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# **Studies on *Clostridium difficile***

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*Clostridium difficile* is the major cause of nosocomial diarrhoea in the UK and is associated with high morbidity and mortality rates. There has been a large increase in cases of *C. difficile* associated disease (CDAD) in the last decade and it is thought that the emergence of the hypervirulent strain (ribotype 027) has contributed towards this rise. A major factor in the control and prevention of the disease is adequate cleaning of the clinical environment and disinfection, usually with chlorine based agents. However, the spores of *C. difficile* are highly resistant to many disinfectants. In this study, a suspension test revealed that the most efficacious sporicidal disinfectants within the panel assessed were a chlorine-releasing agent and a peracetic acid-based disinfectant. Further investigation using a standard carrier test revealed that the chlorine-releasing agent was significantly more effective than the peracetic acid disinfectant under 'clean' conditions. However, in the presence of a soil load there was no significant difference between the sporicidal efficacies of the two disinfectants. Peracetic acid based disinfectants might be considered as an alternative, given the lower associated health hazards. In this investigation, the factors affecting the extent and rate of germination of *C. difficile* spores were determined by the loss of heat and chemical resistance, using a suspension test. Sodium taurocholate and chenodeoxycholate in thioglycollate medium were found to stimulate germination. The rate of germination was affected by temperature and pH, but was the same in both aerobic and anaerobic conditions. These findings were used in the assessment of a novel two-stage approach of elimination of *C. difficile* spores: germination followed by exposure to common antimicrobial agents. A germination solution developed in this study was incubated with the spores of *C. difficile* prior to exposure to 70% ethanol or copper metal. Both 70% ethanol and copper were found to significantly reduce the number of germinating spores of *C. difficile*, but were inactive against dormant spores. Novel analogues of a benzylidene carboxamidrazone (compound **1**) with antimicrobial activity against Gram-positive micro-organisms were produced and only one analogue (compound **8**) together with a thiosemicarbazone (compound **9**) and the original compound (**1**) demonstrated bactericidal activity against vegetative cells of *C. difficile*. These studies enhance the current knowledge of *C. difficile* in relation to germination of its spores, the efficacy of common sporicidal disinfectants and the antimicrobial activity of novel compounds with potential for elimination of *C. difficile*. A two-stage germination - disinfection approach incorporating less hazardous antimicrobials e.g. 70% alcohol and copper for reducing the *C. difficile* spore load has also been described.

Key words: *C. difficile*, disinfectant, spore, germination, sodium taurocholate

*For my Grandad Sam*



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## Abbreviations

ADP	-	Adenosine diphosphate
AFLP	-	Amplified fragment length polymorphism
ANOVA	-	Analysis of variance
APCI-MS	-	Atmospheric pressure chemical ionization mass spectroscopy
AP-PCR	-	Arbitrarily primed polymerase chain reaction
BHI	-	Brain Heart Infusion
BS EN	-	British and European Standard
°C	-	Degrees centigrade
Ca	-	Calcium
CCFA	-	Cycloserine cefoxitin fructose agar
CDA	-	<i>Clostridium difficile</i> antitoxin
CDAD	-	<i>Clostridium difficile</i> associated diarrhoea
CDC	-	Center for disease control
CDI	-	<i>Clostridium difficile</i> infection
CDT	-	<i>Clostridium difficile</i> toxin
CFU	-	Colony forming unit
CIP	-	Cleaning in place procedure
cm	-	Centimeters
D/E	-	Dey and Engley
DMSO	-	Dimethyl Sulfoxide
DNA	-	Deoxyribonucleic acid
DST	-	Direct sensitivity testing
DoH	-	Department of Health
DPA	-	Dipicolinic acid
EIA	-	Enzyme immunoassay

FAA	-	Fastidious anaerobe agar
FTIR	-	Fourier transform infrared
g	-	Gram
GI	-	Gastrointestinal
h	-	Hour
HCl	-	Hydrochloric acid
HCW	-	Healthcare worker
HIV	-	Human immunodeficiency virus
HPA	-	Health Protection Agency
HPV	-	Hydrogen peroxide vapour
Ig	-	Immunoglobulin
IL	-	Interleukin
KBr	-	Potassium bromide
KCl	-	Potassium chloride
kDa	-	Kilodalton
L	-	Litre
LFC	-	Level of free chlorine
LPS	-	Lipopolysaccharide
MBC	-	Minimum bactericidal concentration
mg	-	Milligram
MIC	-	Minimum inhibitory concentration
mins	-	Minutes
mL	-	Milliliter
MLST	-	Multilocus sequence typing
mm	-	Millimeter
mM	-	Millimolar
MRSA	-	Meticillin-resistant <i>Staphylococcus aureus</i>

ms	-	Millisecond
MVLA	-	Multi-locus variable number tandem-repeat analysis
NaDCC	-	Sodium dichloroisocyanurate
NCCLS	-	National committee for clinical laboratory standards
NCTC	-	National collection of type cultures
NHS	-	National Health Service
nm	-	Nanometer
NMR	-	Nuclear magnetic resonance
OD	-	Optical density
ONS	-	Office of National Statistics
PaLoc	-	Pathogenicity locus
PCR	-	Polymerase chain reaction
PFGE	-	Pulse field gel electrophoresis
PHLS	-	Public Health Laboratory Service
P <sub>i</sub>	-	Inorganic phosphate
PMC	-	Pseudomembranous colitis
PPM	-	Parts per million
Quat	-	Quaternary ammonium compound
REA	-	Restriction enzyme analysis
RFLP	-	Restriction fragment length polymorphism
RFU	-	Relative fluorescence units
RNA	-	Ribonucleic acid
RPM	-	Revolutions per minute
SASP	-	Small acid soluble protein
SDW	-	Sterile distilled water
SEM	-	Scanning electron microscope
SLP	-	Surface layer protein

ST	-	Sodium taurocholate
TbCl <sub>3</sub>	-	Terbium chloride
TLC	-	Thin layer chromatography
TM	-	Thioglycollate medium
TNF	-	Tumour necrosis factor
UK	-	United Kingdom
μL	-	Microlitre
μm	-	Micrometre
μM	-	Micromolar
USA	-	United States of America
UV	-	Ultra-violet
v/v	-	Volume per volume
WCA	-	Wilkins Chalgren agar
WCB	-	Wilkins Chalgren broth
w/v	-	Weight per volume



# 1 Introduction

## 1.1 *Clostridium difficile*; Brief Background

*Clostridium difficile* is a Gram-positive, spore-forming, obligately anaerobic bacillus. It was first described in 1935, as a component of the faecal flora in healthy infants and was not recognised as a cause of diarrhoea until 1978 (Barbut and Petit, 2001). The name reflects the difficulty that was encountered in culturing the organism (McFarland *et al.*, 1989, Cookson, 2007). *C. difficile* is usually acquired via the faecal-oral route from an exogenous source, such as from an infected individual or indirectly from a contaminated environment (McMaster-Baxter and Musher, 2007). The spores of *C. difficile* are ingested and after survival through the acidic environment of the gut, they may germinate into vegetative cells that are capable of causing disease (Giannasca and Warny, 2004). Alternatively, the organism may sometimes be delivered directly to the bowel via contaminated instruments; such as endoscopes (Worsley, 1998). *C. difficile* is highly infectious and extremely dangerous in compromised and elderly patients and causes disease through the production of potent proteinaceous exotoxins which have activity against intestinal cells. *C. difficile* is frequently associated with outbreaks of nosocomial diarrhoea and accounts for 15-25% of cases of antibiotic-associated diarrhoea (Barbut and Petit, 2001). Furthermore, the infection can progress rapidly to life-threatening conditions including antibiotic-associated pseudomembranous colitis (PMC) (Schroeder, 2005). Indeed, *C. difficile* currently contributes to the death of approximately 6,500 patients per annum in England and Wales (ONS, 2008) which is approximately four times as many as those caused by meticillin resistant *Staphylococcus aureus* (MRSA).

## **1.2 Prevalence of *C. difficile***

Transient colonisation of *C. difficile* occurs in around 70% of neonates, although these babies are rarely symptomatic, despite having high toxin levels. By the age of 2-3 years colonisation falls to less than 3%, which is the normal rate in healthy adults (Barbut and Petit, 2001, Settle, 1996). Interestingly, prevalence rates rise again during old age, with 14% of the elderly having the organism. Incidence is higher in hospitalised patients, with reports of colonisation in over 20% of patients (Settle, 1996). Voluntary surveillance data has shown that approximately 84% of cases of *C. difficile* associated disease (CDAD) occur in patients aged 65 and over. In all age groups there is approximately an equal distribution of cases between males and females, except in the aged 75 and over category where there are significantly more females infected. However, this may be explained by the increased number of elderly females in the population (HPA, 2007).

### **1.2.1 Reporting of *C. difficile* cases**

Reporting of new cases of *C. difficile* became mandatory in England in January 2004 and all NHS trusts must now report all cases of CDAD, defined by a toxin positive stool sample in patients aged 65 and over. All patients aged 65 and over with diarrhoeal symptoms are to be tested and those below the age of 65 should be tested when clinically relevant, however, reporting of cases in patients below the age of 2 years is not required (HPA, 2007). During 2004 there were 43,672 reports of *C. difficile*, in the UK, an increase of 98% from 2001. However, it is not known how much of this increase is due to an improvement in reporting or an actual increase in incidence of *C. difficile* (Berrington, 2004). The rise in CDAD has been most prominent amongst older patients, especially those in the age group of 75 years and over (HPA, 2005). It is thought that outbreaks are likely to become more common in

the future as the average age of hospitalized patients increases. The number of cases of CDAD had continued to rise and peaked in 2006, with 55,620 cases of *C. difficile* reported in the UK, with the following year showing a 9% decrease in reported cases. However, the HPA emphasize that the decrease has only occurred in this last year and therefore may not represent a general decline in the disease (figure 1.1) (HPA, 2006, HPA, 2007, HPA, 2008). The number of death certificates mentioning *C. difficile* was 6480 in England and Wales in 2006; a 72% increase from the previous year (ONS, 2008).

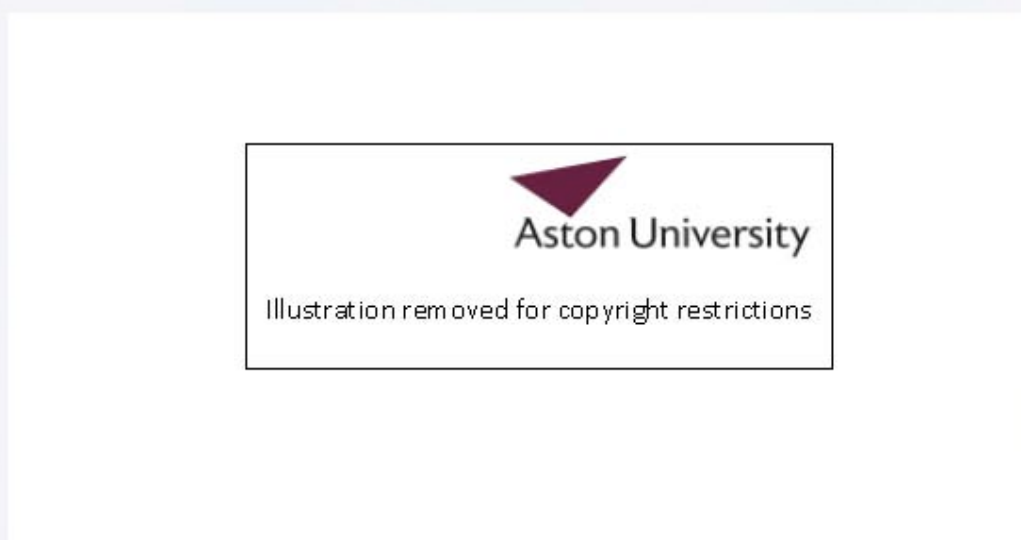


**Figure 1.1** Number of reports of *C. difficile* infection in patients aged 65 years and over in England, Northern Ireland and Wales from 2000 to 2007 (HPA, 2008).

Certain changes to the mandatory surveillance system were made on January 1<sup>st</sup> 2008 by the Chief Medical Officer from the Department of Health (DoH). These amendments included the submission of data regarding the date of admission, onset of diarrhoea and contact with other healthcare facilities. The '28-day rule' was also designed for counting the number of episodes of CDAD a patient acquires. Therefore, *C. difficile* positive samples from the same patient more than 28 days



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apart are regarded as separate episodes, regardless of the number of positive samples within the 28-day period or the location of the patient. These changes aim to facilitate the identification of patients with recurrent episodes of CDAD and the location of infection (DoH, 2008).

### **1.2.2 Outbreaks of *C. difficile***

A largely publicized outbreak of *C. difficile* occurred on the 6<sup>th</sup> of June 2005 at Stoke Mandeville hospital, NHS Trust, UK. Three hundred people were found to be infected and 12 people died from a hyper-virulent strain of *C. difficile* (ribotype 027). In response to this outbreak a report by the Healthcare Commission was issued detailing the factors which contributed to this and a previous outbreak at the hospital (Anon, 2006a). These factors included a shortage of side-rooms, buildings in a poor state of repair, management focus on government waiting times, shortage of nurses, lack of hand-washing facilities and a poor standard of cleanliness. Other outbreaks of *C. difficile* which prompted investigations by the Healthcare Commission included those at Maidstone and Tunbridge Wells hospital, NHS Trust, UK, between October 2005 and September 2006. During this period over 500 people were infected and it was estimated that *C. difficile* was either definitely or probably the main cause of around 60 deaths. An inadequate surveillance system led to the first outbreak being unidentified. The contributing factors were found to be almost exactly the same as those outlined in the Stoke Mandeville outbreaks. In addition, at the Kent and Sussex hospital, beds were very close together, with only a 30cm space in between at some points and in 2006 the trust was rated in the worst 20% of all trusts for cleanliness of wards, toilets and washing facilities (Anon, 2007).

Cookson (2007) stated that similar factors were also listed in reports following outbreaks in Québec, Canada and that the issue of patient transfer between cities may also have contributed to the spread of disease (Cookson, 2007). Between 2002



and 2005, in Québec, it was noted that there was a rise in both the severity and the number of cases of CDAD. Connected with these infections were a high relapse rate and a greater resistance to treatment. At the same time, similar increases in CDAD were also seen across America, with 8 outbreaks of infection being identified in 6 states (Bartlett, 2006).

Surveillance of CDAD outbreaks in Belgium was started in June 2006 and in that month 11 hospitals were identified as having outbreaks of CDAD. In April of the same year, 11 hospitals in the Netherlands had outbreaks and in August of 2006, 9 hospitals in Northern France suffered outbreaks of CDAD. In the Netherlands and France the transfer of patients between hospitals and between care homes and hospitals was identified as the possible cause for the spread of disease (Cookson, 2007).

The financial burden of CDAD on the NHS is substantial. A prospective case-control study in a Cambridge hospital found that the estimated additional cost of each case of *C. difficile* infection was over £4000 and the majority of this cost was due to prolonged hospital stay (Wilcox *et al.*, 1996). Other factors which contribute towards the overall cost include antibiotic treatment, ward closures and lost bed days (Spencer, 1998).

### **1.3 Hypervirulent toxinotype III NAP1/ 027 strain**

The PCR ribotype 027 strain, also known as toxinotype III and pulse field gel electrophoresis (PFGE) type NAP1, of *C. difficile* is responsible for many outbreaks of CDAD in the UK, other European countries and America and is reported to cause a greater severity of disease and a higher mortality rate than other strains of *C. difficile* (Carter *et al.*, 2007, Warny *et al.*, 2005). Warny *et al.* (2005) found that this hypervirulent strain produced 16 times more toxin A and 23 times more toxin B than

control strains and possessed the genes for the binary toxin (Warny *et al.*, 2005). It is thought that an 18 base pair deletion in the *tcdC* gene, which negatively regulates toxin expression may be responsible for the increased toxin production (Monaghan *et al.*, 2008). However, the Health Protection Agency has stated that research has not yet shown a link between the 027 strain and increased disease severity (Anon, 2006b). Cookson (2007) also noted that there has been no evidence of a relation between disease severity and stool toxin concentration (Cookson, 2007). In a recent study, disease severity of CDAD patients infected with different ribotypes of *C. difficile* were compared and it was concluded that the ribotype 027 strain did not demonstrate increased virulence compared to other ribotypes (Morgan *et al.*, 2008).

Recent isolates which have been identified as ribotype 027 are resistant to fluoroquinolones and it is thought that the acquisition of this resistance (due to increased use of fluoroquinolones) has contributed to the emergence of this strain (McDonald *et al.*, 2005, Warny *et al.*, 2005). A recent study has also found an increased sporulation rate of this strain compared to others (Akerlund *et al.*, 2008).

In the UK, fresh concerns were raised over the emergence of ribotype 027 after three Leicestershire hospitals revealed that CDAD was responsible for at least 49 deaths in 2006. In the same year there was an inquiry into an outbreak of *C. difficile* in Maidstone hospital, Kent in which 20 patients died. Prior to these outbreaks, between 2004 and 2005 outbreaks of the epidemic strain (027) were found to be the cause 33 deaths at Stoke Mandeville hospital, Buckinghamshire (Anon, 2006a).

#### **1.4 Community acquired *C. difficile* associated diarrhoea**

There are increasing reports of community acquired CDAD (Johal *et al.*, 2004, Kuijper *et al.*, 2007). A patient is described as having community acquired CDAD if the onset of symptoms has occurred in the community or within 48 hours after

admission to a healthcare facility, as long as onset has occurred more than 12 weeks after release from a hospital or another clinical environment (McDonald *et al.*, 2007). However, the definition of community associated CDAD unfortunately varies (McFarland *et al.*, 2007). Data from the General Practitioner database in the UK, showed that the number of cases of community acquired CDAD in 2004 was 22 per 100 000 cases; an increase from less than 1 per 100 000 in 1994 (Dial *et al.*, 2005). A study by Beaugerie *et al.* (2003) found that there was a fairly high frequency of community acquired *C. difficile* cases in patients which were taking a course of chemotherapy (estimated at 2700 per 100 000 exposures to antibiotics) (Beaugerie *et al.*, 2003). However, *C. difficile* was not found to be the main cause of antibiotic-associated diarrhoea in out-patients. Patients with community acquired *C. difficile* are generally younger, have not been exposed to antibiotics and many have other chronic bowel conditions (McFarland, 2008). It is not known how *C. difficile* is acquired in the community, however it has been suggested that sources may include domesticated animals (Arroyo *et al.*, 2005a) spores in the soil, contamination from other family members and contaminated food (Arroyo *et al.*, 2005a, McFarland *et al.*, 2007).

## **1.5 Acquisition of disease and risk factors**

*C. difficile* has been recovered from most surfaces and fomites within hospital wards and acquisition of the organism occurs primarily in the hospital setting. A study by Fekety *et al.* (1981) demonstrated that *C. difficile* contamination is common in the hospital environment, particularly around infected patients. In the study, the organism was recovered from beds, toilets, floors, mops and furniture. *C. difficile* has also been isolated from the stools and hands of asymptomatic hospital staff (Fekety *et al.*, 1981). Studies have shown that between 11 and 29% of environmental



samples from healthcare facilities were positive for *C. difficile* (Verity *et al.*, 2001, Dubberke *et al.*, 2007). Dubberke *et al.* (2007) found that samples were more likely to be positive for *C. difficile* if they had been taken from a room housing a patient with *C. difficile*, however around a third of samples taken from rooms containing patients asymptomatic for *C. difficile* infection were positive. Roberts *et al.* (2008) were able to culture *C. difficile* from air samples in the hospital environment, suggesting that *C. difficile* spores could be spread throughout the clinical setting through the air (Roberts *et al.*, 2008).

Spores of *C. difficile* are either ingested via the faecal-oral route from the hands of healthcare workers, from the contaminated environment or may be delivered directly into the bowel via contaminated equipment such as endoscopes (Worsley, 1998). Once inside the bowel, in order for the organism to colonise the gut of a healthy person, the flora must be altered in some way, as it is usually inhibitory towards *C. difficile*. The most common cause of disruption to the gut flora is the use of broad spectrum antibiotics. Any type of antibiotic which affects the flora of the gut is capable of inducing *C. difficile* infection, however certain groups in particular have been implicated; such as the cephalosporins, clindamycin and penicillins (Worsley, 1998, McFarland, 2008, Barbut and Petit, 2001). Other risk factors include age, nasogastric intubation, use of cytotoxic and immunosuppressive agents, surgery, use of antacids, concurrent illness and alterations in gut motility, prolonged hospital stay and admission from another hospital (Anon, 1994, Starr *et al.*, 2003). Kenneally *et al.* (2007) also found that CDAD was associated with an extended period of stay in hospital and in particular a prolonged stay in an intensive care unit (Kenneally *et al.*, 2007). Some reports have suggested that the intestinal flora in the elderly and in neonates is less inhibitory. Colonisation is usually transient and this may be due to the inadequate virulence of the infecting strain or the insufficient degree of compromise of the gut flora to allow establishment and full expression of virulence



(Borriello, 1990). A small percentage of patients have been found to be asymptomatic carriers of both epidemic and non-epidemic strains of *C. difficile* and it is thought that these patients have the potential to contribute significantly towards the spread of *C. difficile* infection (Riggs *et al.*, 2007).

Recently there seems to be an emergence of disease occurring in previously low risk groups, such as children above the age of two years and young peripartum women (McFarland, 2008).

## **1.6 Gastrointestinal physiology**

The colon is densely populated with around  $10^{12}$  bacterial cells per gram of contents; most of which are anaerobic bacteria. This complex microbial community is fundamental to the health of the host as it provides important nutrients and interacts with host cells. These communities of bacteria also prevent potentially pathogenic bacteria from colonizing the gut (colonization resistance) and therefore act as an important defence barrier (Donskey, 2004). Gut bacteria are supplied with nutrients from the diet, which have survived passage through the stomach and from components of the intestine itself, such as mucin. These nutrients are digested by bacteria via anaerobic metabolism to produce carbon dioxide, hydrogen, methane and short chain fatty acids. The products of this metabolic process have a major impact on both the gut environment and on the host (Flint *et al.*, 2007).

It is also suggested that the gut bacteria respond to changes in the diet; especially to carbohydrates and as a result alter the gut environment. In particular, it is known that fermentation of non-digestible carbohydrate results in a decrease of the luminal pH. As well as the influence of diet, host physiology and medication also affect the composition of gut microflora and it is thought that it may be in a constant state of change (Flint *et al.*, 2007).

As *C. difficile* mainly affects the elderly population it is important to understand the changes that occur in the gut during aging. The immune response in the elderly is greatly affected by malnutrition (Lesourd, 2006) and therefore reduced immunity causes this population to become more at risk from infection. Coupled with this are changes in the dominant species of bacteria which are found in the gut of elderly populations. A decrease in both the total number and species diversity of bacteroides and bifidobacteria has been shown in the elderly. Bacteroides are considered to be responsible for most of the digestion of polysaccharides in the colon and therefore changes to this species may have a large impact on host metabolic activity and on other bacteria which are part of a cross-feeding network. Bifidobacteria and other fermentative bacteria produce short chain fatty acids which lower the pH of the intestinal lumen, protecting against pathogenic bacteria and therefore a reduction in these bacteria may be detrimental. Numbers of other species of bacteria which increase during aging include fusobacteria, eubacteria, clostridia and facultative anaerobes. Fusobacteria and eubacteria ferment amino acids and transform bile acids respectively and both produce various harmful substances in the process and therefore an increase in the number of these bacteria may have disadvantageous effects on health. All of these changes in bacterial species dominance along with alterations in dietary intake may contribute towards increased susceptibility to infections such as *C. difficile* (Woodmansey, 2007).

Figure 1.2 shows the different species of bacteria which are present in the digestive system of a healthy adult.



Illustration removed for copyright restrictions

(Ridlon *et al.*, 2006)

**Figure 1.2 The digestive system, showing the number and species of bacteria, luminal pH and the circulation of bile salts**

### **1.6.1 Bile acids and enterohepatic circulation**

Bile acid synthesis from cholesterol occurs in the hepatocytes of the liver, where the two primary bile acids; cholic acid and chenodeoxycholic acid are produced. These primary bile acids are conjugated to taurine or glycine by the liver, producing taurocholate and glycocholate respectively, which become bile salts by ionisation. The bile salts are secreted into the bile which is stored and concentrated in the gall bladder (Ridlon *et al.*, 2006). The gall bladder secretes bile into the duodenum after a meal and here the maximal concentration of bile salts in a healthy adult in the duodenum is 12.6mM for glycocholate, 6.9mM for taurocholate and 0.7mM for

cholate, deoxycholate, chenodeoxycholate and lithocholate (Leverrier *et al.*, 2003). In the small intestine the bile salts act as detergents, aiding the digestion and absorption of lipids and lipid-soluble vitamins, before being absorbed by active transport in the distal ileum. Once in the bloodstream, the bile salts are complexed to plasma proteins which circulate back to the liver. Active transporters on the surface of hepatocytes remove bile salts from the blood which are then secreted back into the bile. This process is known as enterohepatic circulation (figure 1.2). During this cycle, bile salts are metabolized (deconjugation and hydroxyl group oxidation) by bacteria in the small intestine. Some bile salts escape active transport by the ileum and pass to the large intestine, where they are converted to deoxycholic acid and lithocholic acid by microbial biotransforming reactions (Ridlon *et al.*, 2006). The structures of the bile salts are shown in figure 1.3.





**Figure 1.3 Structures of bile salts (Sorg and Sonenshein, 2008)**

## **1.7 Pathogenesis of *C. difficile* associated disease**

The pathogenesis of CDAD can be considered broadly in six stages, these include: entry, adhesion, multiplication, avoidance of host defences, damage to the host and release (Poxton *et al.*, 2001, Poxton, 2005).

### **1.7.1 Entry**

Spores of *C. difficile* almost always enter the body from an exogenous source as the organism is not detected in the majority of people and a single strain is usually responsible for an outbreak (Cartmill *et al.*, 1992). The spores of *C. difficile* are ingested and survive passage through the stomach to the intestine or may be delivered directly into the intestine via contaminated endoscopes (Worsley, 1998). As the spores pass through the G.I. tract, they encounter bile salts which have been released from the gall bladder. Recent research has suggested that the bile salts stimulate germination of the spores into vegetative cells (Sorg and Sonenshein, 2008).

### 1.7.2 Adherence

Once inside the gut, the vegetative cells of *C. difficile* must adhere to the surface of the intestinal mucosa. The adherence mechanisms of *C. difficile* are not fully understood, however studies have shown a positive correlation between virulence and mucosal adherence (Waligora *et al.*, 2001). *C. difficile* binds to extracellular matrix proteins on host tissue, such as fibronectin, fibrinogen, collagen and vitronectin. It is thought that the following structures may play a role in colonisation: the capsule, proteolytic enzymes, the surface layer proteins P36 and P47, a cell-surface protein with adhesive properties; Cwp66, the flagella, consisting of the flagellin FliC, and the flagellar cap protein FliD, fibronectin binding protein 68 (Fbp68) and a member of the Hsp60 family of chaperones GroEL (Pechine *et al.*, 2005).

Studies have demonstrated direct binding of *C. difficile* cells to HT-29 and Caco-2 colonic epithelial cell lines, human colonic epithelial cells and to extracellular matrix proteins (Eveillard *et al.*, 1993, Cerquetti *et al.*, 2002, Drudy *et al.*, 2001). It has been suggested that the surface layer proteins (SLPs) in particular are probably involved in binding. Two SLPs named high and low molecular weight SLPs cover the surface of the bacterial cells, forming a regular, crystalline array. Studies have suggested that the high molecular weight SLPs are more involved in binding and have shown strong binding of *C. difficile* SLPs to human epithelial cells and intestinal tissue (Calabi *et al.*, 2002, O'Brien *et al.*, 2005).

### 1.7.3 Multiplication

In order to cause disease *C. difficile* must multiply in order for there to be sufficient numbers of the organism to produce toxin. As the use of broad spectrum antibiotics eliminates much of the normal flora of the intestine, *C. difficile* is able to multiply without much interference by competing organisms (Donskey, 2004). Some studies

have also shown that more toxin is produced by *C. difficile* when exposed to sub-lethal concentrations of antibiotic (Drummond *et al.*, 2003).

#### **1.7.4 Evasion**

It is suggested that *C. difficile* uses its wide array of SLPs to evade the immune response of the host along with degradative enzymes and capsules; however none of these have been proven to be involved in evasion (Poxton, 2005). SLPs are the predominant surface protein of *C. difficile* and consist of high molecular weight proteins (48-56 KDa) and low molecular weight proteins (37-45 KDa), which are derived from a single gene product. The degree of variation in these proteins has been demonstrated to be high between different strains of *C. difficile* (McCoubrey and Poxton, 2001). A study by Drudy *et al.* (2004) has demonstrated that SLPs are highly immunogenic and that human sera contain detectable levels of antibody directed against SLPs. In particular, it was found that patients with a single episode of CDAD had significantly higher IgM antibodies directed against SLP between days 3 to 9 of the disease, compared with those who later developed recurrent CDAD. In addition it was also established that a low level of IgM antibody against SLP on the third day of an initial episode of CDAD resulted in a 25-fold increased risk of a recurrent episode of CDAD (Drudy *et al.*, 2004).

#### **1.7.5 Damage to the host**

Once the organism has attached itself to the gut wall, if it is pathogenic it will produce toxins. The two toxins that most toxigenic *C. difficile* strains produce are toxin A and toxin B. It is estimated that around 3% of isolates of *C. difficile* in the UK are toxin A negative and toxin B positive (A-B+). It was originally thought that both toxins were needed to cause disease; however recent studies have shown that A-B+ strains are

pathogenic (Alfa *et al.*, 2000, Barbut *et al.*, 2002).

#### **1.7.5.1 *C. difficile* toxins**

Toxin A and B were originally termed enterotoxin and cytotoxin respectively as it was observed that toxin A caused accumulation of fluid in intestinal loop models and toxin B caused cytopathic effects on tissue culture monolayers. However, it is now understood that the mode of action of both toxins is very similar; with both causing cell death by disrupting the actin cytoskeleton, after being endocytosed by the host cell (Poxton *et al.*, 2001).

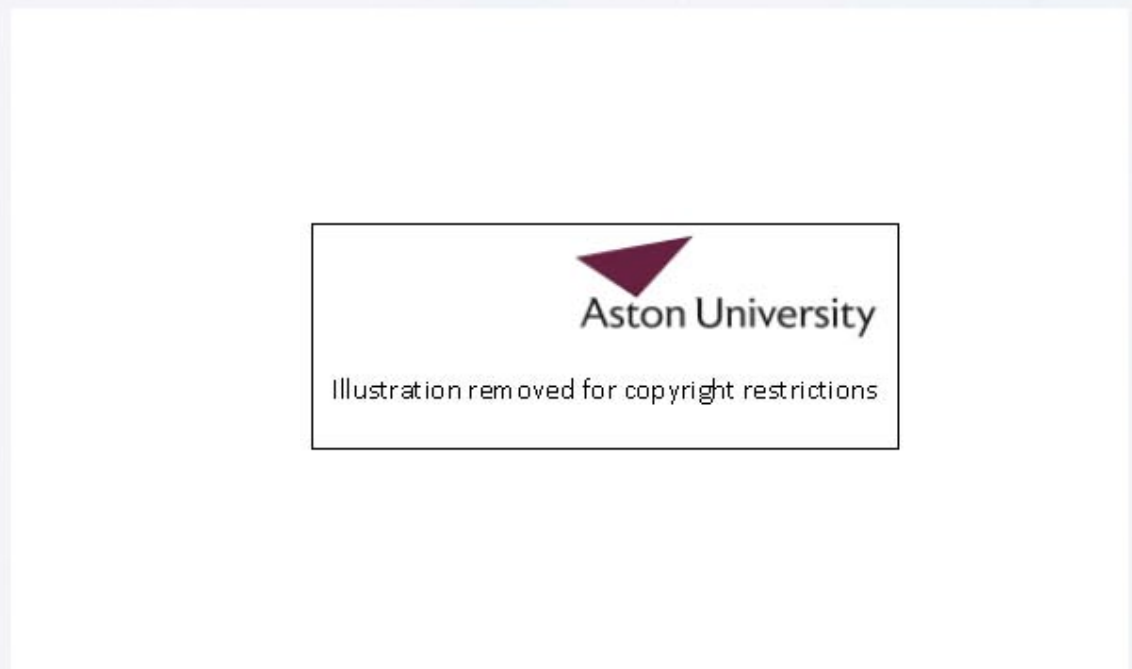
Synthesis of toxins A and B is cell density dependent and when there are sufficient cell numbers, toxin is produced. After entrance to the cytosol via passage through an intracellular compartment, the toxins act on the actin cytoskeleton. They cause cell rounding, as cell processes retract due to the disassembly of filamentous F-actin and an increase in G-actin. Before cell rounding occurs, the toxins act enzymatically to modify Rho proteins which regulate fiber assembly and actin polymerization. This process of modification is called monoglucosylation (Just *et al.*, 1995b, Just *et al.*, 1995a). The loss of functional Rho proteins and the breakdown of actin filaments causes a disruption of the barrier function, by opening the tight junctions between intestinal epithelial cells (Chaves-Olarte *et al.*, 1997). This increases permeability in the intestine and causes watery stools, which is characteristic of *C. difficile* antibiotic-associated diarrhea (figure 1.4) (Poxton *et al.*, 2001).

Toxin A and B also induce apoptosis of enterocytes. It is thought that toxin B triggers apoptosis by inhibiting cell anchorage and cell spreading and by inactivation of Rho proteins. Toxin A is suggested to induce apoptosis by the prevention of anchorage of epithelial cells to the basement membrane (Fiorentini *et al.*, 1998).



Both toxins also activate the immune system by stimulating the release of TNF $\alpha$  and activating macrophages and monocytes to release IL-8. This induces the movement of neutrophils from the blood circulation to the tissue, creating a chemotactic gradient that causes migration of neutrophils to the site of mucosal inflammation (figure 1.4).

Although both toxins have similar structures and mechanisms of action, they differ largely in their potencies. Toxin B is reported to be approximately 1000 times more potent than toxin A (Chaves-Olarte *et al.*, 1997). It is thought that the increased enzymatic activity of toxin B (at least 100 times higher) may contribute towards the difference in potencies (Poxton *et al.*, 2001).



**Figure 1.4 Pathogenesis of *C. difficile* toxins A and B on the host (modified from Poxton *et al.*, 2001)**

As toxins cause death of epithelial cells and activation of immune responses, a fluid consisting of fibrin and neutrophils escapes from blood vessels into the colonic lumen. The 'burst' of this fluid into the lumen is known as a volcanic lesion or

summit. Other histological changes may follow including diffuse cell death and ulceration (colitis) and development of a pseudomembrane consisting of mucin, fibrin, leukocytes and cell debris (Mylonakis *et al.*, 2001) (figure 1.5).



**Figure 1.5 Appearance of colon from a patient with pseudomembranous colitis (PMC); a consequence of *C. difficile* infection (Mylonakis *et al.*, 2001).**

#### **1.7.5.2 Genetic arrangement and structure of toxins A and B**

The genes encoding the two toxins of *C. difficile* (*tcdA* and *tcdB*) are located in the pathogenicity locus (PaLoc) along with genes encoding the proteins TcdC, TcdE and TcdR (figure 1.6). TcdE is thought to be integral in the release of the toxin into the environment. TcdR is fundamental for the initiation of transcription from the *tcdA* and *tcdB* promoters. TcdR acts as an RNA polymerase  $\sigma$  factor and initiates expression of itself. In contrast, TcdC is a negative regulator of toxin synthesis which acts by destabilizing the TcdR-containing holoenzyme-*tcdA* promoter complex. Deletions in this gene result in truncated proteins and it is thought that this may explain the increased level of toxin production in epidemic strains which carry these deletions (e.g. ribotype 027) (Dupuy *et al.*, 2008).



**Figure 1.6 Pathogenicity Locus of *C. difficile* (Dupuy *et al.*, 2008)**

Transcription and translation of the genes in the PaLoc results in the production of *C. difficile* toxins A and B. Toxin A has a molecular mass of 308kDa and toxin B 270kDa. The toxins are 50% identical at the amino acid level and each contain three functional domains. The C-terminal is the receptor binding domain and the middle section is the transmembrane domain, which is believed to be involved in the translocation of the toxin into the cytosol. The N-terminus codes for the enzymatic and cytotoxic effects of the toxins (figure 1.7) (Poxton *et al.*, 2001)



**Figure 1.7 Structure of the *C. difficile* toxins A (~308 KDa) and B (~270 KDa) (Poxton *et al.*, 2001)**

It has been found that some strains, including ribotype 027 produce an additional toxin; a binary toxin, termed CDT. This binary toxin is an actin-specific ADP-ribosyltransferase, which consists of two subunits; CDTa and CDTb. CDTa is responsible for the enzymatic activity of the toxin, where as CDTb is involved in the binding of the toxin to receptors on the surface of host cells. Once bound, the toxin is endocytosed and causes disruption of the cytoskeleton by catalyzing ADP-ribosylation of actin (Carter *et al.*, 2007). It is difficult to determine the role of this toxin in the pathogenesis of disease as only a few strains produce it. However, it is thought that this toxin could be an additional virulence factor due to its cytopathic effects on cell lines and the high degree of similarity displayed at the amino acid level to other clostridial toxins (Terhes *et al.*, 2004).

#### **1.7.6 Release of *C. difficile* spores and cells**

*C. difficile* diarrhoea is often explosive and therefore the release of its spores and cells is easily accomplished (Poxton, 2005). Spores of *C. difficile* act as a reservoir of infection and allow the organism to survive in aerobic conditions, contaminating the surrounding environment (Alfa *et al.*, 2008). Patients and HCW may easily acquire the organism by contact with a contaminated surface and the cycle may then begin again, thus spreading the infection.

### **1.8 Host natural defences**

A study by Pechine *et al.* (2005) found that during an episode of CDAD, *C. difficile* adhesions induce an immune response which may play a role in the defence mechanisms of the host. Sera from patients with CDAD and sera from a control group were compared for antibody titers directed against *C. difficile* toxins A and B



and surface proteins. It was found that in the CDAD group antibodies directed against four surface antigens of *C. difficile* decreased in turn and were significantly lower than those in the control group. However, there was no significant difference in antibody levels for toxin A and B between the control and test group (Pechine *et al.*, 2005).

Other studies have focused on immune responses directed against non-toxic antigens. Drudy *et al.* (2004) studied serum antibody levels in patients with CDAD, carrier patients and in a control group. There was no significant difference in serum IgM, IgG or IgA antibodies between the three groups tested, except in patients with recurrent episodes of CDAD. In patients that had suffered a relapse, levels of IgM antibody were much lower (Drudy *et al.*, 2004). Another study by Mulligan *et al.* (1993) found higher serum IgA and IgM antibody levels directed against somatic cell antigens in asymptomatic carriers, than in patients with CDAD (Mulligan *et al.*, 1993), however these results were not significant. Similar results were found in a study by Warny *et al.* (1994), who recorded lower serum IgG and faecal IgA antitoxin A antibody titers in patients with relapsing CDAD, in comparison with those suffering from a single episode of CDAD (Warny *et al.*, 1994). The authors also suggested that the patients suffering relapses have defective antibody responses to *C. difficile* toxin due to their underlying illness (Giannasca and Warny, 2004). To test if relapsing patients had a defective antibody response to *C. difficile* toxin as a result of their illness a study was conducted involving six chronic relapsing children. Treatment with intravenous gamma globulin resulted in an increase in IgG, but not IgA antitoxin A and a resolution of symptoms. These results suggest that a deficiency of IgG anti-toxin A may predispose children to the development of chronic relapsing *C. difficile*-induced colitis (Leung *et al.*, 1991).

## **1.9 Clinical Manifestations of *C. difficile* associated disease**

The severity of CDAD is widely variable; the spectrum includes diarrhoea, colitis without pseudomembrane formation, PMC, fulminant colitis and death (Worsley, 1998).

A mild form of the disease is associated with lower abdominal cramps, but no systemic symptoms. A moderate form of the disease is characterized by abdominal pain and profuse diarrhoea; usually without bleeding. Other symptoms include fever, malaise, nausea and anorexia. There may also be evidence of patchy or diffuse colitis and faecal leukocytes (Mylonakis *et al.*, 2001). Around 1-3% of patients develop fulminant colitis, along with toxic megacolon, perforation and death. These potentially fatal complications may be accompanied with a decrease or absence of diarrhoea, due to loss of muscle tone and ileus, which may be deceiving. Other pathological features may include chronic diarrhoea, fever, hyperpyrexia, hypoalbuminaemia and fluid accumulation. Some patients demonstrate mucosal thumbprinting (wide bands transversely spanning folds of the colon and associated with thickening of the colon) (Kawamoto *et al.*, 1999). Endoscopy often reveals multiple yellow plaques of varying diameter, raised above the mucosa (Oldfield, 2004). In a minority of patients, a reactive form of arthritis may develop one to four weeks after developing colitis (Keating and Vyas, 1995).

## **1.10 Laboratory Diagnosis**

Laboratory diagnosis of *C. difficile* infection is based on detection of toxin in the faeces of patients and culture of the faecal sample. Unfortunately laboratories differ throughout the UK in that some only use a single toxin detection test or a single immunoassay, where as others use both toxin detection and culture (Barbut *et al.*,

2003). Current UK guidelines suggest the use of enzyme immunoassays or cell cytotoxic assays to detect *C. difficile* toxin (Anon, 2004). Culture is not recommended and it is thought that this may be due to the associated cost and specialized training required for culturing *C. difficile* samples (Fitzpatrick *et al.*, 2008).

Culture is performed on selective cycloserine cefoxitin fructose agar (CCFA) plates, sometimes with the addition of sodium taurocholate as this enhances the recovery of *C. difficile* spores (Bowman and Riley, 1988). Culture is a very sensitive method; however it lacks specificity due to the possibility of isolation of non-toxigenic strains and is a slow process; requiring up to 48 hours of incubation (Delmee *et al.*, 2005). However, Delmee *et al.* (2005) found that culturing recovered 3.4% (335 samples in total) of toxigenic *C. difficile* stool samples which would not have been identified with toxin detection alone.

Toxin testing is more specific than culturing as only toxigenic strains are isolated. It involves inoculating a cell culture (usually HeLa cells) with filtrate of a stool suspension and then observing any cytopathic effects. Enzyme immunoassays (EIA) are also used as they produce results very rapidly; however they have slightly less sensitivity and specificity (Mohan *et al.*, 2006). In a recent study the sensitivities of four enzyme immunoassays (three toxin-detecting EIA and one detecting glutamate dehydrogenase) were compared to culture together with a real-time PCR method for detecting the *tcdC* gene in stool samples. All enzyme immunoassays were found to have unacceptably low sensitivities, compared to culture, whereas the PCR method was found to have high sensitivity and specificity (Sloan *et al.*, 2008). Another study also found real-time PCR involving amplification of the *tcdB* gene to be the method of choice, with greater specificity than enzyme immunoassays (van den Berg *et al.*, 2007). Rapid and accurate diagnosis of *C. difficile* patients is vital in order to isolate and treat patients quickly and effectively.

## 1.11 Phenotypic and genotypic characterisation of *C. difficile*

The first methods used to type *C. difficile* strains were based on phenotypic characteristics, such as antibiograms and serotyping. However, these methods have low discriminatory power and more powerful methods are needed to understand the epidemiology of *C. difficile* beyond a local level (Brazier, 1998). Molecular typing methods are therefore generally preferred as they provide higher levels of typeability and stability of marker expression. Restriction endonuclease analysis (REA) and Restriction fragment length polymorphism (RFLP); which involves restriction endonuclease digestion followed by gel electrophoresis, are both very reproducible and discriminatory methods. However, they are also labour intensive and have generally been replaced by methods based on the polymerase chain reaction (PCR) (Brazier, 1998).

Arbitrarily primed PCR (AP-PCR) has been used to type *C. difficile*, however it lacks reproducibility and a more discriminatory method, known as PCR ribotyping is generally preferred. PCR ribotyping involves using primers which target specific regions of the RNA operon of the organism (Brazier, 1998). This method has been adapted and amplified and is used routinely by the Anaerobe Reference Laboratory in Cardiff, UK. A library has assembled comprising of over 200 distinct ribotypes from over 3000 strains (Brazier, 2001). In the UK the three most predominant ribotypes are 106, 027 and 001 (HPA, 2006). In 1999 the Anaerobe Reference Unit stated that PCR ribotype 1 was the cause of approximately 55% of CDAD in England and Wales (Stubbs *et al.*, 1999). However, a recent report by the Health Protection Agency has now found that the number of infections due to type 001 has declined and that ribotype 027 is now responsible for the majority of cases of CDAD (41.2%) in England (HPA, 2008).

Pulsed field gel electrophoresis (PFGE) is a highly discriminatory method that



allows the whole genome to be analyzed after digestion by restriction enzymes. However, it is slow, expensive and complex and therefore PCR ribotyping is usually the preferred choice.

Other typing methods include sequence typing, toxinotyping and an alternative PCR target; the flagellin gene *flicC*. A study by Kato *et al.* (2005) found that it was possible to type strains identified in causing frequent outbreaks in Japan by sequencing the surface layer protein gene (*slpA*) of a single PCR ribotype (Kato *et al.*, 2005). This method has been shown to be reliable, reproducible and useful when culturing is not appropriate. Recently, a large collaborative study compared seven typing techniques for *C. difficile*, including surface layer protein A gene sequence typing, multilocus variable-number tandem-repeat analysis (MLVA), amplified fragment length polymorphism (AFLP), PCR ribotyping, REA, multilocus sequence typing (MLST) and PFGE. All methods were capable of detecting outbreak strains, however, only MLVA and REA displayed sufficient discriminatory power to distinguish between strains from different outbreaks. There have been issues raised as to the use of MLVA in strain tracking as size differences produced by capillary electrophoresis between laboratories have been found, making comparisons difficult (Killgore *et al.*, 2008).

Typing of *C. difficile* strains is particularly important as it allows the geographical spread of outbreaks to be traced, the number of people infected by a particular strain can be identified and it allows recognition of certain virulent types (Delmee *et al.*, 2005, Killgore *et al.*, 2008).

## 1.12 Sporulation and spore structure

### 1.12.1 Sporulation

Spores are produced in response to starvation, through a process known as sporulation, which comprises several stages. The process begins with the uneven division of a cell, producing two sister cells; one distinctly larger than the other. The next stage is the completion of the spore septum, separating the two cells, before engulfment of the small pre-spore by the larger mother cell. A cortex layer (modified form of cell wall) is then produced between the membranes of the pre-spore and at this stage the thick, proteinaceous spore coat is also formed on the outer surface of the spore. The next stage involves maturation of the spore, with the development of resistance properties and dormancy. Finally the mature spore is released by lysis of the mother cell (Errington, 1993).

Spores are capable of surviving extremes in pH, temperature, radiation, desiccation and some toxic chemicals and are able to monitor their environment, so that when conditions are once again acceptable for growth, they can germinate back into vegetative cells. In general spores are able to survive far longer than growing cells of the same species. The reason for this is that spores are equipped with certain structural features which enable them to withstand harsh environmental conditions. These features include a thick proteinaceous spore coat, with reduced permeability to hydrophilic agents, reduced water content in the spore core, small acid-soluble proteins (SASP) which protect spore DNA against damage and repair of damaged DNA during spore germination (Loshon *et al.*, 2001). Spores of *C. difficile* have been found to survive up to 56 days, in temperatures of 4°C and 20°C (Freeman and Wilcox, 2003).

### 1.12.2 Spore structure

The general structure of a spore includes the central core surrounded by several layers (starting from the innermost layer to the outer layer); the inner membrane, germ cell wall, cortex, outer membrane, coats and the exosporium (Setlow, 2006) (figure 1.8). The proteinaceous exosporium in clostridial species is thought to be involved in spore attachment. Panessa-Warren *et al.* (1997) used Scanning Electron Microscopy (SEM) to demonstrate the attachment of activated clostridial spores to agar by delicate extensions of the exosporium (Panessa-Warren *et al.*, 1997). The spore coat is a complex protein structure, consisting of several layers and containing many disulphide bonds (Gould, 2006). It protects the spore from certain chemicals and lytic enzymes; however it does not aid in the resistance to radiation, heat or other chemicals. The outer membrane also does not provide protection against heat or radiation, as removal of it has little effect on the resistance of the spore. Little is known about the exact role of the outer membrane, although it is fundamental to the structure of the spore (Setlow, 2006). Beneath the outer membrane is the spore cortex which consists of peptidoglycan. This structure is fundamental to the formation of the spore and is responsible for the dehydration of the spore core. During germination, the cortex must be degraded in order for the expansion and outgrowth of the spore core to occur. Underlying the cortex is the germ cell wall; another layer consisting of peptidoglycan, but structurally different from that of the cortex. Beneath the germ cell wall is the inner membrane which acts as the main permeability barrier to chemicals, especially DNA-damaging substances. In the early stages of germination this membrane expands from its compressed state during dormancy and doubles in size and lipid molecules contained in the membrane become mobile. At the very centre of the spore is the core which contains the spores DNA, tRNA, ribosomes and most of its enzymes. The very low water content of the core is a major factor contributing to its resistance to heat and chemicals (Setlow,

2006).



**Figure 1.8 Spore structure (Modified from Setlow, 2006)**

### **1.13 Spore germination**

Germination is generally triggered by certain nutrients, known as germinants. Germinants are usually single sugars, amino acids or purine nucleosides; however mixtures of nutrients can also trigger the germination process. Other germinants include bile salts, lysozyme, cationic surfactants (e.g. dodecylamine) and high pressure. The germinant has to overcome the barriers of the spore coat and the cortex layers in order to trigger germination. It has been suggested that receptor proteins are present on the inner membrane and that the germinant must bind to these receptors in order to stimulate germination. Germinant receptors in *Bacillus* species and their operons have been studied extensively; however, those in *C. difficile* are yet to be identified. Experiments with germination mutants in *B. subtilis* suggested that spores possess germinant-specific receptors. As most species that produce spores contain more than one receptor operon it has been suggested that spores possess many types of receptor that enable the spore to become stimulated



by different germinants (Moir, 2006).

Once a spore has been triggered by a germinant, it will continue to germinate, even if the germinant is removed. When the germinant binds it is suggested that allosteric changes of the membrane proteins occur as evidence suggests that metabolism of the germinant and bulk transport do not occur. Germination has also been shown to occur more rapidly when spores have been subjected to a mild heat treatment in the presence of other germinants (Gould, 2006).

The process of germination occurs in two stages; in the first stage, initially, hydrogen ions, zinc ions and monovalent cations are released. This is followed by a release of dipicolinic acid (DPA) with divalent cations. The release of DPA from the spore core is then replaced with water, thus hydrating the core. At this point the core is insufficiently hydrated for enzymatic action or mobility of proteins. The second stage is the hydrolysis of the peptidoglycan cortex and finally the swelling of the core and expansion of the germ cell wall, due to additional water uptake. Enzyme action is now restored after sufficient hydration of the spore core, allowing macromolecular synthesis, which converts the germinated spore into a growing cell (Setlow, 2003).

#### **1.13.1 Germination of *C. difficile* spores**

There has been little research into the germination of Clostridial spores, in particular *C. difficile*, unlike the germination of *Bacillus* spores, which has been studied extensively. Until recently, most knowledge of the germination of *C. difficile* spores had originated from improvements to increase the recovery of *C. difficile* from environmental samples (Wilcox *et al.*, 2000, Wilson *et al.*, 1982, Wilson, 1983). Wilson *et al.* (1982) first reported that incorporation of 0.1% sodium taurocholate into cycloserine cefoxitin fructose agar (CCFA), in place of egg yolk enhanced recovery of *C. difficile*. A year later Wilson described how the purity of the sodium taurocholate

used affected the percentage germination of *C. difficile* spores, with the purest form causing maximum germination. In this study Wilson also stated that cholate and desoxycholate stimulated germination in both the agar medium and in broth. When *C. difficile* spores were incubated overnight in CCFA broth with taurocholate and desoxycholate (at  $1.9 \times 10^{-3}\text{M}$ ) they stimulated 90 and 82% germination respectively (measured by phase contrast microscopy). Spores incubated with sodium taurocholate alone in distilled water did not germinate and sodium taurocholate was the only bile salt that both stimulated germination, but did not inhibit vegetative cells. Other studies have described the increased recovery of *C. difficile* spores on agar containing lysozyme (Wilcox *et al.*, 2000, Nakamura *et al.*, 1985). Pre-exposure of samples to alkaline thioglycollate before plating onto lysozyme-containing agar is also reported to increase recovery of *C. difficile* spores (Kamiya *et al.*, 1989, Nakamura *et al.*, 1985) However, Wilcox *et al.* (2000) found thioglycollate to have no beneficial effect, only lysozyme incorporation and cooked meat broth enrichment was found to increase recovery of *C. difficile* (Wilcox *et al.*, 2000).

Studies by Freeman *et al.* (2003) and Freeman *et al.* (2005) found that cefotaxime and clindamycin respectively induced germination of *C. difficile* spores in a gut model. Cefotaxime exposure caused rapid germination of *C. difficile* spores, however germination was not seen during clindamycin exposure; it was only 8 days after the antibiotic was withdrawn that germination was observed. Freeman *et al.* (2005) suggest that in order for germination and subsequent toxin production to occur, *C. difficile* spores must be exposed to a CDAD-precipitating antibiotic and that its concentration must be below the MIC for *C. difficile* (Freeman *et al.*, 2003, Freeman *et al.*, 2005).

The only studies to date which have investigated germination of *C. difficile* in detail are that by Sorg and Sonenshein (2008) and by Paredes-Sabja *et al.* (2008). Sorg and Sonenshein (2008) found that taurocholate was the only bile salt to induce

rapid germination of *C. difficile* spores. Taurocholate and glycine were described as co-germinants as together they stimulated germination in *C. difficile* spores, but alone they were ineffective. Cholate and glycocholate were also found to enhance colony formation in *C. difficile*; however in solution they were not capable of inducing rapid germination. Deoxycholate was found to inhibit growth of *C. difficile* vegetative cells and therefore the authors suggest that in the normal healthy gut the bacterial flora prevent the growth of *C. difficile* by converting cholate derivatives to deoxycholate.

In contrast, Paredes-Sabja *et al.* (2008) found that none of the bile salts or the 20 amino acids tested had a significant effect on the germination of *C. difficile* spores. However, potassium chloride (KCl), dodecylamine, inorganic phosphate ( $P_i$ ) and a 1:1 chelate of DPA and  $Ca^{2+}$  all stimulated germination of *C. difficile* spores. The authors therefore suggest that in the body, *C. difficile* spores germinate by sensing  $P_i$  in the duodenum and KCl in the colon.

## **1.14 Treatment of *C. difficile* associated disease**

### **1.14.1 Antibiotics**

Although there has been a substantial amount of research into the diagnosis, control and epidemiology of CDAD, *C. difficile* continues to be a leading cause of nosocomial infectious diarrhoea. At present, treatment of *C. difficile* has not advanced very far in the last ten years; however, some promising new options are currently being explored. The slow progress may be due to the difficulty in determining the efficacy of existing and new drugs. This difficulty arises from the heterogeneous nature of hospital acquired infection, the presence or absence of colitis/ pseudomembranous colitis and the wide variation in the diagnostic methods used (Wilcox, 1998).



The need to find new, alternative treatments for CDAD has arisen from the apparent high relapse rate when initial, conventional therapy is discontinued, increased usage of antibiotics, the increased incidence of CDAD, selection and overgrowth of vancomycin-resistant enterococci (by both oral vancomycin and metronidazole) (Al-Nassir *et al.*, 2008) and the need to reserve vancomycin for treatment of MRSA. Most relapses are due to re-infection with new strains of *C. difficile*, whilst other recurrences of infection may be due to the survival of spores from the original bacteria that were not killed by the antibiotic. If the microflora of the gut has not had sufficient time to re-establish itself, it is susceptible to overgrowth and spores may germinate, colonise the intestine once again and produce damaging toxins. Some patients are more likely than others to suffer a relapse, such as the elderly and those who have recently undergone abdominal surgery (Young *et al.*, 1986).

The initial treatment of CDAD is the termination of the precipitating antibiotic whenever possible or the switch to a narrow spectrum antibiotic. In cases of mild diarrhoea this may be adequate to resolve symptoms; however, most require further treatment with one of two antibiotics; metronidazole or vancomycin. Specific treatment is indicated when symptoms persist after discontinuation of the precipitating antibiotic, or when cessation of the inciting antibiotic is not possible due to the underlying condition or systemic symptoms. It can be a very difficult to decide when to commence treatment as infection usually occurs in elderly patients and it is not possible to predict who will recover quickly, or who will have protracted disease and so delaying treatment may have very grave consequences (Wilcox, 1998).

Hydration and electrolyte replacement therapy may also be required in conjunction with antibiotics in severe cases and in the young and elderly. Antiperistaltic drugs, such as loperamide and diphenoxylate are advised against as they may cause toxic megacolon and slow removal of the organism from the



intestine (McFarland, 2005). Another factor of successful treatment is the rapid diagnosis of *C. difficile*. In severe cases of CDAD, such as those with fulminant colitis, surgery may also be a viable treatment option (Thompson, 2008).

#### **1.14.2 Metronidazole**

Oral metronidazole (400 mg) has been used to treat initial onset of CDAD, over a period of 7-10 days, three times a day (Wilcox, 1998). Wenisch *et al.* (1996) found that in a randomized trial, 94% of patients with an initial *C. difficile* infection recovered after treatment with metronidazole. However, subsequent trials have shown an increasing number of failures and recurrences of infection with metronidazole; with 16-38% of patients suffering from treatment failure after 2004 and 28-29% suffering from a relapse of infection in 2005 (Aslam *et al.*, 2005, Balagopal and Sears, 2007). Patients that are least likely to respond to treatment with metronidazole are those that are critically ill (intensive care patients) and those with low albumin levels (Wenisch *et al.*, 1996). Disadvantages in the use of metronidazole are its side effects, the development of resistance and recurrence rates of disease. The side effects that may be experienced include: nausea, metallic taste and in some cases neurotoxic complications (McFarland, 2005).

Peláez *et al.* (2002) reviewed data on the rate of resistance to metronidazole. In 1994 a 6% rate of resistance to metronidazole was reported and in 1998 9% of strains were found to be resistant. Highest rates of resistance were found among isolates from infected HIV patients. It is postulated that this is due to a higher probability of exposure to the antibiotic on previous occasions (Pelaez *et al.*, 2002).

Overall metronidazole has exhibited high efficacy in treatment of initial episodes of initial *C. difficile* infection both orally and intravenously. It is cost efficient and avoids the use of vancomycin, which should be used as a last resort (Pelaez *et*

*al.*, 2002).

### **1.14.3 Vancomycin**

The typical dosage of vancomycin given is 125 mg, four times a day, for 7-10 days (Wilcox, 1998). In a study by Wenisch *et al.* (1996) treatment failure with vancomycin occurred in 6% of patients, with 16% suffering a relapse of infection. In a more recent study by Pepin *et al.* (2005) a larger proportion (28%) of patients had a relapse of CDAD following treatment with vancomycin (Pepin *et al.*, 2005, Aslam *et al.*, 2005). Advantages for its use are that it has a high efficacy against *C. difficile*, it has no systemic absorption and has very few side effects, except for a fairly common rash (McFarland, 2005). Unfortunately, vancomycin is very expensive, with a 10-day course costing approximately £120, compared to only £2 for metronidazole (Wilcox, 1998). Other disadvantages include the growing concern over the selection of vancomycin-resistant *Enterococcus faecium* and the need to reserve its use for treatment of MRSA (Wenisch *et al.*, 1996, McFarland, 2005). Pepin and colleagues found that since the emergence of the 027 strain, there was no difference between the efficacy of vancomycin and metronidazole for the treatment of CDAD (Pepin *et al.*, 2007).

### **1.14.4 Other antibiotics**

Many studies have compared effectiveness of various other antibiotic treatments to combat CDAD, but none have shown significant superiority over vancomycin or metronidazole.

#### **1.14.4.1 Fusidic acid**

Fusidic acid has a narrow spectrum of activity. It works by inhibiting protein

synthesis and is active against a range of Gram-positive bacteria and a small number of Gram-negative bacteria, both aerobic and anaerobic. It is particularly effective against staphylococci, including strains that are resistant to other antibiotics (Collignon and Turnidge, 1999). In a randomized trial fusidic acid treatment gave an initial cure rate of 93% of patients, which was equal to the performance of vancomycin and metronidazole. A higher rate of recurrence was found with fusidic acid (28%) compared with vancomycin (7%) and metronidazole (16%), however this difference was not significant. There was also a higher rate of asymptomatic carriage observed; with 24% carriage in those treated with fusidic acid, compared with only 13% and 16% in those treated with vancomycin and metronidazole respectively (Wenisch *et al.*, 1996).

#### **1.14.4.2 Teicoplanin**

Wenisch *et al.* (1996) described lower rates of recurrence and asymptomatic carriage of *C. difficile* in patients treated teicoplanin, compared to those treated with vancomycin, metronidazole or fusidic acid. However, the report concluded that metronidazole was probably the drug of choice as differences in recurrence rates and carriage rates were not significant and metronidazole was inexpensive.

In 1996 a study involving in-vitro testing of *C. difficile* with several antibiotics found teicoplanin to be four times more potent than vancomycin and it had the lowest MIC range (Wongwanich *et al.*, 1996).

In a recently updated review that compared all randomized, controlled trials that assessed antibiotic treatment for CDAD, it was concluded that teicoplanin was the best choice of treatment. The report suggests that from the evidence teicoplanin is superior over the other antibiotics as it is better at preventing the spread of the organism and has marginal superior effectiveness in terms of symptomatic cure as it

produces the highest rates of biological and symptomatic cure. However, this antibiotic is expensive and is not widely available (Bricker *et al.*, 2005, Nelson, 2007).

#### **1.14.4.3 Bacitracin**

Two early studies that assessed the effectiveness of bacitracin for treatment of *C. difficile* infection in comparison with vancomycin found that the two antibiotics were equally effective at resolving diarrhoea. However, in both trials it was found that bacitracin was less effective at eradicating *C. difficile* and its toxins from patients' stools (Young *et al.*, 1985, Dudley *et al.*, 1986). In the trial by Dudley *et al.* (1986) 29% of patients treated with vancomycin had either the bacterium or its toxins in their stools, compared to 70% of patients treated with bacitracin. Slightly higher recurrence rates were also noted in patients given bacitracin, compared with those receiving vancomycin. In a later study the in-vitro activity of bacitracin against *C. difficile* was found to be low in comparison with other antimicrobials including teicoplanin, metronidazole and vancomycin (Citron *et al.*, 2003).

#### **1.14.4.4 Ramoplanin**

A study by Citron and colleagues (2003) found that ramoplanin displayed excellent *in-vitro* activity against a panel of *C. difficile* isolates, with minimum inhibitory concentration (MIC) values below that of vancomycin and metronidazole. The authors also suggest that ramoplanin may be less damaging to the gut flora as it has shown poor in-vitro activity against most Gram-negative anaerobes.



#### **1.14.5 Effects of sub-minimum inhibitory concentrations (MIC) of antibiotics on growth and toxin production of *C. difficile***

A study by Drummond *et al.* (2003) identified that the relationship between the effects of growth and toxin production was very complicated and was strain-dependent. In most cases the effects of sub-MIC concentrations of antibiotics were the increase in the initial lag period of growth of *C. difficile* (by about 4 hours) and the onset of toxin elaboration (of toxin A) (Drummond *et al.*, 2003).

#### **1.14.6 Non-antibiotic treatments**

The need to find new, alternative treatments for CDAD has arisen from the apparent high relapse rate when initial conventional therapy is discontinued, increased usage of antibiotics, increased incidence of CDAD, selection of vancomycin-resistant *Enterococcus faecium* and the need to reserve vancomycin for treatment of MRSA. Also most antibiotics only target vegetative cells and do not help to restore the normal protective flora of the gut. Other possible therapy options for treatment of CDAD include: probiotics, adsorbents (including ion-exchange resins and certain polymers), immune products, faecal enemas and bowel irrigation (McFarland, 2005).

##### **1.14.6.1 Probiotics**

Probiotics are living organisms in either mono or mixed cultures, which have specific therapeutic properties and inhibit growth of pathogenic bacteria (D'Souza *et al.*, 2002). The normal healthy gut has colonization resistance, which prevents pathogenic bacteria from attaching to the gut and causing disease. When the balance is disrupted due to antibiotics or other disruptive factors such as volatile fatty acids or a decrease in pH, the luminal wall is susceptible to infection by pathogenic

bacteria. Probiotics help to re-colonise the gut with non-pathogenic bacteria, thus preventing the attachment of harmful bacteria. The specific modes of action of probiotics are not fully understood. As well as preventing adherence of pathogens, other possible mechanisms may include: the stimulation of immune responses, the production of antimicrobial substances, effects on mucin secretion, receptor competition, blockage of toxin production and the immunomodulation of gut-associated lymphoid tissue (Surawicz, 2003, Pillai and Nelson, 2008).

*Lactobacillus* species and *Saccharomyces boulardii* are the most common cultures used for probiotics and are found in cultured and fermented food and drinks. Side effects associated with the use of probiotics are rare, but fungaemia and bacteraemia have been reported in patients with severe underlying illness and careful consideration should be given before treating immunocompromised patients with these agents (Pillai and Nelson, 2008, Pham *et al.*, 2008).

In a recent review all studies involving the use of probiotics alone or together with an antibiotic for the treatment of CDAD were evaluated. The authors concluded that none of the studies recommended the use of probiotics alone in the treatment of CDAD and the studies which demonstrated the beneficial effects in the use of probiotics as an adjunct to antibiotic treatment were too small and lacked statistical power (Pillai and Nelson, 2008).

#### **1.14.6.2 Faecal enemas and bowel irrigation**

Several reports have highlighted the successful treatment of recurring CDAD with faecal enemas (Tvede and Rask-Madsen, 1989, Schwan *et al.*, 1984). In the investigation described by Tvede and Rask-Madsen (1989) rectal instillation of a mixture of facultatively aerobic and anaerobic bacteria diluted in sterile saline resulted in rapid loss of *C. difficile* and its toxins from patients stools. Colonisation of

Bacteroides species was also noted that were not present prior to treatment, thus suggesting that their presence may prevent colonization of *C. difficile*. Donor stools are often given by the partner of a patient and the main disadvantages to this treatment are the possibility of infection from the donor stool and the acceptability of this crude method (Thompson, 2008).

#### **1.14.6.3      Adsorbents**

Another form of treatment is the binding of *C. difficile* toxins in the lumen of the gut, before they are able to cause disease. Various different adsorbents have been tested including ion-exchange resins and certain polymers.

#### **1.14.6.4      Ion-exchange resins**

A randomized, placebo-controlled trial involving 38 patients with post-operative diarrhoea found that the binding agent colestipol had no effect on the faecal excretion of *C. difficile* or its toxin. On the basis of their study the authors recommended that colestipol should not be used to treat antibiotic-associated colitis (Mogg *et al.*, 1982). In another study, the binding ability of colestipol and cholestyramine to *C. difficile* toxin was evaluated. Cholestyramine reduced toxin levels in stools due to binding and delayed death in the hamster model of clindamycin-induced cecitis. However, both resins were found to bind to vancomycin and therefore caution was advised when prescribing both resin and vancomycin in combination (Taylor and Bartlett, 1980).

#### **1.14.6.5      Immune products**

The immune response in the host may be a significant predictor of

recurrences of CDAD. A study by Leung *et al.* (1991) found that treatment of chronic relapsing patients with intravenous gamma globulin resulted in an end to their symptoms and a clearing of toxin B from their stools, as well as an increase in IgG antitoxin A levels. Similar results have also been found in other studies, where treatment of adults with intravenous human immunoglobulin has resulted in a resolution to symptoms and an increase in IgG antitoxin A and B levels (Salcedo *et al.*, 1997, Wilcox, 2004).

Randomized clinical trials with immunoglobulin treatment have not, as yet, been conducted. Many studies lack relevant control groups and dosing regimes and preparations of commercial pooled immunoglobulin have not been standardized (McFarland, 2005).

Vaccination against one or both of the toxins is a possible treatment for the future, although the elderly may be least likely to respond to this preventative measure (Thompson, 2008). Recently, a novel human monoclonal antibody directed against toxin A of *C. difficile* has been produced (CDA1), which has shown to be effective in preventing mortality from *C. difficile* disease in hamsters. A phase I study with healthy volunteers has found CDA1 to be well tolerated with increasing doses leading to increasing serum CDA1 concentrations. At present, randomized trials involving patients with CDAD and treatment with CDA1 are being conducted (Taylor *et al.*, 2008).

#### **1.14.6.6 Polymers**

A polyanionic drug named Tolevamer has been tested for its toxin binding ability in the treatment of CDAD. Binding of Tolevamer was found to inhibit the biological activity of both *C. difficile* toxins A and B (Braunlin *et al.*, 2004). Another polymer named Synsorb that consists of oligosaccharide sequences attached to an



inert support has been suggested as a potential therapy for CDAD and pseudomembranous colitis. Results of neutralization experiments have found that Synsorb can effectively neutralize toxin A from stool samples (Heerze *et al.*, 1994). A development from this product is Synsorb 90, which consists of a trisaccharide attached to an inert support. The trisaccharide used is a receptor that toxin A has been found to bind to and Synsorb 90 therefore acts as a 'decoy receptor'. Results showed that when Synsorb 90 was administered to rats, there was a massive reduction in toxin A-associated fluid secretion and permeability (Castagliuolo *et al.*, 1996). Although these studies have found promising results, more research is needed if these agents are to be considered for clinical use.

### **1.15 Prevention of CDAD**

Worsley (1998) identified three key precautions which should be followed in order to prevent CDAD in hospitals, which included: the restricted use of antibiotics, thorough cleaning of the hospital environment and strict adherence to procedures when caring for patients with CDAD.

Following reports of an outbreak of CDAD in Montréal, Valiquette *et al.* (2004) commented that measures to control CDAD must be multi-faceted and identified a list of preventative measures to reduce transmission of infection. Such control measures included surveillance of *C. difficile* infection in order to identify emerging resistance, outbreaks of new strains and the evaluation of intervention procedures. In order to reduce transmission between patients it was identified that there should be isolation of *C. difficile* patients, use of separate equipment from other patients, adequate cleaning protocols, increased hand washing compliance and early diagnosis of infection. Other measures included rapid and effective therapy and protection of the gut flora (Valiquette *et al.*, 2004). A review of key research issues

regarding *C. difficile* infection also identified the importance of education and staffing issues as well as patient transfers and *C. difficile* infection in the community (Zhanel and Hammond, 2005). In addition to these measures the Healthcare Commission listed several recommendations to improving rates of CDAD after serious failings were identified at hospitals in Maidstone and Tunbridge Wells. These recommendations included prescribing the narrowest spectrum antibiotics for the shortest possible time, improvement of staffing levels and staff training regarding infection control, improvement in recording *C. difficile* on death certificates, and appropriate reporting and investigation of incidents and risk analysis (Anon, 2007).

The effectiveness of a multifaceted approach to control CDAD was highlighted in a recent study which reported a large decrease of 66% in the number of cases of CDAD over a 24-month period after a series of interventions. Some of the interventions introduced included daily isolation reports, weekly reports by the infection control director, daily unit rounds with chief nurses, a 10% hypochlorite disinfection protocol and a dual chamber cleaning container to ensure the correction dilution of hypochlorite was delivered. The report emphasized that collaboration between all the staff at the hospital contributed significantly to the success of the interventions (Whitaker *et al.*, 2007).

#### **1.15.1 Contamination and cleaning**

Contamination of the environment with *C. difficile* spores is a major factor in the spread of the organism. After an outbreak of antibiotic-associated colitis in a hospital in Michigan, in 1984, environmental samples were taken from the ward. 31.4% of these samples were positive for *C. difficile* and the majority of these were the same bacteriophage-bacteriocin type (Kaatz *et al.*, 1988).

The choice of cleaning agent and the dilution used are extremely important as

a study by Wilcox and Fawley (2000) found that sporulation of *C. difficile* was enhanced when the organism was cultured in faeces containing sub-inhibitory levels of certain cleaning agents. Spores have been found to be resistant to many conventional cleaning agents (Settle and Wilcox, 1996) and many sporicidal agents are hazardous to the healthcare worker (HCW).

#### **1.15.1.1 Detergents verses disinfectants**

There is much conflict regarding the use of disinfectants to clean housekeeping equipment. The Public Health Laboratory Service (PHLS) (1994) stated that there was no evidence that any disinfectant was more effective in reducing environmental contamination than soap or detergent (Anon, 1994). The issue of microbes building resistance to disinfection has also been raised by authors supporting the use of detergents for environmental cleaning (Russell, 1999, Russell, 2004). Other authors have stated that detergents themselves can become contaminated and that the use of disinfectants has been shown to greatly reduce the level of environmental contamination (Barbut and Petit, 2001, Wilcox and Fawley, 2000). A report found that phosphate buffered hypochlorite was very effective at reducing contamination and when used there was a 98% reduction in surface contamination (Wilcox and Fawley, 2000). The Department of Health (DoH) has recently described (2007) guidelines and procedures that should be followed by HCWs in order to reduce hospital acquired infections such as *C. difficile*. Detailed in the guidelines are the recommendations that hypochlorite together with detergents should be used during outbreaks of infection to reduce environmental contamination with *C. difficile* (Pratt *et al.*, 2007). The theory behind this is that detergents are capable of removing organic material, such as blood; where as the activity of some disinfectants can be compromised in the presence of such material. Therefore, prior

cleaning with detergents can eliminate organic material, which allows disinfectants to work effectively on reducing the bacterial load, as they possess a much greater biocidal activity than detergents (Hota, 2004).

#### **1.15.1.2 Chlorine-based disinfectants**

The effective sporicidal activity of various chlorine-based disinfectants against bacterial spores is widely known (Bloomfield and Uso, 1985, Coates, 1996, Perez *et al.*, 2005, Kreske *et al.*, 2006) and many studies have shown a link between the use of chlorine-based cleaning agents and a decrease in CDAD (Kaatz *et al.*, 1988, McMullen *et al.*, 2007). Fawley *et al.* (2007) compared the efficacy of five cleaning agents against the vegetative cells and spores of *C. difficile* and found that only chlorine-based disinfectants were effective against *C. difficile* spores, compared to a detergent and a hydrogen peroxide-containing agent. In a study by Perez *et al.* (2005) spores of *C. difficile* were inactivated in 30 minutes or less with all chlorine-based agents tested, with the most rapid rate of inactivation occurring within 10 minutes with hypochlorite solutions at 5000mg/LFC (free-chlorine level) (Perez *et al.*, 2005). In contrast Block (2004) found that sodium dichloroisocyanurate, containing 1000ppm of available chlorine produced less than a 1 log reduction in viable spores after 10 minutes exposure.

Unfortunately chlorine is associated with various hazards to the HCW which have been highlighted in occupational health reports: Guidelines issued by the Center for Disease Control (CDC) state that the permissible exposure limit for chlorine is 1ppm and that overexposure can result in irritation of the skin, eyes and mucous membranes (CDC, 1981).



#### **1.15.1.3 Peracetic acid-based disinfectants**

Peracetic acid is an alternative disinfectant which also demonstrates sporicidal activity at 500-10000ppm, with inactivation of bacterial spores occurring in 15 seconds to 20 minutes (Rutala and Weber, 1999). Furthermore, in a study by Block (2004), it was shown that a peracetic acid-based disinfectant achieved over a 6 log<sub>10</sub> reduction factor in *C. difficile* spores in 10 minutes, compared to a chlorine releasing agent which achieved only a 0.7 log<sub>10</sub> reduction in the same period (Block, 2004). The advantages of peracetic acid-based disinfectants are that they produce environmentally friendly by-products and they can be used on a variety of materials and surfaces (Rutala and Weber, 1999). However, these disinfectants have a pungent odour (unless a neutralising agent is used) and there is a risk of skin and eye damage in their concentrated form (Rutala and Weber, 1999, Kitis, 2004).

#### **1.15.1.4 Other cleaning agents**

A study by Wullt *et al.* (2003) found that acidified nitrate and glutaraldehyde demonstrated sporicidal activity against *C. difficile*, with a mean inactivation factor of over 4 in 30 minutes exposure, both in the presence and absence of organic matter. Acidified nitrate is not thought to be hazardous as it is produced by mammalian cells; however, glutaraldehyde has been reported to cause asthma and dermatitis (Wullt *et al.*, 2003).

A new method for decontaminating the clinical environment, involving the use of hydrogen peroxide vapour (HPV) has been described by Boyce (2007). A 10-month trial of HPV in a hospital infected with *C. difficile* ribotype 027 found that all samples collected after decontamination were negative for *C. difficile*. A decrease in the number of new cases of *C. difficile* was also observed during the trial period (Boyce, 2007).

#### **1.15.1.5 Education and feedback, and standards of cleanliness**

Education of the HCW is also important as inadequate cleaning and mistakes in preparation may lead to insufficient removal of surface contamination, thus facilitating the spread of the organism. A study by Eckstein *et al.* (2007) found that after a simple intervention involving the education of house-keeping staff about cleaning, there was a reduction in the level of contamination of the hospital environment. The authors suggest that contamination and cleaning should be monitored and that feedback should be given to housekeeping staff. However, the rates of CDAD were not measured during the study and so it is not clear whether the reduced level of contamination led to a decrease in the number of cases of CDAD (Eckstein *et al.*, 2007).

In order to adequately assess cleaning in hospitals Dancer (2004) recommended that there should be internationally agreed standards, such as those which exist for water, air and food preparation surfaces. Dancer suggests that regular audits could then be carried out which would be based on scientific criteria, instead of current standards which only judge cleanliness in terms of whether an area is 'visibly clean' (Dancer, 2004).

#### **1.15.2 Germination theory: A new approach to cleaning?**

A recent study by Hornstra *et al.* (2007) described the method of germinating spores of *Bacillus cereus* adhered to stainless steel prior to cleaning in order to enhance the effectiveness of cleaning procedures. The principle of this method was that germinating spores become susceptible to sanitizing agents that dormant spores are usually resistant to. In this study the number of spores recovered after cleaning without a germination stage did not decrease from the number in the original inoculum. However, the incorporation of a germination step, prior to cleaning

produced a 3 to 4 log reduction in viable spores recovered, highlighting the effectiveness of this two-stage cleaning approach (Hornstra *et al.*, 2007). Further research must be conducted with other spore-forming bacteria in order to assess the potential of this cleaning strategy.

Another study has looked at the opposite approach; of inhibiting germination of spores to prevent development into vegetative cells which cause disease. The authors suggest that this method which has been applied to the prevention of food spoilage may have the potential to prevent the development of CDAD in vulnerable patients by preventing germination of *C. difficile* spores (Alvarez and Abel-Santos, 2007)

### **1.16 Designing out infection: antimicrobial surfaces**

The antimicrobial properties of metals, in particular copper have been well established for many years. Recently there has been increased interest into metallic coatings and impregnated textiles and surfaces which demonstrate antimicrobial activity (Chung *et al.*, 2008, Galeano *et al.*, 2003, Takai *et al.*, 2002). A recent study by Singh *et al.* (2008) has described the use of a silver impregnated amniotic membrane, possessing antimicrobial activity, as a burn dressing. In the study the dressing produced a 95% reduction in viable counts of various bacteria in 2-4 hours and release of silver was observed for up to 4 days (Singh *et al.*, 2008). In another study, a zeolite coating (containing 2.5% silver ions and 14% zinc ions) applied to stainless steel displayed rapid antimicrobial activity against *S. aureus*; a clinically relevant organism, within one hour of exposure (Bright *et al.*, 2002).

The intrinsic antimicrobial activity of copper has been utilized in the textile industry, in water purification and in paint and building materials (Mehtar *et al.*, 2008). Noyce *et al.* (2006) found that a strain of meticillin resistant *Staphylococcus*

*aureus* (MRSA) at a concentration of  $10^2$  CFU/mL was eliminated after 30 minutes exposure to copper and at a concentration of  $10^7$  CFU/mL was killed after 90 minutes. Stainless steel did not demonstrate any antimicrobial activity and strains of MRSA remained viable for up to 72 hours (Noyce *et al.*, 2006). Other alloys of copper were found to be less effective than pure copper at eliminating bacteria. Faundez *et al.* (2004) also demonstrated that stainless steel did not possess any antimicrobial activity; however, copper was found to be inhibitory towards *Salmonella enterica* and *Campylobacter jejuni*. A significant decrease in viability was observed between two and four hours of exposure to copper at 25°C, with longer periods of exposure required at 10°C (Faundez *et al.*, 2004). Another study that assessed the antibacterial properties of copper and copper alloys, found that copper eliminated *Escherichia coli* O157 in 90 minutes, at room temperature, compared to stainless steel in which *E. coli* remained viable for over 28 days. Copper alloys were also found to be effective, but required longer periods of contact (Wilks *et al.*, 2005).

The only study to assess activity of copper surfaces against *C. difficile* vegetative cells and spores is that by Weaver *et al.* (2008). In this study complete death of *C. difficile* spores was achieved within 24-48 hours and the authors suggest that the use of copper alloys in the clinical environment may contribute towards reducing *C. difficile* infection (Weaver *et al.*, 2008).



## 1.17 Aims and objectives

- Assess the efficacy of cleaning agents currently used in the clinical setting and compare with new and existing agents against the vegetative cells and spores of *C. difficile*.
- Determine the efficacy of sporicidal cleaning agents using a method to adequately simulate the clinical environment and assess the effect of a soil load on their activity
- Explore the physical and chemical factors which affect the germination of *C. difficile* spores and the agents required for germination of *C. difficile*.
- Determine the efficacy of 70% ethanol, stainless steel and copper to eliminate the vegetative cells, dormant spores and germinating spores of *C. difficile*.
- Produce novel compounds and assess their antimicrobial activity against the vegetative cells and spores of *C. difficile*.
- Compare the activity of novel compounds to existing antimicrobial agents against a panel of *C. difficile* isolates.

## 2 Antimicrobial efficacy of disinfectants against the spores and vegetative cells of *C. difficile*

### 2.1 Introduction

Patients with *Clostridium difficile* associated infection (CDI) excrete large numbers of both vegetative cells and spores of *C. difficile* in their faeces which contaminate the clinical environment as well as transiently colonizing the skin of healthcare workers and patients (Wilcox, 2003). An important factor in the prevention and control of CDI is efficient infection control measures which encompass both effective hand hygiene practices and environmental cleaning and disinfection.

Although it is accepted that environmental contamination with *C. difficile* spores is a major factor in the spread of infection, there has been controversy over which hard-surface cleaning products to use. Kaatz *et al.* (1988) and Fawley *et al.* (2007) demonstrated the efficacy of chlorine-based agents in eliminating *C. difficile* spores and Fawley *et al.* (2007) found them to be superior to a detergent (Hospec, Youngs Detergents) and a hydrogen peroxide based disinfectant. However, a systematic review in 2004 by Dettenkofer *et al.* revealed that the use of disinfectants in place of detergents did not result in lower infection rates of nosocomial infections in general or in *C. difficile* rates specifically (Dettenkofer *et al.*, 2004). The DoH recently produced a document detailing guidance on how to prevent *C. difficile* infection; identifying effective environmental decontamination as a fundamental factor in reducing rates of *C. difficile* infection (DoH, 2007). Here, it was specified that chlorine-based disinfectants or other sporicidal agents should be used in order to reduce contamination with *C. difficile* spores. Detailed in the guidelines are the recommendations that hypochlorite together with detergents should be used during outbreaks of infection to reduce environmental contamination with *C. difficile* (Pratt *et al.*, 2007). Indeed, the importance of the choice of cleaning agent and its use at the

correct concentration has been highlighted by Wilcox *et al.* (2000). In this study it was found that sub-inhibitory concentrations of non-chlorine-based cleaning agents including a non-ionic surfactant (Hospec), a quaternary ammonium compound (D4) and a monoethanolamine (D2) induced spore formation in *C. difficile* and therefore, contributed to environmental contamination of *C. difficile* spores.

Recently there has been increased interest into more 'natural' and non-hazardous products (Chen *et al.*, 2002, Velazquez *et al.*, 2007). A number of products for the cleaning and disinfection of hard surfaces based on ascorbic acid have been produced by GDM technologies Ltd, including Citrofresh®, which is promoted as non-toxic and eliminating 99.9% of bacteria upon contact.

In this initial part of the study, the efficacy of Citrofresh® was assessed against vegetative cells and spores of *C. difficile* along with two commonly used hospital cleaning agents, Chlor-clean®; a chlorine-based disinfectant and R2® cleaner; a hard surface cleaning agent containing didecyldimethyl ammonium chloride). In addition, a peracetic acid cleaning agent, Wofasteril®, was also assessed as the advantages of peracetic acid-based disinfectants in terms of their non-corrosive nature and the production environmentally friendly by-products have been highlighted (Rutala and Weber, 1999) together with their sporicidal activity against *C. difficile* spores (Block, 2004). The sporicidal activity of each disinfectant was assessed in accordance with BS EN 13704: 2002 standard guidelines.

## 2.2 Methods

### 2.2.1 Preparation of vegetative cell suspensions of *C. difficile*

Wilkins Chalgren agar (WCA) (Oxoid, UK) was inoculated with *C. difficile* NCTC 11204 and ribotype 027 from frozen bead cultures, stored at -70 °C (Microbank® beads, Pro-Lab Diagnostics, Cheshire, UK) and plates were incubated anaerobically (MiniMACS Anaerobic Workstation, Don Whitley, UK), at 37°C, for 48 hours. A single colony from each plate was then used to inoculate 10mL of Wilkins Chalgren broth (WCB) (Oxoid, UK). Broths were incubated anaerobically for 24 hours, at 37°C and standardised to 10<sup>3</sup> CFU/mL for neutraliser and soil load toxicity assessment and 10<sup>6</sup> CFU/mL for disinfectant efficacy tests. Cultures were standardized with WCB, by measurement of optical density (OD) at 600nm, with reference to a calibration curve (see appendix 1).

### 2.2.2 Preparation of spore suspensions of *C. difficile*

Columbia base blood agar plates (Oxoid, UK) were inoculated with *C. difficile* (ribotype 027 and NCTC 11204) from frozen bead cultures (stored at -70 °C) and were incubated anaerobically, for 72 hours, at 37°C. The plates were then removed and left in aerobic conditions, at room temperature for 24 hours prior to harvesting all colonies into 10mL of 50%(v/v) saline (0.9% w/v) and 50% (v/v) industrial methylated spirits (Shetty *et al.*, 1999). Spore suspensions were filtered through sterile glass wool and stored at 4°C until use.

To determine the concentration of spores in a suspension, both a viable count and a total spore count were performed. For the viable count, the spore suspension was serially diluted in sterilized distilled water (SDW) and 100µL of the diluted sample was spread onto Fastidious Anaerobe Agar (FAA) (Lab M, Lancashire, UK),



containing 0.1% (w/v) sodium taurocholate (ST) ( $\geq 95\%$ , Sigma-Aldrich, UK). Plates were incubated at 37°C in anaerobic conditions for 48 hours after which the CFU/mL was determined. To establish the total spore concentration, 20 $\mu$ L of the spore suspension was delivered onto a Neubauer counting chamber and the number of spores determined by light microscopy using a x40 objective.

### **2.2.3 Preparation of a soil load (to simulate environmental contamination)**

A stock solution of soil load was produced by dissolving 0.3g bovine albumin (Sigma-Aldrich, UK) in 10mL SDW and sterilized using membrane filtration (0.4 $\mu$ m). The stock was prepared at 10x the required concentration.

### **2.2.4 Disinfectant neutraliser**

The neutraliser used was prepared as described by Espigares *et al.* (2003) and contained: 120mL Tween 80, 25mL 40% (w/v) sodium metabisulphate, 15.69g sodium thiosulphate pentahydrate, 10g sodium thioglycollate, 3g L-cysteine and 4g lecithin. This was made up to 1000mL and was adjusted to pH 7, before sterilization at 121°C, for 15 minutes. The neutraliser was stored at room temperature until use.

### **2.2.5 Disinfectants**

The disinfectants assessed were: Citrofresh® (GDM Technologies Ltd, UK), Wofasteril® (Kesla Pharma Ltd, Germany), R2® (Diversey Lever, Canada) and Chlor-clean® (Guest Medical, UK). All disinfectants except R2® (pre-prepared solution) were made up to 1.25x the concentration recommended by manufacturers, as this was required by the BS EN 13704: 2002 guidelines. Each disinfectant was prepared fresh prior to each experiment and was diluted from concentrate using hard

water (600µL hydrated calcium chloride (v/v), 800µL hydrated sodium bicarbonate (w/v) and 98.6mL distilled water).

#### **2.2.6 Assessment of toxicity of the neutraliser and the soil load towards the vegetative cells of *C. difficile***

One hundred microlitres of *C. difficile* NCTC 11204 cell suspension in WCB ( $10^3$  CFU/mL) (as described in 2.2.1) was added to either 800µL soil load or 800µL of neutraliser and 100µL SDW and mixed thoroughly. After 5 minutes 100µL was removed and spread across the surface of a WCA plate. Plates were incubated anaerobically, for 48 hours, at 37°C.

#### **2.2.7 Efficacy of the neutraliser to nullify the activity of the disinfectants**

One hundred microlitres of soil load (described in 2.2.3) was mixed with 900µL disinfectant and a 100µL sample was taken and added to 800µL of neutraliser, which was vortex mixed for 30 seconds. After 5 minutes, 100µL of *C. difficile* NCTC 11204 cell suspension ( $10^3$  CFU/mL) was added to the neutralised solution and mixed thoroughly by inversion. After 30 minutes, 100µL was removed and spread across the surface of a WCA plate. Plates were incubated as described previously. To assess the antimicrobial activity of each disinfectant, 100µL of each agent was mixed with 100µL of *C. difficile* NCTC 11204 cell suspension and left to stand at 37°C, in anaerobic conditions, for 60 minutes. A 100µL aliquot was then spread across the surface of a WCA plate and incubated as described previously.

### **2.2.8 Antimicrobial activity of disinfectants against the vegetative cells of *C. difficile* in the presence and absence of a soil load**

One hundred microlitres of a *C. difficile* cell suspension of ribotype 027 and NCTC 11204 (adjusted to  $10^6$  CFU/mL) was mixed with 100 $\mu$ L soil load (or SDW for the control). After 5 minutes 800 $\mu$ L of disinfectant was added, mixed thoroughly by inversion and left for 60 minutes, at 37°C, in anaerobic conditions (Don Whitley, UK). A 100 $\mu$ L aliquot was then taken and added to 900 $\mu$ L neutraliser and left for 5 minutes, at 37°C, in anaerobic conditions. From the neutralised solution, a 100 $\mu$ L sample was then taken and spread across the surface of a WCA plate and plates were incubated as described previously. This was conducted in anaerobic conditions in order to prevent death or sporulation of the *C. difficile* cells.

### **2.2.9 Antimicrobial activity of disinfectants against the spores of *C. difficile* in the presence and absence of a soil load**

Spore suspensions of *C. difficile* 027 and NCTC 11204 were centrifuged at 13000 rpm for 5 minutes and the supernatant discarded. The pellet was resuspended in SDW and adjusted to a concentration of  $10^4$  or  $10^6$  CFU/mL by serial dilution with SDW. Sporocidal activity of the disinfectants was tested in accordance with the BS EN 13704:2002 standards. Briefly, 100 $\mu$ L of spore suspension was added to 100 $\mu$ L soil load (or SDW, for control purposes) and vortex mixed for 30 seconds. After 5 minutes 800 $\mu$ L of the test disinfectant was added, vortex mixed for 30 seconds and left for 60 minutes at room temperature. A 100 $\mu$ L aliquot was then taken and added to 900 $\mu$ L neutraliser and left for 5 minutes at room temperature. A 100 $\mu$ L sample of the neutralised solution was then spread across the surface of an FAA plate, containing 0.1% (w/v) sodium taurocholate and 5% (v/v) defibrinated horse blood. Plates were incubated anaerobically for 48 hours at 37°C. In line with the BS EN

13704 guidelines, a 3 log reduction (99.9%) of viable spores in the presence and absence of soil load must be achieved in order for the disinfectant to be sporicidal.

#### **2.2.10 Statistical analysis**

Results were analysed using an unpaired t-test (GraphPad InStat 2).



## 2.3 Results

### 2.3.1 Number of *C. difficile* spores in suspension determined by viable and total counts

There was no significant difference ( $P>0.05$ ) between the total and viable counts from the spores suspension of *C. difficile* tested (table 2.1).

**Table 2.1** Viable and total counts of *C. difficile* ribotype 027

Count	Mean CFU/mL of <i>C. difficile</i> (range)
Viable count	$1.15 \times 10^7$ ( $1-1.37 \times 10^7$ )
Total count	$1.46 \times 10^7$ ( $9.1 \times 10^6-1.8 \times 10^7$ )

### 2.3.2 Assessment of toxicity of neutraliser and soil load towards vegetative cells of *C. difficile* and efficacy of neutraliser to nullify the activity of the disinfectants

The neutraliser and soil load were non-toxic towards the cells of *C. difficile* as there was no log reduction in CFU/mL after cells were exposed to either solution (table 2.2). The neutraliser effectively nullified the activity of disinfectants as there was no log reduction in CFU/mL after cells were exposed to the neutralized disinfectants (table 2.2).

**Table 2.2 Toxicity of neutraliser and soil load towards the vegetative cells of *C. difficile* and the efficacy of the neutraliser to nullify the activity of the disinfectants**

Suspension / Solution	Mean CFU/mL of <i>C. difficile</i> (range)
Cell suspension (control)	$6.8 \times 10^3$
Cells and neutraliser	$5.61 \times 10^3$ ( $4.48 - 6.24 \times 10^3$ )
Cells and soil load	$5.6 \times 10^3$ ( $4.64 - 6.12 \times 10^3$ )
Cells and neutralized Citrofresh® with a soil load	$5.01 \times 10^3$ ( $3.72 - 6.6 \times 10^3$ )
Cells and neutralized Wofasteril® with a soil load	$6.44 \times 10^3$ ( $5.92 - 6.96 \times 10^3$ )
Cells and neutralized R2® with a soil load	$5.86 \times 10^3$ ( $5.68 - 6.1 \times 10^3$ )
Cells and neutralized Chlor-clean® with a soil load	$5.31 \times 10^3$ ( $4.4 - 6.72 \times 10^3$ )

### 2.3.3 Antimicrobial efficacy of disinfectants against vegetative cells of *C. difficile*

All disinfectants demonstrated antimicrobial activity against the vegetative cells of *C. difficile* NCTC 11204 and 027, with a >3 log reduction in CFU/mL within 60 minutes of exposure, in both the presence and absence of a soil load (table 2.3).

**Table 2.3** Antimicrobial efficacy of disinfectants against vegetative cells of *C. difficile* ribotype 027 and NCTC 11204 after 1-hour exposure (in the presence and absence of a soil load)

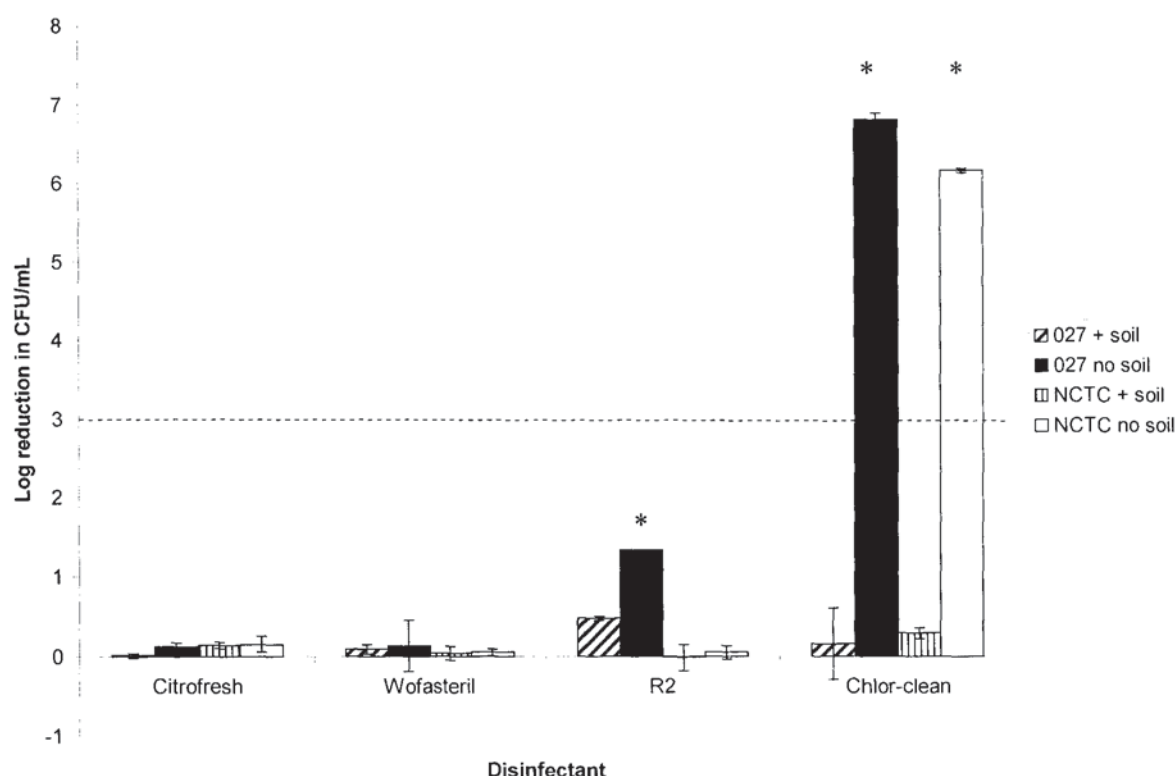
Disinfectant	Mean log reduction in CFU/mL of <i>C. difficile</i> 027 (soil load)	Mean log reduction in CFU/mL of <i>C. difficile</i> 027 (no soil load)	Mean log reduction in CFU/mL of <i>C. difficile</i> NCTC 11204 (soil load)	Mean log reduction in CFU/mL of <i>C. difficile</i> NCTC 11204 (no soil load)
Citrofresh®	3.96	4.03	5.03	6.86
Wofasteril®	3.23	3.43	4.08	6.86
R2®	3.2	3.6	4.64	6.86
Chlor-clean®	3.85	4.51	5.33	6.86

#### **2.3.4 Efficacy of disinfectants against the spores of *C. difficile* (10<sup>6</sup> CFU/mL) in the presence and absence of a soil load.**

In the absence of a soil load the only disinfectant to achieve >3 log reduction in *C. difficile* spores of both strains tested was Chlor-clean®. Following a 1 h exposure to Chlor-clean® a 6.82 and 6.18 log reduction in the spores of *C. difficile* ribotype 027 and NCTC 11204 respectively was achieved (figure 2.1). With the ribotype 027 strain, a significant reduction in CFU/mL ( $P < 0.05$ ) was also produced by R2®, but not with *C. difficile* NCTC 11204.

However, in the presence of a soil load the antimicrobial activity of Chlor-clean® was significantly reduced ( $P < 0.05$ ) and only a 0.16 and 0.12 log reduction in spores ( $P > 0.05$ ) was achieved in ribotype 027 and NCTC 11204 strain respectively. All remaining disinfectants produced <1 log reduction in CFU/mL, which was not

significant ( $P>0.05$ ) after 1 h exposure, in the presence of a soil load (figure 2.1).



\*  $P < 0.05$

**Figure 2.1 Efficacy of disinfectants against the spores of *C. difficile* ribotype 027 and NCTC 11204 ( $10^6$  CFU/mL) after 1 h exposure in the presence and absence of a soil load**

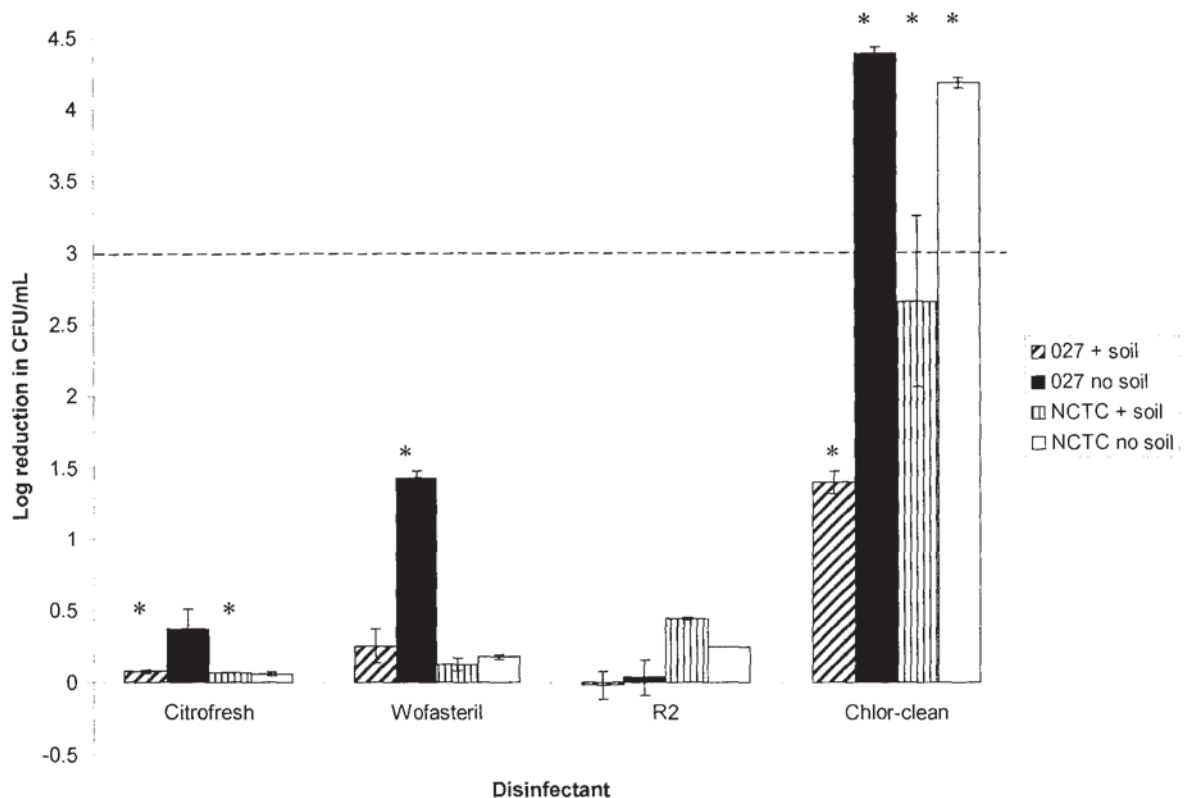
### 2.3.5 Antimicrobial efficacy of disinfectants against spores of *C. difficile* ( $10^4$ CFU/mL) in the presence and absence of a soil load

With a lower concentration of *C. difficile* spores ( $10^4$  CFU/mL), the peracetic acid based disinfectant (Wofasteril®) was more effective; producing a significant log reduction ( $P < 0.01$ ) in both strains of *C. difficile* tested, with a 1.43 log reduction (96% reduction) in remaining ribotype 027 spores. However, the only cleaning agent to



produce >3 log reduction (99.9% reduction) in CFU/mL in both strains of *C. difficile* tested was Chlor-clean® (figure 2.2).

Whilst Chlor-clean® and Citrofresh® produced a significant reduction ( $P < 0.01$ ) in viable spores of *C. difficile* in the presence of a soil load, none of the disinfectants achieved a  $\geq 3$  log reduction (99.9% reduction) in line with BS EN 13704: 2002 (figure 2.2).



\*  $P < 0.05$

**Figure 2.2** Efficacy of disinfectants against the spores of *C. difficile* ribotype 027 and NCTC 11204 ( $10^4$  CFU/mL) after 1 h exposure in the presence and absence of a soil load

## 2.4 Discussion

The environment surrounding hospitalised patients infected with *C. difficile* is often contaminated with the microorganism and its spores (Fekety *et al.*, 1981). The importance of effective environmental disinfection is therefore essential in preventing *C. difficile* infection and has been highlighted in several studies (Hota, 2004, Kramer *et al.*, 2006).

In this current investigation, all disinfectants demonstrated antimicrobial activity against the vegetative cells of both strains of *C. difficile* tested, with all agents achieving >3 log reduction in viable cells in 60 minutes, in both the presence and absence of a soil load. These findings are important as the vegetative cells of *C. difficile* have been found to survive on moist surfaces in room air for up to 6 hours (Jump *et al.*, 2007).

When challenged with a solution containing a high concentration of *C. difficile* spores ( $10^6$  CFU/mL), in the absence of a soil load, the only disinfectant to produce >3 log reduction (>99.9% reduction) in 1 h was the chlorine-based agent, Chlor-clean®. The sporicidal activity of chlorine-releasing agents, such as Chlor-clean® is due to their ability to degrade the spore coat and cortex which rehydrates the protoplast and causes damage to it (Bloomfield and Arthur, 1992). R2® displayed a significant reduction (1.34 log reduction) with ribotype 027 strain; however this was not sufficient to be classed as sporicidal and interestingly there was no significant reduction observed with *C. difficile* NCTC 11204. This may suggest that the reduction observed with ribotype 027 may have been due to laboratory error rather than activity against *C. difficile* spores. R2® comprises didecyldimethyl ammonium chloride and is a quaternary ammonium compound (Quat). The findings in this

investigation concur with previous studies which have also demonstrated that the Quats do not possess any sporicidal activity (Acosta-Gio *et al.*, 2005). With this high concentration of *C. difficile* spores, none of the other remaining disinfectants produced a significant reduction in the number of remaining spores ( $P>0.05$ ).

When challenged with a lower concentration of *C. difficile* spores ( $10^4$  CFU/mL); which is a more realistic concentration of spores that would be encountered in a clinical environment, (as approximately 100 spores and cells are released per gram of faeces (Wilcox, 2003)), Chlor-clean® remained the only agent capable of reducing the spore load by 99.9%. However, Wofasteril® produced a 96% reduction ( $P<0.01$ ) in *C. difficile* ribotype 027 spores in the absence of a soil load. It was expected that this peracetic acid-based disinfectant would demonstrate sporicidal activity as other studies have also found peracetic acid-based disinfectants to be very effective against the spores of *C. difficile* (Block, 2004, Sattar *et al.*, 2006). The mode of action of peracetic acid against spores is still not well understood, however it is thought that it produces damaging organic radicals and denatures proteins (Marquis *et al.*, 1995, Kitis, 2004).

None of the other disinfectants showed significant activity against a low concentration of *C. difficile* spores of both strains tested ( $P>0.05$ ). This was partly expected as according to the manufacturer, Citrofresh® works by destroying the cell membrane of organisms. Although this highly acidic solution is damaging to *C. difficile* cells, the spores of *C. difficile* are highly resistant to acidic environments as they withstand the gastric acid of the stomach and therefore it was expected that the spores would be resistant to this agent.

It is essential to test the efficacy of cleaning agents in the presence of a soil load as cells and spores of *C. difficile* are released within the faeces of patients

which contaminate the clinical environment. Although the chlorine-based agent demonstrated the greatest sporicidal activity against the spores of *C. difficile*, its activity was the most affected in the presence of a soil load, with only a 0.16 log reduction in *C. difficile* ribotype 027 spores in the presence of a soil load, compared to a 6.82 log reduction without a soil load ( $P < 0.01$ ). Although a previous study found sodium dichloroisocyanurate (NaDCC) based disinfectants (such as Chlor-clean®) to be less affected by organic soiling than sodium hypochlorite (bleach) (Bloomfield and Uso, 1985), the activity of the NaDCC based disinfectant in this study was found to be significantly impeded by the presence of a soil load. Indeed, other studies have also found a significant decrease in the activity of chlorine-based disinfectants in the presence of protein and other organic matter (Sagripanti and Bonifacino, 1997, Kida *et al.*, 2003). The pH of the cleaning agent may determine the size of the effect which organic matter may have on its activity. Alkaline detergents are less affected by organic matter and are often used to de-grease and de-soil surfaces; however, they lack sporicidal activity. The more acidic agents such as chlorine-based agents possess powerful sporicidal activity but their activity is significantly reduced in the presence of organic matter.

In conclusion, none of the cleaning agents assessed in this study produced a  $\geq 3$  log reduction in the spores of *C. difficile* in 1 h in both the presence and absence of a soil load. Therefore, within the test parameters used in this part of the investigation none of the agents could truly be classed as sporicidal according to the BS EN 13704:2002 standards. This is of interest as two agents (R2® and Chlor-clean®) which are currently used in UK NHS hospitals, do not pass the criterion for a sporicidal agent against *C. difficile*. The findings of this chapter therefore provide further evidence to support initial cleaning with a detergent, in order to remove organic matter, followed by disinfection with a chlorine-releasing agent to eliminate



the spores of *C. difficile*. This is in line with the epic2 guidelines, commissioned by the DoH, which recommend the use of hypochlorite and detergent in outbreaks of infection where the spread of disease may be due to the organisms' survival and contamination of the environment (Pratt *et al.*, 2007).

Although the method employed in this chapter acts as an effective screening process and is used as a British Standard to determine the sporicidal activity of disinfectants; it does not simulate the conditions experienced in the clinical environment. Therefore, in the subsequent chapter the two most active agents; Chlor-clean® and Wofasteril® were further assessed using a standardized carrier test system.

### **3 Efficacy of dichloroisocyanurate and peracetic acid against the spores of *C. difficile***

#### **3.1 Introduction**

In the previous chapter of this thesis, the chlorine-based disinfectant, Chlor-clean®, was shown to be the most effective disinfectant of those tested against spores of *C. difficile*. However, the peracetic acid-based agent, Wofasteril®, also exhibited significant sporicidal activity when challenged with a lower spore load. In light of these initial results, both of these disinfectants were selected for further investigation, using a standard carrier test method which simulates contamination of the clinical environment.

Various studies have demonstrated the sporicidal activity of chlorine-based disinfectants against bacterial spores (Bloomfield and Uso, 1985, Coates, 1996, Mayfield *et al.*, 2000, Kreske *et al.*, 2006, Perez *et al.*, 2005) and a correlation between the use of chlorine-based cleaning agents and a decrease in the number of cases of CDAD has been demonstrated (Kaatz *et al.*, 1988, McMullen *et al.*, 2007). Despite the findings of these studies and the recommendations by the DoH, the number of reported cases of CDI continues to increase and CDI is currently the leading cause of healthcare acquired diarrhoea in the UK (HPA, 2007).

The potential hazards associated with using chlorine-releasing agents have also been highlighted in occupational health reports: the Centre for Disease Control (CDC) state in their guidelines that the permissible exposure limit for chlorine is 1ppm and that overexposure to chlorine gas can result in severe irritation of the skin, eyes and mucous membranes. In its liquid form the effects of short-term overexposure of chlorine include burns to the skin and eyes on contact. The effects of long-term or repeated exposure to chlorine include skin irritation and corrosion of

the teeth (CDC, 1981).

Peracetic acid has been shown to inactivate bacterial spores within 20 minutes, with activity between 500 to 10000ppm (Rutala and Weber, 1999). However, the disadvantages of peracetic acid-based disinfectants are the pungent odour (unless a neutralizing agent is used) and risk of skin and eye damage in their concentrated form (Rutala and Weber, 1999, Kitis, 2004).

The rapid action of chlorine-releasing agents is thought to be due to their ability to degrade the spore coat and cortex and therefore rehydrate the protoplast and cause damage to it (Bloomfield and Arthur, 1992). Peracetic acid-based biocides are likely to exert sporicidal activity by the production of organic radicals and denaturation of proteins; however the mode of action of peracetic acid against spores is still not known (Marquis *et al.*, 1995, Kitis, 2004).

In this chapter, the sporicidal activity of Wofasteril® and Chlor-clean® was assessed against spores of *C. difficile* ribotype 027 in the presence and absence of a soil load by a standard carrier test method. The time taken for each disinfectant to eliminate the spores of *C. difficile* was also determined.

## **3.2 Methods**

### **3.2.1 Test organisms**

*C. difficile* ribotype 027 (R20291) was provided by the Anaerobe Reference Laboratory, Cardiff, UK.

### **3.2.2 Microbicide**

Wofasteril® and Alcapur® buffer solution (an odour neutralizing agent, containing >5% sodium hydroxide) were prepared according to the manufacturer's guidelines (Kesla Hygiene AG, Germany). Wofasteril® (0.5% v/v) was prepared by adding one part Wofasteril® containing 40% (v/v) peracetic acid, to three parts diluted alcapur. Chlor-clean® (Guest Medical, UK) was diluted to 1000ppm (surface disinfectant concentration for every day use) by dissolving one tablet in 1L of SDW. Chlor-clean® tablets contain dichloroisocyanurate (NaDCC) and a detergent. According to manufacturers' guidelines, both microbicides were freshly prepared immediately before every experiment.

### **3.2.3 Spore suspensions**

A spore suspension of *C. difficile* ribotype 027 was produced following the method described by Shetty *et al.* (1999) and detailed in the previous chapter (see 2.2.2).

### **3.2.4 Neutraliser**

The neutraliser for NaDCC and peracetic acid was prepared at double strength as follows: 120mL Tween 80 (Sigma-Aldrich, UK), 25mL of 40% (w/v) sodium metabisulphite (FSA, Loughborough, UK), 15.69g sodium thiosulphate pentahydrate (BDH Ltd., Poole, UK), 10g sodium thioglycollate (Sigma-Aldrich, UK), 3g L-cysteine



(Sigma-Aldrich, UK), 4g lecithin (Fisher, Loughborough, UK), was made up to 1000mL with distilled water. The pH was adjusted to 7.0 using sodium hydroxide concentrated pellets and the neutraliser was then sterilised by autoclaving at 121°C for 15 minutes (Espigares *et al.*, 2003).

### **3.2.5 Assessment of neutraliser efficacy and toxicity**

To determine the efficacy of the neutraliser for inactivation of NaDCC and peracetic acid, 100µL of each disinfectant was added to 900µL of neutraliser. This was vortex mixed thoroughly and after 30 minutes 10µL of spore suspension, containing  $1 \times 10^6$  CFU/mL spores was added. After 24 h 100µL of the inoculated solution was spread onto FAA, incorporating 0.1% (w/v) taurocholate and 5% (v/v) horse blood) and plates were incubated at 37°C, in anaerobic conditions for 48 h. After incubation the number of CFU/mL was determined. To ensure that the neutraliser was not sporicidal 10µL of spore suspension was added to 990µL of neutraliser (SDW for the control). The solution was vortex mixed thoroughly and after 24 h was serially diluted and 100µL was spread onto a FAA plate (incorporating 0.1% taurocholate and 5% horse blood) and incubated as described above. Results were analysed using a one-way Analysis of Variance (ANOVA) Test and the Tukey-Kramer Multiple Comparisons Test (GraphPad InStat 2).

### **3.2.6 Rate of neutralisation of disinfectants**

To determine whether the neutraliser nullified the activity of both disinfectants at the same rate, 1mL of either peracetic acid or NaDCC was added to 9mL of neutraliser at time 0 and a 1mL sample was taken at 0, 1, 2, 3, 4 and 5 minutes. Each sample was added to 10µL of spore suspension ( $1 \times 10^6$  CFU/mL) and vortex mixed thoroughly. Each sample was left for 3 h, and then serially diluted before plating

100µL onto FAA (incorporating 0.1% (w/v) sodium taurocholate and 5% (v/v) horse blood). The plates were incubated at 37°C in anaerobic conditions for 48 h and the number of CFU/mL determined.

### **3.2.7 Soil load to simulate environmental contamination**

The soil load used to simulate a contaminated clinical environment comprised both protein and carbohydrate and was adapted from Perez *et al.* (2005). Three hundred and forty microlitres of spore suspension was combined with 35µL of 5% (w/v) tryptone (Oxoid, Basingstoke, UK), 100µL of 0.4% (w/v) bovine mucin (Sigma-Aldrich, UK) and 25µL of 5% (w/v) albumin from bovine serum (Sigma-Aldrich, UK), all in phosphate buffered saline (Sigma-Aldrich, UK), containing 0.05% (w/v) magnesium sulphate (FSA, Loughborough, UK).

### **3.2.8 Sporicidal time-kill studies**

The method used was modified from Perez *et al.* (2005). A *C. difficile* ribotype 027 spore load of  $1 \times 10^5$  CFU/mL was prepared by diluting the stock spore suspension with SDW. Ten microlitres of the test spore suspension was then placed onto a sterile stainless steel carrier disk (1cm<sup>2</sup>) and dried under a flow cabinet for one hour, at room temperature. The inoculated disk was then placed flat (inoculated side up) across the internal base of a sterile bijou. Fifty microlitres of the microbicide (prepared as detailed above) or SDW (for the control) was added to the disk. This was sufficient microbicide to completely cover the entire surface of the disc. Each bijou tube was then sealed to ensure the microbicide did not dry out. Multiple disks were inoculated to allow for duplicate sampling and the rate of kill of both disinfectants was assessed over a one hour period at 0, 3, 6, 9, 12, 15, 30 and 60 minutes. At each time point, the disks were entirely covered with 950µL of

neutraliser, allowed to stand for 30 min and then vortex mixed thoroughly for one minute. After serial dilutions the 1mL aliquot was transferred into a sterile Petri dish into which 15mL of molten FAA (incorporating 0.1% (w/v) sodium taurocholate and 5% (v/v) horse blood), cooled to 47°C, was added and mixed thoroughly. The plates were subsequently incubated at 37°C in anaerobic conditions for 48 h and the number of CFU/mL determined.

### **3.2.9 Statistical analysis**

Results were analysed using the Repeated Measures ANOVA Test and Fishers Post-Hoc analysis (Statistica 6.0 package).

### 3.3 Results

#### 3.3.1 Neutraliser non-toxicity and efficacy

The neutraliser used in this investigation did not demonstrate any sporicidal activity against *C. difficile* 027 as there was no significant difference ( $P>0.05$ ) in the number of colonies recovered between the control and the spore suspension exposed to neutraliser (table 3.1). The neutraliser effectively nullified the sporicidal activity of both disinfectants as there was no significant difference between the spore suspensions exposed to neutraliser alone and spore suspensions exposed to neutralised disinfectant (table 3.1). The neutraliser nullified both disinfectants at the same rate: NaDCC (mean 3 minutes, range 1-5); peracetic acid (mean 3 minutes, range 2-4).



**Table 3.1 Efficacy of the neutraliser to nullify the sporicidal activity of NaDCC and peracetic acid and non-toxicity of the neutraliser towards the spores of *C. difficile***

Test	Mean CFU/mL recovered (range)	P value
Control (spores in water)	$1.79 \times 10^6$ ( $1.4 - 2.26 \times 10^6$ )	
Spores and neutraliser	$1.15 \times 10^6$ ( $1.07 - 1.31 \times 10^6$ )	>0.05
Neutralised NaDCC and spores	$1.01 \times 10^6$ ( $6.5 \times 10^5 - 1.49 \times 10^6$ )	>0.05
Neutralised Peracetic acid and spores	$1.14 \times 10^6$ ( $9.7 \times 10^5 - 1.33 \times 10^6$ )	>0.05
NaDCC and spores (neutralised after 24 hours to prevent carry over effect of disinfectant on agar)	0	<0.01
Peracetic acid and spores (neutralised after 24 hours)	0	<0.01

### 3.3.2 Efficacy of peracetic acid and NaDCC in the absence of a soil load

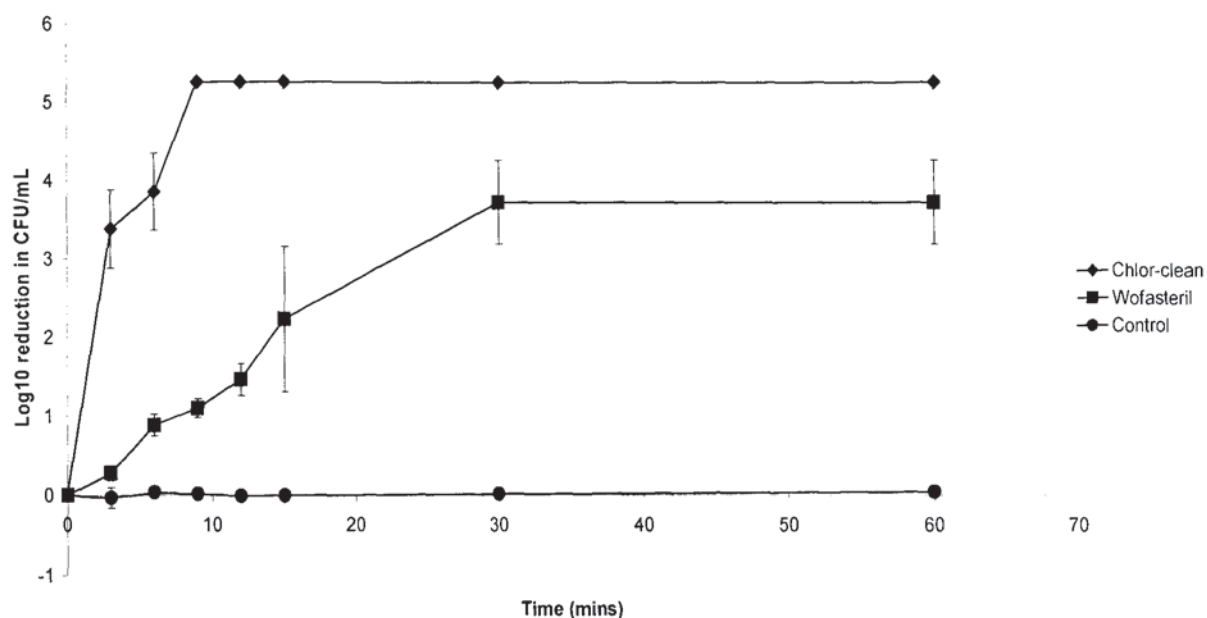
Both peracetic acid and NaDCC demonstrated sporicidal activity against spores of *C. difficile* 027 on stainless steel surfaces in the absence of a soil load, however NaDCC demonstrated a significantly higher rate of inactivation compared to peracetic acid ( $P < 0.05$ ) (Table 3.2 and Figure 3.1). NaDCC achieved a  $>3 \log_{10}$

reduction (99.9% reduction of spore load) in 3 minutes, whilst 30 minutes was required to achieve the same level of reduction with peracetic acid under the same conditions. NaDCC was significantly more active than the peracetic acid-based disinfectant for up to 9 minutes of exposure (table 3.2). A contact time of 9 minutes was required to reduce the spore load of *C. difficile* to below detection levels with NaDCC. With peracetic acid, 25 CFU/mL (mean average) were still recovered after 60 minutes.

**Table 3.2 Activity of NaDCC and peracetic acid against the spores of *C. difficile* ribotype 027 in the absence of a soil load**

Time (minutes)	Peracetic acid (recovered CFU/mL)	Log <sub>10</sub> reduction	NaDCC (recovered CFU/mL)	Log <sub>10</sub> reduction	P value
0	1.34 x 10 <sup>5</sup>	0	1.8 x 10 <sup>5</sup>	0	
3	7.15 x 10 <sup>4</sup>	0.27	75	3.38	<0.01
6	1.75 x 10 <sup>4</sup>	0.88	25	3.39	<0.01
9	1.06 x 10 <sup>4</sup>	1.1	0	5.26	<0.01
12	4.6 x 10 <sup>3</sup>	1.46	0	5.26	0.175
15	7.75 x 10 <sup>2</sup>	2.24	0	5.26	0.804
30	25	3.73	0	5.26	0.994
60	25	3.73	0	5.26	0.996

Overall P value= <0.01



**Figure 3.1** Sporicidal activity of dichloroisocyanurate (NaDCC) and peracetic acid in the absence of a soil load against the spores of *C. difficile* ribotype 027

### 3.3.3 Efficacy of peracetic acid and NaDCC in the presence of a soil load

In the presence of a soil load there was no significant difference found between the sporicidal activities of either disinfectant during the course of the experiment (table 3.3). A  $>3 \log_{10}$  reduction in *C. difficile* spores was achieved in 60 minutes with both disinfectants (table 3.3 and figure 3.2).

### 3.4 Discussion

In this part of the study, the efficacy of the chlorine releasing agent, Chlor-clean® and the peracetic acid-based agent, Wofasteril® against the spores of *C. difficile* was investigated. In the absence of a soil load, NaDCC (1000ppm) demonstrated rapid sporicidal activity against the spores of *C. difficile* ribotype 027, producing a  $>3 \log_{10}$  reduction (99.9% reduction in spore load) in 3 minutes, and  $>5 \log_{10}$  reduction in 9 minutes. In concurrence with the findings of the previous chapter, peracetic acid demonstrated significantly lower activity ( $P<0.05$ ) against *C. difficile* spores compared to NaDCC under these conditions. However,  $>3 \log_{10}$  reduction (99.9% reduction in spore load) was achieved in 30 minutes. These results do not concur with those found by Block (2004), who observed a  $<1 \log_{10}$  reduction of *C. difficile* spores on stainless steel when exposed to NaDCC (1000ppm) for 10 minutes and a  $6 \log_{10}$  reduction with a peracetic acid-based disinfectant. However, differences in the starting inocula, the manufacturing source of the disinfectants and in the methods employed by the two studies must be taken into consideration when comparing these results. Indeed, the level of reduction of *C. difficile* spores produced by peracetic acid was considerably higher when using the method in this current chapter, compared to the method used in the previous chapter. It has previously been reported that inconsistencies in the degree of reduction demonstrated by biocides are prevalent when using only one method to determine sporicidal activity (Bloomfield *et al.*, 1994, Block, 2004) and therefore it is not surprising that differences have occurred between the results of the two methods employed.

A key finding of this part of the study showed that whilst NaDCC demonstrated a significantly more effective sporicidal activity compared to the peracetic acid-based disinfectant under clean conditions, NaDCC still required a



contact time of 9 minutes to eliminate spores of *C. difficile* to below detection levels. Under routine clinical conditions however, chlorine-based disinfectants are unlikely to be in contact with contaminated surfaces for this period of time before being removed by wiping. The contact time between disinfectant and hard surface is clearly an important factor and therefore needs to be taken into account when cleaning hard surfaces.

In the presence of a soil load, which is reflective of a clinical environment, the sporicidal activity of NaDCC was reduced. At 3 minutes approximately 50% of *C. difficile* spores remained on the stainless steel surface and an exposure time of up to 60 minutes was required to produce  $>3 \log_{10}$  reduction (99.9% reduction in spore load) in *C. difficile* spores. This finding concurs with the previous chapter and other studies that have recorded a decrease in activity of NaDCC in the presence of organic material (Coates, 1996). The sporicidal activity of peracetic acid was not significantly different ( $P>0.05$ ) to that of NaDCC in the presence of a soil load and an exposure time of up to 60 minutes was also required to produce a  $>3 \log_{10}$  reduction in CFU/mL.

Although NaDCC demonstrated increased activity against *C. difficile* spores in the absence of a soil load, compared to peracetic acid, these conditions do not reflect the clinical environment and it is unlikely that *C. difficile* spores would be present on surfaces without any accompanying organic matter. The findings of this chapter therefore support the important issue raised in the previous chapter of reducing the organic load by surface cleaning prior to disinfection in order for disinfectants to work more efficiently. Furthermore, surface disinfectants, for example NaDCC, should remain in contact with the surface for at least 9 minutes before being removed.

Alternatively, as there was no significant difference in the sporicidal activity of

either disinfectant in soiled conditions and given the occupational health hazards associated with the use of chlorine-releasing agents, the results of this study suggest that a peracetic acid-based disinfectant (where the pungent odour can be neutralised with an appropriate buffer) may offer a suitable alternative to the health care worker when decontaminating the clinical environment.

It must also be noted that hospital surfaces consist of many different materials other than stainless steel and therefore the rates of sporicidal action of disinfectants may differ when applied to different surfaces (Block, 2004, Perez *et al.*, 2005). There may also be some variation between different strains of *C. difficile*, however ribotype 027 is currently the most significant strain in the UK as it is now responsible for the majority of cases of *C. difficile* infection (HPA, 2008).

In terms of cost, there is little difference between the two disinfectants. Wofasteril® is approximately 8-20€ (~£6-15) per litre and Alcapur® 1.50-2.50€ (~£1.12-1.87) per litre, depending on the size of the container. One litre of Wofasteril® concentrate makes 200L of cleaning agent, if used at 0.5%; therefore, it costs 4-9 pence to make 1L of diluted Wofasteril®/Alcapur® solution. Chlor-clean® costs approximately 4 pence to make 1L of diluted solution (information obtained from the manufacturers).

With regards to safety aspects, the acidic, corrosive nature of Wofasteril® alone is neutralised by the addition of Alcapur®, which raises the pH of the disinfectant mixture from approximately pH 3 to pH 9.0 - 9.5. The mixture of Wofasteril® and Alcapur® at this pH is non-corrosive to metal surfaces. Furthermore, tests relating to the occupational hazards of peracetic acid alone have shown that solutions up to 0.01% are well tolerated on the urinary bladder epithelium of rabbits. However, higher concentrations of peracetic acid caused partly focal, partly diffuse haemorrhagic necrotizing urocystitides (Wutzler *et al.*, 1987). Other studies found that the combination of 2.5% Wofasteril® and 7.5% Alcapur® caused irritation of the

skin of rats and rabbits and it is therefore expected that this combination would also cause irritation of the mucous membranes (information from the manufacturer: Kesla Pharma Wolfen GMBH, Germany). Safety issues in relation to lower working concentrations (0.5% v/v, as in this study) of buffered Wofasteril® have not been tested to date and need investigating in further clinical studies.



## 4 Chemical and physical factors influencing the germination of *C. difficile* spores

### 4.1 Introduction

Patients with CDI excrete large numbers of *C. difficile* spores and cells in their faeces (Wilcox, 2003), which readily contaminate the surrounding environment and serve as a reservoir of infection. Previous chapters in this thesis have demonstrated the efficacy of various cleaning agents and disinfectants against the spores and vegetative cells of *C. difficile*. Spores of *C. difficile* are ingested from the environment and infection may then develop in susceptible patients. Following ingestion, the irreversible process of spore germination occurs in the small intestine giving rise to the metabolically active vegetative forms of *C. difficile* which produce proteinaceous exotoxins associated with disease in the large intestine (Keynan *et al.*, 1964, Poutanen and Simor, 2004, Moir, 2006).

Germination occurs in several stages including initiation of germination following exposure to a specific germinant; loss of heat and chemical resistance, along with hydrogen and zinc ions and monovalent cations and the release of the calcium and dipicolinic acid (DPA) complex. This is followed by degradation of the cortex and small acid-soluble proteins (SASPs), allowing re-hydration of the cell and the resumption of metabolic activity (De Vries, 2004, Moir, 2006).

Until recently, there has been limited research into the factors associated with germination of *C. difficile*. Indeed, most knowledge of *C. difficile* germination has arisen following techniques to improve the recovery of *C. difficile* from environmental samples (Arroyo *et al.*, 2005b, Wilcox *et al.*, 2000, Wilson *et al.*, 1982, Wilson, 1983). Various studies have demonstrated improved recovery of *C. difficile* by the incorporation of sodium taurocholate into agar medium (Wilson *et al.*, 1982, Wilson,



1983, Buggy *et al.*, 1985) or lysozyme; both with and without pre-exposure to thioglycollate (Kamiya *et al.*, 1989, Nakamura *et al.*, 1985, Wilcox *et al.*, 2000). Furthermore, Wilson, (1983) stated that the bile salts cholate and desoxycholate stimulated germination in both agar and broth medium when incubated overnight (Wilson, 1983).

A recent study by Sorg and Sonenshein, (2008) investigated the effects of bile salts on the germination of *C. difficile* spores in much greater depth. They found that taurocholate was the only bile salt to induce rapid germination of *C. difficile* spores in solution. Other cholate derivatives (cholate and glycocholate) enhanced colony formation (and therefore germination), but this was only seen when incorporated into agar medium, with a long incubation period. It was also found that both taurocholate and glycine were required for germination of *C. difficile* spores. The authors suggested that *C. difficile* spores germinate in the jejunum, where the concentration of nutrients such as glycine and primary bile salts is high, and then pass to the ileum and eventually to the caecum, which is anaerobic. It is proposed that in the caecum of a healthy adult, microorganisms convert cholate derivatives into deoxycholate. As deoxycholate is inhibitory towards *C. difficile*, the cells are prevented from growing and colonizing the gut, thus preventing infection. However, if the gut flora is disrupted considerably, especially by the intake of broad-spectrum antibiotics, the organisms responsible for converting cholates into deoxycholate are depleted and therefore the gut environment is far less inhibitory towards *C. difficile*. The concentration of cholate derivatives may also be considerably higher due to the lack of conversion, therefore encouraging germination, growth and colonization of *C. difficile* (Sorg and Sonenshein, 2008).

Another recent study by Paredes-Sabja *et al.* (2008) found conflicting results to that of Sorg and Sonenshein (2008), with the incorporation of bile salts having little effect on spore germination. They also concluded that inorganic phosphate and

potassium chloride (KCl) stimulated germination of *C. difficile* spores and suggest that spores which enter the human body are triggered to germinate by the presence of phosphate in the duodenum and KCl in the colon (Paredes-Sabja *et al.*, 2008).

Physical factors which may affect germination of *C. difficile* including pH, temperature and atmosphere have not yet been explored.

The aims of this chapter were to investigate the chemical and physical factors which influence germination of *C. difficile* spores. The potential of sodium taurocholate, cholate, deoxycholate, glycocholate and chenodeoxycholate to initiate germination was determined along with optimal concentrations to achieve maximum germination. Furthermore the effectiveness of glycine and sodium taurocholate as co-germinants was studied. The effect of common physical factors including temperature, pH and atmosphere upon the rate and extent of germination of *C. difficile* spores was also investigated.

## **4.2 Methods**

### **4.2.1 Strains of *C. difficile***

The *C. difficile* reference strain, NCTC 11204 and *C. difficile* ribotype 027 R20291 (Anaerobe Reference Laboratory, Cardiff) were used.

### **4.2.2 Preparation of spore suspensions**

Spore suspensions were prepared following the method by Shetty *et al.* (1999), as described in chapter 2 of this thesis (section 2.2.2). Before use, spore suspensions were centrifuged at 13000rpm for 5 minutes (Spectrafuge 24D, Labnet, UK). The supernatant was then discarded and the pellets were re-suspended and diluted accordingly with SDW. Spore suspensions were observed using the LIVE/DEAD® BacLight™ stain (Invitrogen- Molecular Probes, UK) with epifluorescence microscopy to confirm that any remaining vegetative cells were dead.

### **4.2.3 Demonstration of germination of *C. difficile* spores by fluorescence monitoring of DPA release**

The method of fluorescence monitoring used was based on that of Kort *et al.* (2005). A solution containing 100µM DPA, with 100µM terbium chloride hexahydrate (TbCl<sub>3</sub>) in 20mM Tris buffer (pH 7.5) was filter sterilized (0.45µm pore size) and 100µL of filtered solution was placed in three wells of a black microtiter plate (Appleton Woods, UK). In the adjacent column, three wells were filled with 100µL of 100µM TbCl<sub>3</sub> in 20mM Tris buffer pH 7.5, for the control. The plate was read using a plate reader (Spectra Max Gemini EM, Molecular Devices) with excitation wavelength set at 270nm and emission at 475-600nm (Kort *et al.*, 2005).

A calibration curve of DPA concentration against Relative Fluorescence Units (RFU) was determined by producing a stock solution of 640µM DPA dissolved in water.



This was diluted with either 20mM Tris buffer (pH 7.5), thioglycollate medium alone or thioglycollate containing 6.9mM sodium taurocholate ( $\geq 95\%$ , Sigma Aldrich, UK), 12.6mM glycocholate ( $\geq 97\%$ ), 0.7mM cholate ( $\geq 99\%$ ) and 0.7mM chenodeoxycholate (minimum 97%), to give a concentration of 64 $\mu$ M DPA. One hundred microlitres of each solution was transferred into three wells of a black microtiter plate and serial dilutions (1:2) using the appropriate medium were performed across the plate to give a concentration range of 64-0.5 $\mu$ M of DPA. To each well 100 $\mu$ L of double strength 20 $\mu$ M TbCl<sub>3</sub> in 400mM sodium acetate buffer (pH 5.0) was added. The final concentration of DPA in the wells therefore ranged from 0.25-32 $\mu$ M. The fluorescence emission from each well of the plate was read using the Spectra Max Gemini EM plate reader (excitation wavelength= 270nm, emission=450-520nm)

#### **4.2.4 Demonstration of germination of *C. difficile* spores by measurement of optical density (OD)**

Five wells of a transparent polystyrene flat bottom microtiter plate (Appleton Woods, UK) were filled with 100 $\mu$ L of *C. difficile* NCTC 11204 spore suspension ( $10^6$  CFU/mL) and 100 $\mu$ L of double strength thioglycollate medium, containing 6.9mM sodium taurocholate (ST); the maximal duodenal concentration in healthy adults (Leverrier *et al.*, 2003). The two control columns contained 100 $\mu$ L of sterile distilled water in place of either the thioglycollate medium or the spore suspension. The plate was read immediately after the germinant was added to the spore suspension using the plate reader (Anthos Labtec Instruments, Austria) at wavelengths of 450nm and 600nm. Subsequent readings were taken at 15, 20, 30, 40, 50 and 60 minutes.



#### **4.2.5 Demonstration of germination of *C. difficile* spores by measurement of loss of resistance to ethanol**

The ethanol shock method was based on that of Levinson and Hyatt (1966). Briefly, 250µL volumes of *C. difficile* spore suspension ( $10^6$  CFU/mL) were placed in microcentrifuge tubes. At time 0, 250µL of double strength thioglycollate medium (Oxoid, UK) containing 6.9mM ST, was added to each microcentrifuge tube at room temperature. After 0, 5, 10, 20, 30, 40, 50 and 60 minutes the contents of each tube were placed into 1.17mL of 100% ethanol. After one hour incubation, each solution was transferred into 8.33mL of Wilkins Chalgren broth (WCB) (Oxoid, UK). Serial dilutions were performed and 1mL samples were placed in sterile Petri dishes, before the addition of 15mL of molten FAA containing 0.1% (w/v) ST. Plates were incubated at 37°C, anaerobically for 48 hours and the CFU/mL was calculated.

#### **4.2.6 Demonstration of germination of *C. difficile* spores by measurement of loss of heat resistance**

The heat shock method used was based on that from Levinson and Hyatt (1966). Briefly, 250µL volumes of *C. difficile* NCTC 11204 spore suspension ( $10^6$  CFU/mL) were placed in microcentrifuge tubes. At time 0, 250µL of double strength thioglycollate medium containing 6.9mM ST, was added to each tube at room temperature. After 0, 5, 10, 20, 30, 40, 50 and 60 minutes, the contents of the microcentrifuge tubes were transferred into 9.5mL of WCB, equilibrated to either 70°C or chilled on ice. Heat treated samples were kept at 70°C for 10 minutes. Serial dilutions were performed and 1mL samples were placed in sterile Petri dishes, before the addition of 15mL of molten FAA with 0.1% ST. Plates were incubated at 37°C, anaerobically for 48 hours and the CFU/mL was calculated.

#### **4.2.6.1 Determination of the optimum concentrations of bile salts required for germination of *C. difficile* spores**

The same method to determine loss of heat resistance was used in this experiment; however, spore suspensions were exposed to each bile salt solution for one hour before heat shocking. The bile salts tested included: sodium taurocholate, cholate, glycocholate, deoxycholate ( $\geq 98\%$ ) and chenodeoxycholate (Sigma Aldrich, UK), at concentrations of 0.001, 0.01, 0.1, 1, 10 and 100mM. (Deoxycholate did not dissolve at 100mM and therefore only 0.001-10mM deoxycholate was tested).

#### **4.2.6.2 Rate of germination in anaerobic and aerobic conditions and the effect of temperature**

The ethanol shock method was carried out at 20°C and 37°C aerobically and at 37°C in anaerobic conditions. In all anaerobic tests, the spore suspensions, germinant solutions and broth were incubated in the anaerobic chamber (37°C) for one hour before the experiment was started in order for the solutions to equilibrate.

#### **4.2.6.3 Effect of pH on the rate of germination of *C. difficile* spores**

The pH of the thioglycollate medium containing 6.9mM ST was adjusted with either hydrochloric acid or sodium hydroxide before autoclaving. The pH of the sterilized medium was then measured after one hour incubation in anaerobic conditions. The pH range tested was from pH 5.5 to pH 8.5. The ethanol shock method was implemented in this test as it was performed in the anaerobic cabinet. All solutions used in the experiment (i.e. the germinant, spore suspension, broth, ethanol and water) were left in the anaerobic cabinet for one hour to equilibrate before testing commenced. The pH of the germinant solutions was monitored to ensure they remained stable in the anaerobic environment.

#### **4.2.6.4 Determination of components required for germination of *C. difficile* spores**

The heat shock method was implemented in this test. Spore suspensions of *C. difficile* NCTC 11204 were exposed to different components of the germination media for 60 minutes prior to heat shocking. The following combinations were mixed with 6.9mM taurocholate: thioglycollate medium (TM), sodium thioglycollate only (not the medium), TM without glucose, TM without glucose or yeast, TM without glucose, yeast or tryptone and TM without glucose, tryptone, yeast or cysteine. (Thioglycollate medium contains the following: tryptone (15g/L), yeast extract (5g/L), sodium chloride (2.5g/L), sodium thioglycollate (0.5g/L), L-cysteine (0.5g/L), resazurin (0.001g/L), agar (0.75g/L), glucose (5.5g/L)).

One percent (w/v) lysozyme was dissolved in 1mL SDW, filter-sterilised (0.45µm) and added to 9mL sterilized thioglycollate medium (final concentration of 0.1% lysozyme). 0.1% (w/v) dodecylamine was dissolved in 10mM Tris-HCl buffer (pH 7.5) (Vepachedu and Setlow, 2007) and filter-sterilised (0.45µm) and 0.1% (w/v) taurocholate was dissolved in thioglycollate medium and sterilized by autoclaving. Each germinant was incubated with *C. difficile* NCTC 11204 for 60 minutes before heat shocking and culturing (as described previously).

#### **4.2.6.5 Efficacy of glycine and taurocholate as co-germinants of *C. difficile* spores**

The method used to measure heat resistance was implemented in this test. Spore suspensions were exposed to thioglycollate medium containing 6.9mM ST or glycine (0.2% w/v) dissolved in sterilised distilled water, containing 6.9mM ST for 60 minutes.

#### **4.2.7 Visualisation of germination by membrane integrity staining**

The method used by Hashimoto *et al.* (1969) to visualize germination in *Bacillus cereus* and *B. megaterium* spores was adapted: 1mL of *C. difficile* NCTC 11204 spores was centrifuged at 1300rpm for 5 minutes and the supernatant discarded. The pellet was resuspended in 100µL of sterile distilled water. After vigorous mixing, 5µL of the spore suspension was spread on a glass coverslip and allowed to dry at room temperature. The dried spore suspension was then covered with 100µL of methanol for 15-20 seconds. One microlitre of LIVE/DEAD® BacLight™ stain (containing 1.67mM SYTO® 9 and 18.3mM propidium iodide) (Invitrogen- Molecular Probes, UK) was then added to 100µL of either thioglycollate medium alone, or thioglycollate medium with 6.9mM ST. Ten microlitres of the solution containing the stain was placed on a glass microscope slide and the coverslip containing the dried spore film (sample face down) was then mounted on the slide over the drop of thioglycollate and pressed firmly (Hashimoto *et al.*, 1969). The slide was immediately examined by phase contrast and epifluorescence microscopy with a Zeiss Axioskop microscope (100x/1.30 Plan NEOFLUAR oil immersion objective), fitted with a Zeiss AxioCam HRc and AxioVision 3.1 software. Pictures were cropped in the same area and the auto-contrast tool was performed on all pictures, using Adobe Photoshop, version 7.0.

#### **4.2.8 Statistical analysis**

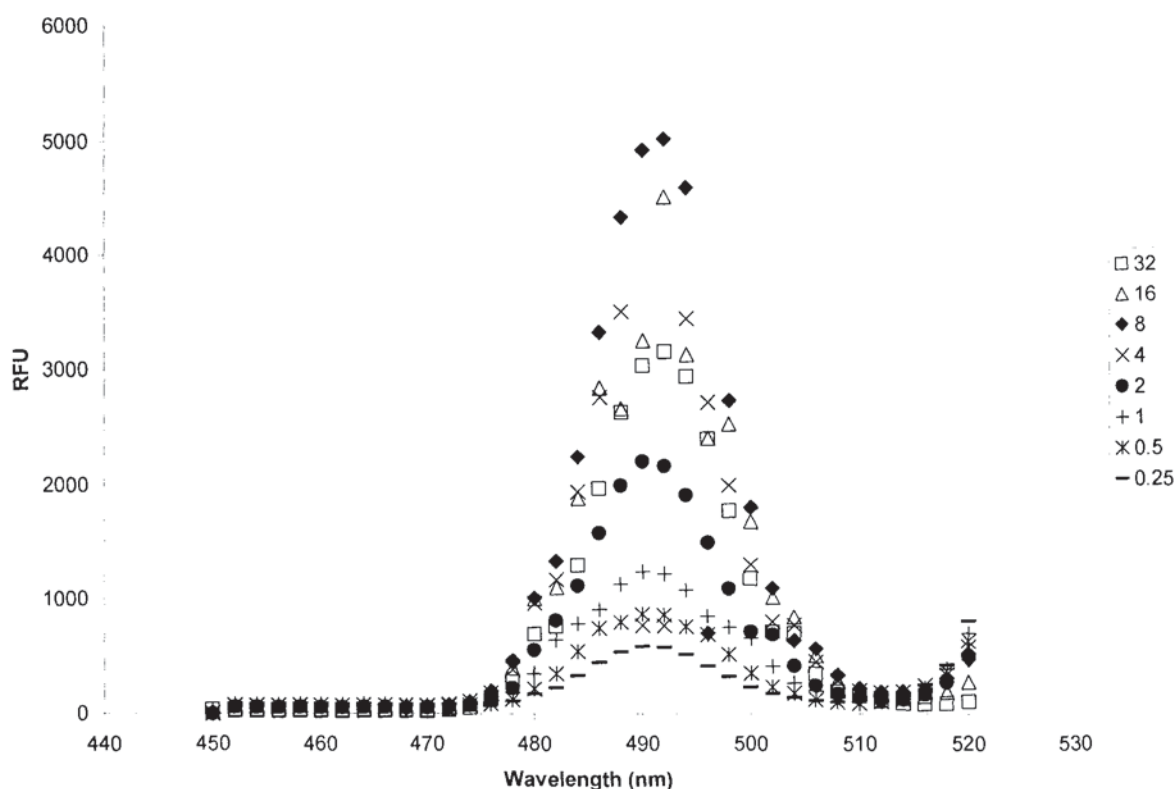
Data were analysed using either an unpaired t-test or a one-way ANOVA with Tukey Kramer multiple comparisons test (GraphPad InStat, version 3.06)



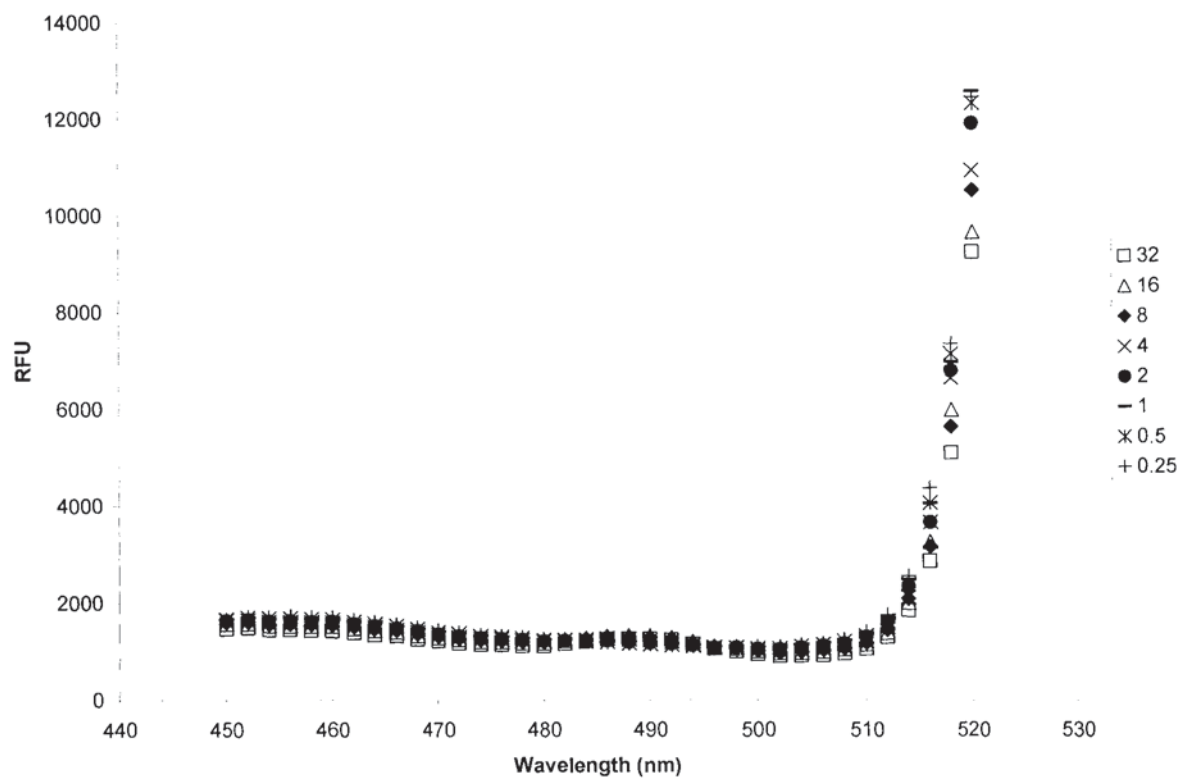
## 4.3 Results

### 4.3.1 Measurement of germination by the fluorescence of dipicolinic acid (DPA) released from germinating spores

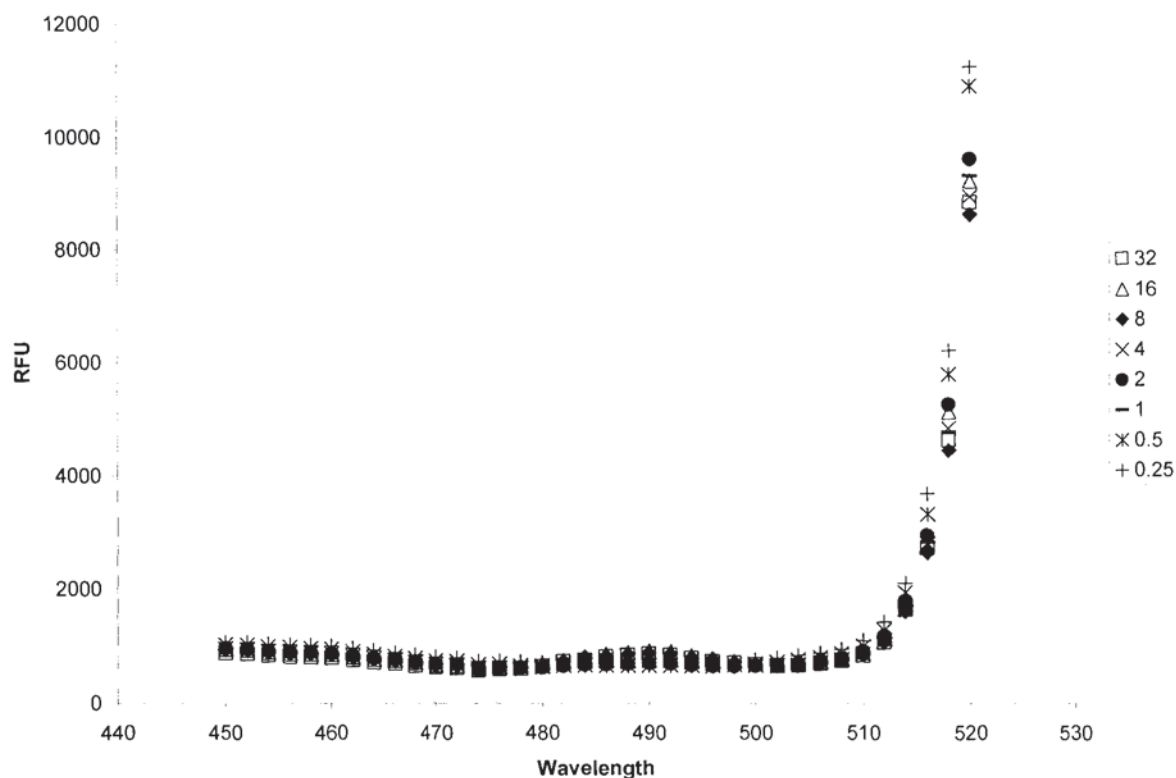
Each concentration of DPA dissolved in buffer solution produced a peak of fluorescence at a wavelength of between 479 and 510nm. The level of fluorescence increased in a concentration dependent manner with DPA, except when DPA concentration reached above 8 $\mu$ g/mL (figure 4.1). However, when DPA was dissolved in thioglycollate medium containing bile salts the fluorescence was quenched and there was no peak observed at any concentration of DPA (figure 4.2). Quenching also occurred when the bile salts were omitted from the thioglycollate medium (figure 4.3). As quenching occurred this method could not be used in further tests.



**Figure 4.1** Fluorescence emission spectra of varying concentrations of DPA (μM) in buffer, excitation wavelength 270nm.



**Figure 4.2** Fluorescence emission spectra of DPA ( $\mu\text{M}$ ) in thioglycollate medium containing sodium taurocholate, glycocholate, chenodeoxycholate and cholate, (excitation wavelength 270nm).



**Figure 4.3** Fluorescence emission spectra of varying concentrations of DPA ( $\mu\text{M}$ ) in thioglycollate medium, (excitation wavelength 270nm).

#### 4.3.2 Measurement of germination of *C. difficile* spores by optical density (OD)

At either wavelength tested, no difference in OD was observed after 60 minutes incubation of *C. difficile* spores with thioglycollate medium containing ST, compared with the control solutions (table 4.1). This method therefore, proved to be unsuccessful in measuring the germination of *C. difficile* spores in this investigation.

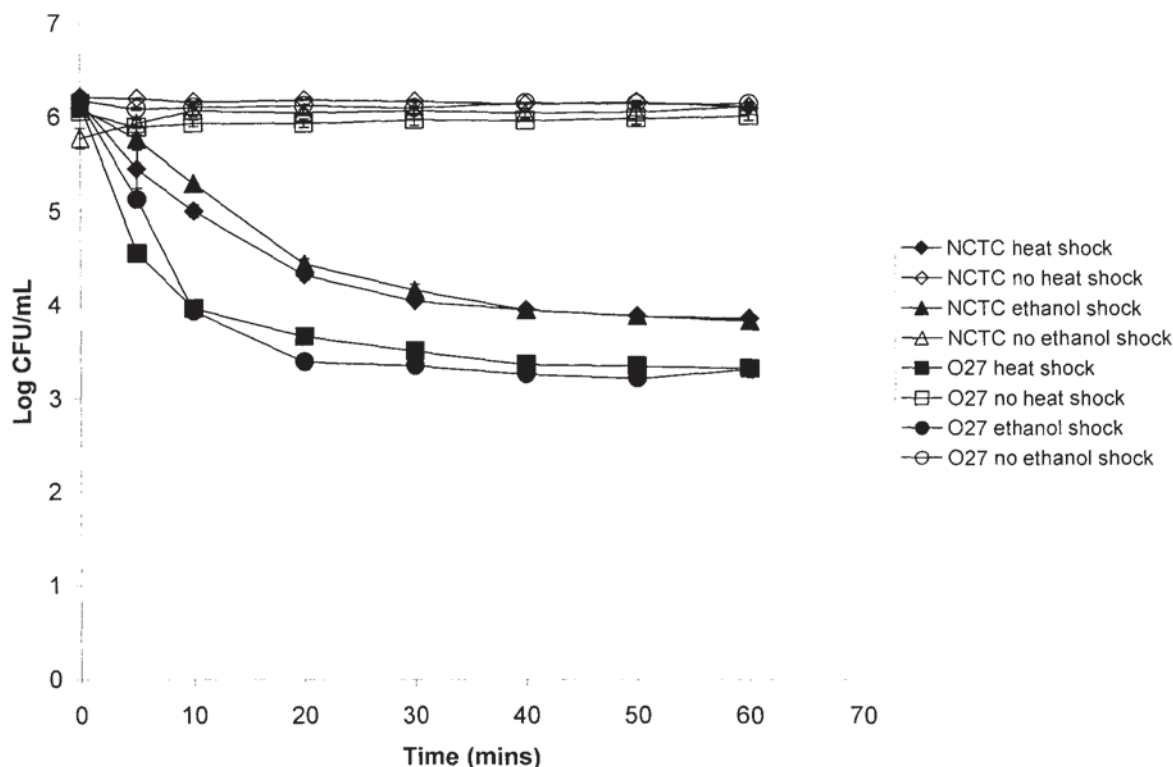
**Table 4.1 Effect of germinating *C. difficile* spores (NCTC 11204) on optical density of suspension**

Test suspension	Time 0	Time	Time 0	Time
	(620nm)	60mins (620nm)	(450nm)	60mins (450nm)
Spores with thioglycollate and taurocholate	0.151	0.148	0.246	0.247
Spores in water	0.152	0.143	0.198	0.197
Thioglycollate and taurocholate in water	0.069	0.065	0.125	0.121

#### **4.3.3 Rate of germination of *C. difficile* spores by measurement of the loss of heat and chemical resistance**

*C. difficile* spores exposed to 6.9mM ST in thioglycollate medium demonstrated sensitivity to both heat and ethanol (figure 4.4). *C. difficile* ribotype 027 demonstrated a significantly faster rate and higher level of spore germination compared to NCTC 11204 within the first 5-40 minutes of exposure to the germinant ( $p < 0.05$ ). However, after 40 minutes exposure, there was no significant difference in the level of germination between either strains ( $P > 0.05$ ); a log reduction in spores of *C. difficile* ribotype 027 of 2.78 and 2.9 was achieved with heat and ethanol shock respectively whilst a log reduction of 2.21 in spores after heat shock and of 2.15 after ethanol shock was demonstrated in *C. difficile* NCTC 11204. There was no significant difference at any point, between the loss of heat and chemical resistance in either strain ( $P > 0.05$ ).



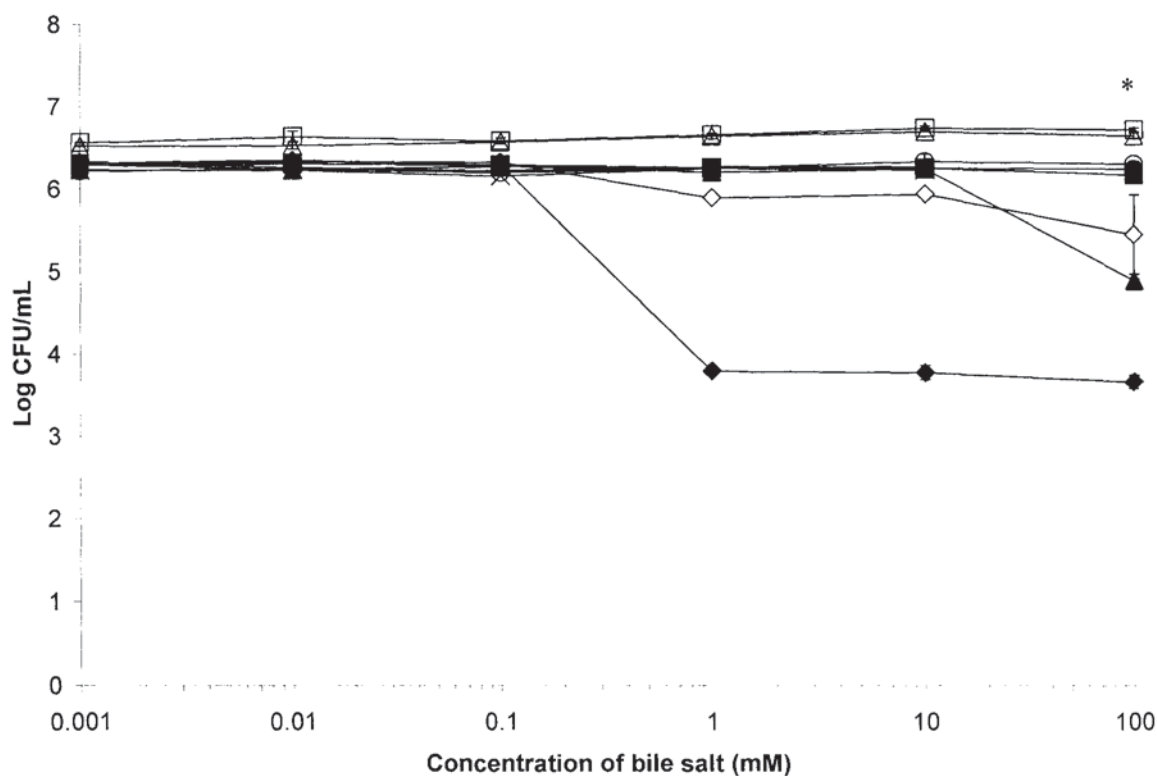


**Figure 4.4 Viability of *C. difficile* NCTC 11204 and ribotype 027 spore suspensions following exposure to germinant and exposure to heat or ethanol shock**

#### 4.3.3.1 Determination of the optimum concentrations of bile salts required for germination of *C. difficile* spores

Sodium taurocholate (ST) and chenodeoxycholate were the only members of the bile salts studied found to initiate germination of *C. difficile* NCTC 11204 spores, as measured by loss of heat resistance. Concentrations of the optimum germinant (ST) between 0.1 and 100mM were required to achieve a 2.49-2.62 log reduction in spores (figure 4.5) compared to chenodeoxycholate where a 1.28 log reduction was observed at 100mM. The optimum concentration of ST to achieve maximum germination was 1-10mM. Higher concentrations (100mM) of ST demonstrated an inhibitory effect on *C. difficile* spores as a 0.89 log reduction was observed in the control (ST and ice). A minimum concentration of 0.1mM ST was required to initiate germination in both *C. difficile* NCTC 11204 and *C. difficile* ribotype 027. Following

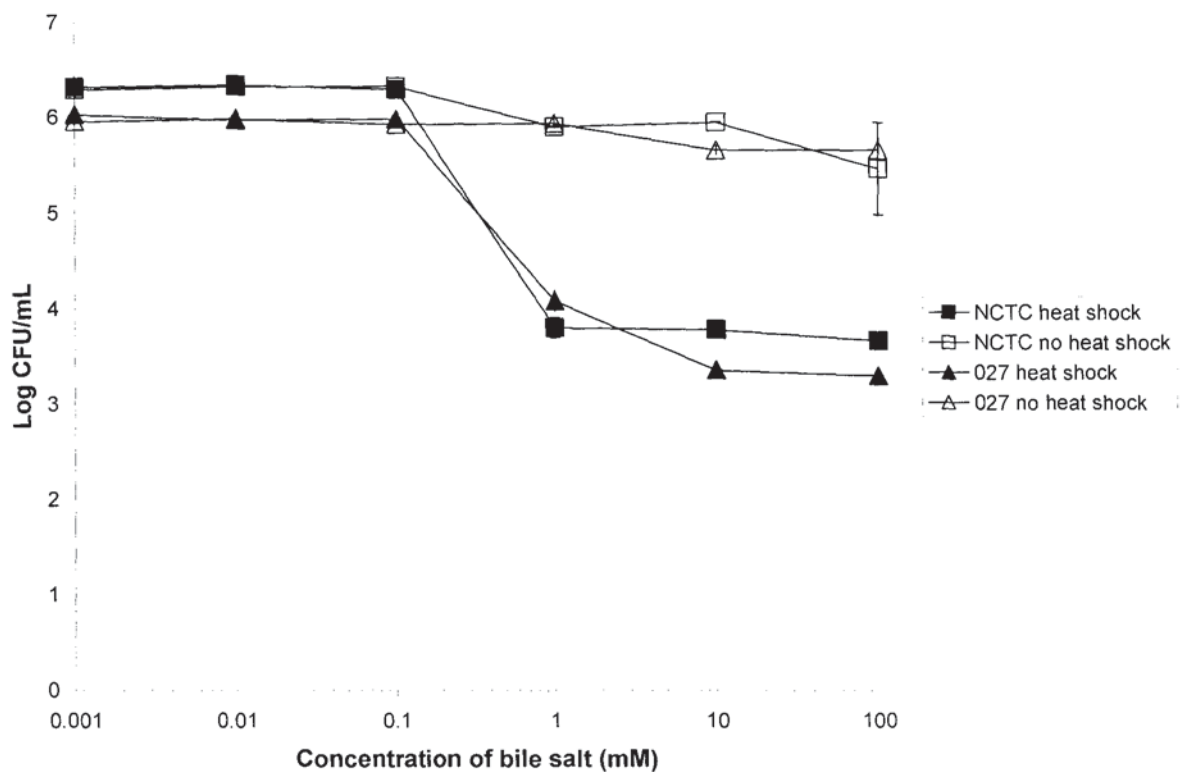
exposure to ST at concentrations of 1, 10 and 100mM, heat shocking yielded a 2.49 - 2.62 log reduction in spores of *C. difficile* NCTC 11204 and a 1.93 - 2.71 log reduction in spores of *C. difficile* ribotype 027 (figure 4.6).



\* No data for 100mM deoxycholate

Filled diamonds: ST and heat shock, open diamonds: ST and ice, filled triangles: chenodeoxycholate and heat shock, open triangles: chenodeoxycholate and ice, filled squares: glycocholate and heat shock, open squares: glycocholate and ice, filled circles: cholate and heat shock, open circles: cholate and ice, straight line: deoxycholate and heat shock, cross: deoxycholate and ice.

**Figure 4.5** Effect of different bile salts on the germination of *C. difficile* spores (strain NCTC 11204) shown by viability following heat shock



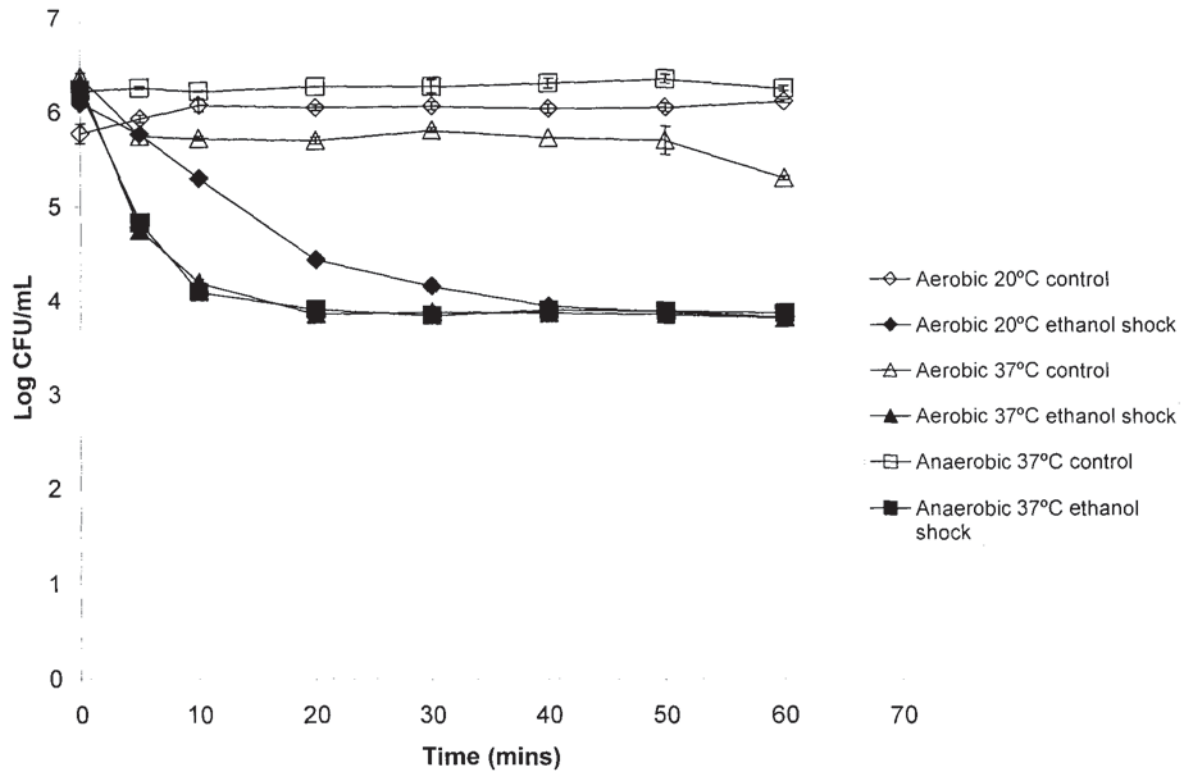
**Figure 4.6** Effect of ST concentration in the germination medium on the spores of *C. difficile* NCTC 11204 and ribotype 027 shown by viability following heat shock

#### 4.3.3.2 Rate of germination under anaerobic and aerobic conditions and the effect of temperature

There was no significant difference in the rate of spore germination in *C. difficile* NCTC 11204 and ribotype 027 under either anaerobic or aerobic conditions ( $P > 0.05$ ) (figures 4.7 and 4.8). The initial rate of germination in both strains of *C. difficile* was significantly influenced by temperature with a faster rate of germination occurring at 37°C compared to 20°C ( $P < 0.05$ ) within the first 5- 20 minutes (for *C. difficile* ribotype 027) and 5-30 minutes (for *C. difficile* NCTC 11204).

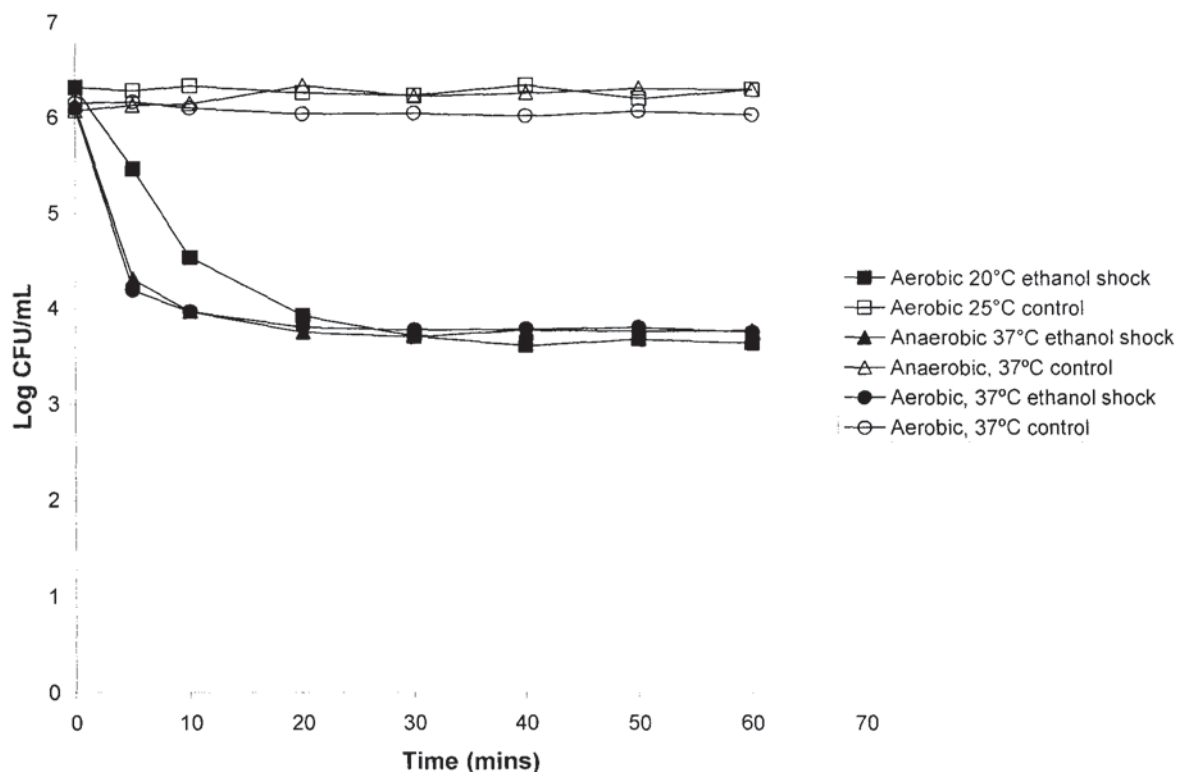
At 37°C, in both aerobic and anaerobic conditions, a 1.5-1.9 log reduction in spores was achieved within the first five minutes for both strains, whilst at 20°C a 0.33-0.85 log reduction was observed. However, following 60 minutes exposure to the

germinant there was no significant difference in the level of germination in both strains at 20°C and 37°C.



**Figure 4.7** Effect of aerobic and anaerobic conditions and temperature on the germination of *C. difficile* NCTC 11204 spores shown by viability following 70% ethanol shock

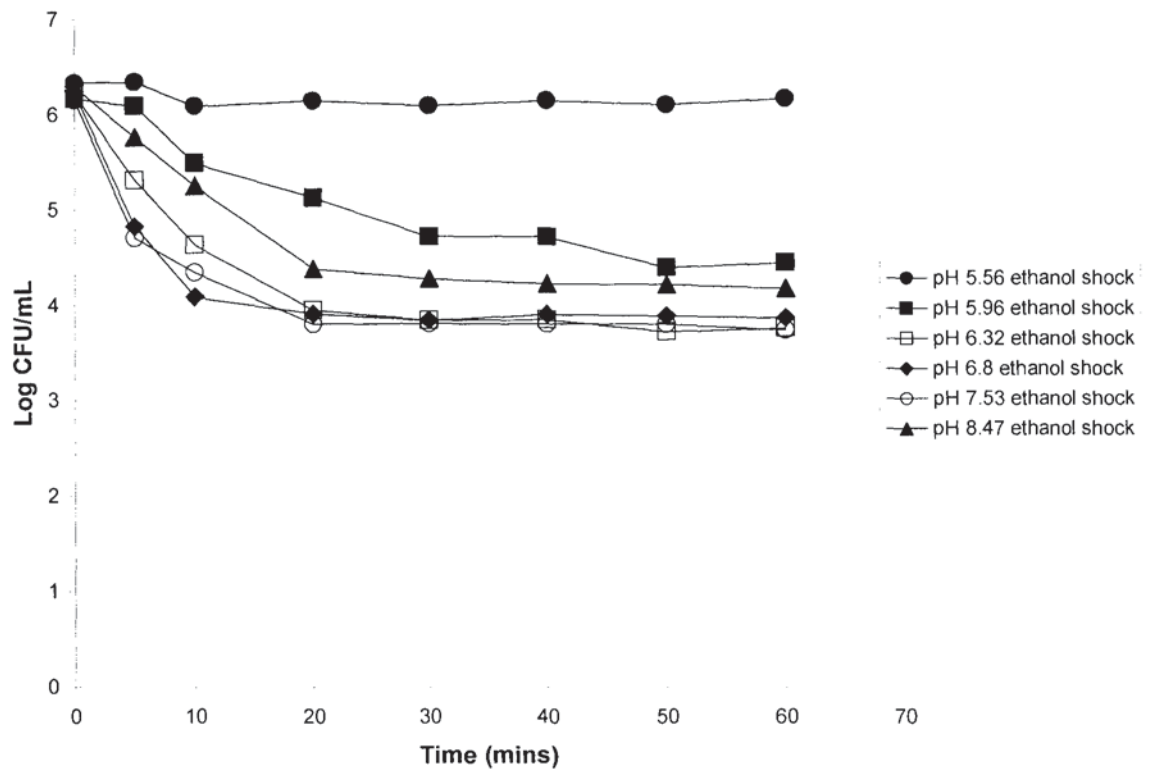




**Figure 4.8** Effect of aerobic and anaerobic conditions and temperature on the germination of *C. difficile* 027 spores shown by viability following 70% ethanol shock

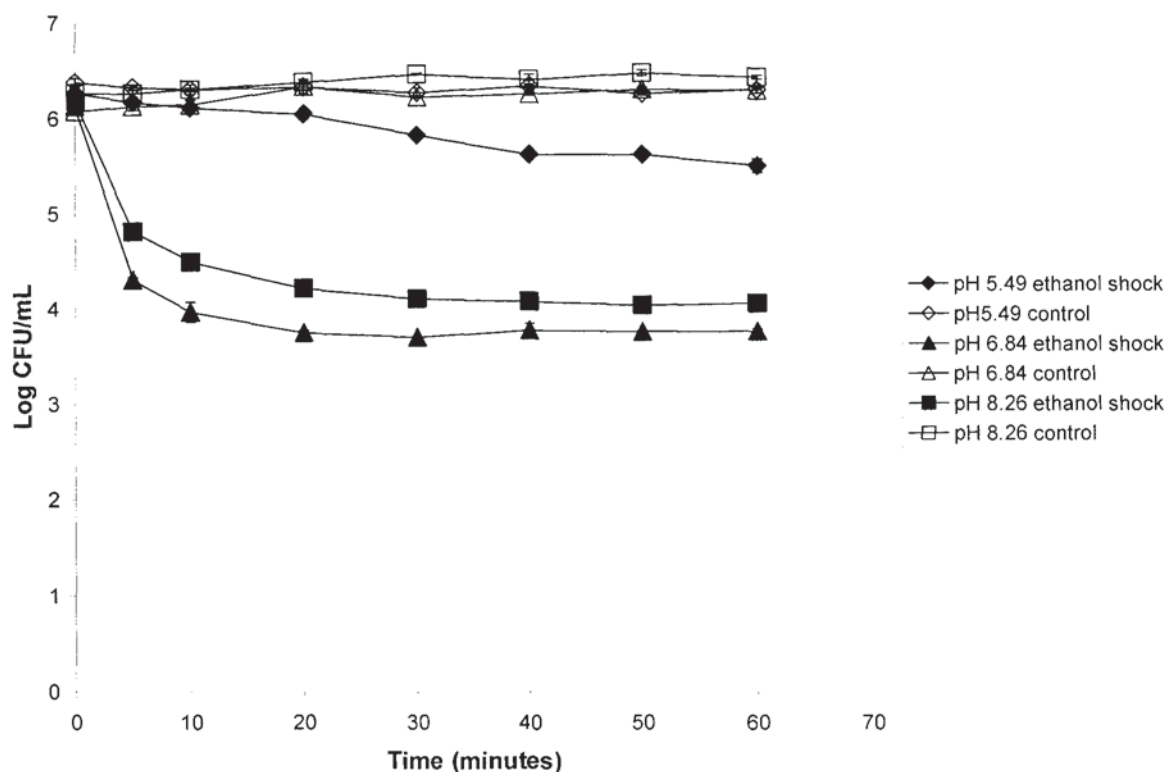
#### 4.3.3.3 Effect of pH on the rate and extent of germination

The rate and extent of *C. difficile* spore germination in both strains was significantly reduced in acidic conditions (pH 5.49-6.84), compared to neutral/ alkaline conditions (pH 6.8-8.47) ( $P < 0.05$ ), with the maximum rate and extent of germination occurring between pH 6.32 and pH 7.53 (figure 4.9 and 4.10). At pH 5.56 there was a 0.16 log reduction in CFU/mL of the NCTC 11204 strain observed after 60 minutes incubation, compared to a 2.36 and 2.12 log reduction at pH 6.8 and pH 8.47 respectively (figure 4.9). Similarly, with *C. difficile* ribotype 027, after 1 hour incubation of spores at pH 6.84, there was a 2.36 log reduction in CFU/mL, compared to a log reduction of 2.12 and 0.75 at pH 8.26 and pH 5.49 respectively (figure 4.10).



(All corresponding controls for each pH without ethanol shock produced no log reduction over time, but are not shown for clarity).

**Figure 4.9** Effect of germinant pH on the germination of *C. difficile* NCTC 11204 spores in anaerobic conditions shown by viability following 70% ethanol shock

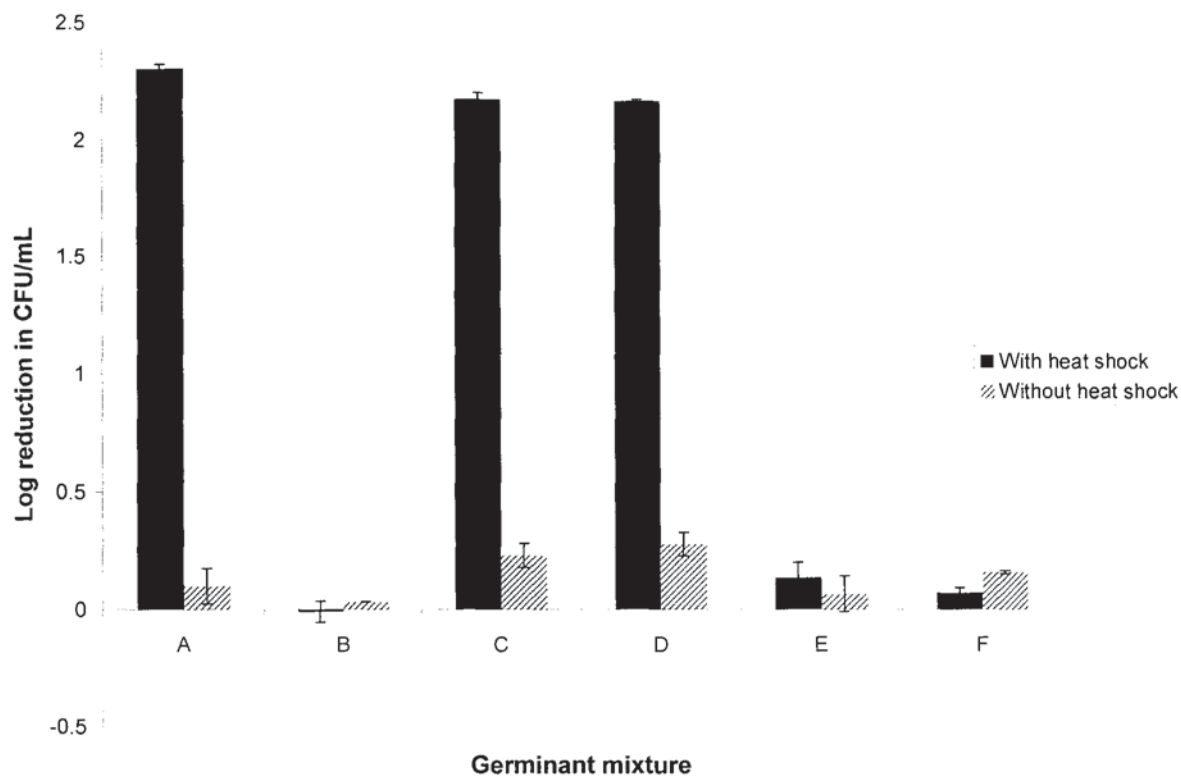


**Figure 4.10** Effect of pH of the germination medium on the germination of *C. difficile* ribotype 027 shown by viability following 70% ethanol shock

#### 4.3.3.4 Determination of the components required for germination of *C. difficile* spores

*C. difficile* spores exposed to ST (6.9mM) with all components of the thioglycollate medium gave a log reduction in CFU/mL of 2.3. Similar log reduction values in CFU/mL were observed after spores were exposed to ST and thioglycollate medium without glucose or yeast. However, there was no log reduction in CFU/mL after *C. difficile* spores were exposed to ST and sodium thioglycollate alone, or when tryptone was removed from the thioglycollate medium (figure 4.11).

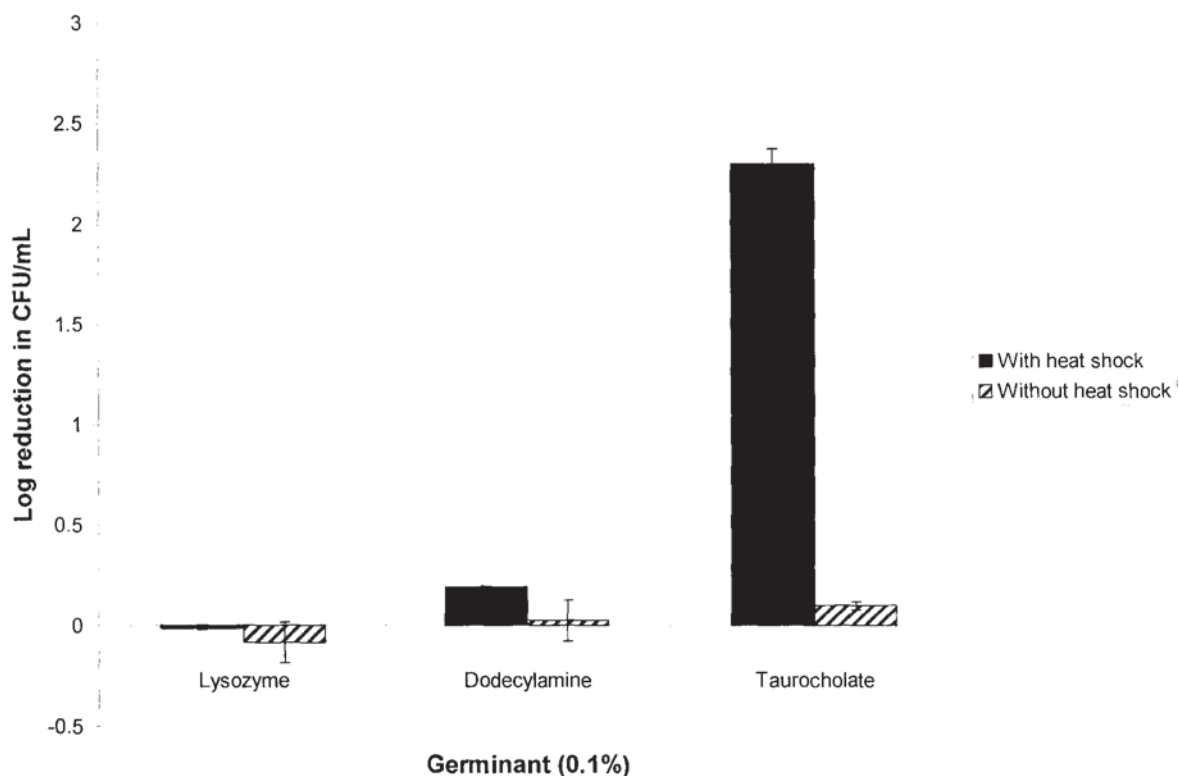
Exposure of *C. difficile* spores to either lysozyme or dodecylamine produced less than a 0.2 log reduction in CFU/mL after heat shocking, compared to a 2.3 log reduction in CFU/mL with ST (figure 4.12).



A= Thioglycollate medium, B= Sodium thioglycollate C= Thioglycollate medium without glucose, D= Thioglycollate medium without glucose or yeast, E= Thioglycollate medium without glucose, yeast or tryptone, F= Thioglycollate medium without glucose, tryptone, yeast or cysteine. (A - F also contained 6.9mM ST)

**Figure 4.11 Determination of the components of the germination medium required for germination of *C. difficile* NCTC 11204 spores**

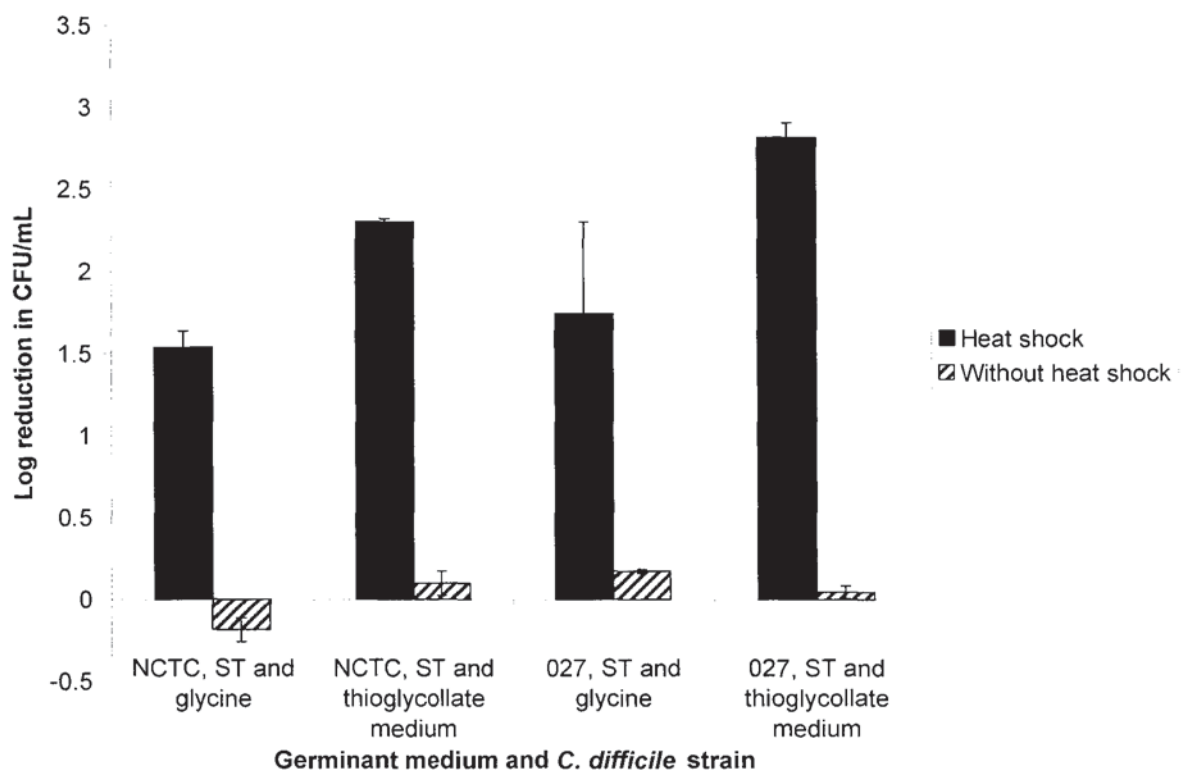




**Figure 4.12 Efficacy of germinants on the spores of *C. difficile* NCTC 11204 after 1 h exposure**

#### **4.3.3.5 Efficacy of glycine and taurocholate as co-germinants of *C. difficile* spores**

Glycine, an amino acid contained in tryptone, together with ST (6.9mM) produced a log reduction of 1.54 and 1.74 in the NCTC 11204 strain of *C. difficile* and ribotype 027 respectively. However, after exposure to ST, with all the components of the thioglycollate medium, heat shocking produced significantly higher ( $P < 0.03$ ) log reductions in CFU/mL of 2.3 and 2.82 with strain NCTC 11204 and ribotype 027 respectively (figure 4.13).



**Figure 4.13** Log reduction in CFU/mL of *C. difficile* NCTC 11204 and ribotype 027 spores after 60 minutes exposure to different germination media

#### **4.3.4 Observation of germination by membrane integrity staining and phase contrast microscopy**

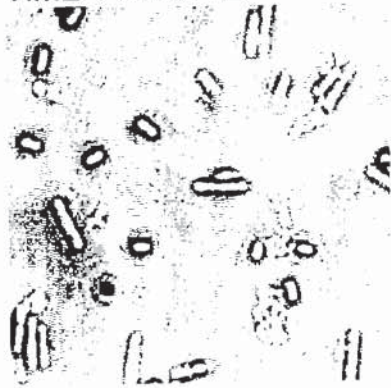
Germination of spores of *C. difficile* in the presence of ST was confirmed by membrane integrity staining (with fluorescence microscopy) and phase contrast microscopy. At 1 minute exposure to 6.9mM ST in thioglycollate medium, spores appeared phase bright compared to phase dark dead vegetative cells and after 20 minutes exposure there was only slight swelling of the spores. However, after 160 minutes, spores became phase dark and were swollen and more rounded in appearance (figure 4.14). The control spores remained phase bright and did not become swollen or change in shape.

Membrane integrity staining showed that after 20 minutes exposure to 6.9mM ST in thioglycollate medium, slight, peripheral green fluorescence of *C. difficile* spores could be observed, with dead vegetative cells stained red (Figure 4.15). At 160 minutes exposure, spore germination was clearly visible and confirmed by the presence of swollen, brightly fluorescent terminal spores and free spores of *C. difficile*. Only slight, peripheral staining of spores exposed to thioglycollate medium only (control) was observed after 160 minutes.

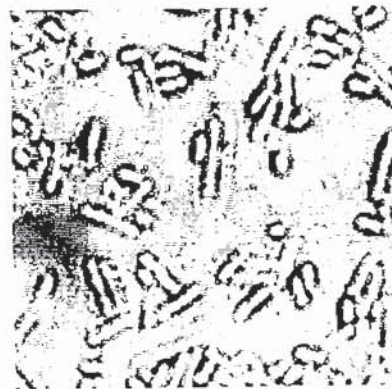
CONTROL  
(Thioglycollate medium only)

GERMINATION MEDIA  
(6.9mM ST in thioglycollate medium)

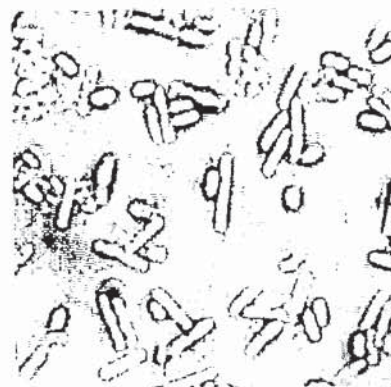
TIME 1 MINUTE



TIME 20 MINUTES



TIME 160 MINUTES



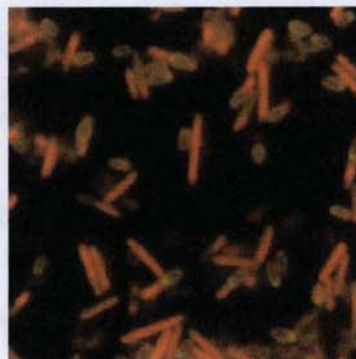
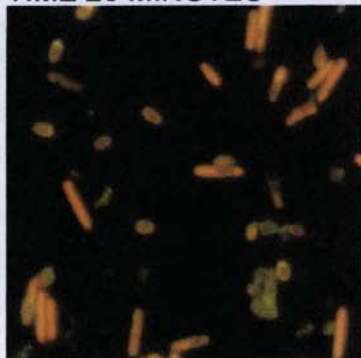
**Figure 4.14** Spore suspensions exposed to germinant medium and observed under phase contrast microscopy (x100 magnification, 67ms exposure time)



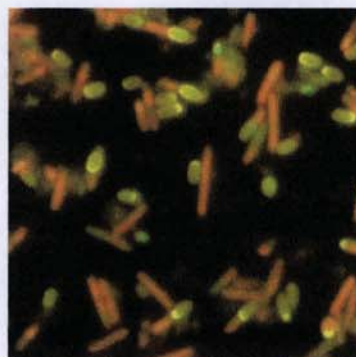
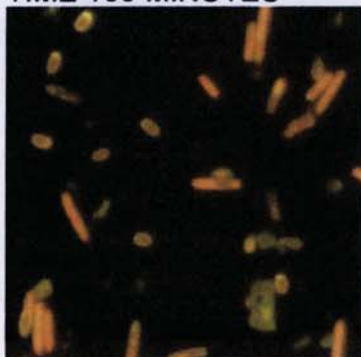
CONTROL  
(Thioglycollate medium only)

GERMINATION MEDIA  
(6.9mM ST in thioglycollate medium)

TIME 20 MINUTES



TIME 160 MINUTES



**Figure 4.15** Spore suspensions exposed to germinant medium and observed under fluorescence microscopy (x100 magnification, 1341ms exposure time)

## 4.4 Discussion

Determining germination of *C. difficile* spores by measuring DPA fluorescence and reduction in optical density (OD) was ineffective in this investigation. DPA fluorescence was quenched when the thioglycollate medium was added to the solution and there was no reduction in OD after incubation of the germinant with *C. difficile* spores, compared to control solutions. However, the indirect measure of germination by loss of resistance to heat or ethanol was successful. Loss of both heat and chemical (70% ethanol) resistance in *C. difficile* spores (NCTC 11204 and 027) occurred at similar rates following exposure to 6.9mM ST in thioglycollate medium. The sequence of events that occurs once germination has been triggered varies between different species of bacteria, however loss of heat and chemical resistance occurs in the very early stages of germination (Levinson and Hyatt, 1966, Rowley and Feeherry, 1970) making these factors useful indicators of spore germination.

The chemical factors influencing *C. difficile* spore germination investigated in this current study were the bile salts. Sodium taurocholate (ST) and chenodeoxycholate were the only bile salts found to initiate germination in *C. difficile* with ST showing the greatest activity as a germinant at concentrations between 0.1 and 100mM (optimum 1-10mM). The normal physiological concentration of ST, in the duodenum is 6.9mM and 1.2mM in the jejunum (Leverrier *et al.*, 2003), which coincide with the optimum range for germination of *C. difficile* spores as demonstrated in this current investigation. These results concur with the recent findings of Sorg and Sonenshein (2008) who demonstrated that taurocholate (at 1%, which equates to ~ 20mM) was the only bile salt capable of inducing rapid germination of *C. difficile* spores in solution. Previous studies have also indicated that glycocholate, cholate and deoxycholate are also capable of stimulating

germination; however, extensive periods of exposure are required (Wilson, 1983, Sorg and Sonenshein, 2008). The results of this thesis however, do not concur with those of Paredes-Sabja *et al.* (2008), who found that bile salts had very little effect on the germination of *C. difficile* spores. This may be due to the heat shocking of the spores at 80°C for 10 minutes in the study by Paredes-Sabja *et al.* (2008) before exposure to the bile salts, which may have affected the ability of the spores to germinate.

In this investigation there was very little variability in germination between different strains of *C. difficile* following exposure to the germinant. Heat shocking produced between a 1.93 - 2.7 log reduction in CFU/mL after exposure to 1, 10 and 100mM ST in both strains, with less than a 1 log reduction at 0, 0.001, 0.001 and 0.1mM.

Interestingly, there was no difference in the rate or extent of germination of *C. difficile* spores when germinated under aerobic or anaerobic conditions. These findings concur with those of Paredes-Sabja *et al.* (2008) who also found little difference between germination in aerobic and anaerobic conditions with *C. difficile* spores. The ability of *C. difficile* spores to germinate in both aerobic and anaerobic conditions may be a key factor for the microorganism to achieve maximum germination within the human body. Indeed, the point at which ST is at optimal concentration for germination in the digestive system is in the duodenum and jejunum, which are aerobic environments. However, in the anaerobic conditions of the colon, which are necessary for *C. difficile* to proliferate and cause disease, the concentration of ST is below that required for spore germination.

The fact that *C. difficile* spores readily germinate under aerobic conditions

may also potentially be exploited within the clinical setting in developing new strategies to eliminate spores from the environment. It is widely known that germinating spores demonstrate an increased susceptibility to common antimicrobial agents compared to dormant spores. Incorporation of a germination solution into the clinical environment, perhaps as part of a two-stage disinfection procedure, may potentially reduce the resistance of *C. difficile* spores, thus rendering them susceptible to common hard surface disinfectants, for example 70% isopropyl alcohol and 2% chlorhexidine/70% isopropyl alcohol. This 'germinate / exterminate' approach may avoid the need to use more hazardous sporicidal agents including bleach and peracetic acid. Indeed, a two-stage cleaning process was previously described by Hornstra *et al.* (2007) with germinating spores of *Bacillus cereus* and is discussed in the following chapters of this thesis in relation to *C. difficile* spores.

Increasing the temperature from 20°C to 37°C had a significant effect on the rate but not the extent of germination of *C. difficile* spores. A faster rate of germination was observed at 37°C (body temperature) between 0 and 40 minutes with the NCTC 11204 strain and between 0 and 20 minutes with ribotype 027 strain than from either strain at 20°C (room temperature). This finding is also of importance if strategies to germinate *C. difficile* spores within the environment are developed as the germinant must be in contact with the spores for at least 30 minutes to ensure maximal germination in the air.

The pH at which *C. difficile* spores were incubated with the germinant had a significant effect on both the rate and the extent of germination. Maximum germination of both test strains was achieved between pH values of 6.32 and 7.53. These results are in agreement with those of Paredes-Sabja *et al.* (2008) who found that the pH optimum for germination of KCl-induced *C. difficile* spores was between



6 and 7.5. Germination was only marginally reduced at an alkaline pH (8.26-8.47), whilst acidic conditions significantly reduced the germination process. As ingested spores of *C. difficile* pass through the gastrointestinal tract, they encounter a range of pHs; from the highly acidic environment of the stomach to the duodenum where bile salts are secreted from the gall bladder. The pH of the duodenum is between 5.7 and 6.4 and the concentration of ST in the duodenum is around 6.9mM (optimum for germination). As the spores enter the jejunum, the pH increases to between 5.9 and 6.8, which presents a more favourable environment for germination.

Treatments which increase gastric pH such as proton pump inhibitors and gastric acid suppressors have been described as risk factors for development of CDAD (Dubberke *et al.*, 2007, Jump *et al.*, 2007). Jump *et al.* (2007) found that vegetative *C. difficile* cells could survive in gastric contents if the pH was above or equal to 5. They suggested that treatment with proton pump inhibitors may lead to increased survival of vegetative cells and germinated spores in the intestine, therefore increasing the number of *C. difficile* cells capable of colonizing the gut and causing disease.

Sodium thioglycollate, glucose, yeast and cysteine were not essential for germination of *C. difficile* spores as the spores were still able to germinate in their absence. However, the removal of tryptone (containing many amino acids) from the germination media significantly reduced the level of germination of *C. difficile* spores, indicating that one or more amino acids were essential for germination. Stimulation of germination was partly restored with the addition of a single amino acid; glycine to the germination media. Glycine as a chemical initiator of germination has been previously shown to trigger the process in spores of *Clostridium botulinum* (Ward and Carroll, 1966). Furthermore, in *C. difficile*, it has been described as a co-

germinant, together with ST (Sorg and Sonenshein, 2008). In this study, glycine and ST were also found to stimulate germination, but to a lesser extent than with ST and the full thioglycollate medium. This finding suggests that other amino acids may indeed be required, in addition to glycine and ST, to produce optimum levels of germination. When considering potential germination techniques as part of a 'germination-based' disinfection regime in the clinical setting, other constituents in addition to glycine and ST may be required to develop the optimal germination solution. Further work should concentrate on establishing which additional components are essential to achieve maximum germination of *C. difficile* spores.

Lysozyme and dodecylamine were ineffective at stimulating germination of *C. difficile* spores in the 60 minute test period, compared to ST. Other studies have shown increased recovery rates of *C. difficile* spores (and therefore enhanced germination) with the incorporation of lysozyme into agar medium, with and without pre-treatment with alkaline thioglycollate (Kamiya *et al.*, 1989, Wilcox *et al.*, 2000). This indicates that lysozyme may require a longer incubation to induce germination in *C. difficile*. Dodecylamine has been demonstrated to be an effective germinant in *Bacillus* species and is able to exert its effects without the need for a receptor (Setlow, 2003, Moir, 2006), however; in this investigation it did not stimulate germination of *C. difficile* spores within the test period. Conflicting results were found by Paredes-Sabja *et al.* (2008) who demonstrated that *C. difficile* spores germinated in the presence of dodecylamine. However, in their study, both spores and germinant were incubated at 60°C which may have had an effect on the germination capabilities of the spore suspension.

*C. difficile* spores appeared phase bright and dead vegetative cells appeared phase dark under phase contrast microscopy prior to the addition of a germinant.

After 160 minutes exposure to ST in thioglycollate medium, spores appeared to have swollen and became phase dark. However, after 160 minutes exposure to thioglycollate medium alone with no germinant, the spores did not change in appearance and remained phase bright. Many other studies use the proportion of phase dark to phase bright spores as a measure of the proportion of spores that have germinated (Caipo *et al.*, 2002, Giorno *et al.*, 2007, Plowman and Peck, 2002). When the LIVE/DEAD® BacLight™ stain was incorporated into the spore suspensions, the dead vegetative cells were stained red, where as there was only a faint peripheral green staining of the dormant spores. Melly *et al.* (2002) also found that the nucleic acids in the dormant spores were not stained by the BacLight™ stain and suggested that this was probably due to the permeability barrier of the inner spore membrane (Melly *et al.*, 2002). Setlow *et al.* (2002) speculated that the peripheral staining of the spores could be due to staining of the peptidoglycan (Setlow *et al.*, 2002). After a 160 minute exposure to the germinant solution in this study, spores of *C. difficile* appeared swollen and fluoresced apple green, where as the control spores retained only a faint peripheral stain.

From this investigation it is clear that many different chemical and physical parameters affect the rate and level of germination of spores. The spore population is heterogenous (Caipo *et al.*, 2002) and therefore, not all spores in a population germinate at the same rate. In all experiments incorporated in this study, a small proportion of spores did not germinate (0.1-0.5%), even after 1 hour exposure to the germinant solution. Hornstra *et al.* (2007) also found that a small proportion of *Bacillus cereus* spores did not germinate when exposed to a germinant and describe these spores as 'highly dormant'. Woese *et al.* (1968) described a model for the kinetics of germination which attempts to account for the two different populations of spores that are apparent in the presence of a germinant in which one group



germinates and the other remains in a dormant state. They suggest that spores contain different amounts of a 'germination substance' and that germination occurs when this substance reaches a critical level (Woese *et al.*, 1968). Caipo *et al.* (2002) found that population density effected germination in *B. megaterium* spores and suggest that this may be due to quorum sensing between the bacterial spores.

In conclusion, this study provides an insight into some of the chemical and physical factors that influence germination of *C. difficile* spores and adds knowledge to the current, relatively limited, understanding of the process. Further work is clearly warranted in this area, particularly if the germination process is exploited to develop novel strategies for the treatment and prevention of CDI.



## **5 Antimicrobial efficacy of 70% (v/v) ethanol against vegetative cells and dormant spores of *C. difficile*, and spores exposed to 1% (w/v) sodium taurocholate in thioglycollate medium**

### **5.1 Introduction**

Spores of *C. difficile* are able to persist on surfaces for extended periods as they are resistant to the hard surface disinfectants commonly used within the clinical setting, for example, 70% (v/v) industrial methylated spirits. Consequently, the more hazardous, less widely used sporicidal agents e.g. hypochlorite are required to effectively eliminate the spores from the environment (Wheeldon *et al.*, 2008, Wutzler *et al.*, 2005). There is therefore a need for new, less hazardous sporicidal disinfectants to be developed or novel disinfection strategies to reduce the spore load of *C. difficile* within the environment.

Hornstra *et al.* (2007) recently investigated the efficacy of a two-stage cleaning procedure with the spore-former *Bacillus cereus*, which involved exposing its spores to a germination solution (containing L-alanine and inosine) prior to a cleaning in place (CIP) procedure. This two-stage approach produced a 3 to 4 log reduction in contaminating spores compared to the CIP alone, which did not produce any log reduction.

In chapter 4 of this thesis it was demonstrated that *C. difficile* spores could be effectively germinated in air, at room temperature by the addition of a germinant solution containing 0.1-100mM (~0.05%-5%) sodium taurocholate (ST) in thioglycollate medium and that these germinating spores were susceptible to both heat and 70% (v/v) ethanol.

As the previous chapter utilized the susceptibility of germinating spores to ethanol as a measure of germination, tests were only conducted in suspension.

However, when assessing the efficacy of ethanol as an antimicrobial for use on hard surfaces, it is important to use an appropriate method in order to simulate the conditions of the clinical environment. Therefore, in this chapter a carrier test method was adopted to assess the efficacy of 70% (v/v) ethanol against dormant and germinating spores of *C. difficile*. Furthermore, the results from chapter 4 of this thesis also demonstrated that the optimum concentration of ST to initiate germination was 1% (w/v). In light of this, 1% (w/v) ST was incorporated into the germination solution in this part of the investigation. The aims of this the study were to determine the optimum exposure time to the germinant to render spores of *C. difficile* susceptible to 70% (v/v) ethanol and the period of contact required with 70% (v/v) ethanol in order to produce a significant reduction in the spore load.

## **5.2 Methods**

### **5.2.1 Bacterial strains**

*C. difficile* NCTC 11204 and ribotype 027 R20291 (Anaerobe Reference Laboratory, Cardiff, UK) were stored on Microbank® beads (Pro-Lab Diagnostics, Cheshire, UK) and kept at -70°C until required.

### **5.2.2 Spore suspensions**

Spore suspensions of *C. difficile* were produced following the method described by Shetty *et al.* (1999) and given in detail in chapter 2, section 2.2.2 of this thesis.

### **5.2.3 Neutraliser**

For neutralization of 70% (v/v) ethanol in this section of work, the neutraliser described in chapter 3 of this thesis, section 3.2.4, was used.

### **5.2.4 Efficacy of the neutraliser to nullify the effect of 70% (v/v) ethanol and cytotoxicity of the neutraliser against vegetative cells of *C. difficile***

To determine the neutralizing efficacy of the neutraliser, 100µL of 70% (v/v) ethanol (SDW for the control) was added to 900µL of neutraliser and was mixed thoroughly by repeated inversion. After 30 minutes 100µL of a *C. difficile* cell suspension (NCTC 11204), standardized to 10<sup>6</sup> CFU/mL (by dilution with WCB and reference to a calibration curve) was added. A 100µL sample was then taken after 60 minutes and spread onto a WCA plate. Plates were incubated anaerobically, for 48 hours, at 37°C.

To ensure the neutraliser was non-toxic to vegetative cells of *C. difficile*, 900µL of neutraliser (SDW for the control) was added to a standardized *C. difficile*

NCTC 11204 cell suspension ( $10^6$  CFU/mL) and mixed thoroughly by repeated inversion. One hundred microlitre samples were taken at 0, 30 and 60 minutes and spread onto a WCA plate. Plates were incubated as described above.

#### **5.2.5 Antimicrobial efficacy of 70% (v/v) ethanol against vegetative cells of *C. difficile* cells**

To confirm antimicrobial efficacy of the ethanol used in this part of the study, initial studies were undertaken against vegetative cells of *C. difficile* which have known susceptibility to ethanol. The vegetative *C. difficile* cell suspension was prepared as described in chapter 2, section 2.2.1 of this thesis. Nine hundred microlitres of 70% (v/v) ethanol (SDW for the control) was added to 100 $\mu$ L of *C. difficile* cell suspension NCTC 11204 ( $10^6$  CFU/mL) in a microcentrifuge tube and mixed thoroughly by repeated inversion for 30 seconds. After 30 minutes incubation (to allow for ethanol to exert its cytotoxic effects) in anaerobic conditions with the lids closed, at 37°C, the entire 1mL sample was transferred to 9mL of neutraliser (section 3.2.4) and mixed thoroughly by inversion for 30 seconds. A 100 $\mu$ L sample was taken and spread onto a WC agar plate. Plates were incubated anaerobically, at 37°C, for 48 hours and the number of CFU/mL were calculated.

#### **5.2.6 Susceptibility of germinating *C. difficile* spores to 70% (v/v) ethanol**

The carrier test method used was adapted from (Perez *et al.*, 2005). Stainless steel coupons (1cm<sup>2</sup>) were inoculated with 10 $\mu$ L of *C. difficile* NCTC 11204 and ribotype 027 spore suspension ( $10^6$  CFU/mL). The suspensions were allowed to dry for 30 minutes under a laminar air flow cabinet and then placed into separate sterile bijoux tubes (contaminated side up). Fifty microlitres of thioglycollate medium, containing 1% (w/v) ST was delivered onto the contaminated surface of each disk for 5, 10, 20



and 30 minutes. Nine hundred and fifty microlitres of either 70% ethanol (v/v) or saline (0.9% w/v) containing 0.1% (v/v) Tween 80 was then added to each tube. After 0, 30, 60 and 90 minutes, the contents of each tube was transferred into 9mL of sterile neutraliser (Espigares *et al.*, 2003). Each neutralized solution was vortex mixed thoroughly for 1 minute before 1mL samples were added to sterile Petri dishes prior to the addition of 15mL of molten FAA (containing 5% (v/v) defibrinated horse blood and 0.1% (w/v) ST). The plates were mixed thoroughly and once dried were incubated anaerobically, at 37°C for 48 hours and the number of CFU/mL determined.

#### **5.2.7 Statistical analysis**

Statistical analysis of data was performed using a one-way ANOVA and Tukey Kramer post-hoc test using the InStat® package (GraphPad Software Inc., version 3.06)

## 5.3 Results

### 5.3.1 Toxicity of 70% (v/v) ethanol and neutraliser against vegetative cells of *C. difficile* and efficacy of neutraliser to nullify the effects of 70% (v/v) ethanol

Ethanol (70% v/v) eliminated all vegetative cells of *C. difficile* within the study time period. No viable cells were recovered after 30 minutes incubation, compared to the control in which  $4.3 \times 10^6$  CFU/mL remained (Table 5.1).

There was no significant difference between the log reduction in viable cells in the neutralized suspension compared to controls (0.124 log reduction;  $P > 0.05$ ) demonstrating the efficacy of the neutraliser to nullify the effects of 70% (v/v) ethanol. Furthermore, the neutraliser was non-toxic towards the vegetative cells of *C. difficile* (0.212 log reduction in CFU/mL after 60 minutes incubation with the neutraliser (table 5.2)).

**Table 5.1 Toxicity of 70% (v/v) ethanol against vegetative cells of *C. difficile* NCTC 11204**

Test suspension	Time 0 Mean CFU/mL (range)	Time 30 mins Mean CFU/mL (range)	Average log reduction in CFU/mL
<i>C. difficile</i> cells and 70% (v/v) ethanol	$3.33 \times 10^6$ ( $2.7 - 4.3 \times 10^6$ )	0	6.52
<i>C. difficile</i> cells and SDW	$4.37 \times 10^6$ ( $2.8 - 5.8 \times 10^6$ )	$4.3 \times 10^6$ ( $3.1 - 5.2 \times 10^6$ )	0.01

**Table 5.2 Efficacy of the neutraliser to nullify the activity of 70% (v/v) ethanol and toxicity against the cells of *C. difficile***

Test suspension	Time 0 Mean CFU/mL (range)	Time 60 mins Mean CFU/mL (range)	Average log reduction in CFU/mL
<i>C. difficile</i> cells and neutralized ethanol	1.7 x 10 <sup>6</sup> (1.51 - 2.04 x 10 <sup>6</sup> )	1.28 x 10 <sup>6</sup> (1.12 – 1.47 x 10 <sup>6</sup> )	0.124
<i>C. difficile</i> cells, neutraliser and SDW	6.17 x 10 <sup>6</sup> (5.2 - 6.8 x 10 <sup>6</sup> )	2.53 x 10 <sup>6</sup> (1.9 – 2.9 x 10 <sup>6</sup> )	0.39
<i>C. difficile</i> cells and neutraliser	1.7 x 10 <sup>6</sup> (1.51 - 2.04 x 10 <sup>6</sup> )	1.04 x 10 <sup>6</sup> (8 x 10 <sup>5</sup> – 1.34 x 10 <sup>6</sup> )	0.212
<i>C. difficile</i> cells and SDW	6.17 x 10 <sup>6</sup> (5.2-6.8 x 10 <sup>6</sup> )	4.63 x 10 <sup>6</sup> (3.3- 5.4 x 10 <sup>6</sup> )	0.124

### 5.3.2 Susceptibility of germinating spores of *C. difficile* to 70% (v/v) ethanol

There was no significant reduction in the number of dormant spores of *C. difficile* exposed to ethanol compared to spores exposed to SDW ( $P>0.05$ ; figures 5.1-5.4) within 90 minutes. These results confirm the lack of sporicidal activity of 70% (v/v) ethanol.

Pre-exposure of *C. difficile* spores of both strains to the germinant (1% ST (w/v) in thioglycollate medium) for a 5 minute time-period was insufficient to induce a significant reduction ( $P>0.05$ ) during exposure to ethanol (figure 5.1).

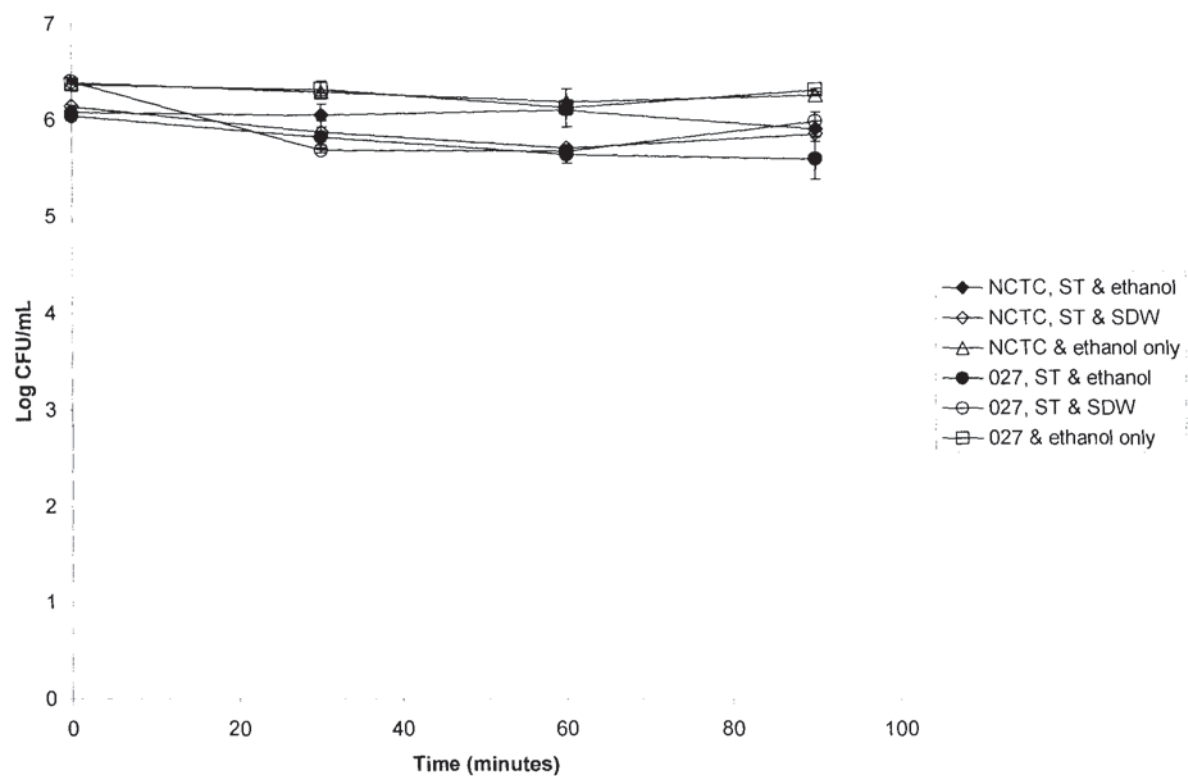
Prior incubation with the germinant for a ten minute period with subsequent ethanol exposure yielded a 0.36 log reduction in the number of remaining *C. difficile* NCTC 11204 spores, which was significant ( $P<0.05$ ) in the first 30 minutes of exposure to ethanol or SDW (Figure 5.2). With ribotype 027, the number of remaining spores after exposure to the germinant followed by ethanol or SDW was

significantly less ( $P<0.05$ ) than after 90 minutes exposure to ethanol alone. Prior incubation with the germinant for ten minutes with subsequent ethanol exposure yielded a 1.51 log reduction in the number of remaining spores compared to a 0.05 log reduction after 90 minutes incubation with ethanol alone (figure 5.2).

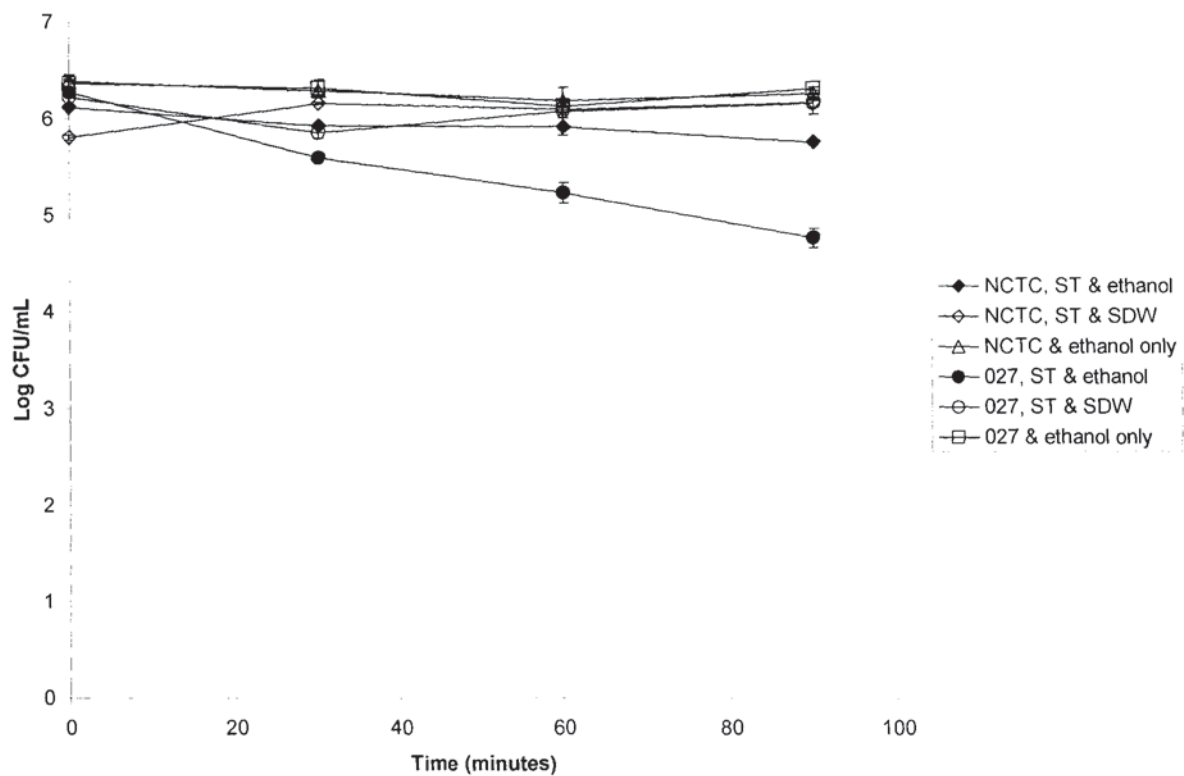
Twenty minutes incubation of *C. difficile* spores with the germinant produced a significant log reduction ( $P<0.001$ ) of 2.92 and 2.21 in the NCTC 11204 and 027 strains respectively in the first 30 minutes of exposure to ethanol (figure 5.3). There was also a significant decrease ( $P<0.05$ ) in the number of *C. difficile* spores recovered after incubation with the germinant followed by exposure to SDW; although this decrease was significantly smaller compared to that with both the germinant and ethanol ( $P<0.05$ ). For example, prior exposure to the germinant yielded a 3.1 log reduction in the NCTC 11204 strain after 90 minutes incubation with ethanol, compared to 0.37 log reduction after exposure to the germinant followed by SDW.

Similarly, with 30 minutes prior exposure to the germinant, there was a significant reduction ( $P<0.01$ ) in the number of spores recovered, with a log reduction of 1.85 and 2.15 in the NCTC 11204 and 027 strains respectively within the first 30 minutes of treatment with ethanol (figure 5.4). With the NCTC 11204 strain there was a significant difference ( $P<0.05$ ) at 30-90 minutes in the numbers of spores recovered between spores which were exposed to both the germinant and ethanol and spores which were exposed to the germinant and SDW. With ribotype 027 strain, a greater reduction was found in the number of spores recovered after exposure to the germinant and SDW and therefore there was no significant difference ( $P>0.05$ ) at any point between spores exposed to the germinant and ethanol and spores exposed to the germinant and SDW.

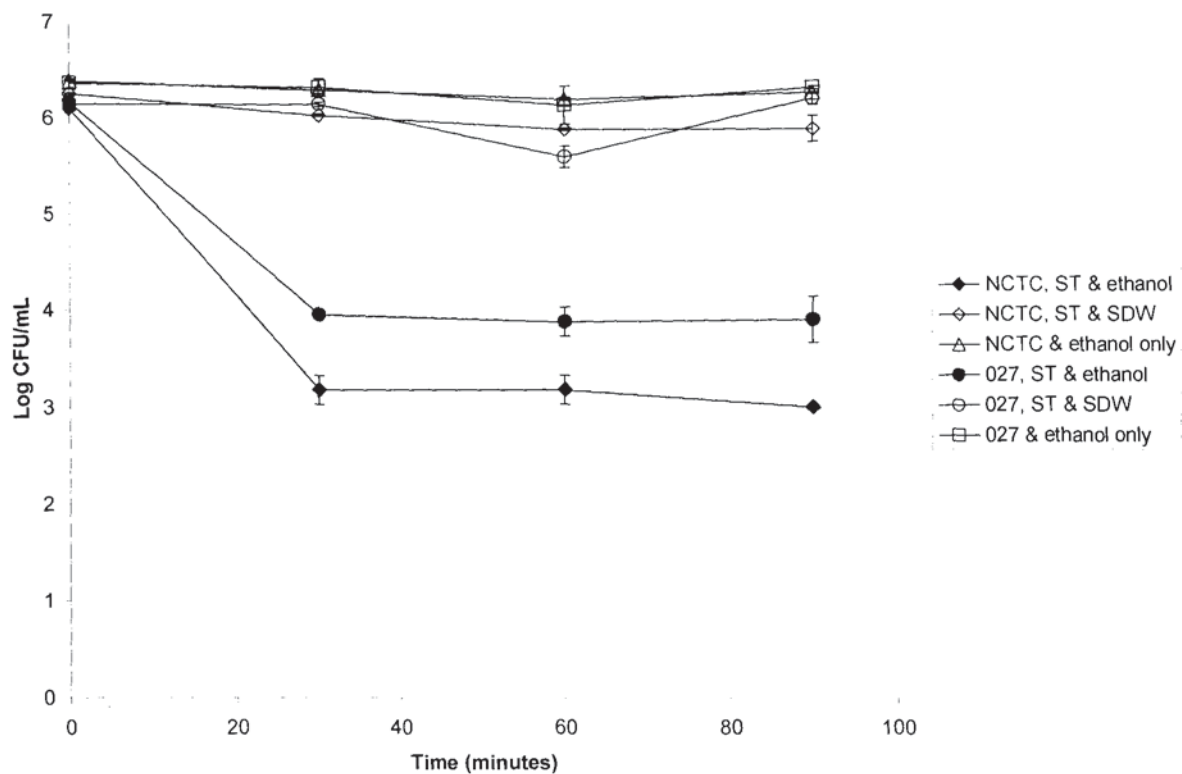




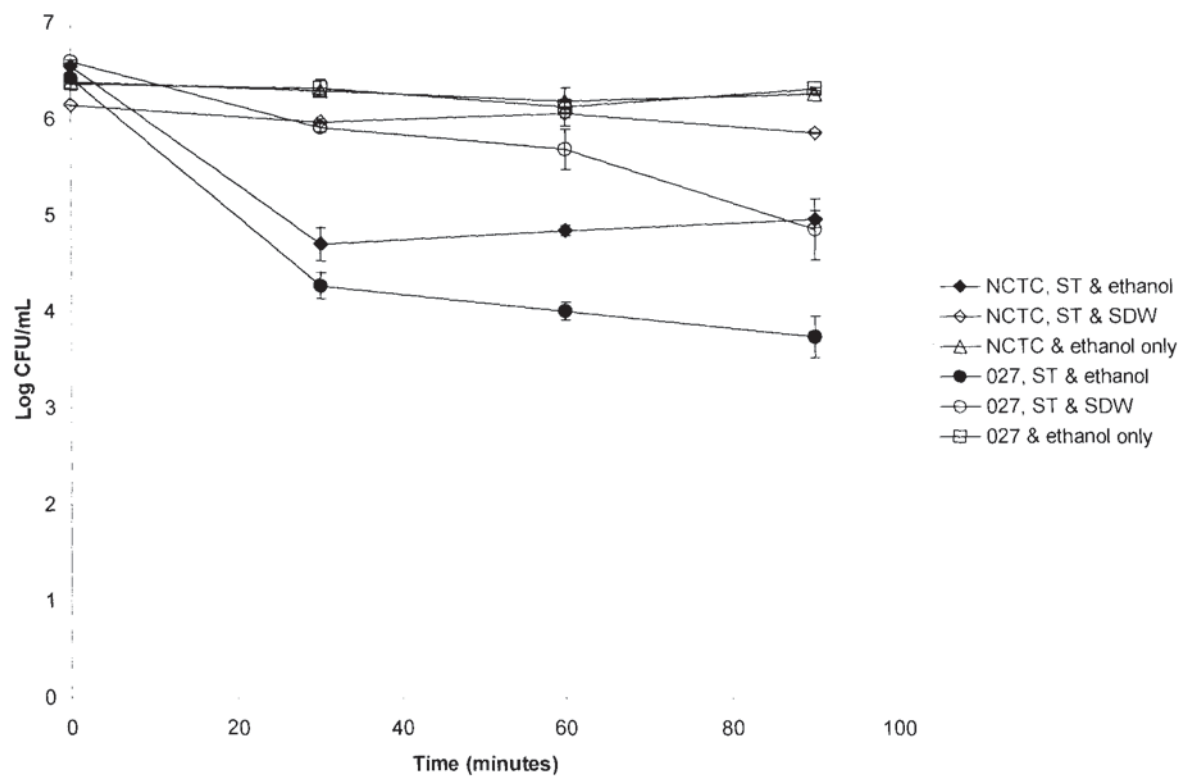
**Figure 5.1** *C. difficile* NCTC 11204 and ribotype 027 spores exposed to 70% (v/v) ethanol following 5 minutes exposure to 1% (w/v) ST in thioglycollate medium



**Figure 5.2** *C. difficile* NCTC 11204 and 027 spores exposed to 70% (v/v) ethanol following 10 minutes exposure to 1% (w/v) ST in thioglycollate medium



**Figure 5.3** *C. difficile* NCTC 11204 and ribotype 027 spores exposed to 70% (v/v) ethanol following 20 minutes incubation with 1% (w/v) ST in thioglycollate medium



**Figure 5.4** *C. difficile* NCTC 11204 and ribotype 027 spores exposed to 70% (v/v) ethanol following 30 minutes incubation with 1% (w/v) ST in thioglycollate medium



## 5.4 Discussion

The first part of the investigation was designed to assess the antimicrobial activity of 70% (v/v) ethanol against the dormant spores and confirm its activity against vegetative cells of *C. difficile*. The results show that whilst 70% (v/v) ethanol is effective at rapidly eliminating vegetative cells of *C. difficile* it has no activity against its dormant spores. These findings concur with those of Best *et al.* (1994) and Kampf and Kramer, (2004), who reported that ethanol possesses very rapid and effective bactericidal activity but practically no sporicidal activity (Best *et al.*, 1994, Kampf and Kramer, 2004).

However, it is well accepted that germinating spores of bacteria are more susceptible to antimicrobials (Hornstra *et al.*, 2007, Pol *et al.*, 2001). The time of exposure of the spores of *C. difficile* to the germinant is an important factor relating to reduced resistance to antimicrobials. Prior exposure of the spores of *C. difficile* to 1% (w/v) ST in thioglycollate medium for a period of 5 minutes in this study was insufficient to initiate germination in either strain of *C. difficile*, as treatment with 70% (v/v) ethanol produced no significant log reduction in CFU/mL ( $P > 0.05$ ). However, ten minutes prior exposure with the germinant was sufficient to induce a  $\leq 1$  log reduction (but significant reduction:  $P < 0.05$ ) in the number of *C. difficile* NCTC 11204 spores in the first 30 minutes of exposure to ethanol or SDW. Pre-exposure for 10 minutes to the germinant with *C. difficile* ribotype 027 also produced a reduction (of up to 1.51 log) in remaining spores; however this only became significant after 90 minutes incubation with either ethanol or SDW, compared to ethanol exposure alone. Ten minutes incubation with the germinant is therefore also inadequate to germinate the spores sufficiently to eliminate the spore load.

Interestingly, a reduction in the number of remaining spores after incubation with the germinant without exposure to ethanol also indicates that some of the germinating spores of *C. difficile* become susceptible to the aerobic environment alone which concurs with the findings of previous studies. A recent study by Jump *et al.* (2007) showed that vegetative cells of *C. difficile* are rapidly killed in air on dry surfaces. Furthermore, in the study by Weaver *et al.* (2008) which investigated the survival of *C. difficile* on metal surfaces, it was shown that the number of cells recovered was significantly reduced in the first three hours of exposure to the air on the surface of stainless steel.

Exposure of *C. difficile* spores to the germination medium for 20 minutes produced similar results to those exposed to the germinant for 30 minutes, with a significant reduction in the number of remaining spores ( $P < 0.05$ ). With *C. difficile* ribotype 027, a log reduction of 2.21 and 2.15 was produced in the first 30 minutes of exposure to 70% (v/v) ethanol after pre-incubation with the germinant for 20 and 30 minutes respectively. Therefore, a contact time of between 20 to 30 minutes with the germinant followed by ethanol exposure is required to produce maximum reduction of *C. difficile* spores adhered to stainless steel in aerobic conditions. These findings are similar to those of Hornstra *et al.* (2007) who found that a time frame of 5 to 60 minutes was required to induce germination of *B. cereus* spores adhered to stainless steel prior to a cleaning in place procedure.

As with a ten minute incubation period with the germinant, there was a significant ( $P < 0.05$ ) reduction in the number of spores recovered after 20 minutes incubation with subsequent exposure to SDW compared to spores exposed to ethanol alone. However, after 30 minutes incubation with the germinant alone there was no significant difference ( $P > 0.05$ ) in the number of spores (027 strain) recovered compared to ethanol exposure alone. This finding therefore demonstrates that the use of an antimicrobial, such as 70% (v/v) ethanol after the germination step is

required for maximum elimination of spores following short exposure times to the germinant. The principle of exposing dormant spores of *C. difficile* to an appropriate germinant to render them more susceptible to antimicrobial agents, for example ethanol, may also avoid the need to use sporicidal agents including hypochlorite and peracetic acid which are frequently more hazardous to the HCW (Wutzler *et al.*, 2005, Wheeldon *et al.*, 2008). Furthermore, ethanol is effective against a wide range of clinically relevant organisms, including meticillin-resistant *Staphylococcus aureus* (MRSA) and works very rapidly (Suzuki *et al.*, 1997, Kampf and Kramer, 2004).

In this study there was insufficient time to investigate the effects of a soil load on the activity of the germinant solution and 70% (v/v) ethanol. This would be important if this 'germinate / exterminate' approach was to be considered as a potential strategy towards decontaminating the clinical setting. Spores of *C. difficile* are inevitably mixed with organic matter as they are released in the faeces of infected patients. Therefore, any cleaning agents that are inactivated in the presence of organic matter would not be effective in reducing *C. difficile* contamination in the clinical setting. Walder *et al.* (1989) found the surface cleaning efficacy of ethanol (70% (v/v) or higher) was low when used against organisms in the presence of blood and serum and organisms became fixed to the test surface (Walder *et al.*, 1989). Further studies are therefore warranted to ensure that the efficacy of 70% (v/v) ethanol is not reduced in the presence of organic matter and the efficacy of other cleaning agents should also be investigated in place of 70% (v/v) ethanol, which might be less affected by a soil load.

In accordance with the findings in the previous chapter of this thesis, there was a proportion of spores which did not germinate during incubation with the germinant in air, which could be due to quorum sensing of the spore population

(Caipo *et al.*, 2002). Further studies should therefore focus on achieving maximum germination by possibly targeting quorum sensing in *C. difficile* and the use of other antimicrobials to achieve maximum elimination of contaminating spores.



## **6 Antimicrobial efficacy of copper surfaces on vegetative cells and the dormant and germinating spores of *C. difficile***

### **6.1 Introduction**

In the previous chapter of this thesis it was demonstrated that the number of contaminating spores of *C. difficile*, which were dried onto a stainless steel surface, could be significantly reduced by the use of a two-stage cleaning process, involving germination of spores, followed by killing with 70% ethanol. In this chapter the same principle of 'germinate to exterminate' is applied, but the antimicrobial agent being investigated is copper metal.

Recently, research into the antimicrobial efficacy of metal surfaces including stainless steel, zeolite (silver/zinc), brass, bronze and in particular copper has escalated (Wilks *et al.*, 2005, Wilks *et al.*, 2006, Galeano *et al.*, 2003, Bright *et al.*, 2002, Yoon *et al.*, 2007, Mehtar *et al.*, 2008, Weaver *et al.*, 2008). Several studies have been undertaken to assess the antimicrobial properties of these metals and many clearly demonstrate their potential application for reducing the microbial bioload of vegetative microorganisms, including *Staphylococcus aureus* (Bright *et al.*, 2002) and MRSA (Mehtar *et al.*, 2008). The only study to date which has assessed the sporicidal activity of copper metal is that by Weaver *et al.* (2008), in which complete death of *C. difficile* spores was observed in 24-48 hours when exposed to various copper alloys. However, it is well known that when bacterial spores are exposed to a suitable germinant they become significantly more sensitive to antimicrobials. Indeed, in the previous chapter of this thesis the spore load of *C. difficile* was reduced by approximately 2 log orders following stimulation with a germinant (1% (w/v) sodium taurocholate) and subsequent exposure to 70% (v/v) ethanol, which has no activity against dormant spores. In this chapter, the ability of

copper metal to reduce the viable load of vegetative cells, dormant and germinating spores of two strains of *C. difficile* (NCTC 11204 and ribotype 027) was investigated. The viability of *C. difficile* on copper was compared to stainless steel which is commonly used for surfaces in the clinical setting.

## **6.2 Methods**

### **6.2.1 Microorganisms**

*C. difficile* NCTC 11204 and *C. difficile* 027 R20291 (Anaerobe Reference Laboratory, Cardiff, UK) were stored on Microbank® beads (Pro-Lab Diagnostics, Cheshire, UK) and kept at -70°C until required.

### **6.2.2 Preparation of vegetative cell suspensions of *C. difficile***

Cell suspensions of *C. difficile* were produced following the method described in chapter 2, section 2.2.1 of this thesis.

### **6.2.3 Preparation of spore suspensions of *C. difficile***

Spore suspensions of *C. difficile* were produced following the method by Shetty *et al.* (1999) and described in chapter 2, section 2.2.2 of this thesis.

### **6.2.4 Preparation of *C. difficile* spore germinant**

One percent (w/v) sodium taurocholate (~20mM) was established in preliminary studies as being within the optimal concentration range to achieve maximum germination of spores of *C. difficile* (chapters 4 and 5). A 10mL germinant solution was prepared with double strength thioglycollate medium dissolved in distilled water, containing 2% (w/v) sodium taurocholate. The germination solution was autoclaved at 121°C for 15 min and stored at room temperature.

### **6.2.5 Preparation of a soil load**

The soil load used comprised of both protein and carbohydrate to simulate a contaminated clinical environment and was adapted from Perez *et al.* (2003). It is described in detail in chapter 3, section 3.2.7 of this thesis.

### **6.2.6 Preparation of copper and stainless steel coupons**

Coupons (1cm<sup>2</sup>) of copper (UNS C19700, Copper Development Association, USA) and stainless steel (GTSS Engineering Supplies, Coventry, UK) were prepared according to the method of Noyce *et al.* (2006). Briefly, coupons were degreased and cleaned with acetone prior to immersion in 70% (v/v) ethanol and flaming to sterilize and were stored in a sterile container until use.

### **6.2.7 Preparation and assessment of D/E neutraliser (for copper neutralization)**

#### **6.2.7.1 Preparation of D/E neutraliser**

Five-hundred mL of D/E neutraliser (BD Biosciences, USA) was prepared in line with the manufacturer's instructions and stored at 4°C until required (Dey and Engley Jr., 1994).

#### **6.2.7.2 Assessment of copper neutralization and non-toxicity**

In order to assess the copper neutralization effect of D/E neutraliser, an overnight cell suspension of *C. difficile* prepared in WCB was standardized to a concentration of approximately 5x10<sup>6</sup> CFU/mL, as determined by measurement of OD at 600nm. Fifty microlitres of the suspension was mixed with 50µL of D/E neutraliser and inoculated onto the surface of a copper coupon which was inside a sterile bijou



bottle. In addition, 50 $\mu$ L of *C. difficile* suspension was mixed with 50 $\mu$ L of SDW and inoculated onto the surface of second copper coupon inside a bijou bottle. After 60 minutes anaerobic incubation, at 37°C, 900 $\mu$ L of SDW was added to each bijou and mixed thoroughly. After appropriate dilutions, a 100 $\mu$ L sample was spread onto a WCA plate and plates were incubated anaerobically, at 37°C, for 48 hours.

To ensure D/E neutraliser was non-toxic towards *C. difficile*, 100 $\mu$ L of *C. difficile* cell suspension was mixed with 900 $\mu$ L of D/E neutraliser and dispensed into a sterile micro centrifuge tube. Finally, 100 $\mu$ L of *C. difficile* cell suspension was mixed with 900 $\mu$ L of SDW in a sterile micro centrifuge tube. After 60 minutes anaerobic incubation, serial dilutions were performed and a 100 $\mu$ L sample was inoculated onto a WCA plate. Plates were incubated at 37°C, anaerobically, for 48h and the number of CFU determined. All neutralization experiments were performed in duplicate.

#### **6.2.8 Assessment of antimicrobial efficacy of copper metal and stainless steel against vegetative cells and dormant spores of *C. difficile***

Sufficient coupons of copper and stainless steel were inoculated with 10 $\mu$ L of each test strain of *C. difficile* (10<sup>6</sup> CFU/mL) inside sterile glass bottles. In order to assess the antimicrobial activity of copper against vegetative cells of *C. difficile*, assays were undertaken at 37°C in anaerobic conditions, whilst the efficacy of copper against dormant and germinating spores of *C. difficile* was carried out in air at room temperature (to simulate the clinical environment). At 30 minute intervals over a 3 h time period, 990 $\mu$ L of D/E neutraliser and 10 glass beads (3mm diameter, Merck, UK) were added to the appropriate test universal tubes, vortex mixed for one minute and left to stand at room temperature (for spores) or 37°C in anaerobic conditions (for vegetative cells) for 30 minutes, with the bottle lids in place. (This time period

allowed for adequate neutralization). Each suspension was diluted in SDW if appropriate and mixed with 15mL of molten FAA supplemented with 0.1% (w/v) ST. For tests involving vegetative cells, samples were inoculated onto WCA. All plates were incubated at 37°C in anaerobic conditions for 48 h. Experiments were performed in duplicate.

#### **6.2.9 Assessment of antimicrobial efficacy of copper metal and stainless steel against spores of *C. difficile* exposed to 1% (w/v) sodium taurocholate**

Sufficient coupons of copper and stainless steel were prepared to assess the activity of these metals on spores of *C. difficile* exposed to 1% (w/v) ST in thioglycollate medium over a 3 h period with 30 minute sampling times. Ten microlitres of each spore suspension was mixed with 10µL of 2% (w/v) sodium taurocholate in double strength thioglycollate medium and inoculated onto the surface of copper and stainless steel coupons which were placed in sterile bijoux bottles. At the sampling times described previously, 980µL of D/E neutraliser and 10 glass beads were added to the bottles which were then vortex mixed for one minute and allowed to stand at room temperature for 30 minutes. All spore suspensions were then diluted and cultured as previously described.

#### **6.2.10 Statistical Analysis**

Statistical analysis of data was performed using an unpaired t-test (two-tailed), using the InStat® package (GraphPad Software Inc., version 3.06) and repeated measures ANOVA, with Fisher LSD post hoc test, using the Statistica package (StatSoft Inc, version 6.0).

## 6.3 Results

### 6.3.1 Efficacy and non-toxicity of D/E neutraliser

Results for the efficacy and non-toxicity of D/E neutraliser are shown in table 6.1. D/E neutraliser was shown to neutralize the antimicrobial activity of the copper coupons whilst being non-toxic towards *C. difficile* cells. The negative values for log reduction indicate some growth during the incubation period in the presence of the neutraliser.

**Table 6.1 Efficacy of D/E neutraliser to nullify the activity of copper and toxicity against *C. difficile* cells NCTC 11204**

Test components	Average log reduction in CFU/mL after 60mins exposure
Copper, D/E neutraliser and cell suspension	-0.45
Cell suspension and copper	6.06
D/E neutraliser and cell suspension	-0.08
Cell suspension and SDW	0.124

### 6.3.2 Efficacy of copper metal and stainless steel against the vegetative cells of *C. difficile*

Stainless steel did not demonstrate any antimicrobial activity against the vegetative cells of *C. difficile*, and no log reduction in CFU/mL was observed after 30 minutes exposure. Copper metal demonstrated significantly enhanced ( $p < 0.05$ ) antimicrobial activity against vegetative cells of *C. difficile*, achieving a  $>6$  log reduction in CFU/mL in 30 minutes (table 6.2).

**Table 6.2 Antimicrobial efficacy of copper and stainless steel against vegetative cells of *C. difficile***

Time (min)	Mean <i>C. difficile</i> CFU/mL remaining (range)			
	Stainless steel		Copper	
	NCTC	027	NCTC	027
0	7.6 x 10 <sup>6</sup> (6.3-8.3)	5.6 x 10 <sup>6</sup> (5.5-5.6)	1.2 x 10 <sup>6</sup> (1.-1.4)	4.6 x 10 <sup>6</sup> (3.6-5.6)
30	7.3 x 10 <sup>6</sup> (6.8-7.8)	3.2 x 10 <sup>6</sup> (2.4-4.0)	0 *	0 *

\* P<0.05

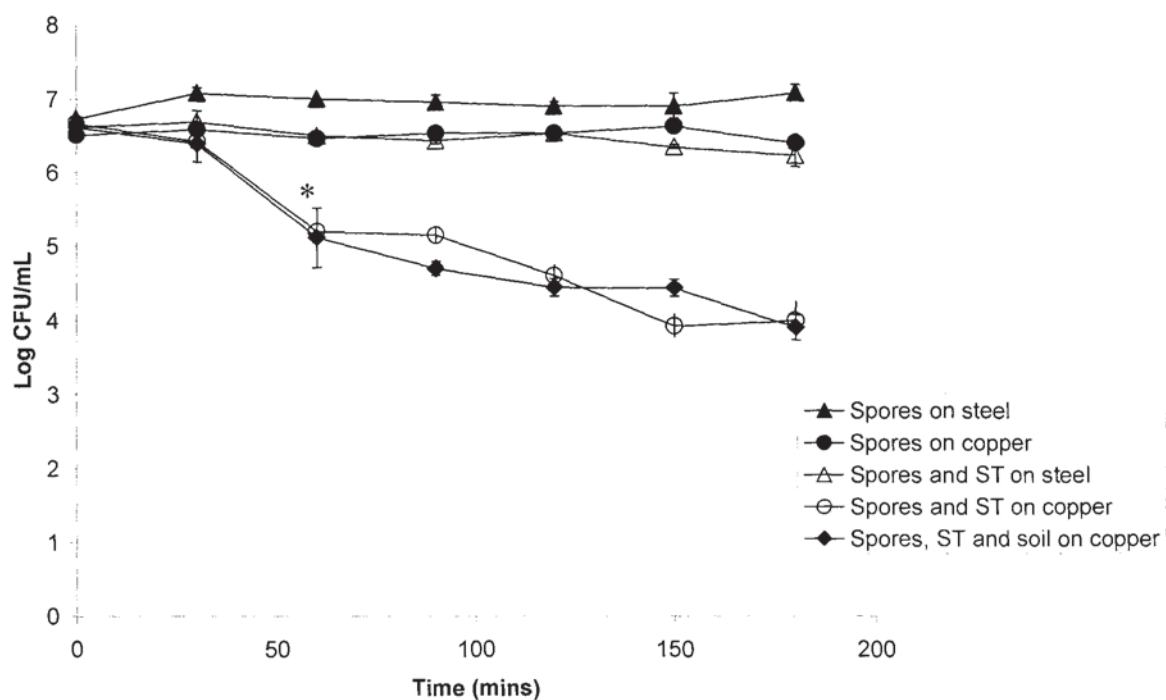
### **6.3.3 Efficacy of copper metal and stainless steel against dormant and germinating spores of *C. difficile***

Stainless steel did not demonstrate antimicrobial activity against either dormant or germinating spores of *C. difficile* NCTC 11204 and 027, as no reduction in viability was observed following 3 h exposure (P>0.05) (figures 6.1 and 6.2). Furthermore, there was no reduction in viability of dormant spores of either strain of *C. difficile* on copper metal within the 3 hour study period. However, the viability of spores of both *C. difficile* NCTC 11204 and ribotype 027 exposed to the germination solution was significantly reduced (p <0.05) within 60 minutes, compared to the viability of spores with the germinant solution on stainless steel. At 3 hours a 2.67 (99.79% reduction) and 2.96 (99.87% reduction) log reduction in germinating *C. difficile* NCTC 11204 and 027 spores respectively was achieved on copper metal.



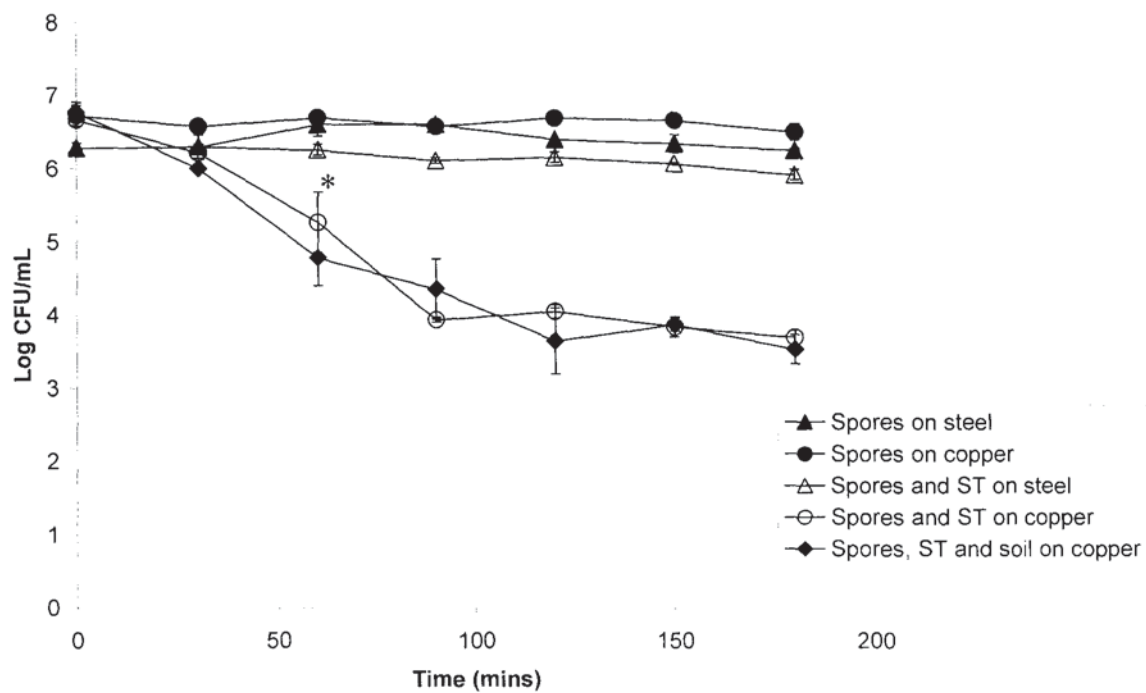
### 6.3.4 Effect of a soil load on the antimicrobial activity of copper

In both strains of *C. difficile* there was a significant decrease in CFU/mL following incubation of spores with the germinant solution and a soil load on the surface of copper ( $P<0.05$ ) within the first 60 minutes of exposure. A log reduction of 2.71 and a 3.22 was achieved for NCTC 11204 and 027 strain respectively, after 3 h exposure to copper (figures 6.1 and 6.2). This reduction in CFU/mL in both strains of *C. difficile* was not significantly different to the reduction achieved in the absence of a soil load ( $P>0.05$ ).



\*  $P<0.05$

**Figure 6.1** Antimicrobial and sporicidal effects of copper and stainless steel on dormant and germinating spores of *C. difficile* NCTC 11204



\*  $P < 0.05$

**Figure 6.2** Antimicrobial and sporicidal effects of copper and stainless steel on dormant and germinating spores of *C. difficile* ribotype 027

## 6.4 Discussion

In line with other recent studies that have evaluated the efficacy of copper against nosocomial pathogens including MRSA and *Pseudomonas aeruginosa*, (Mehtar *et al.*, 2008) copper metal, in this investigation, exhibited rapid antimicrobial activity against the vegetative cells of *C. difficile* compared to stainless steel which remained inert.

In the 3 h study period incorporated in this investigation, both copper and stainless steel surfaces were ineffective at reducing the numbers of viable dormant *C. difficile* spores and no log reduction was observed. However, in the recent study by Weaver *et al.* (2008) it was shown that a >5 log reduction in dormant spores of *C. difficile* was achieved following a 24 - 48 h exposure to pure copper. This finding clearly indicates that copper metal is indeed effective at eliminating dormant spores of *C. difficile* but an extended exposure and contact period is required. In the current investigation, a 3 h time period was chosen to assess the antimicrobial efficacy of copper metal against *C. difficile* as this is a more realistic and clinically practical time frame. Whilst copper metal alone was ineffective at reducing the number of spores of *C. difficile* in a 3 h period, the introduction of a specific germinant (1% (w/v) ST in thioglycollate medium) significantly enhanced the kill of *C. difficile* spores on copper metal with a 1.47 log reduction of NCTC 11204 spores and a 1.39 log reduction of 027 spores within 60 minutes in the absence of a soil load. Furthermore at 3 h there was a log reduction of 2.67 (99.79%) and 2.96 (99.87%) in the NCTC 11204 and 027 strains respectively. Germinating spores of *C. difficile* on stainless steel were not eliminated in 3 h, thus highlighting the antimicrobial efficacy of copper metal. The application of specific germinants to eliminate spores in the presence of antimicrobials has also been adopted in a previous research investigation by Hornstra *et al.* (2007) and is described in Chapter 5. The findings of these studies demonstrate that the addition of a germinant to a spore suspension results in

susceptibility to antimicrobial agents to which dormant spores are usually resistant. The incorporation of a germination step may be of particular importance with *C. difficile* as it has been found that the use of sub-inhibitory levels of certain cleaning agents can induce spore formation in *C. difficile* (Wilcox and Fawley, 2000).

The mechanism of action of copper is not completely understood as yet, but it is suggested that it exerts its cytotoxic effects by the production of hydroperoxide radicals which degrade proteins by the oxidation of amino acid residues and the degradation of sulphydryl groups and possibly cause membrane damage. Copper ions are also released which can bind DNA and may disrupt electron transport, thus preventing metabolic activity in the cell (Gant *et al.*, 2007, Weaver *et al.*, 2008)

It is essential that assessment of the true antimicrobial efficacy of an agent for use in the clinical setting is undertaken in the presence of a soil load to simulate environmental contamination. Indeed, in the clinical environment *C. difficile* spores are highly likely to be surrounded with organic material, as they are excreted in the faeces of patients. In this investigation, the incorporation of a soil load (containing albumin, tryptone and mucin) into the *C. difficile* spore suspension did not demonstrate any inhibitory effect on the antimicrobial activity of copper metal. Both the rate and extent of reduction in CFU/mL of *C. difficile* spores on exposure to copper and a germinant in the presence of a soil load was not significantly lower compared to that in the absence of a soil load ( $P>0.05$ ). These findings are important as they demonstrate that even in the presence of a soil load; copper metal is still effective at rapidly reducing the number of viable spores of *C. difficile* in the presence of a specific germinant. However, in a recent study it was shown that a build up of bacterial cells (*Staphylococcus aureus*) and organic matter (bovine serum albumin) on copper surfaces occurs after several cycles of soiling and cleaning (Airey and Verran, 2007). As this current investigation assessed the antimicrobial effects of copper alone, without the incorporation of a cleaning agent, further *in vitro*



studies may be warranted to assess the effect of multiple cleaning and soiling cycles on the antimicrobial activity of copper.

The incorporation of copper metal surfaces into the clinical environment is currently being widely considered as one approach to 'design out' healthcare acquired infection in modern medicine. Whilst copper metal has been shown to effectively eliminate less hardy nosocomial pathogens including MRSA, *P. aeruginosa* (Mehtar *et al.*, 2008) and vegetative cells of *C. difficile*, extended contact times of 24-48 h are required to eliminate its resilient spores (Weaver *et al.*, 2008). However, the novel approach of introducing a specific germinant to significantly enhance the susceptibility of *C. difficile* spores to copper metal warrants consideration if copper surfaces are adopted in clinical practice.

## **7 Production of novel compounds and their antimicrobial activity against the spores and vegetative cells of *C. difficile***

### **7.1 Introduction**

A series of benzylidenecarboxamidrazones were originally produced at Aston University to assess their anti-tuberculosis properties. One compound in particular was found to possess antimicrobial activity against many Gram-positive organisms associated with hospital acquired infections, including *Staphylococcus aureus* and coagulase-negative staphylococci, but was inactive against Gram-negative organisms such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Rathbone *et al.*, 2006). Unfortunately the active compound was also found to be very toxic against human leucocytes and therefore unsuitable for therapeutic applications. The compound is also highly lipophilic and therefore has reduced solubility in water. However, N-oxide analogues of this compound are less lipophilic and have greater water solubility, therefore, the initial active compound and several N-oxide analogues were chosen for testing against *C. difficile*; a Gram-positive organism.

The antimicrobial activity of thiosemicarbazones has also been investigated in which a 5-nitrofuranyl group attached via an ethylene spacer is suggested to be important in its activity (Costello *et al.*, 2008). A thiosemicarbazone with these structural features was therefore also tested against *C. difficile*.

The importance of finding new antimicrobials which are active against *C. difficile* has increased recently after the emergence of the hypervirulent ribotype 027 strain and its resistance to fluoroquinolones (Pepin, 2008, Spigaglia *et al.*, 2008) and

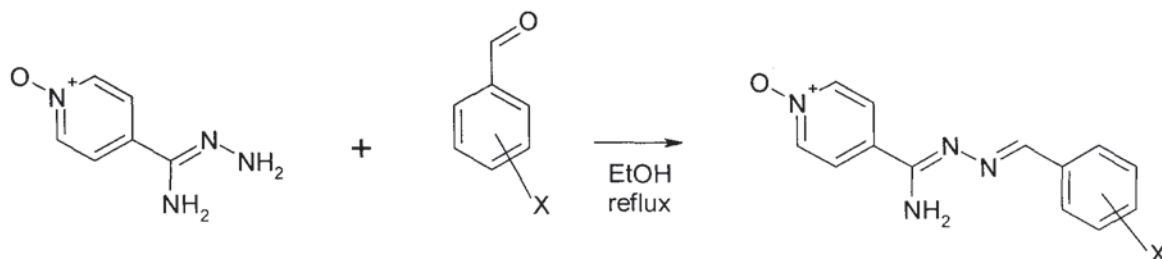
due to the increasing number of cases of *C. difficile* infection each year (HPA, 2007).

The aims of this chapter were three-fold: firstly to produce a series of analogues of the active benzylidenecarboxamidrazone, previously produced at Aston University and to determine their purity. Secondly, the antimicrobial activity of these compounds and other compounds previously produced at Aston University was assessed against the vegetative cells and spores of *C. difficile*. Thirdly, the activity of these compounds was compared to vancomycin; a widely antimicrobial agent with efficacy against the vegetative cells of *C. difficile*.

## 7.2 Methods

### 7.2.1 Chemicals

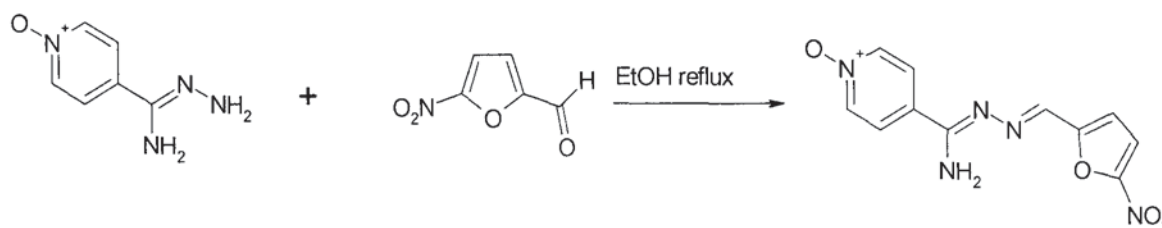
Six benzaldehydes were combined with a carboxamidrazone to produce N-oxide analogues (**2 – 7**) of compound **1** (figure 7.3); a compound previously produced at Aston University which had demonstrated antimicrobial activity against Gram-positive bacteria (figure 7.1).



**Compounds 2-7**

**Figure 7.1** Chemical synthesis of *N*<sup>1</sup>-benzylidene- [pyridine (N-oxide)]- 4 - carboxamidrazones (compounds **2 - 7**)

Another compound (**8**) was also produced by combining the same carboxamidrazone with a furaldehyde (figure 7.2)

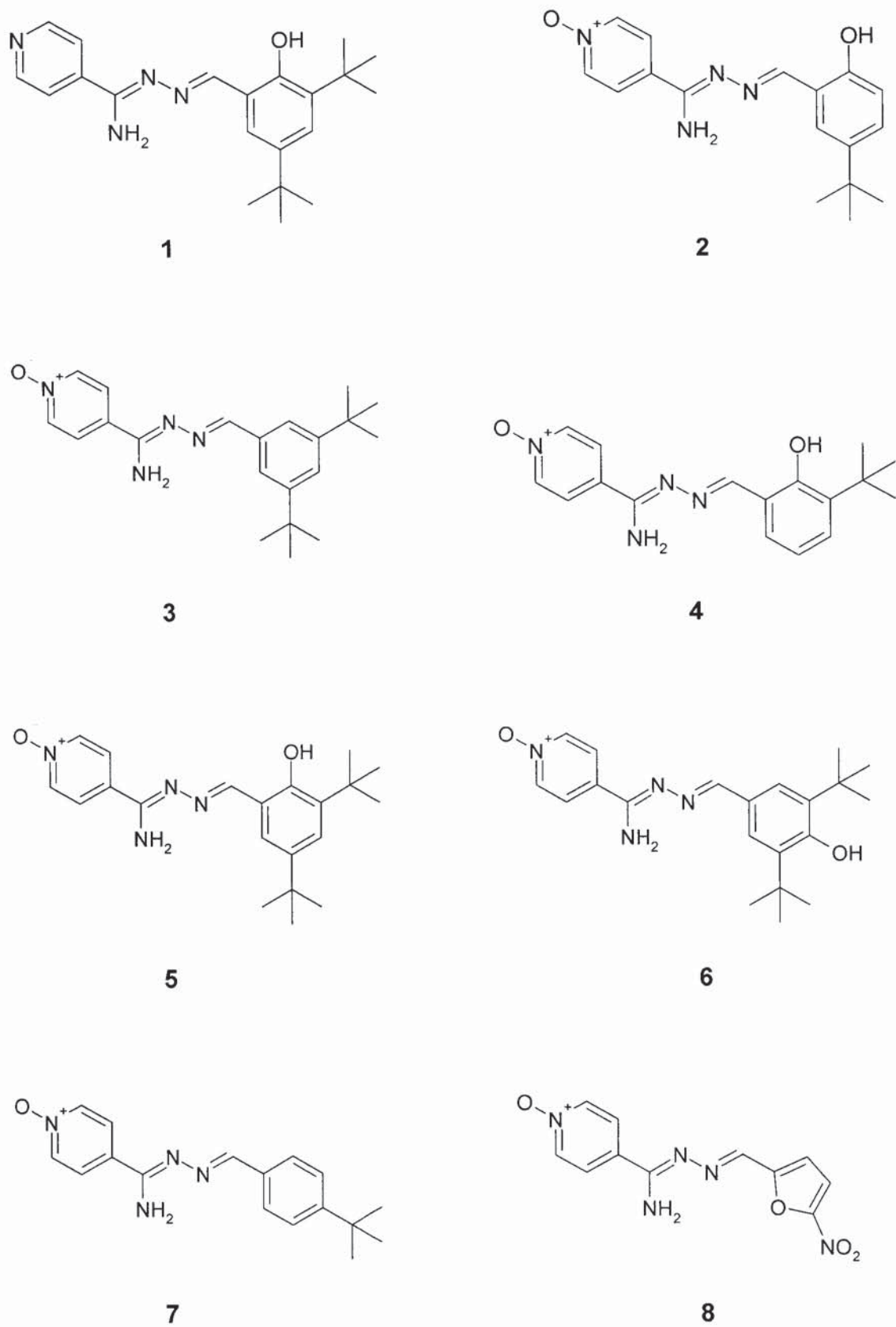


**Compound 8**

**Figure 7.2** Chemical synthesis of compound **8**

The structures of compound **1** and the N-oxide analogues that were produced are shown in figure 7.3. The names of the resulting arylidenecarboxamidrazones are given in appendix 2.





**Figure 7.3** Structure of compound 1 and its N-oxide analogues (2 – 8)

### 7.2.2 Preparation of *N*<sup>1</sup>-Arylidene-pyridine-4-carboxamidrazone-4-N-oxides

A mixture of the pyridine carboxamidrazone N-oxide and an appropriate aldehyde (1.1 – 1.3 molar equivalents) in ethanol (20 mL/g of carboxamidrazone N-oxide) was stirred at reflux for 18h. The starting materials dissolved once heating commenced. After cooling, the precipitated material was collected by filtration, washed with a little cold ethanol (20 - 40mL) and dried under vacuum. The material obtained at this point was generally found to contain a single component as judged by thin layer chromatography. If necessary the material was purified by recrystallisation (Billington *et al.*, 1998).

### 7.2.3 Assessment of compound purity

To establish the purity of the compounds thin layer chromatography (TLC), atmospheric pressure chemical ionisation mass spectrometry (APCI-MS), Fourier transform infrared (FTIR) and nuclear magnetic resonance spectroscopy (NMR) were performed along with the determination of melting points for each compound. (All data on the purity and melting points of each compound can be found in appendix 2).

#### 7.2.3.1 Thin Layer Chromatography

The crude products were analysed by thin layer chromatography on silica UV<sub>254</sub> aluminum backed plates, eluting with ethyl acetate: methanol (3:1). The components were visualized under UV light at 254nm.

### **7.2.3.2 Mass Spectrometry- Atmospheric Pressure Chemical Ionisation (APCI) and Electrospray**

Approximately 1-2mg of each compound was dissolved in acetonitrile with heat and ultrasound waves if required in mass spectrometry tubes. APCI-MS was conducted using a Hewlett-Packard 5989B quadrupole instrument, connected to an electrospray 59987A unit with an APCI accessory and automatic injection, with a Hewlett-Packard 1100 series autosampler.

### **7.2.3.3 Fourier Transform Infrared**

A background scan was performed first for comparison with the sample scan. Approximately 1-2mg of each compound was mixed with approximately 200-300mg of potassium bromide (KBr) and ground in a mortar and pestle. The mixture was placed in the press, the vacuum was connected and a pressure of 10 tons was applied for approximately 30 seconds. The disc was carefully extracted and the infrared scan was conducted using a Mattson 3000 FTIR spectrophotometer.

### **7.2.3.4 Proton NMR**

Proton NMR spectra were obtained on a Bruker AC 250 instrument, operating at 250MHz as solutions in  $d_6$ -DMSO and referenced from  $\delta$ DMSO = 2.50 ppm unless otherwise stated.

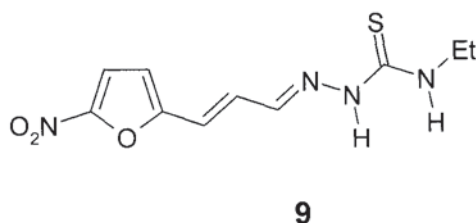
### **7.2.3.5 Melting Points**

Approximately 1-2mg of compound was compressed between two cover-slips and observed under a Reichert-Jung Thermo Galen hot stage microscope. The sample was heated slowly and it was observed at which point the compound began to melt.

This was repeated three times for each compound and the melting points were corrected using calibration compounds.

#### **7.2.4 Antimicrobial activity of arylidenecarboxamidrazones and a thiosemicarbazone against the vegetative cells of *C. difficile***

The antimicrobial activity of compound **1** and its seven analogues together with a thiosemicarbazone (**9**) (figure 7.4) was assessed against the vegetative cells of *C. difficile* by agar diffusion and the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) of each compound were determined.



**Figure 7.4** A thiosemicarbazone previously produced at Aston University (Tims and Rathbone, unpublished results, 2003, Aston University).

##### **7.2.4.1 Agar diffusion test to assess the antimicrobial activity of 9 novel compounds against the vegetative cells of *C. difficile***

The sensitivity of *C. difficile* vegetative cells to 9 compounds was determined by the agar diffusion method based on NCCLS guidelines (NCCLS, 2003). Compounds were prepared by dissolving 5.12mg in 1mL of dimethyl sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO.) and diluted with SDW to a concentration of 256µg/mL. An overnight cell suspension of *C. difficile* NCTC 11204 was adjusted to a concentration of  $10^5$  CFU/mL using WCB by reference to a calibration curve. A sterile cotton swab was immersed in the cell suspension and was spread evenly over the surface of a direct sensitivity testing (DST) agar plate, ensuring complete



coverage. Four holes (6mm diameter) were cut into the agar into which 75µL of dissolved compound (256µg/mL) was delivered. DST plates were incubated at 4°C for approximately 30 minutes to allow for diffusion of the compounds before being transferred to an anaerobic chamber, at 37°C for 48 hours. The zones of inhibition produced by each compound were then examined.

#### **7.2.4.2 Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antimicrobial compounds and vancomycin against *C. difficile* vegetative cells**

MICs and MBCs of compounds and vancomycin were determined by the microbroth dilution method (Rosenblatt *et al.*, 1979, McVay and Rolfe, 2000) against 31 clinical isolates (City Hospital, Birmingham, NHS Trust), NCTC 11204 strain and ribotype 027 of *C. difficile* (Anaerobe Reference Laboratory, Cardiff, UK). Compounds were prepared by dissolving 5.12mg of each compound in 1mL of DMSO and diluting with WCB to give a concentration of 512µg/mL. Two hundred microlitres of this solution was added to the first well of a transparent, round-bottomed microtiter plate (Appleton Woods, UK). Dilutions in the ratio of 1:2 were then performed across the plate using WCB so that all wells contained 100µL of compound and concentrations ranged from 0.5-512µg/mL.

The antibiotic solution was prepared by dissolving 64mg of vancomycin (Sigma-Aldrich, UK) in 1mL of SDW, diluting with WCB and dispensing 100µL into each well of a microtiter plate so that the concentration ranged from 64-0.5µg/mL.

The microtiter plates containing the antimicrobials were left to equilibrate in anaerobic conditions, at 37°C for 30 minutes before the addition of 100µL of a *C. difficile* cell suspension standardized to 10<sup>6</sup> CFU/mL by the measurement of OD at 600nm and reference to a calibration curve. The final concentrations of compounds

and antibiotic in the wells ranged from 0.25 to 215µg/mL and 0.25 to 32µg/mL respectively, with  $10^5$  organisms per well.

The microtiter plates were incubated at 37°C, for 48 hours under anaerobic conditions. The MIC was determined as the lowest concentration of antimicrobial agent inhibiting the total growth of *C. difficile* cells. After incubation, the content of each well (200µL) was spread onto a separate WCA plate. Plates were incubated for 48 hours, at 37°C, under anaerobic conditions. The MBC was determined as the lowest concentration of the antimicrobial agent at which 99.9% of organisms in the original inoculum were killed. All tests were performed in triplicate.

#### **7.2.5 Determination of the activity of compounds 1, 8 and 9 against the spores of *C. difficile***

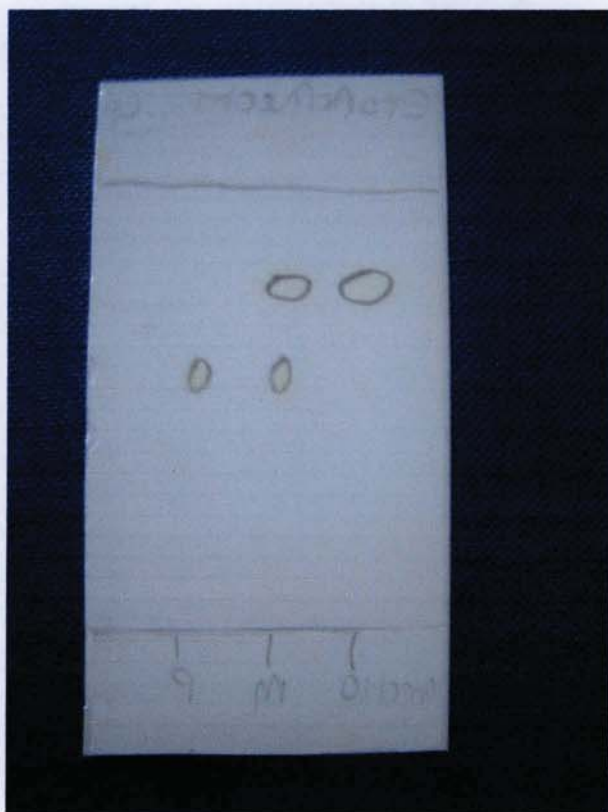
Spore suspensions were made according to the method by Shetty *et al.* (1999), as described in chapter 2, section 2.2.2 of this thesis. One hundred microlitres of each compound (512µg/mL) was added to three wells of a microtiter plate (100µL of saline for the control) before the addition of 100µL of *C. difficile* spore suspension (either NCTC 11204 strain or ribotype 027), diluted to a concentration of  $10^6$  CFU/mL using SDW. The solutions were mixed thoroughly and the microtiter plate was incubated for 5 hours, at room temperature, in aerobic conditions. After 5 hours, the content of each well (200µL) was placed in 9.8mL of SDW. One milliliter samples were then taken and after serial dilutions, were placed in a sterile Petri dish before the addition of 15mL of molten FAA (containing 0.1% ST and 5% defibrinated horse blood). Plates were incubated anaerobically for 48 hours, at 37°C and the number of CFU/mL were calculated.

### **7.2.6 Statistical analysis**

The MIC and MBC data of the antimicrobials were analysed using the Friedman nonparametric repeated measures test and Dunn's multiple comparison post test (GraphPad InStat 2 package).

### 7.3 Results

The crude material produced from the reaction scheme in figure 7.2 was recrystallised from ethanol in order to obtain compound **8**. All compounds were demonstrated to be pure by the methods used and the characterization results are given in detail in appendix 2. An example of a TLC plate showing the purified compound, together with the aldehyde is shown in figure 7.5.

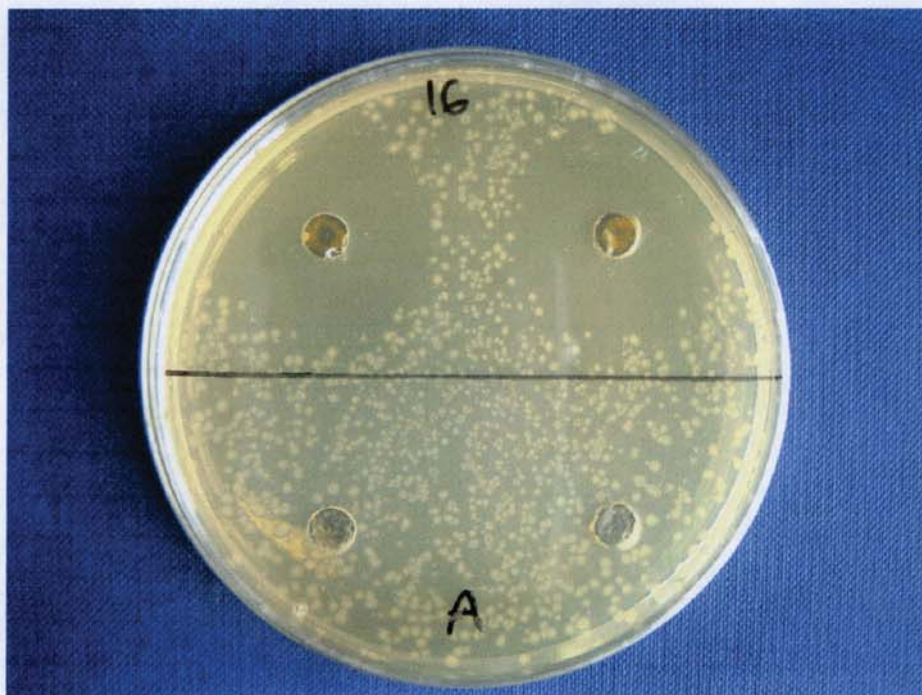


**Figure 7.5** TLC plate showing separation of the aldehyde and product in a mixture (two spots on the central line) and the singular spots of the product alone (left) and the aldehyde alone (right)



### 7.3.1 Antimicrobial activity of arylidenecarboxamidrazones and a thiosemicarbazone determined by agar diffusion

Only compounds **1**, **8** and **9** demonstrated antimicrobial activity against the vegetative cells of *C. difficile* by agar diffusion, with the zones of inhibition being clearly visible (figure 7.6).



**Figure 7.6** Agar diffusion plate showing the effect of compound **8** (upper two wells of the plate) and compound **4** (lower wells) on the growth of *C. difficile* vegetative cells

### 7.3.2 Determination of MICs and MBCs of compounds 1, 8 and 9 and vancomycin against the vegetative cells of *C. difficile*

The three compounds chosen for further testing (1, 8 and 9) were found to be active against all 33 isolates of *C. difficile* tested, along with vancomycin (data courtesy of Lauren Green) (Table 7.1). The MICs of vancomycin were significantly lower than the MICs of the three compounds against the 33 isolates of *C. difficile* tested ( $P < 0.001$ ) and there was a much smaller range of MIC values with vancomycin. However, there was no significant difference found between the MICs for each compound against *C. difficile* ( $P > 0.05$ ).

The MBCs of vancomycin were also significantly lower than those of the compounds against the vegetative cells of *C. difficile* ( $P < 0.001$ ) and a significant difference was also found between the MBC values of compound 9 and those of compound 8; with the MBC data for compound 9 being lower than that of compound 8 ( $P < 0.01$ ).

**Table 7.1 MICs and MBCs of antimicrobial agents against the vegetative cells of *C. difficile***

Compound or antibiotic	Mean MIC in µg/mL (range)	Mean MBC in µg/mL (range)
1	13.94 (4 to 256)	14.79 (4 to 256)
8	21.94 (2 to 128)	34.36 (2 to 256)
9	7.25 (0.25 to 32)	7.73 (0.25 to 32)
Vancomycin	1.27 (0.5 – 4)	1.92 (0.5 – 4)

### 7.3.3 Activity of compounds 1, 8 and 9 against the spores of *C. difficile*

None of the compounds tested demonstrated any sporicidal activity against the spores of *C. difficile* (NCTC 11204 and ribotype 027). There was a log reduction in CFU/mL of <0.1 in all of the test suspensions after 5 hours aerobic incubation with each compound (table 7.2).

**Table 7.2** Sporicidal activity of antimicrobial compounds against the spores of two strains of *C. difficile*

Compound (256µg/mL)	Log reduction in	Log reduction in
	CFU/mL of <i>C. difficile</i> NCTC 11204 after 5 h exposure	CFU/mL of <i>C. difficile</i> ribotype 027 after 5 h exposure
1	-0.071	0.079
8	-0.042	0.044
9	0.005	-0.024



## 7.4 Discussion

The target compounds (**2** - **8**) were prepared in one step following the reaction schemes shown in figures 7.1 and 7.2. The yields ranged from 42% to 90% and all were represented as a single spot on a TLC plate, indicating purity. The compounds also exhibited NMR spectra, IR spectra and mass spectra consistent with their proposed structures.

Of the nine compounds tested only two novel compounds (**1** and **8**) together with a thiosemicarbazone (**9**) demonstrated antimicrobial activity against the vegetative cells of *C. difficile*. Each of these active compounds demonstrated antibacterial activity against all isolates of *C. difficile* tested with a mean MIC of 13.94, 21.94 and 7.25 in compound **1**, **8** and **9** respectively. Vancomycin, however, demonstrated superior activity against the vegetative cells of *C. difficile*, with a significantly lower mean MIC of 1.27 ( $P < 0.001$ ) and a smaller range of MIC values. There was no significant difference between the three compounds in terms of their antimicrobial activity against *C. difficile* ( $P > 0.05$ ), except between the MBC data of compound **8** and compound **9**, with a significantly lower bactericidal concentration required with compound **9** than with compound **8** ( $P < 0.01$ ).

Other studies have found the range of MIC values for vancomycin against *C. difficile* strains to be very similar to those found in this study (0.5-4 µg/mL). For example, Citron and colleagues found that the MIC range for vancomycin against 18 strains of *C. difficile* was 0.5-4 µg/mL (Citron *et al.*, 2003). Similarly, in another study involving 110 toxigenic strains of *C. difficile* the MIC range for vancomycin was found to be 0.06-4 µg/mL (Hecht *et al.*, 2007).

Vancomycin works by binding to a pentapeptide which is involved in the translocation of precursors of peptidoglycan to the cell wall. The precursors are not



able to reach the transglycosylase in the cytoplasmic membrane and the extension of the glycan chains is prevented, therefore preventing cell wall synthesis (Courvalin, 2006).

The mode of action of compounds **1**, **8** and **9** is yet to be determined, however it has been noted that the 5-nitrofuranyl group attached via an ethylene spacer in the thiosemicarbazone (compound **9**) and hydrophobic groups attached to the benzylidene moiety of benzylidenepyridylcarboxamidrazones (compounds **1** and **8**) may be important in the antimicrobial activity of these compounds (Billington *et al.*, 2000, Costello *et al.*, 2008). Given the susceptibility of Gram-positive organisms and the resistance of Gram-negatives to the compounds, one hypothesis is that they may be unable to penetrate through the outer lipopolysaccharide (LPS) and proteinaceous membrane of Gram-negative organisms.

Although the MIC and MBC values of the compounds were significantly lower than those of vancomycin against *C. difficile*, the compounds may be less damaging to the indigenous flora than antibiotics as the Gram-negative bacteria would be largely unaffected by their use. However, many Gram-positive bacteria which are beneficial to the host such as lactobacilli and bifidobacteria would still be eliminated. The toxicity of these compounds has not been determined as yet and therefore further investigation is required if they were to be considered as a possible treatment option. Further work regarding separation of the E/Z isomer mixture of compound **8** would also have to be undertaken if the compounds were considered for treatment as the current mixture would be unacceptable for use.

None of the compounds demonstrated any activity against the spores of *C. difficile*, with less than a 0.1 log reduction with all compounds after 5 hours exposure. This was expected given the resistance of Gram-negative organisms to these

compounds as the spores of *C. difficile* are highly resistant to many chemicals and therefore very powerful agents, such as hypochlorite and glutaraldehyde are required to eliminate them (Wullt *et al.*, 2003, Fawley *et al.*, 2007). However, another potential use for these antimicrobial agents may be as part of a cleaning process. Previous chapters of this thesis have shown that spores may become susceptible to various antimicrobial agents after exposure to a germinant (1% sodium taurocholate). Therefore, a germinant solution together with a compound solution may act to eliminate the spores of *C. difficile*. However, further work is again required to determine the efficacy of these compounds against the germinating spores of *C. difficile* and the safety hazards associated with their use.

## 8 Final Discussion

In 2007 there were 50,392 cases of *C. difficile* infection in England, Northern Ireland and Wales (HPA, 2008). Despite this being a decrease of 9% from the previous year, infections associated with *C. difficile* remain a significant cause of morbidity and mortality. It is thought that the emergence of a hypervirulent strain of *C. difficile*; ribotype 027 and increased resistance to fluoroquinolones (Razavi *et al.*, 2007, Spigaglia *et al.*, 2008) has exacerbated the problem together with resistance of *C. difficile* spores to many common cleaning agents and disinfectants (Worsley, 1998, Vonberg *et al.*, 2008). This thesis has investigated the effectiveness of a panel of antimicrobial agents against the spores and vegetative cells of *C. difficile* together with the physical and chemical factors influencing germination of its spores.

A major factor in preventing the spread of *C. difficile* is effective cleaning of the hospital environment. There has been much controversy over which cleaning agent to use, with some studies advocating the use of detergents (Anon, 1994, Worsley, 1998), while others state that disinfectants should be used to decontaminate the environment (Barbut and Petit, 2001, Wilcox and Fawley, 2000). It is however, widely acknowledged that detergents do not possess any sporicidal activity (Fawley *et al.*, 2007, Gerding *et al.*, 2008) and the choice of cleaning agent and the concentration used are of particular importance as sub-inhibitory levels of some agents have been shown to induce sporulation (Wilcox and Fawley, 2000). Government reports advise the use of detergents to remove organic matter followed by disinfection with hypochlorite (Pratt *et al.*, 2007). Indeed reduced activity of chlorine-based agents in the presence of organic matter has been described previously (Rutala and Weber, 1997). However, there are a number of occupational hazards associated with the use of chlorine-based agents (Rutala and Weber, 1997)



and there is therefore a need for alternative disinfectants which are effective but less hazardous towards the HCW.

Studies on currently available disinfectants reported in this thesis using a British and European standard suspension test method showed that only the chlorine-releasing agent, Chlor-clean®, was capable of reducing the number of viable *C. difficile* spores by 99.9% (3 log cycles) within one hour. The peracetic acid-based agent, Wofasteril®, was capable of producing a 96% reduction, but only against a reduced starting inoculum of *C. difficile* spores. The other disinfectants assessed including a quaternary ammonium compound (R2®) commonly used in hospitals in the West Midlands, UK, and a solution based on ascorbic acid (Citrofresh®) were largely ineffective against *C. difficile* spores. As expected, in the presence of a soil load the activity of Chlor-clean® was significantly compromised and none of the other cleaning agents tested in the presence of a soil load possessed the criterion for definition as a sporicidal agent against *C. difficile*. These findings therefore support the suggested cleaning process recommended by the UK Government of removing organic matter firstly with a detergent, followed by disinfection with a chlorine-based agent. However, it is still concerning that none of the agents tested, including two cleaning products used in hospitals in the West Midlands, UK passed the criteria to be defined as sporicidal against *C. difficile*.

Further investigation into the efficacy of the most active cleaning products tested against the spores of *C. difficile* was conducted using a carrier test method which more adequately simulates the clinical environment. The importance of using such methods when determining disinfectant efficacy has been highlighted previously (Reybrouck, 1992). Reybrouck (1992) found that there was no correlation between suspension tests and practice tests, however the practice tests; such as the quantitative carrier test, correlated well with practice. In this thesis, the chlorine-



based agent demonstrated significantly higher efficacy against the spores of *C. difficile* under 'clean' conditions than the peracetic acid-based agent; with a 5 log reduction in 9 minutes, compared to a 1 log reduction in the same time frame. These results suggest that even the most effective agent must be left in contact with the spores for a minimum of 9 minutes, which is unlikely to occur in the healthcare setting, before being wiped away. When tested in the presence of a soil load there was no significant difference between the sporicidal efficacies of either agent against the spores of *C. difficile*, with both agents requiring up to 60 minutes to achieve a  $\geq 3$  log reduction in the number of viable spores. As the spores of *C. difficile* are undoubtedly present with organic matter in the clinical environment (due to their release in the faeces of patients) this further supports the use of detergents to remove organic matter before exposure to a disinfectant. Alternatively, given the hazards associated with the use of chlorine-based agents, peracetic acid disinfectants, particularly those with neutralisers to eliminate its odour, may offer a suitable alternative as it has equivalent activity in the presence of a soil load.

Other studies have demonstrated that hydrogen peroxide vapour has activity against the spores of *C. difficile* (Boyce *et al.*, 2008, Shapey *et al.*, 2008). However, this is a very invasive product; requiring evacuation of the whole ward when used. Clearly, there is a need for new effective disinfectants, which act rapidly, are not greatly affected by organic matter and are not highly hazardous or invasive. Additionally, staff must be educated on the exposure time required for existing disinfectants to act. Alternatively, novel strategies, for example germination of *C. difficile* spores prior to exposure of commonly used, less hazardous hard surface disinfectants may offer one way forward in preventing the spread of *C. difficile*.

Spores of *C. difficile* which contaminate the environment act as a reservoir of infection, however, they are only capable of causing disease if they germinate into

toxin-producing cells once inside the body. To date, only two studies have looked at germination of *C. difficile* spores in detail, with both studies reaching conflicting conclusions. In the first study by Sorg and Sonenshein (2008), it was concluded that the bile salt sodium taurocholate and the amino acid glycine act as co-germinants, stimulating germination of *C. difficile* spores. The findings within this thesis largely concur with those of Sorg and Sonenshein (2008), as it was determined that glycine and sodium taurocholate were fundamental for germination; however, the results also suggest that other amino acids may also be required to reach maximum levels of germination in *C. difficile* spores. It was also found that of the bile salts tested only sodium taurocholate was capable of stimulating germination of *C. difficile* spores between 1 and 100mM and chenodeoxycholate at 100mM. Further investigations should therefore focus on testing a panel of amino acids together with glycine and sodium taurocholate and chenodeoxycholate to determine which amino acids enhance *C. difficile* germination.

Conversely, Paredes-Sabja *et al.* (2008) found that the incorporation of bile salts into the growth medium had little effect on the germination of *C. difficile* spores but Brain Heart Infusion (BHI) broth stimulated germination of *C. difficile* spores. Paredes-Sabja *et al.* (2008) also found that none of the 20 amino acids tested initiated germination of *C. difficile* spores; instead, potassium salts were required. However, a major difference in their study, compared to this thesis and the study of Sorg and Sonenshein (2008) was the heat shocking of spore suspensions at 80°C for 10 minutes, before exposure to potential germinants (Paredes-Sabja *et al.*, 2008).

Interestingly, the results of this thesis showed that there was no difference in the rate or level of germination in anaerobic conditions, compared to aerobic conditions. This may be important when considering germination of the organism within the human body, as the optimum concentration of bile salts is in the



duodenum, which is an aerobic environment (Leverrier *et al.*, 2003). The pH of the germinant solution had a significant effect on both the rate and level of germination. It is well established that a range of different pH environments exist throughout the G.I tract, from the highly acidic stomach to the more neutral conditions of the duodenum and jejunum; where the concentration of bile salts is optimum (Ridlon *et al.*, 2006). The optimum levels of germination occurred between pH 6.32 - 7.53 and this may therefore support studies which have demonstrated an increased risk of developing CDAD in patients on proton pump inhibitor medications that elevate gastric pH (Dial *et al.*, 2004, Aseeri *et al.*, 2008). The level of germination of *C. difficile* spores was the same at 37°C compared to 20°C, indicating that *C. difficile* spores can be effectively germinated at room temperature. However the rate of germination was higher at 37°C, indicating that the optimum conditions for germination are inside the host, at body temperature.

These findings enhance existing knowledge of germination in *C. difficile* and may contribute towards ways of preventing the development and spread of disease. For example, if spores of *C. difficile* were prevented from germinating into toxin-producing cells in the body, then infection and disease may be prevented. The potential of this method for preventing disease by spore-formers such as *C. difficile* has been described by Alvarez and Abel-Santos (2007) and the efficacy of nisin to inhibit spore outgrowth during germination of *Bacillus anthracis* spores has been recently highlighted (Gut *et al.*, 2008). Further investigation into the inhibitory effects of nisin on the germination of *C. difficile* spores is therefore required as the antimicrobial effects of nisin on *C. difficile* cells have already been reported together with an effective way of delivering nisin to the gut (Bartoloni *et al.*, 2004, Ugurlu *et al.*, 2007).

Alternatively, as the rate and level of germination of *C. difficile* spores remained the same in both aerobic and anaerobic conditions and the level of

germination was equal in both room temperature and body temperature, it may be possible to target spores in the environment rendering them more susceptible to widely-used antimicrobial agents.

The concept of incubating spores with a germinant, followed by exposure to an antimicrobial has been effectively demonstrated with bacillus spores (Hornstra *et al.*, 2007). This two stage concept was applied to *C. difficile* spores using a carrier test method to simulate environmental conditions. One percent sodium taurocholate (~20mM) was selected as the concentration for use as this is within the optimum range as determined in this thesis together with either 70% (v/v) ethanol or pure copper metal as the antimicrobial agents. This study demonstrated that the spores of *C. difficile* required 20-30 minutes incubation with 1% sodium taurocholate in thioglycollate medium before exposure to 70% ethanol for at least a further 30 minutes in order for maximal reduction (2-3 log orders) in the number of viable spores. Although this method was effective at reducing the number of viable spores, the incubation times required are lengthy and are probably not realistic for use in the hospital environment. As demonstrated in other studies (Woese *et al.*, 1968, Hornstra *et al.*, 2007), there was also a small percentage of spores that did not germinate and were therefore not killed by 70% ethanol. Caipo *et al.* (2002) suggest that differences between spores in the time taken to germinate may be due to quorum sensing within the spore population. Despite these disadvantages this disinfection method does avoid the use of more hazardous chemicals such as chlorine and glutaraldehyde. Furthermore, alternative antimicrobials could also be explored in addition to 70% ethanol, for example, 2% (w/v) chlorhexidine in 70% (v/v) isopropyl alcohol. If this method was to be considered for use in clinical practice, it would have to be assessed in the presence of organic matter to ensure that the activity of the germinant and the antimicrobial were not greatly affected. Future studies could also focus on improving the percentage of spores which germinate,



possibly by using higher purities of sodium taurocholate or by targeting quorum sensing of the organism.

Although 70% ethanol and the germinant medium were not effective when applied simultaneously, the application of the germinant solution to copper metal (contaminated with *C. difficile* spores) resulted in a significant reduction in viable spores. Copper metal alone did not demonstrate antimicrobial activity towards the dormant spores of *C. difficile* and stainless steel did not demonstrate any antimicrobial activity against either dormant or germinating spores. However, with the incorporation of the germinant solution, copper metal was capable of producing a 2-3 log reduction in 3 hours, with >1 log reduction in 60 minutes. Importantly, the activity of copper was not reduced by the presence of a soil load and therefore any organic matter that is likely to accompany the spores of *C. difficile* in a clinical environment is unlikely to affect the antimicrobial efficacy of copper metal if used in the clinical setting.

Copper metal has previously been shown to demonstrate good activity against a range of organisms including MRSA (Noyce *et al.*, 2006) and this research suggests that with the aid of a germinant solution, this surface could also possess activity against the spores of *C. difficile*. Future research could investigate the potential of incorporating a germinant, such as sodium taurocholate tethered to the copper surface thus developing a 'germination surface'. Another significant finding resulting from the germination studies in this thesis was the reduction in the number of remaining spores when an antimicrobial was not used. This suggests that the germinating spores become susceptible to the aerobic environment alone. Future studies could potentially focus upon the production of a wipe impregnated with a germinant solution together with the incorporation of copper surfaces or an antimicrobial solution in order to reduce the spread of infection of *C. difficile* in the

clinical environment. However, if this was successful there may be significant barriers such as educating the public and HCW on the use of a germinant to reduce the spread of *C. difficile* infection as the disinfectants that are currently used have been in use for many years and there may be considerable reluctance to change. The current exposure time required for the germinant and antimicrobial to act is also too long to be of value in clinical practice. Further work should therefore focus upon optimizing the germination solution and reducing the exposure time necessary for the antimicrobial to eliminate the spores of *C. difficile*.

The development of novel, non-toxic compounds with activity against *C. difficile* is also important as despite various interventions and Government schemes, *C. difficile* infection continues to be a widespread problem, causing nearly 6500 deaths in 2006 in England and Wales (HPA, 2008, ONS, 2008). A series of analogues of a benzylidene-carboxamidrazone compound previously developed at Aston University that demonstrated antimicrobial activity against Gram-positive organisms, was produced and assessed in a preliminary investigation in this thesis. The precursor compound, **1**, together with one analogue and a thiosemicarbazone demonstrated good antimicrobial activity against a panel of *C. difficile* strains, but their activity was inferior compared to that of vancomycin. Whilst the initial data is promising, significant further testing of these compounds is required including toxicity testing.

None of the compounds tested demonstrated any activity towards the spores of *C. difficile* and therefore would not be considered as a suitable sporicidal agent. However, as these compounds possess activity against vegetative cells of *C. difficile*, potentially they may be used in conjunction with a germinant solution to reduce the number of contaminating *C. difficile* spores. Further investigation would also be required to ensure the safety of these compounds and to determine their efficacy against *C. difficile* spores when used with a germinant solution and in the

presence of a soil load.

Most of the findings of this thesis contribute towards measures for the prevention of infection due to *C. difficile*. However, it must be emphasized that prevention should be a multi-faceted approach in order to be successful (Valiquette *et al.*, 2004). For example, the production of a novel disinfectant or cleaning regime with high efficacy against *C. difficile* might not be successful in practice without adequate education of the cleaning staff. Other authors have suggested several measures that should be adhered to in order to prevent *C. difficile* infection including: isolation of *C. difficile* patients, prescribing narrow spectrum antibiotics, adequate cleaning, surveillance of infection, increased compliance of hand-washing protocols, reduction in patient transfers, early diagnosis, rapid treatment and education and collaboration between all staff members (Valiquette *et al.*, 2004, Anon, 2007, Whitaker *et al.*, 2007).

This research presented in this thesis has explored the efficacy of a panel of cleaning agents and novel compounds against the spores and cells of *C. difficile*, and provided a significant insight into the factors influencing the germination of its spores. A potential two-stage cleaning process has been identified involving germination of *C. difficile* spores, followed by exposure to an antimicrobial, with an aim to contribute towards mechanisms which prevent the spread of infection in the clinical environment.



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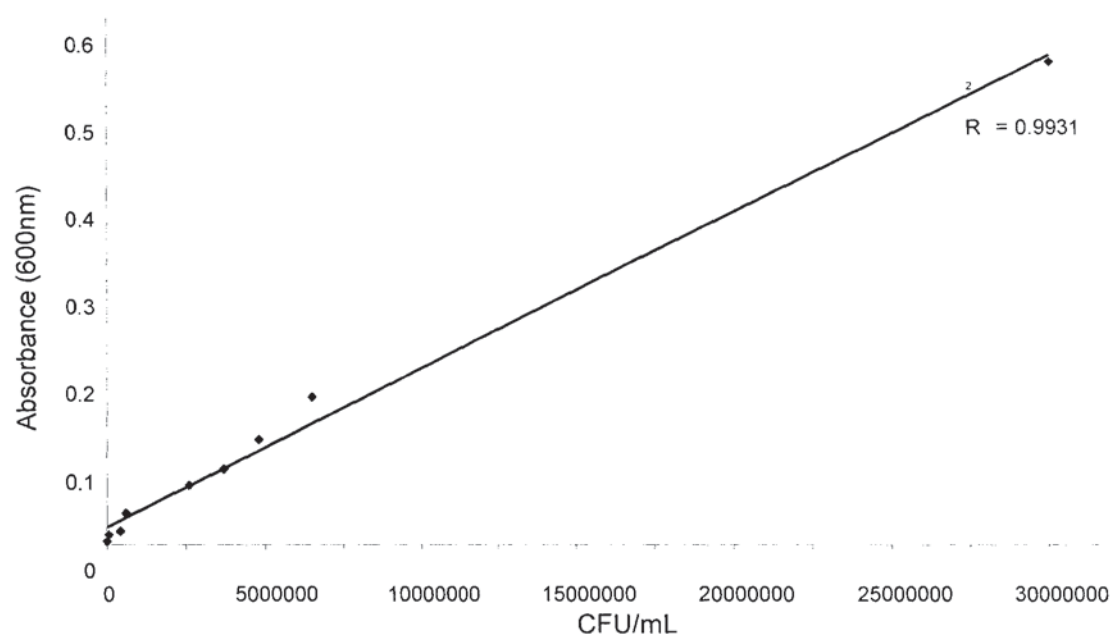
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## Appendices

### Appendix 1: Determination of CFU/mL of *C. difficile* cells in relation to Optical Density

A universal containing 10mL of Wilkins Chalgren broth was inoculated with a colony of *C. difficile* (NCTC 11204) from a 48 hour culture plate. The inoculated broth was incubated at 37°C, under anaerobic conditions and after 48 hours, a 1mL sample was taken from the culture and the optical density (OD) was measured at 600nm. A 100µL sample was also taken from the culture and after appropriate serial dilutions, with Wilkins Chalgren broth, 100µL was transferred onto a well-dried Wilkins Chalgren agar plate in five 20µL drops, using the Miles-Misra method (Miles and Misra, 1938). The culture was then diluted to 1:2, 1:4, 1:6, 1:8, 1:10, 1:20, 1:50, 1:100, 1:1000 and 1:10000 using Wilkins Chalgren broth. The OD<sub>600</sub> of each of the dilutions was measured and 100µL of each solution was plated onto Wilkins Chalgren agar as described above, using the Miles and Misra method. Agar plates were incubated in anaerobic conditions for 48 hours at 37°C and the number of colonies was counted.

**Figure A 1 Relationship between optical density and CFU/mL of *C. difficile***



$$Y = 2E - 08x + 0.0196$$

## Appendix 2: Characterisation of novel compounds

### Compound 2:

*N*<sup>1</sup>-(2-Hydroxy-5-tert-butylbenzylidene)pyridine-4-carboxamidrazone-4-N-oxide

Yellow crystalline solid. 51% yield.

MP 212.9 – 215.4 °C

MS (APCI +ve) *m/z* = 313 (*M*+H)<sup>+</sup>

IR (KBr disc)  $\nu$  = 3459, 3273, 3107, 2955, 1628, 1592, 1532, 1485, 1396, 1244 (N-O) cm<sup>-1</sup>

<sup>1</sup>H NMR (D6-DMSO;  $\delta$ DMSO = 2.50ppm) 1.28 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>), 6.86 (d, 1H, *J*=8.6Hz, Ar 3'-H), 7.13 (bs, 2H, NH<sub>2</sub>), 7.33 (dd, 1H, *J*=8.6, 2.5Hz, Ar 4'-H), 7.63 (d, 1H, *J*=2.5Hz, Ar 6'-H), 7.94 (d, 2H, *J*=7.3Hz, Ar 2-H and Ar 6-H), 8.29 (d, 2H, *J*=7.2Hz, Ar 3-H and Ar 5-H), 8.67 (s, 1H, N=CH), 10.60 (bs, 1H, OH) ppm

### Compound 3:

*N*<sup>1</sup>-(3,5-Di-tert-butylbenzylidene)pyridine-4-carboxamidrazone-4-N-oxide

Yellow crystalline solid. 58% yield.

MP 213.8 – 215.8 °C

MS (APCI +ve) *m/z* = 353 (*M*+H)<sup>+</sup>, 335 (*M*-H<sub>2</sub>O)<sup>+</sup>

IR (KBr disc)  $\nu$  = 3450, 3282, 2957, 1617, 1533, 1498, 1441, 1395, 1359, 1346, 1253 (N-O) cm<sup>-1</sup>

<sup>1</sup>H NMR (D6-DMSO;  $\delta$ DMSO = 2.50ppm) 1.33 (s, 18H, 2 x C(CH<sub>3</sub>)<sub>3</sub>), 7.13 (bs, 2H, NH<sub>2</sub>), 7.45 (t, 1H, *J*=1.7Hz, Ar 4'-H), 7.72 (d, 2H, *J*=1.8Hz, Ar 2' and 6'-H), 7.95 (d, 2H, *J*=7.1Hz, Ar 2-H and Ar 6-H), 8.30 (d, 2H, *J*=7.1Hz, Ar 3-H and Ar 5-H), 8.46 (s, 1H, N=CH) ppm



**Compound 4:**

*N*<sup>1</sup>-(2-Hydroxy-3-tert-butylbenzylidene)pyridine-4-carboxamidrazone-4-N-oxide

Yellow crystalline solid. 51% yield.

MP 195.0 – 197.2 °C

MS (APCI +ve) *m/z* = 313 (M+H)<sup>+</sup>

IR (KBr disc)  $\nu$  = 3549, 3458, 3307, 3190, 3087, 3064, 2970, 1642, 1600, 1531, 1495, 1426, 1393, 1233 (N-O), 1184 cm<sup>-1</sup>

<sup>1</sup>H NMR (D6-DMSO;  $\delta$ DMSO = 2.50ppm) 1.43 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>), 6.87 (t, 1H, J=7.7Hz, Ar-H), 7.17 (bs, 2H, NH<sub>2</sub>), 7.27-7.35 (overlapping m, 2H, 2 x Ar-H), 7.95 (d, 2H, J=7.0Hz, Ar 2-H and Ar 6-H), 8.30 (d, 2H, J=7.0Hz, Ar 3-H and Ar 5-H), 8.65 (s, 1H, N=CH), 11.77 (s, 1H, OH) ppm

**Compound 5:**

*N*<sup>1</sup>-(3,5-Di-tert-butyl-2-hydroxybenzylidene)pyridine-4-carboxamidrazone -4-N-oxide

Yellow crystalline solid. 68% yield.

MP 277.2 – 278.9 °C

MS (APCI +ve) *m/z* = 369 (M+H)<sup>+</sup>

IR (KBr disc)  $\nu$  = 3458, 3273, 3114, 2955, 1635, 1622, 1542, 1500, 1438, 1399, 1356, 1247 (N-O), 1177 cm<sup>-1</sup>

<sup>1</sup>H NMR (D6-DMSO;  $\delta$ DMSO = 2.50ppm) 1.28 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>), 1.43 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>), 7.12 (bs, 2H, NH<sub>2</sub>), 7.30 (d, 1H, J=2.4Hz, Ar'-H), 7.33 (d, 1H, J=2.4Hz, Ar'-H), 7.94 (d, 2H, J=7.3Hz, Ar 2-H and Ar 6-H), 8.30 (d, 2H, J=7.3Hz, Ar 3-H and Ar 5-H), 11.57 (s, 1H, N=CH) ppm

**Compound 6:**

*N*<sup>1</sup>-(3,5-Di-*tert*-butyl-4-hydroxybenzylidene)pyridine-4-carboxamidrazone-4-*N*-oxide

Yellow crystalline solid. 42% yield

MP 220.4 – 223.1 °C

MS (APCI +ve) *m/z* = 369 (*M*+*H*)<sup>+</sup>

IR (KBr disc)  $\nu$  = 3624, 3608, 3445, 3260, 2950, 1618, 1486, 1439, 1423, 1227 (N-O), 1171 cm<sup>-1</sup>

<sup>1</sup>H NMR (D6-DMSO;  $\delta$ DMSO = 2.50ppm) 1.42 (s, 18H, 2 x C(CH<sub>3</sub>)<sub>3</sub>), 6.94 (bs, 2H, NH<sub>2</sub>), 7.34 (bs, 1H, OH), 7.62 (s, 2H, Ar 2' and 6'-H), 7.92 (d, 2H, *J*=7.1Hz, Ar 2-H and Ar 6-H), 8.28 (d, 2H, *J*=7.1Hz, Ar 3-H and Ar 5-H), 8.37 (s, 1H, N=CH) ppm

**Compound 7:**

*N*<sup>1</sup>-(4-*tert*-butylbenzaldehyde)pyridine-4-carboxamidrazone-4-*N*-oxide

Yellow crystalline solid. 90% yield

MP 216.1 – 218.2°C

MS (APCI +ve) *m/z* = 297 (*M*+*H*)<sup>+</sup>, 279 (*M*-H<sub>2</sub>O)<sup>+</sup>

IR (KBr disc)  $\nu$  = 3450, 3324, 3095, 3060, 3029, 2962, 2944, 2898, 2864, 1616, 1527, 1500, 1445, 1400, 1362, 1345, 1330, 1320, 1245 (N-O), 1195 cm<sup>-1</sup>

<sup>1</sup>H NMR (D6-DMSO;  $\delta$ DMSO = 2.50ppm) 1.31 (s, 9H, C(CH<sub>3</sub>)<sub>3</sub>), 7.14 (bs, 2H, NH<sub>2</sub>), 7.46 (d, 2H, *J*=8.4Hz, Ar 3'-H and Ar 5'-H), 7.83 (d, 2H, *J*=8.4Hz, Ar 2'-H and Ar 6'-H), 7.94 (d, 2H, *J*=7.3Hz, Ar 2-H and Ar 6-H), 8.29 (d, 2H, *J*=7.3Hz, Ar 3-H and Ar 5-H), 8.42 (s, 1H, N=CH), 10.12 (bs, 1H, NH) ppm

**Compound 8:**

*N*<sup>1</sup>-(5-Nitro-2-furylidene)pyridine-4-carboxamidrazone-4-*N*-oxide *E/Z* isomer mixture

The product obtained was a mixture of *E/Z* isomers (2.4/1 ratio) about the N=C-furyl

double bond.

Orange solid. Yield 79%.

MS (APCI +ve)  $m/z$  = 276 ( $M+H$ )<sup>+</sup>, 258 ( $M-H_2O$ )<sup>+</sup>

IR (KBr disc)  $\nu$  = 3432, 1625, 1509, 1466, 1321, 1247 (N-O), 1172  $\text{cm}^{-1}$

<sup>1</sup>H NMR (D6-DMSO;  $\delta_{\text{DMSO}}$  = 2.50ppm)

Major product E isomer: 7.39 (d, 1H,  $J=3.9\text{Hz}$ , furyl H-3), 7.51 (bs, 2H,  $\text{NH}_2$ ), 7.82 (d, 1H,  $J=3.9\text{Hz}$ , furyl H-4), 7.97 (d, 2H,  $J=7.1\text{Hz}$ , Ar 2-H and Ar 6-H), 8.31 (d, 2H,  $J=7.0\text{Hz}$ , Ar 3-H and Ar 5-H), 8.36 (s, 1H,  $\text{N=CH}$ ) ppm

Minor product Z isomer: 7.59 (d, 1H,  $J=3.9\text{Hz}$ , furyl H-3), 7.62 (bs, 2H,  $\text{NH}_2$ ), 7.77 (d, 1H,  $J=3.8\text{Hz}$ , furyl H-4), 7.86 (s, 1H,  $\text{N=CH}$ ), 8.00 (d, 2H,  $J=7.3\text{Hz}$ , Ar 2-H and Ar 6-H), 8.33 (d, 2H,  $J=7.0\text{Hz}$ , Ar 3-H and Ar 5-H) ppm

## Appendix 3: Presentations and publications

### Poster presentations

SFAM Summer conference, Apex International, Edinburgh, 3-6th July 2006:

Efficacy of novel antimicrobial compounds against *Clostridium difficile*.

L.J. Wheeldon, T. Worthington, D.L. Rathbone, A.C. Hilton, P.A. Lambert, T.S.J. Elliott

6<sup>th</sup> International Conference of Hospital Infection Society, Amsterdam, Netherlands, 16-18<sup>th</sup> October 2006:

Susceptibility of Germinating *Clostridium difficile* Spores to 70% Ethanol

L.J. Wheeldon, T. Worthington, A.C. Hilton, P.A. Lambert, T.S.J. Elliott

18<sup>th</sup> European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, 19-22<sup>nd</sup> April 2008:

Susceptibility of *Clostridium difficile* Spores To Copper: The Germination Theory

L.J. Wheeldon, T. Worthington, C.J. Lowden, A.C. Hilton, P.A. Lambert, T.S.J. Elliott



## Published Papers

**Wheeldon LJ**, Worthington T, Hilton AC, Elliott TS, Lambert PA.

Physical and chemical factors influencing the germination of *C. difficile* spores.

J Appl Microbiol. 2008 Dec;105(6):2223-2230

**Wheeldon LJ**, Worthington T, Lambert PA, Hilton AC, Lowden CJ, Elliott TS.

Antimicrobial efficacy of copper surfaces against spores and vegetative cells of *Clostridium difficile*: the germination theory.

J Antimicrob Chemother. 2008 Sep;62(3):522-5.

**Wheeldon LJ**, Worthington T, Hilton AC, Lambert PA, Elliott TS.

Sporicidal activity of two disinfectants against *Clostridium difficile* spores.

Br J Nurs. 2008 Mar 13-26;17(5):316-20.

Costello C, Karpanen T, Lambert PA, Mistry P, Parker KJ, Rathbone DL, Ren J,

**Wheeldon L**, Worthington T.

Thiosemicarbazones active against *Clostridium difficile*.

Bioorg Med Chem Lett. 2008 Mar 1;18(5):1708-11.

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