As this was a retrospective study, analysis of factors associated with antimicrobial resistance prevalence particularly for culture-confirmed CoNS bacteraemia in the haematology patient group could not be corroborated with therapeutic sequels. The lack of both clinical and follow-up microbiological data availability prevented the definitive assessment of treatment outcomes in relation to prescribed antimicrobials and therapy duration.

In conclusion, *S. epidermidis* was the predominant species isolated from blood cultures of patients with haematological malignancies presenting with CoNS-associated bacteraemia and those comprising skin-colonising isolates of the pre-admission patient group. This study presented strong evidence that in-patient CoNS isolates compared to the control patient group were significantly associated with an increased proportion expressing multi-drug and methicillin-resistance, concomitant with the production of the major virulence factor, slime ($p < 0.0001$). There was little evidence of vancomycin resistance, however the significant levels of teicoplanin resistance detected particularly among the in-patient isolates serves as a good marker for emerging resistance due to antibiotic pressure related to high vancomycin use in the haematology unit. The spectrum of *in-vitro* activity exhibited by daptomycin against all species-specific CoNS isolates in this study suggest that it has the potential to be a viable

alternative treatment option for CoNS-associated bacteraemia in the high-risk haematology patient group.

Significant overall differences in phenotypic and genotypic patterns were observed amongst and between CoNS species-specific isolates representing bacteraemia and those skin-colonising isolates considered as potential pathogens. Hence the definitive findings of this study do not support the hypothesis that CoNS skin-colonising isolates may be the primary source of CoNS-associated bacteraemia in the haematology and BMT patient group.

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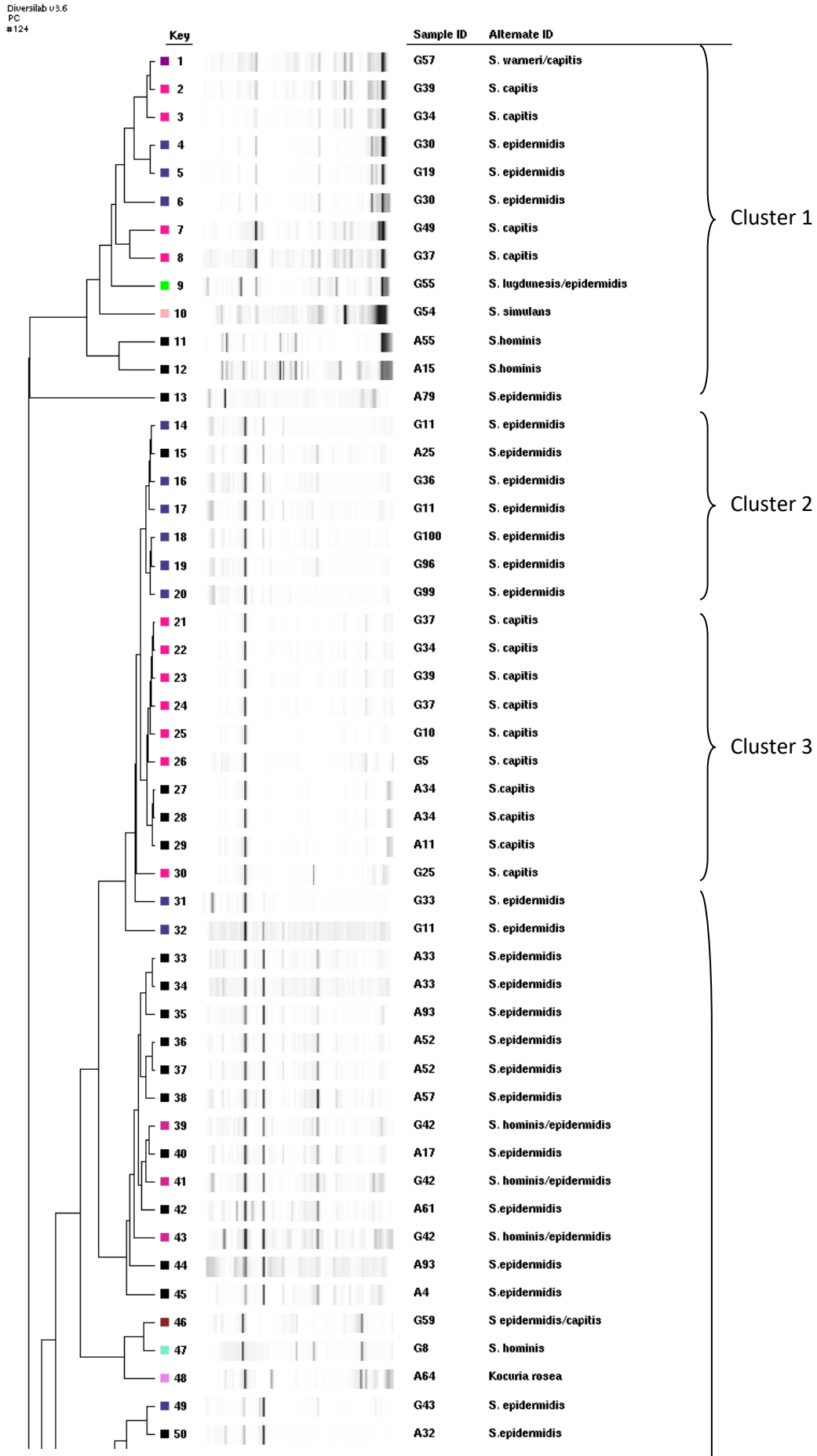
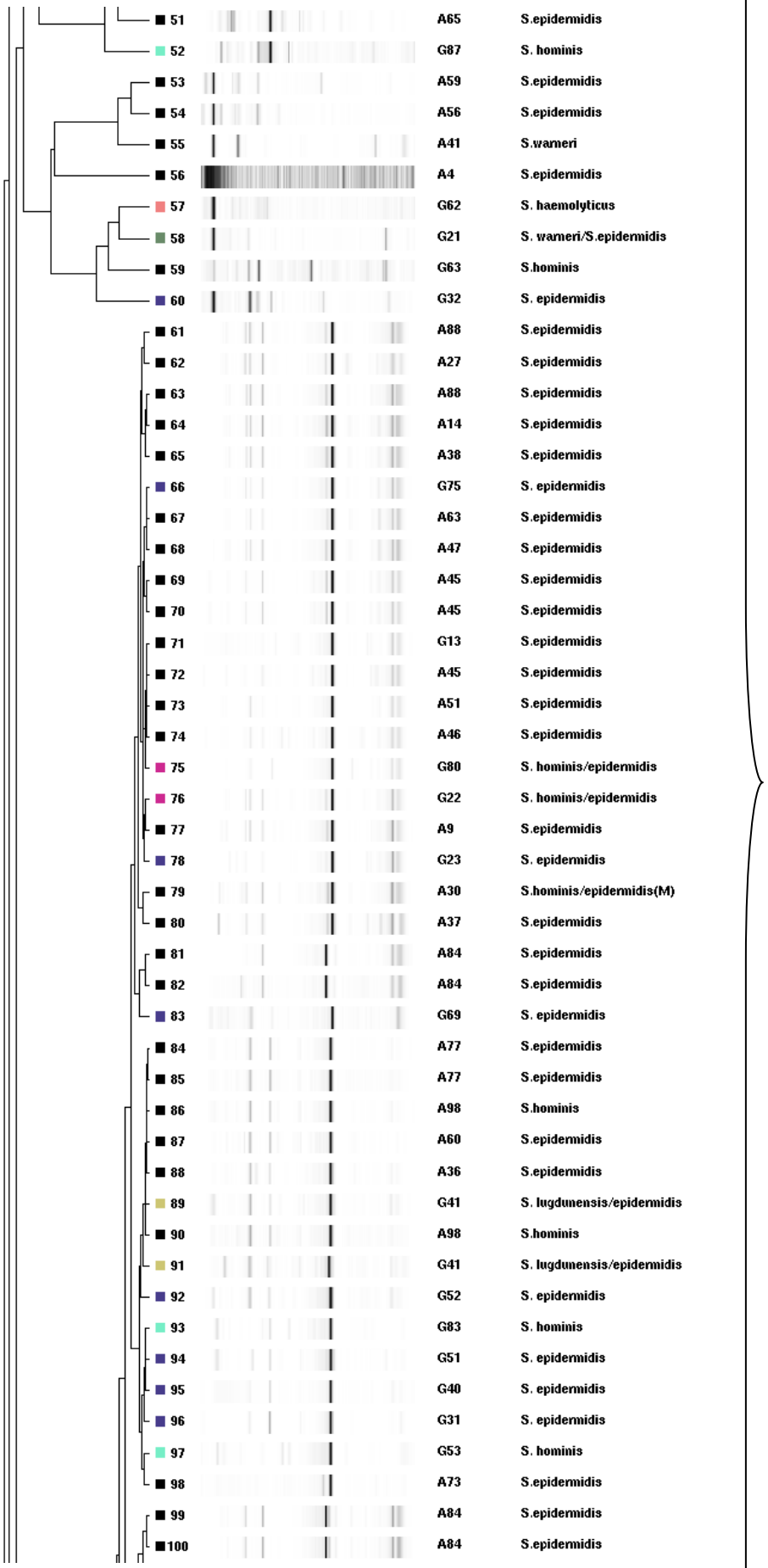
Appendices

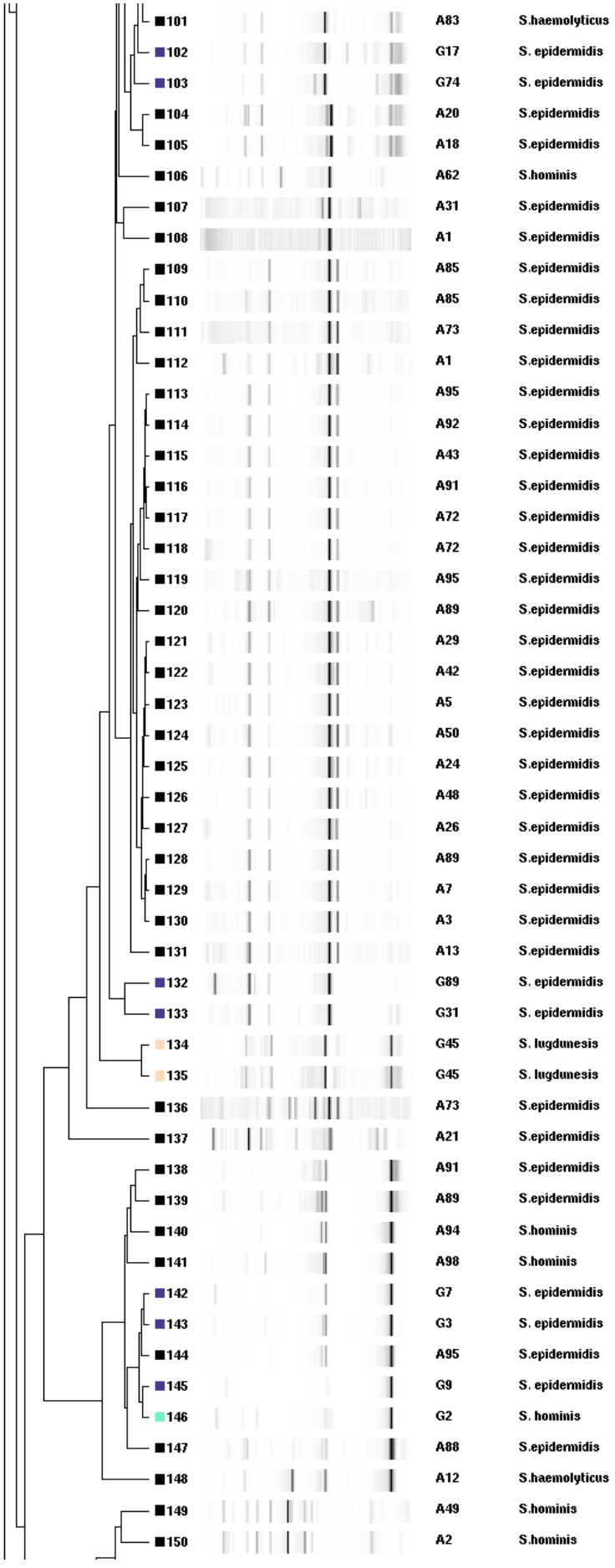
Appendix 1

The dendrogram created using the DiversiLab analytic tools based Pearson's Correlation calculation reveals similarities between the fingerprint profiles of all the CoNS species isolates obtained through rep-PCR genotyping methodology. The similarity scale bar located below the dendrogram is representative of percentage similarity with a cut-off value set at >95%.

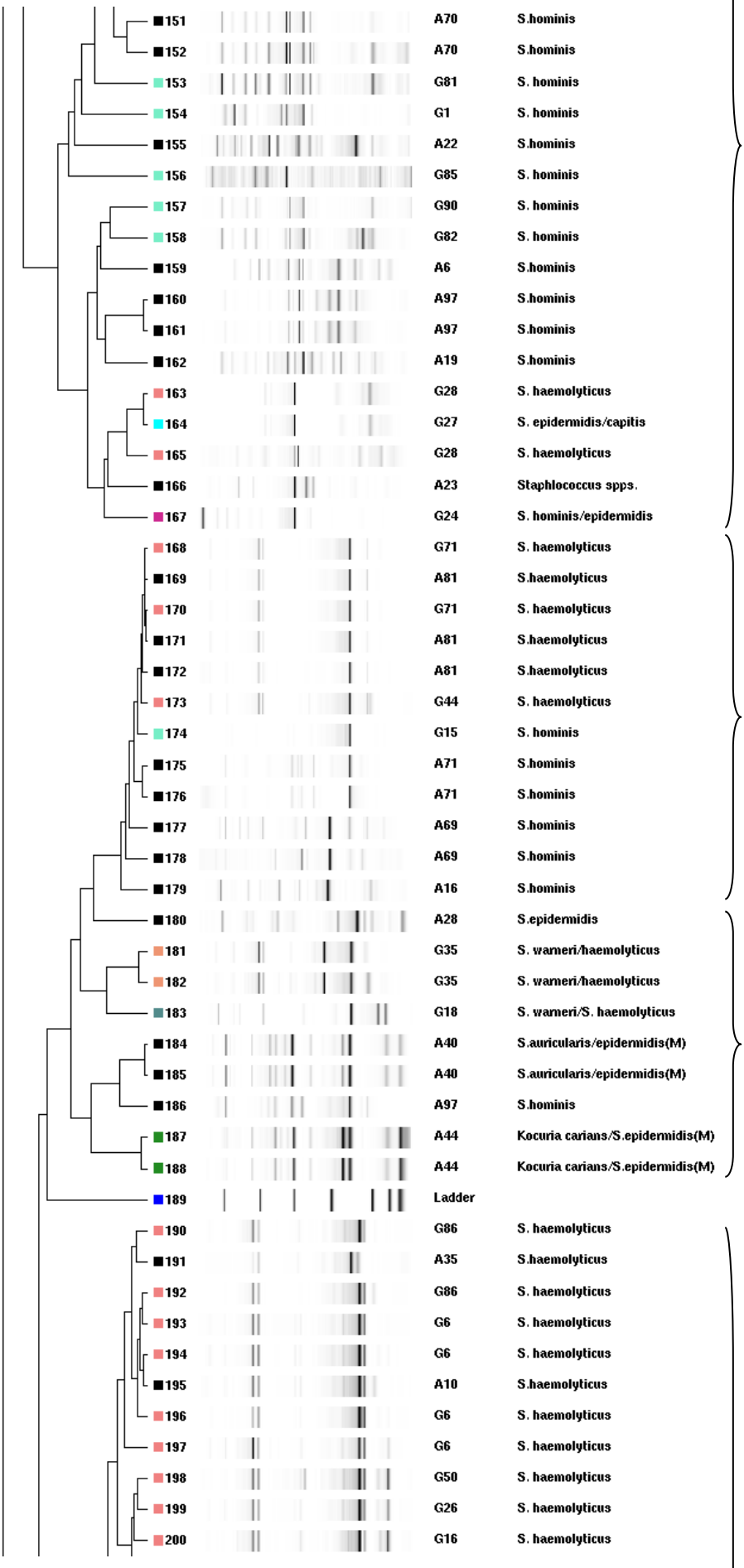

Diversilab v3.6
PC
#124
Key
Sample ID
Alternate ID
1 G57 S. warneri/capitis
2 G39 S. capitis
3 G34 S. capitis
4 G30 S. epidermidis
5 G19 S. epidermidis
6 G30 S. epidermidis
7 G49 S. capitis
8 G37 S. capitis
9 G55 S. lugdunesis/epidermidis
10 G54 S. simulans
11 A55 S.hominis
12 A15 S.hominis
13 A79 S.epidermidis
14 G11 S. epidermidis
15 A25 S.epidermidis
16 G36 S. epidermidis
17 G11 S. epidermidis
18 G100 S. epidermidis
19 G96 S. epidermidis
20 G99 S. epidermidis
21 G37 S. capitis
22 G34 S. capitis
23 G39 S. capitis
24 G37 S. capitis
25 G10 S. capitis
26 G5 S. capitis
27 A34 S.capitis
28 A34 S.capitis
29 A11 S.capitis
30 G25 S. capitis
31 G33 S. epidermidis
32 G11 S. epidermidis
33 A33 S.epidermidis
34 A33 S.epidermidis
35 A93 S.epidermidis
36 A52 S.epidermidis
37 A52 S.epidermidis
38 A57 S.epidermidis
39 G42 S. hominis/epidermidis
40 A17 S.epidermidis
41 G42 S. hominis/epidermidis
42 A61 S.epidermidis
43 G42 S. hominis/epidermidis
44 A93 S.epidermidis
45 A4 S.epidermidis
46 G59 S epidermidis/capitis
47 G8 S. hominis
48 A64 Kocuria rosea
49 G43 S. epidermidis
50 A32 S.epidermidis
Cluster 1
Cluster 2
Cluster 3

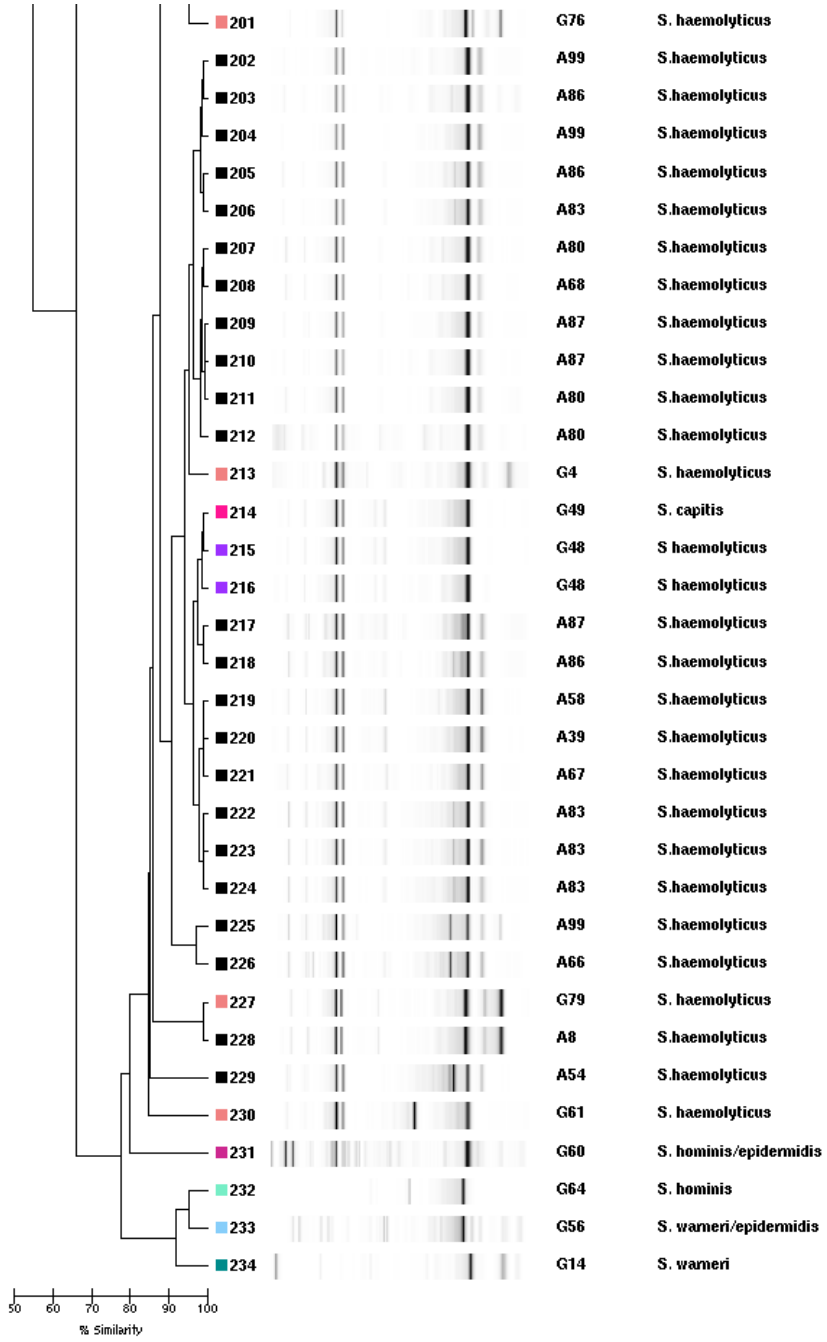

51 A65 S.epidermidis
52 G87 S. hominis
53 A59 S.epidermidis
54 A56 S.epidermidis
55 A41 S.warneri
56 A4 S.epidermidis
57 G62 S. haemolyticus
58 G21 S. warneri/S.epidermidis
59 G63 S.hominis
60 G32 S. epidermidis
61 A88 S.epidermidis
62 A27 S.epidermidis
63 A88 S.epidermidis
64 A14 S.epidermidis
65 A38 S.epidermidis
66 G75 S. epidermidis
67 A63 S.epidermidis
68 A47 S.epidermidis
69 A45 S.epidermidis
70 A45 S.epidermidis
71 G13 S.epidermidis
72 A45 S.epidermidis
73 A51 S.epidermidis
74 A46 S.epidermidis
75 G80 S. hominis/epidermidis
76 G22 S. hominis/epidermidis
77 A9 S.epidermidis
78 G23 S. epidermidis
79 A30 S.hominis/epidermidis(M)
80 A37 S.epidermidis
81 A84 S.epidermidis
82 A84 S.epidermidis
83 G69 S. epidermidis
84 A77 S.epidermidis
85 A77 S.epidermidis
86 A98 S.hominis
87 A60 S.epidermidis
88 A36 S.epidermidis
89 G41 S. lugdunensis/epidermidis
90 A98 S.hominis
91 G41 S. lugdunensis/epidermidis
92 G52 S. epidermidis
93 G83 S. hominis
94 G51 S. epidermidis
95 G40 S. epidermidis
96 G31 S. epidermidis
97 G53 S. hominis
98 A73 S.epidermidis
99 A84 S.epidermidis
100 A84 S.epidermidis

Cluster 4


101 A83 S.haemolyticus
102 G17 S. epidermidis
103 G74 S. epidermidis
104 A20 S.epidermidis
105 A18 S.epidermidis
106 A62 S.hominis
107 A31 S.epidermidis
108 A1 S.epidermidis
109 A85 S.epidermidis
110 A85 S.epidermidis
111 A73 S.epidermidis
112 A1 S.epidermidis
113 A95 S.epidermidis
114 A92 S.epidermidis
115 A43 S.epidermidis
116 A91 S.epidermidis
117 A72 S.epidermidis
118 A72 S.epidermidis
119 A95 S.epidermidis
120 A89 S.epidermidis
121 A29 S.epidermidis
122 A42 S.epidermidis
123 A5 S.epidermidis
124 A50 S.epidermidis
125 A24 S.epidermidis
126 A48 S.epidermidis
127 A26 S.epidermidis
128 A89 S.epidermidis
129 A7 S.epidermidis
130 A3 S.epidermidis
131 A13 S.epidermidis
132 G89 S. epidermidis
133 G31 S. epidermidis
134 G45 S. lugdunesis
135 G45 S. lugdunesis
136 A73 S.epidermidis
137 A21 S.epidermidis
138 A91 S.epidermidis
139 A89 S.epidermidis
140 A94 S.hominis
141 A98 S.hominis
142 G7 S. epidermidis
143 G3 S. epidermidis
144 A95 S.epidermidis
145 G9 S. epidermidis
146 G2 S. hominis
147 A88 S.epidermidis
148 A12 S.haemolyticus
149 A49 S.hominis
150 A2 S.hominis

Cluster 5


151 A70 S.hominis
152 A70 S.hominis
153 G81 S. hominis
154 G1 S. hominis
155 A22 S.hominis
156 G85 S. hominis
157 G90 S. hominis
158 G82 S. hominis
159 A6 S.hominis
160 A97 S.hominis
161 A97 S.hominis
162 A19 S.hominis
163 G28 S. haemolyticus
164 G27 S. epidermidis/capitis
165 G28 S. haemolyticus
166 A23 Staphlococcus spps.
167 G24 S. hominis/epidermidis
168 G71 S. haemolyticus
169 A81 S.haemolyticus
170 G71 S. haemolyticus
171 A81 S.haemolyticus
172 A81 S.haemolyticus
173 G44 S. haemolyticus
174 G15 S. hominis
175 A71 S.hominis
176 A71 S.hominis
177 A69 S.hominis
178 A69 S.hominis
179 A16 S.hominis
180 A28 S.epidermidis
181 G35 S. wameri/haemolyticus
182 G35 S. wameri/haemolyticus
183 G18 S. wameri/S. haemolyticus
184 A40 S.auricularis/epidermidis(M)
185 A40 S.auricularis/epidermidis(M)
186 A97 S.hominis
187 A44 Kocuria carians/S.epidermidis(M)
188 A44 Kocuria carians/S.epidermidis(M)
189 Ladder
190 G86 S. haemolyticus
191 A35 S.haemolyticus
192 G86 S. haemolyticus
193 G6 S. haemolyticus
194 G6 S. haemolyticus
195 A10 S.haemolyticus
196 G6 S. haemolyticus
197 G6 S. haemolyticus
198 G50 S. haemolyticus
199 G26 S. haemolyticus
200 G16 S. haemolyticus
Cluster 6
Cluster 7
Cluster 8



The dendrogram illustrates the rep-PCR profiles of the 233 CoNS isolates tested from both patient groups. This includes all samples that were retested for unresolved rep-PCR fingerprints and for reproducibility checks. The rep-PCR reference molecular ladder is represented at position 189 (cluster 8).

Conferences Attended

Federation of Infection Societies Conference. 16th to 18th November 2011 Manchester, England

European Congress of Clinical Microbiology and Infectious Diseases. 23rd Conference 27th to 30th April 2013. Berlin, Germany

Microbe 2104. Conference 19th to 21st September 2014. Sheffield, England

European Congress of Clinical Microbiology and Infectious Diseases. 25th Conference 25th to 28th April 2015. Copenhagen, Denmark

European Congress of Clinical Microbiology and Infectious Diseases. 27th Conference 22nd to 25th April 2017. Vienna, Austria

European Congress of Clinical Microbiology and Infectious Diseases. 28th Conference 21st to 24th April 2018. Madrid, Spain

Microbe 2018. Conference. 21st to 23rd September 2018. Sheffield, England

List of Poster Presentations

Rahila Chaudhry, Tony Worthington, Anthony Hilton, Ira Das. Characterisation of Coagulase-negative staphylococcal blood isolates from a haematology and bone marrow transplant unit; phenotypic characterisation, glycopeptide susceptibility and comparison of in-vitro activity of newer agents. Conference of the Federation of Infection Societies. November 2011 Manchester, England

Rahila Chaudhry, Tony Worthington, Anthony Hilton, Ira Das. Phenotypic characterisation and antibiotic susceptibility of clinical isolates of coagulase-negative staphylococci recovered from blood cultures of patients on a haematology and bone marrow transplantation unit. European Congress of Clinical Microbiology and Infectious Diseases. 27th to 30th April 2103 Berlin, Germany

Rahila Chaudhry, Tony Worthington, Anthony Hilton, Ira Das. Association of antimicrobial resistance with virulence traits in coagulase-negative staphylococci recovered from blood cultures of patients on a haematology unit in comparison to control group of patients. European Congress of Clinical Microbiology and Infectious Diseases. 25th to 28th April 2015. Copenhagen, Denmark

Rahila Chaudhry, Tony Worthington, Anthony Hilton, Ira Das. Comparison of phenotypic and genotypic characteristics of coagulase-negative staphylococci isolates recovered from blood cultures of patients on a haematology unit with those from the pre-admission patient group. European Congress of Clinical Microbiology and Infectious Diseases. 22nd to 25th April 2017. Vienna, Austria

Federation of infection societies Conference 2011

Characterisation of Coagulase-negative staphylococcal blood isolates from a haematology and bone marrow transplant unit; phenotypic characterisation, glycopeptide susceptibility and comparison of *in-vitro* activity of newer agents

University Hospitals Birmingham NHS NHS Foundation Trust

Rahila Chaudhry[1], Tony Worthington[2], Anthony Hilton[2], Ira Das[1], [1]Department of Clinical Microbiology and Infection Control, University Hospitals Birmingham NHS Foundation Trust, Birmingham B15 2TH, UK. [2]School of Life and Health Sciences, Aston University, Birmingham, UK.

Introduction

- Coagulase negative staphylococci (CoNS) are the most frequently isolated organisms from blood stream infections in haematology and bone marrow transplantation units.
- CoNS are usually multi-drug resistant and at present the glycopeptide vancomycin, remains the drug of choice for treatment[1]. The widespread use of glycopeptides in recent years has led to the emergence of CoNS isolates with decreased susceptibility to both the glycopeptides[2].
- Reduced glycopeptide susceptibility in *Staphylococcus aureus* isolates has been reported to have poor treatment outcomes. However, reports relating to prevalence of reduced glycopeptide susceptibility in CoNS and their association with clinical outcomes are scanty[3-4].
- The prevalence of reduced glycopeptide susceptibility amongst the CoNS and its association with clinical outcome is infrequently reported.

Aims

- To investigate susceptibility of CoNS recovered from the blood cultures of bactereamic haematology and BMT patients to vancomycin and teicoplanin using Vitek2® and E-tests.
- In addition, susceptibility to linezolid, daptomycin and tigecycline was also determined.

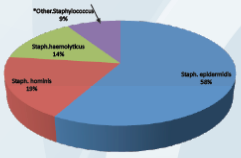
Methods

- CoNS isolates were obtained from blood cultures processed by the BactAlert® (bioMérieux) automated blood culture system and processed according to laboratory standard operating procedures based on HPA standard methods[5].
- Antimicrobial and biochemical profiles of the isolates were determined using automated Vitek2® (bioMérieux) system.
- CoNS isolates were saved on beads (Microbank™ beads ProLab-Diagnostics) at -20°C for further investigation. After recovery on blood agar, vancomycin, teicoplanin, daptomycin and tigecycline minimum inhibitory concentrations (MIC's) were determined using the standard E-test strips (bioMérieux), protocol according to manufacturer's recommended procedure.
- EUCAST approved interpretative criteria were applied for susceptibility categorization[6].

Results

- Seventy four isolates of CoNS, collected from 11th April 2010 to 25th August 2011 were investigated.
- *Staphylococcus epidermidis* (58%) was the most frequently recovered species followed by *Staphylococcus hominis* (19%).

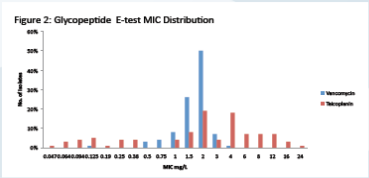
Fig 1: Distribution of CoNS species on Haematology and BMT unit



*Other Staphylococcus species include:

Staph. capitis	*Staph. auricularis*
Staph. warneri	*non-reactive-Staphylococcus*
Kocuria. variana	*Kocuria. rosea*

- 6 (8%) and 18 (25%) of the CoNS isolates were resistant to vancomycin (MIC >2mg/L) and teicoplanin (MIC >4mg/L) respectively.



- Comparatively, susceptibility results obtained with Vitek2® system, 1% (1) were reported as resistant to vancomycin whereas with E-test, resistance was detected in 8% (6) CoNS isolates.
- Resistance detection for teicoplanin was almost comparable; 18 isolates (26%) and 16 (21%) were resistant with E-test and Vitek2® respectively.
- There is a significant difference in glycopeptide MIC distribution with automated Vitek2® in comparison with those obtained E-tests.

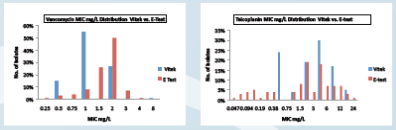


Figure 3: MIC Distribution illustrating Vancomycin borderline susceptibility and resistance at 2mg/L and >2mg/L respectively and Telcoplanin borderline susceptibility at 4mg/L and resistance at >4mg/L

- 37 (50%) of the CoNS isolates exhibited borderline susceptibility to vancomycin(MIC=2mg/L) by E-test in comparison to 20 (27%) by Vitek2®.
- In comparaison, borderline susceptibility to teicoplanin (MIC=4mg/L) was exhibited in 14 (18%) isolates and 22 (30%) by E-test and Vitek2® respectively
- All isolates were susceptible to linezolid and daptomycin whereas resistance to tigecycline was observed in 16% of the CoNS isolates.

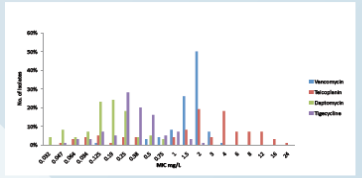


Figure 4: Comparative MIC distribution of the glycopeptides with the newer antimicrobials, daptomycin and tigecycline

Conclusion

- The majority of isolates were of the species *Staphylococcus epidermidis*.
- A significant proportion of CoNS blood culture isolates demonstrate resistance or borderline resistance to the glycopeptides.
- Reduced susceptibility to tigecycline was identified in a small proportion of the CoNS isolates.
- Newer agents such as linezolid and daptomycin may offer improved treatment options in patients presenting with bacteraemia due to CoNS.

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4. Biavasco F *et al.* Glycopeptide resistance in Coagulase-negative staphylococci. *Eur J Clin Microbiol Infec Dis* 2000 19:403-417
5. Health protection agency: www.hpa_standardmethods.org.uk/pdf.sops.asp
6. European Committee on Antimicrobial Susceptibility Testing (EUCAST) Clinical Breakpoint Table v.1.3 2011-01-05

Phenotypic characterisation and antibiotic susceptibility of clinical isolates of coagulase-negative staphylococci recovered from blood cultures of patients on a haematology and bone marrow transplantation unit.

Rahila Chaudhry[1], Tony Worthington[2], Anthony Hilton[2], Ira Das[1] [1] Department of Clinical Microbiology and Infection Control, Queen Elizabeth Hospital Birmingham, Birmingham B15 2WB, UK, [2] School of Life and Health Sciences, Aston University, Birmingham B4 7ET, UK

Introduction

- Coagulase negative staphylococci (CoNS) have emerged as the major cause of nosocomial blood stream infections in haematology and bone marrow transplantation units[1].
- *Staphylococococcus epidermidis* is the most common CoNS species associated with nosocomial blood stream infection followed by *S.hominis and S. haemolyticus*. These microorganisms are common causative agents of intravascular catheter-associated infections in neutropaenic patients[2] .
- Glycopeptide antibiotics, in particular vancomycin, has been to date considered the drug of choice for treatment of CoNS infections[3]. The widespread use of glycopeptides in recent years has led to the emergence of CoNS isolates with decreased susceptibility to both of the glycopeptides, vancomycin and telcoplanin[4-5].
- Reports relating to prevalence of reduced glycopeptide susceptibility amongst CoNS and their association with clinical outcomes especially in the immunocompromised host remain limited[6].

Objectives

- The aim of this study was to investigate the minimum inhibitory concentrations (MIC's) of a panel of antimicrobial agents, including vancomycin and telcoplanin, against well-characterised CoNS recovered from the blood cultures of patients on a haematology unit.
- The susceptibility to newer antimicrobial agents including daptomycin, tigecycline and linezolid was assessed and correlated with the CoNS phenotype.

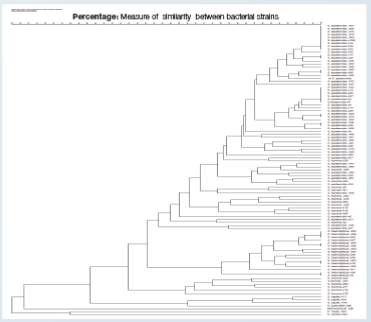
Methods

- CoNS isolates were obtained from blood cultures processed by the BactAlert® (bioMérieux) automated blood culture system and processed according to the laboratory standard operating procedures based on HPA standard methods[7].
- Susceptibility and MIC's of CoNS to all antimicrobials were determined using the Vitek®2 (bioMérieux), automated system, however susceptibility to vancomycin, telcoplanin, daptomycin and tigecycline was also assessed with the standard E-test agar diffusion method; protocol according to manufacturer's recommended procedure.
- EUCAST approved interpretative criteria were applied for susceptibility categorization[8].
- Phenotypic characterisation of the isolates through biochemical profiling was undertaken using Vitek®2 system.
- Statistical analysis; Chi-square test was used for categorical variables and Dice Coefficient with UPGMA.

Results

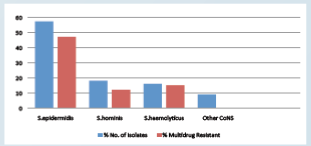
- One hundred isolates of CoNS, collected from 11th April 2010 to 15th March 2012 were investigated.
- *Staphylococcus epidermidis* (57%) was the most frequently recovered species followed by *Staphylococcus hominis* (18%) and *Staphylococcus haemolyticus* (16%).

Fig 1: Phenotypic relatedness of 100 CoNS: Dendrogram based on Vitek®2 Biochemical analysis using Dice coefficient UPGMA method



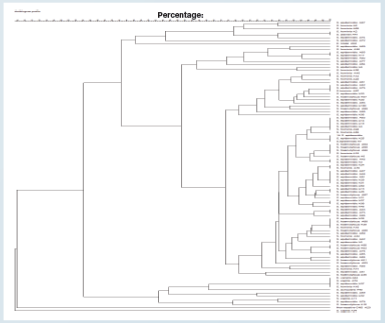
- Although a high degree of relatedness was observed amongst individual CoNS species, significant differences in the biochemical profiles was observed among individual strains of a particular CoNS species type.
- Resistance to the ß-lactams in particular methicillin and other groups of antibiotics was widespread amongst the three CoNS species.

Fig 2: Comparative CoNS species distribution frequency to multidrug resistance



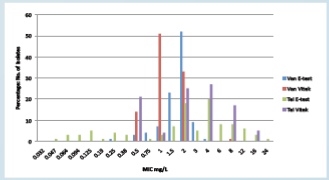
- 78% of the CoNS isolates were methicillin resistant and 74% of these demonstrated multidrug resistance (≥ 6 antimicrobials)
- There was a significant association between *S. epidermidis* and its propensity to be multidrug resistant ($p<0.05$)

Fig 3: CoNS species relatedness to antibiogram profile analysis; dendrogram based on Vitek®2 panel of 18 antibiotics using UPGMA methodology.



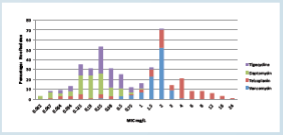
- A great degree of diversity in antimicrobial susceptibility was detected amongst the strains of particular CoNS species.
- Glycopeptide resistance was similar within the three dominant CoNS species with a higher incidence of telcoplanin resistance compared to vancomycin.

Fig 4: Glycopeptide MIC distribution: Comparison of E-test with those obtained with the Vitek®2



- There is a significant difference in glycopeptide MIC distribution with automated Vitek®2 in comparison with those obtained by E-tests.
- 10% and 18% of the isolates were resistant to vancomycin and telcoplanin (MIC >2mg/L and >4mg/L respectively) by E-test in comparison to 1% and 22% resistance reported by Vitek®2.
- Similarly 53% exhibited borderline susceptibility to vancomycin (MIC 2mg/L) by E-test in comparison to 33 % borderline susceptibility reported by Vitek.
- Borderline Vitek telcoplanin susceptibility detection at MIC 4mg/L was higher (27%) compared to 20% with E-test.

Fig 5: Comparative MIC distribution of the glycopeptides with the newer antimicrobials daptomycin and tigecyclins.



- All isolates were susceptible to daptomycin (MIC ≤1mg/L) however 13% of the isolates were tigecycline resistant (MIC ≤0.5mg/L)

Conclusion

- *Staphylococcus epidermidis* was the predominant species isolated associated with both methicillin and multidrug resistance.
- Overall no similarity in antibiogram was detected amongst the strains of a particular species of CoNS, suggesting diversity of strains associated with bacteraemia in patients in our Haematology unit.
- A significantly high proportion of CoNS isolates demonstrated resistance or borderline resistance to the glycopeptides with MICs near the upper limit of the susceptibility range.
- All isolates were susceptible to daptomycin and linezolid where as reduced susceptibility to tigecycline was identified in a small proportion of the CoNS isolates.
- Newer agents such as linezolid and daptomycin may therefore offer improved treatment options in patients presenting with CoNS bacteraemia.

References

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7. Health Protection Agency: www.hpa_standardmethods.org.uk/pdf.sops.ap
8. European Committee on Antimicrobial Susceptibility Testing (EUCAST) Clinical Breakpoint Table Version 11.1 May 2011 & May 2012

Acknowledgment

This study has been supported by an unconditional educational grant from Norvartis Pharmaceuticals UK Ltd.

Association of antimicrobial resistance with virulence traits in Coagulase-negative Staphylococci recovered from blood cultures of patients from a Haematology unit in comparison to control group of patients

Rahila Chaudhry[1], Tony Worthington[2], Anthony Hilton[2], Ira Das[1], [1]Department of Clinical Microbiology and Infection Control, Queen Elizabeth Hospital Birmingham, Birmingham B15 2WB, UK
[2]School of Life and Health Sciences, Aston University, Birmingham B4 7ET, UK

Introduction

- *Staphylococcus epidermidis* is the most common Coagulase negative staphylococcal (CoNS) species associated with nosocomial blood stream infections in haematology and bone marrow transplantation units.[1]
- Reports relating to the incidence of antimicrobial resistance in nosocomial CoNS are rising but their association with clinical outcomes especially in the immunocompromised host remain limited.[2,3]
- The efficacy of drug resistance has been directly related to the expression of various virulence traits such as extracellular enzymes which play an important part in staphylococcal pathogenesis.[4]
- Virulence factors produced by *Staphylococcus aureus* are well documented, but little is known about the toxigenic potential of CoNS and their pathogenic association with antimicrobial resistance except for the well documented role of biofilms.[5]
- Investigation of important virulence factors have intensified with regards to new approaches to the treatment and prevention of staphylococcal infections.
- Phenotypically expressed traits may be predictive of therapeutic outcomes.[6]

Objectives

- The aim of this study was to investigate the association of antimicrobial resistance with virulence factors amongst blood culture isolates of well-characterised CoNS from haematology in-patients and those from pre-admission control group of patients.
- The presence of four of the commonly expressed virulence factors in the CoNS isolates, were assessed and correlated with the phenotypes.

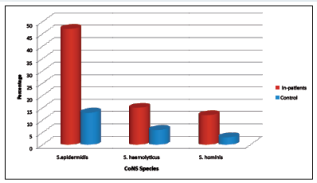
Methods

- CoNS isolates were obtained from routine blood cultures of patients admitted to the Haematology unit. These were processed by the BactAlert® (bioMérieux) automated blood culture system according to the standard laboratory operating procedures.[7]
- Control group CoNS were obtained from groin screen swabs of pre-admission patients.
- Susceptibility of CoNS, to all antimicrobials was determined using the Vitek®2 (bioMérieux) automated system.
- EUCAST approved interpretative criteria was applied for susceptibility categorization.[8]
- Phenotypic characterisation of the isolates through biochemical profiling was undertaken using Vitek®2 system.
- Four well established virulence traits expressed by CoNS including Exopolysaccharide (Slime), Haemolysin, Protease and Lipase were investigated using previously described agar based test protocols.[9,10]
- Statistical analysis; Principal Component Analysis using Statistica-version 10 package.

Results

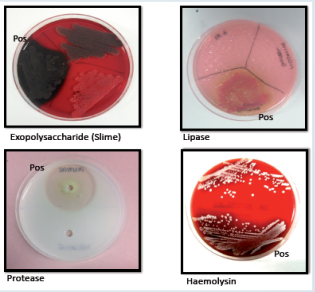
- In both groups, *S. epidermidis* was the most frequently recovered species (57% in-patient and 32% control group) followed by *S. hominis* and *S. haemolyticus*.
- Seventy-eight percent of the bacteraemic and 24% of the control isolates demonstrated multi-drug resistance.

Fig1: Group Comparison of CoNS species distribution with Multi-drug resistance



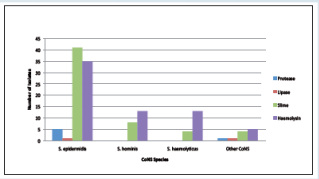
- Multi-drug resistance was highest in *S. epidermidis* both from the in-patient and control groups, 47% and 13% respectively.
- Slime and Haemolysin were the most commonly expressed virulence factors amongst both groups.

Fig.2: Four commonly expressed Virulence Factors in CoNS



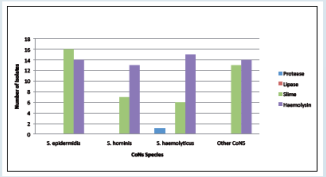
- Seventy-two percent of the in-patient isolates of *S. epidermidis* and 50% of the control group isolates expressed slime.
- Haemolysin production by *S. haemolyticus* was highest in the in-patient and control group of patients; 81% and 88% respectively.

Fig 3: Haematology Patient Group: Virulence factors expressed in CoNS Species



- Lipase expression was absent in the control isolates and poorly expressed among the in-patient isolates (2%).
- 6% of the in-patient isolates expressed Protease compared to 1% in control group isolates.

Fig 4: Control Group: Virulence factors expressed in CoNS Species



- Statistical analysis: Principal Component Analysis(PCA) was carried out using Statistica-version 10 package.
- Principle component factor analysis reveals a lack of association or correlation between antibiotic groups and virulence traits in the control patient isolates.

Fig 5: Extraction Principle Components Analysis: Control Group - Antibiotics and Virulence Factors

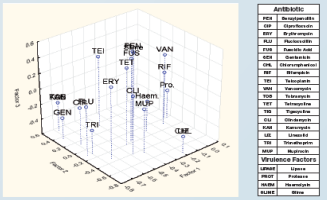
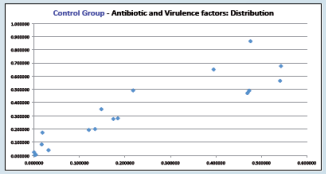


Fig 6: PCA factor analysis – Control patient group



- In the in-patient group, PCA factor analysis revealed a degree of clustering with multi-drug resistance.
- Of the virulence factors, Slime and Haemolysin production showed some association with Rifampicin resistance but no association with multi-drug resistance.

Fig 7: Extraction Principle Components Analysis: Haematology Patient Group - Antibiotics and Virulence Factors

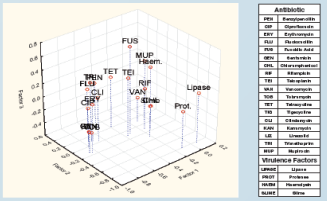
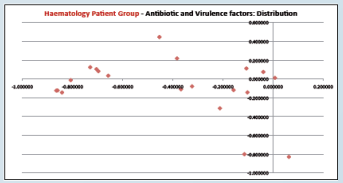
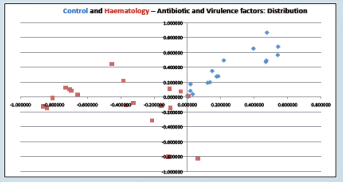


Fig 8: PCA factor analysis – Haematology in-patient group



- Comparative PCA factor analysis indicates a lack of clustering between the two patient groups.

Fig 9: Comparative PCA factor analysis of Control and Haematology patient groups



- The distribution of antimicrobial resistance with virulence factors is distinctly dissimilar in both groups.

Conclusion

- *S. epidermidis*, the predominant species isolated from both the in-patient and control groups, expressed the highest proportion of multi-drug resistance and slime production. Haemolysin production was highest among *S. haemolyticus* in both groups.
- Correlation of antibiotic resistance with virulence traits was not observed in the control group. Only minor correlation with some antibiotic resistance was observed in the haematology patient group.
- Antibiotic resistance correlation with virulence, which may play a significant role in the pathogenesis of infection was not observed in this study.

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Comparison of Phenotypic and Genotypic Characteristics of Coagulase-Negative Staphylococci Isolates Recovered from Blood Cultures of Patients on a Haematology Unit with those from the Pre-admission Patient Group

Rahila Chaudhry[1], Tony Worthington[2], Anthony Hilton[2], Ira Das[1]

[1]Department of Clinical Microbiology and Infection Control, Queen Elizabeth Hospital Birmingham, Birmingham B15 2GW, UK [2]School of Life and Health Sciences, Aston University, Birmingham B4 7ET, UK

Introduction

- Bloodstream infections due to coagulase negative staphylococci (CoNS) are the most common cause of bacterial nosocomial infections associated with high rates of morbidity in patients with haematological disorders.[1]
- CoNS species, in particular *Staphylococcus epidermidis* are ubiquitous commensals of the healthy human skin and mucosa. These have emerged as important opportunist pathogens associated with clinically significant bacteraemia especially in patients with intravascular devices and underlying immunocompromised states.[2]
- These opportunistic infections are usually associated with multi-drug resistant CoNS strains possessing determinants which facilitate survival on skin and device surfaces. Establishing the source of these opportunistic pathogens is therefore critical in prevention and management strategy.[3]
- Recent progress in genomic and molecular epidemiology has provided an interesting insight into the flexibility of normal CoNS skin commensals generating novel phenotypic and genotypic variants that are associated with opportunistic infections in immunocompromised patients.[4]
- Phenotypically expressed traits in specific isolates of CoNS may be predictive of therapeutic outcomes; however these traits may not be able to distinguish different strains of the same species expressing the same characteristics. These clonally related strains of CoNS can be distinguished further by their genetic fingerprint thus providing a link between potential sources and clinical infection.[5]

Objectives

- This study aimed to address the hypothesis that skin colonising strains of CoNS, through cross infection, may be associated with bacteraemic infection in patients on the haematology and bone marrow transplant (BMT) unit.
- The purpose of this study was to investigate the phenotypic and genotypic diversity of CoNS isolates recovered from blood cultures of patients on a haematology and BMT unit and skin isolates from the Pre-admission patient (control) group.
- The phenotype was assessed and correlated with the genotype of both the bacteraemic CoNS isolates and the skin colonising isolates from the Pre-admission patient group as the putative source of infection.

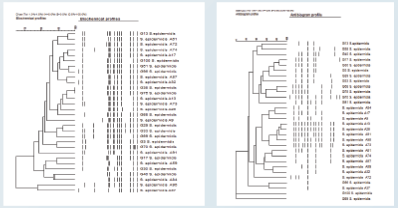
Methods

- CoNS isolates were obtained from blood cultures processed by the BactAlert® (bioMérieux) automated blood culture system. Control group CoNS were obtained from groin skin area screen swabs of Pre-admission patients.
- 50 out of 200 (25%) of the predominant species were randomly selected from both the In-patient and control patient groups and investigated for phenotypic and genotypic characteristics.
- Phenotypic characterisation through biochemical profiling and susceptibility of CoNS to a panel of 18 antimicrobials was undertaken using the Vitek®2 (bioMérieux) automated system.
- Genotypic characterisation was carried out using Public Health England reference laboratory standardised PFGE protocol. DNA samples were digested with *Sma*I endonuclease, separated using the Biorad CHEF DRII instrument followed by visualisation of DNA banding patterns.[6,7]
- Statistical data analysis: Principal Component Analysis(PCA) was carried out using Statistica-version 10 package.
- Phenotypic and genotypic CoNS species isolate comparisons were carried out by calculation of the Dice correlation coefficient and clustering was performed by the unweighted pair group method of arithmetic averages (UPGMA) to construct dendrograms using the Bionumeric Software.

Results

- In both patient groups, 50 out of the 200 of the predominant species of CoNS isolates were randomly selected for investigation.
- *S. epidermidis* was the most frequently recovered species (57% in bacteraemic patient group and 32% in control group).
- Phenotypic characterisation based on PCA analysis of biotypes and antibiograms of the 30 isolates of *S. epidermidis* revealed a significant lack of association between isolates from the two patient groups.

Fig 1: UPGMA dendrogram analysis of biotype profiles and antibiograms of the 30 *S. epidermidis* strains of the predominant CoNS species isolated from the haematology and BMT unit and the Pre-admission control group. In-Patient group isolates denoted by (A-number) and the skin isolates from the control group by (G-number). The scale bar at the top represents percentage similarity with the cut-off value set at 95%.



- A high degree of phenotypic dissimilarity was also observed between the five strains of *S. hominis* and *S. haemolyticus*, the second most predominant of the CoNS species in both patient groups.
- Genotypic characterisation based on PCA analysis of PFGE profiles revealed a significant lack of association between the three predominant CoNS species isolates from the two patient groups.

Fig 2: UPGMA dendrogram analysis and comparison of PFGE profiles of the 30 *S. epidermidis* strains of the predominant CoNS species isolates from the haematology and BMT unit. In-Patient group (A-number) and those from the Pre-admission control group of patients (G-number). The scale bar at the top represents percentage similarity with the cut-off value set at >90%.

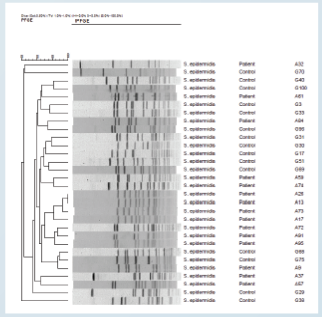
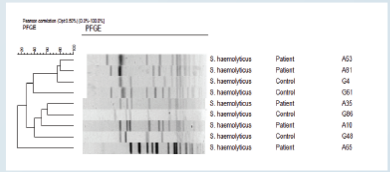
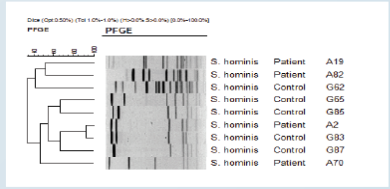


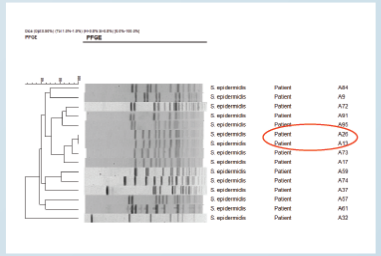
Fig 3: UPGMA dendrogram analysis and comparison of PFGE profiles among strains of *S. haemolyticus* and *S. hominis* isolated from the haematology and BMT unit. In-Patient group isolates denoted as (A-number) and the skin isolates from the Pre-admission control group of patients by (G-number). The scale bar at the top representing percentage similarity with the cut-off value set at >90%.





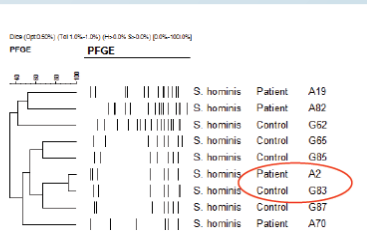
- Comparison of PCA analysis of the PFGE profiles of the 30 isolates of *S. epidermidis* and 10 isolates each of *S. hominis* and *S. haemolyticus* from the two patient groups, demonstrated a significant lack of strain relatedness.
- A degree of genetic relatedness was however observed between two *S. epidermidis* isolates from the In-patient group.

Fig 4: UPGMA dendrogram analysis of PFGE profiles of *S. epidermidis* isolates from the haematology and BMT unit In-patient group. Patient isolates A26 and A13 were the same clonal type, additionally only strain A73 revealed a close relationship with these two strains.



- 100% genetic relatedness was observed in two out of the fifteen (13%) bacteraemic isolates of *S. epidermidis*. Conversely, correlation with the phenotype was not observed.
- Two isolates of *S. hominis*, one from each of the patient groups demonstrated close relatedness, revealing >95% genetic similarity by PCA analysis but with no phenotypic correlation.

Fig 5: UPGMA dendrogram comparative analysis of PFGE profiles of *S. hominis* isolates from the In-patient and control patient groups, demonstrating close genetic relatedness between patient strain A2 and skin isolate strain G85.



- Genotypic and phenotypic relatedness was not observed between strains of *S. haemolyticus* from both patient groups.
- Overall there was no significant correlation between the phenotype and genotype of the three predominant CoNS species isolates from both patient groups.

Conclusion

- The study demonstrates a high degree of phenotypic and genotypic diversity amongst strains of the same CoNS species in both patient groups.
- There was a lack of association between skin colonising strains of *S. epidermidis*, *S. hominis* and *S. haemolyticus* and those bacteraemic strains recovered from the in-patient group.
- This study supports the hypothesis that skin colonising strains are unlikely to be implicated as the primary source of CoNS related bacteraemia in the haematology and BMT patient group.

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