Evaluation Of Molecular Typing Methods For Methicillin Resistant *Staphylococcus aureus*

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ABSTRACT

Effective control of methicillin resistant *Staphylococcus aureus* (MRSA) depends upon a thorough knowledge of its epidemiology. Typing of isolates can show the Infection Control Team whether there is an outbreak caused by spread of a single organism or, alternatively, a series of sporadic unrelated MRSA. The optimum typing method has not yet been defined. Bacteriophage typing, the mainstay for many years, is unreliable. Pulsed-field gel electrophoresis (PFGE) is highly discriminatory and is regarded as the "gold standard" but it is expensive, time consuming and requires expertise. Methods based on the polymerase chain reaction (PCR) may offer a compromise, as results are rapid, reasonably discriminatory and reproducible.

The aim of this study was to systematically evaluate a selection of methods for molecular typing of MRSA, for use in the routine microbiology laboratory. This was carried by evaluating each of the molecular typing systems for typability, reproducibility, discriminatory ability, stability, cost effectiveness and turn around time.

A total of 152 MRSA isolates were tested, comprising of two sets; outbreak/connected isolates and a diverse, non-epidemiologically connected set.
A group of 36 isolates were from outbreaks from hospitals in Oxford, Aberdeen and Birmingham. The remaining 116 isolates were sporadic isolates from hospitals in the UK and epidemic MRSA stains (EMRSA 1-16).

The methods evaluated were PFGE; repetitive element sequence based PCR methods involving amplification using repMP3 primer derived from *Mycoplasama pneumoniae*, Shine-Dalgarno-transposon 916 spacer amplification, inter-IS256 fragment amplification, and amplification of the 16S-23S rRNA intergenic spacer region; ribotyping and binary typing Results were analysed using the program Gel Compar II (Applied Maths, Belgium). The degree of homology was determined by DICE coefficient and clustering correlation by UPGMA, with a 1.2% position tolerance.

There are no standard guidelines for the interpretation of molecular typing generated profiles except those proposed by Tenover *et al*. Isolates were assigned groups according to two sets of criteria; a one band difference or a difference of 7 or more bands according to Tenover's' criteria. The resulting groups were used to determine reproducibility, epidemiological concordance and Simpson's Index of diversity.

PFGE was found to be the best method in terms of exhibiting excellent typability, reproducibility and epidemiological concordance, and good stability and discriminatory ability when Tenover's criteria are applied.
Of the Rep-PCR methods, RS-PCR was found to show excellent typability and epidemiological concordance and good reproducibility and stability.

PFGE and RSPCR are the most suitable methods for use in the routine microbiology laboratory.

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LIST OF ABBREVIATIONS

6-APA	6 - Amino-Penicillinic Acid
AP-PCR	Arbitarily Primed - PCR
bp	Base Pair
CHEF	Contour Clamped Homogeneous Electrophoresis
DNA	Deoxyribo-Nucleic Acid
EMRSA	Epidemic Methicillin Resistant *Staphylococcus aureus*
ET	Electrophoretic Type
FIGE	Field Inversion Gel Electrophoresis
IS	Insertion Sequence
kb	Kilo Base
mRNA	Messenger RNA
MIC	Minimal Inhibitory Concentration
MLEE	Multi Locus Enzyme Electrophoresis
MLST	Multi Locus Sequence Typing
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
OD	Optical Density
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
rRNA	Ribosomal RNA
RAPD	Randomly Amplified Polymorphic DNA
REA	Restriction Enzyme Analysis
REP-PCR	Repetitive Extragenic Palindromic - PCR
RFLP-PCR	Restriction Fragment Length Polymorphisms - PCR
RNA	Ribo-Nucleic Acid
RS-PCR	Ribosomal Spacer - PCR
RTD	Routine Test Dilution
SDS-PAGE	Sodium Do-decyl Sulphate- Polyacrylamide Gel Electrophoresis
SSC-*mecA*	Staphylococcal Cassette Chromosome *mec*
ST	Sequence Type
tRNA	Transfer RNA
UPGMA	Un-weighted Paired Group Method using Arithmetic averages
UV	Ultra-Violet

1.0 INTRODUCTION AND BACKGROUND

Staphylococci are commonly found as normal flora on the skin, skin glands and mucous membranes. They are responsible for superficial bacterial infections such as boils, pustules and infected minor wounds, however they can also cause more serious infections, such as endocarditis, osteomyelitis and septic shock, in both hospitalised and non-hospitalised individuals.

The first anti-staphylococcal antibiotic was penicillin, discovered by Fleming in 1929 and first used therapeutically in 1941. Since then, penicillins, and other β-lactams have been used extensively and successfully as antibiotics in the treatment of bacterial infections (Dyke and Gregory, 2000) Strains of *S. aureus* soon emerged which expressed resistance to penicillin due to production of the enzyme β-lactamase. This enzyme inactivates penicillin by hydrolysis of the β- lactam ring. During the 1950s, virulent strains of *S. aureus* emerged as a major nosocomial pathogen in many parts of the world (Shanson, 1981).

To combat the action of β-lactamase, newer antibiotics were developed by enzymatically deacylating benzylpenicillin to produce 6-aminopenicillinic acid (6-APA). The 6-APA treated with various acyl halides produced a variety of semi-synthetic penicillins, including methicillin, which are only slowly hydrolysed by staphylococcal β-lactamase. Introduction of methicillin, accompanied by improved infection control measures, led to a general decrease in the frequency of nosocomial staphylococcal infections. However, methicillin resistant *Staphylococcus aureus* (MRSA) soon emerged and extensive spread was observed in the late 1970s through to the 1980s.

MRSA is resistant to all β-lactam agents including cephalosporins and carbapenems. Vancomycin and teicoplanin are widely regarded, as the definitive choice of therapy, however there is concern regarding reduced susceptibility to these agents (Hiramatsu *et al.*, 1997; Martin *et al.*, 1997) Some strains remain susceptible to fluoroquinolones, trimethoprim/ sulfamethozole, gentamicin, and rifampicin.

MRSA is of growing concern in the hospital environment as it is readily transmitted by patients and health care workers, colonized or infected with MRSA. The rising incidence of MRSA infections increases morbidity and cost to the Health Service, as these organisms are difficult and expensive to treat.

1.1 Methicillin Resistance

The first MRSA containing the *mec* determinant were isolated in 1960, shortly after the introduction of methicillin into clinical use. Staphylococcal resistance to methicillin is expressed by a chromosomal gene, *mec*A that encodes for the penicillin binding protein (PBP), with a low affinity for β-lactamase stable penicillins. These are enzymes anchored in the cytoplasmic membrane (Dyke and Gregory, 2000[a]). The methicillin resistance determinant, *mec,* provides staphylococci with an intrinsic resistance against all β- lactams (Iandolo, Bannantine and Stewert, 2000).

Mec is carried on a mobile genetic element, the staphylococcal cassette chromosome *mec* (SSC*mec*), of which four forms, differing in size and genetic composition, have been described. *Mec* consists of *mec*A, the structural gene for PBP2a; *mec*I and *mec*RI, regulatory elements controlling *mec*A transcription and 20 to 45kb of *mec*-associated DNA. *Mec* is integrated at a specific site in the genome of *S. aureus*, between the *spa* (protein A) and *pur*A (adenine requirement) genes located on the *sma*I-G fragment of *S. aureus* 8325. However, the

nature and origin of this element or the mechanisms by which it is transferred are unknown. Other β-Lactamases, which are transposon and plasmid encoded, are widely disseminated. In contrast, the *mec* determinant is restricted to a few clonal lineages and seems to favour clonal over horizontal spread.

The evolutionary origins of MRSA are poorly understood. However, as a result of evolutionary and population studies, it has been suggested (Kreiswirth *et al.*, 1993) that all MRSAs were descendents of a single *S. aureus* strain that acquired *mec*A. Recent studies suggest that MRSA is very divergent, due to horizontal transmission of *mec*A among *S. aureus* strains. Musser and Kapur, (Musser and Kapur, 1992) have cited three lines of evidence supporting the idea of horizontal transfer and recombination of *mec* rather than chromosomal divergence following resistance acquisition by a single precursor cell.

1.2 Epidemiology of MRSA

MRSA is responsible for both endemic and epidemic hospital acquired infections. It is the single most commonly isolated organism recovered from surgical site infections and bloodstream infections. Serious nosocomial infections due to MRSA infections prolong hospitalisation. Patients are often isolated and special precautions undertaken by health care workers, resulting in increased hospital costs. In addition to methicillin resistance, resistance to other antibiotics is also high.

MRSA may be introduced into the hospital environment by colonized or infected patients or, more rarely health care workers. From the original source, the organism can spread to other patients. Nasal carriage is the usual reservoir for, occurring in approximately 30% of the population. Most infecting MRSAs are from the patients' own nose. A high proportion of

nasal carriers will also carry MRSA on their hands and other areas of the skin (Wenzel and Perl, 1995).

1.3 Transmission of MRSA

Personnel may transiently contaminate their hands while caring for patients colonized or infected with MRSA and hence transmit the organism to other patients without becoming nasal carriers. Those who have areas of dermatitis colonization or infection are particularly likely to transmit the organism to patients. Contaminated clothing, gowns and environmental surfaces are also implicated in the transmission of MRSA, but are not considered to be as important as person-to-person spread.

Transmission may be direct, indirect or airborne. Direct transmission occurs through skin-to-skin contact with susceptible individuals. Indirect transmission takes place when a susceptible host encounters an intermediate, such as gloves, or an inanimate object (medical equipment, for example) that are contaminated with the pathogen.

Airborne transmission is most likely to occur when patients have large draining wounds, or areas of dermatitis that are colonized or infected with the organism. Transmission of nosocomial MRSA is affected by factors such as the characteristics of the strain involved, host factors of the patients at risk, antibiotic utilization policies, and infection control measures implemented in the hospital (Cooke *et al.,* 1986).

Certain strains of MRSA (epidemic MRSA or EMRSA) encountered in hospitals in Britain have spread through hospitals despite the use of measures that have effectively controlled transmission of other strains. The characteristics responsible for rapid spread of such organisms are not clear, although it has been suggested that strains that produce large amounts of coagulase or those with multiple copies of the protein A gene may be more likely to cause

outbreaks (Frenay *et al.*, 1994). Host factors that increase the chances of acquiring MRSA in hospitals include location on a high-risk ward, prior surgical procedures, prolonged hospitalisation, and the presence of indwelling vascular catheters or prosthetic devices. Previous antimicrobial therapy increases the patients' risk of acquiring MRSA.

1.4 Hospital Infection Control

Many different infection control strategies have been used by hospitals to limit the spread of MRSA within hospitals, but no single strategy has been accepted as appropriate for all hospitals. Many hospitals use a set of basic measures for limiting the spread of methicillin sensitive *S. aureus* (MSSA), but implement additional surveillance and preventative measures when dealing with MRSA. In the UK, hospitals implement expanded surveillance and control measures if MRSA strains of demonstrated epidemic potential, i.e. 'EMRSA's, are encountered.

In 1990 guidelines were drawn up by the Working Party of the British Society for Antimicrobial Chemotherapy, to establish infection control measures for EMRSA-1 which was the most prevalent at the time. However, EMRSA –3, 15 and 16 are now the main strains found in hospitals in England and Wales. The incidence of EMRSA-15 and 16 has increased dramatically over recent years (Ayliffe *et al.*, 1998).

All staff and patients on an affected ward are screened for MRSA if a single case occurs in an intensive care unit or if two or more cases occur on a standard ward, and any individuals who are found to be MRSA carriers are treated with intranasal mupirocin. This strategy requires considerable laboratory resources and results in frequent use of mupirocin in affected hospitals. An additional problem is the emergence of mupirocin resistance. Mupirocin resistance may be low-level (MIC 8-25mg/L; of doubtful therapeutic significance) or high

level (MIC >256mg/L) due to acquisition of the *mup*A gene. Mupirocin resistance is often related to prolonged or repeated courses of therapy or cross colonisation of resistant strains during the course of outbreaks.

1.5 Typing of MRSA

The spread of MRSA is, in the majority of cases, clonal, i.e. the same strain is passed from person to person. However, sporadic cases are encountered.

Controlling MRSA is one of the primary goals of most infection control programs. The prevention and control of MRSA will depend on a thorough understanding of its epidemiology (see section 1.2). Bacterial strain typing has become an important clinical tool to investigate suspected outbreaks and to evaluate nosocomial transmission. A pre-requisite for this is a reliable typing scheme that can accurately show the relationship between bacteria isolated from different sources.

Numerous typing methods focus on discriminating MRSA isolates. Historically, techniques such as biotyping, serotyping, antibiotic susceptibility testing and bacteriophage typing have been used. These are all methods and are limited by the capacity of the bacteria to change unpredictably in their ability to express a particular characteristic in a changing environment. Some are also limited by their inability to type some strains, the lack of reproducibility and discriminatory power.

Procedures based on DNA analysis offer a more stable and universal approach to typing microorganisms (Williams *et al.*, 1999). As a result a number of DNA- based methods have been introduced. Examples include, plasmid analysis, restriction endonuclease analysis of chromosomal DNA (ribotyping, insertion-sequence typing, pulsed-field gel electrophoresis (PFGE)) and Polymerase Chain Reaction (Restriction Fragment Length Polymorphisms

(RFLP)-PCR, Arbitarily Primed (AP) -PCR, and Repetitive extragenic palindromic (Rep) - PCR).

At present there is no consensus regarding the best method to use for typing MRSA (Weller, 2000; Weber and Pfaller, 1997). An effective typing method should be highly discriminatory, reproducible, standardized, based on a single stable feature, widely available, inexpensive, user friendly and have performed satisfactorily in an epidemiological investigation (Weller, 2000).

Improvements in DNA analysis technology have resulted in the development of more universal and sensitive typing schemes for medically important microorganisms (Williams *et al.*, 1999). One of the main drawbacks of DNA-based methods is the difficulty associated with analysis and standardization of the complex banding profiles frequently seen on gels. This is being addressed by the recent introduction of computer based gel documentation systems and associated software (Weber and Pfaller, 1997).

2.0 LABORATORY PROCEDURES FOR EPIDEMIOLOGICAL ANALYSIS OF MRSA

Random isolates of the same species often differ in multiple characteristics, whereas the progeny of any particular isolate are typically indistinguishable or highly similar (Shopsin and Kreiswirth, 2001). The central hypothesis motivating typing studies is that a set of isolates obtained from an epidemiological cluster, for example during the course of an outbreak, are directly descended from a common precursor and as such will share characteristics by which they can be differentiated from unrelated isolates. Strain typing exploits the genetic diversity within a bacterial species to differentiate and identify the particular strain relevant to the epidemiological problem. Current molecular techniques can disclose subtle alterations and detect single genetic events within the bacterial chromosome. MRSA represents a *S. aureus* subset of unique epidemiological importance, particularly in health care institutions. However, strain-typing of MRSA has proved a particular challenge, much of which is related to their limited genetic diversity.

A convenient basis for classifying typing systems is to divide them into phenotypic techniques i.e. those, which detect characteristics expressed by the micro-organism, and genotypic techniques - those that involve direct DNA-based analyses of chromosomal or extra-chromosomal genetic elements.

2.1 Characteristics of Typing Methods

The ability of molecular typing systems to distinguish epidemiologically unrelated isolates is due to the genetic variation of the chromosomal DNA. It is assumed in an outbreak situation that isolates representing the outbreak strain will have the same genotype and epidemiological unrelated isolates will have a different genotype. A high degree of variation is reflected by differentiation of unrelated strains by a variety of typing techniques. However, little genetic diversity can be difficult to differentiate resulting in a limited number of strain types, even when newer molecular typing techniques are applied (Tenover, 1997).

All typing systems can be characterised in terms typability, reproducibility, discriminatory ability, ease of performance, and ease of interpretation.

Table 2.1.1: Characteristics of Typing Systems

Typing System	Proportion of Strains Typable	Reproducibility	Discriminatory Power	Ease of Interpretation	Ease of Performance
Biotyping	Majority	Poor	Poor	Moderate	Easy
Antimicrobial Susceptibility	Majority	Good	Poor	Easy	Easy
Serotyping	Moderate	Good	Fair	Moderate	Moderate
Plasmid Fingerprinting	Moderate	Good	Good	Moderate	Moderate
Phage Typing	Moderate	Poor	Good	Moderate	Moderate
REA of chromosomal DNA with conventional electrophoresis	Majority	Good	Good	Difficult	Moderate
RFLP analysis with DNA probes	Majority	Excellent	Moderate to Difficult	Moderate to difficult	Difficult
PFGE	Majority	Excellent	Excellent	Moderate	Moderate
Rep-PCR	Majority	Good	Good	Moderate	Moderate

Adapted from reference (Tenover 1997)

Typability refers to the ability of a technique to assign an unambiguous result (type) to each isolate. A reproducible method is one that yields the same result upon repeat testing of a

strain. Poor reproducibility may be due to technical variation in the method. Biological variation occurring during *in-vitro* or *in-vivo* passage of the organism reflects poor stability of the measured feature. The discriminatory power of the technique refers to its ability to differentiate among epidemiologically unrelated isolates, ideally assigning each isolate to a different type.

Ease of performance is measured by the cost of specialised reagents and equipment, the technical complexity of the method, and the effort required to learn and implement the technique in the laboratory. Finally, ease of interpretation refers to the effort and experience required to obtain useful, reliable typing information using a particular method.

At present, there is no standard guideline that may be applied to the interpretation of molecular typing results; however, guidelines for the interpretation of PFGE have been suggested by Tenover (Tenover 1995). These guidelines state that the electrophoretic profile must have a minimum of ten distinct fragments, and can only be applied to epidemiological studies of potential outbreaks spanning a short period of time (1 to 3 months). Variations in the number of bands differences in the profiles generated are used to group the isolates into indistinguishable, closely related, possibly related and different (see table 21.2). This grouping is established by accounting the number of band differences to genetic events, such as point mutations, resulting in the loss or gain of a restriction site; an insertion, a deletion or a chromosome inversion. A single genetic event i.e. 2-3 band differences, groups the isolates as closely related. The greater the number of genetic differences (4-7 band differences), the less they are likely to be related.

Table 2.1.2: General Principles For The Interpretation Of Molecular Typing Analysis (Summary of Ref: Tenover 1995)

Microbiological Interpretation Based on Typing Result	No. of Genetic Differences Compared With Outbreak Strain	Typical No. of Fragment Differences Compared to Outbreak Strain	Epidemiological Correlation
Indistinguishable	0	0	Isolate is part of the outbreak
Closely Related	1	2-3	Isolate probably is part of the outbreak
Possibly Related	2	4-6	Isolate possibly is part of the outbreak
Different	3	≥7	Isolate is not part of the outbreak

2.2 Phenotypic Techniques for MRSA

Typing methods that assess phenotypic differences are essentially limited by the tendency of microorganisms to alter the expression of the responsible genes. Such changes may occur unpredictably or in response to various environmental stimuli. Methods that assess polymorphic phenotypes, such as serotyping and bacteriophage typing, require specific reagents for each individual type.

2.2.1 Biotyping

Biotyping refers to the pattern of metabolic activities expressed by an isolate and may include specific biochemical reactions, colonial morphology, and environmental tolerances (e.g. the ability to grow on certain media or at extremes of pH or temperatures) (Arbeit, 2000). Such characteristics have classically been used for taxonomy. In some cases, biotyping is routinely and reliably performed using automated or modular systems designed for species

identification. Attempts to use biochemical tests for strain differentiation of *S. aureus* have been unsatisfactory.

2.2.2 Antimicrobial Susceptibility Testing

Analysing the antimicrobial susceptibility patterns can sometimes be helpful in determining whether MRSA clusters are caused by a single strain or by multiple unrelated strains and can be the initial indicator of an outbreak situation. Resistance profile determination is an effective first-line test that is cheap, rapid, standardised and readily accessible to the routine microbiology laboratory.

Clinical microbiology laboratories routinely test most bacterial isolates for susceptibility to a range of antimicrobial agents. Both manual and automated methods are widely available. The detection of a new or unusual pattern of antibiotic resistance among isolates cultured from multiple patients is often the first indication of an outbreak (Arbeit, 2000; Weller, 2000).

However, due to the lack of discriminatory ability, genotyping of isolates is often necessary. Antibiotic susceptibility testing has relatively limited epidemiological value because of variation in the individual resistance phenotypes. There are various genetic mechanisms by which a given strain can become abruptly resistant to a particular antibiotic, including point mutations and the acquisition of specific resistance genes via plasmids and transposons from other strains or even species. Since a single plasmid or transposon can carry multiple resistance determinants, resistance to multiple antimicrobial agents may be acquired simultaneously. On the other hand, in the absence of specific selective pressure, such elements may be lost.

2.2.3 Serotyping

Serotyping has not been used extensively for *S. aureus*. Tests have been developed for detecting differences in the capsular polysaccharides and the antigenic properties of coagulase (Weller, 2000). However, only reference laboratories are able to perform reliable serotyping especially for those strains that require cross-absorbed polyclonal antisera or standardized monoclonal antibodies that are not commercially available (Struelens, 1996).

2.2.4 Bacteriophage Typing

For decades, strain typing of *S. aureus* was carried out by phage typing. This technique evolved from observations that some *S. aureus* isolates carried bacteriophages that lysed some, but not all, unrelated isolates (Arbeit, 2000). Typically, the response to a particular phage was consistent for isolates representing the same strain, and thus a panel of diverse phages could be used to identify and differentiate distinct strains of *S.aureus*.

Phage typing of *S. aureus* was standardized by the International Sub-committee on Phage Typing of staphylococci (Weller, 2000). An approved set of phages was recommended which, with minor modifications, has been used worldwide ever since. Currently 23 standard phages are applied to an agar plate covered with the test organism. There is also room for two locally selected phages. Areas of lysis (plaques) caused by each phage are noted as either a strong or a weak reaction. The former, defined as more than 50 plaques, is used to distinguish between isolates. A lower number of plaques representing a weak reaction is recorded but should not influence the final phage type. If a Staphylococcus isolate shows no lysis at the Routine Test Dilution (RTD), then a concentration 100 times greater (RTD x 100) is employed. Unfortunately, standardization of the phages used has not led to uniformity of practice and interpretation.

For many years, phage typing was the method of choice for the investigation of MRSA epidemiology. The necessity for keeping stocks of phages and the propagating strains has confined it to larger laboratories and reference facilities. The main disadvantage of phage typing is the high proportion of modern isolates which are non-typable. Typically, this is 20-30 % but can be as high as 75%.

2.2.5 Electrophoretic Protein Typing and Immunoblotting

Variations in the types and structure of the proteins expressed by microorganisms can be detected by several different methods (Arbeit, 2000). Electrophoretic protein typing is performed by isolating materials, including proteins, glycoprotein conjugates, and lipopolysacccharides, from whole cell or cell surface preparations, separating the materials by sodium dodecyl suphate-polyacrylamide gel electrophoresis (SDS-PAGE), and staining the proteins in the gel to determine the resulting pattern. If the proteins are radiolabelled before isolation, the pattern can be detected by autoradiography. In the method *immunoblotting,* the electrophoresed bacterial products are transferred (blotted) onto a nitrocellulose membrane and then exposed either to antisera raised against specific type strains or, to pooled human sera which contains broadly reactive antibodies. The bound antibodies can then be detected using commercially available enzyme-labelled anti-immunoglobulins.

These techniques have been effective in epidemiological investigations of *S. aureus.* Immunoblotting has been used by only a few investigators and has not been widely adopted for several reasons. Firstly, the method is technically demanding, and the effect of technical factors, such as extraction procedures and the source of the primary antibody, have not been fully defined. Secondly, protein expression can vary in response to subtle changes in bacterial

growth conditions such as temperature, media composition, and pH. The patterns detected are very complex, so that comparisons among multiple strains can be difficult and the significance of small differences is uncertain.

In multi-locus enzyme electrophoresis (MLEE), isolates are analysed for differences in the electrophoretic mobilities of a set of housekeeping proteins. Cell extracts containing the soluble metabolic enzymes are electrophoresed in non-denaturing starch gels. For each enzyme analysed, the gel is stained with a specific colorimetric substrate, such that the position of the enzyme is detected by the appearance of a visible reaction product. Variations in the electrophoretic mobility of the enzyme, referred to as electromorphs, typically reflect amino acid substitutions that alter the charge of the protein and thereby identify allelic variations in the chromosomal genes encoding the enzyme. Combinations of the electromorphs are designated electrophoretic types (ETs) and each distinct ET is considered to represent a multilocus genotype. Although individual enzymes may be absent (null) in particular isolates, evaluation of multiple metabolic enzymes ensures that all isolates are typable. MLEE has been used effectively to analyse the population genetics of bacterial species and in outbreak situations (Struelens, 1996; Opal *et al.,* 1990).

2.3 Genotypic Techniques for Typing MRSA

The preparation of restriction digests of DNA and electrophoresis are the essential elements in all genotypic techniques (Arbeit, 2000; Williams *et al.*, 1999).

A restriction endo-nuclease enzyme cuts ('digest') double-stranded DNA at a specific ('restricted') nucleotide recognition sequence. The number and size of the restriction fragments generated by digesting a given piece of DNA are influenced by the recognition sequence of the enzyme and the composition of the DNA. The enzyme used must therefore, be carefully selected, the conditions of the reaction must be defined, the DNA to be cleaved must be as pure as possible and the electrophoretic discrimination and the end-point detection carefully standardised.

In conventional restriction enzyme analysis (REA), bacterial DNA is digested with endonucleases that have relatively frequent restriction sites, generating hundreds of fragments ranging from appropriately 0.5 to 50kb in length. Variations in their DNA sequences alter the number and distribution of restriction sites. All isolates are typable by REA, however, the profiles consist of hundreds of bands that may be unresolved and overlapping, and therefore difficult to compare (Weber and Pfaller, 1997; Arbeit, 2000). The pattern may be confused by restriction fragments derived from plasmids, which can readily contaminate genomic DNA preparations. Thus, isolates that differ only in their plasmid content may be designated as different strains.

The fragments generated by restriction digestion can be separated by size using various types of gel electrophoresis. When comparable DNA from different sources (e.g. different strains)

are digested with the same enzyme, variations in the nucleotide sequences will generate variations in the size and, consequently, electrophoretic mobility, of the fragments.

These differences can be detected directly by staining the electrophoretic gel with ethidium bromide and viewing under ultraviolet (UV) light or indirectly by Southern blotting analysis.

In Southern blotting, the restricted fragments are separated by agarose gel electrophoresis and transferred ('blotted') onto a nitrocellulose or nylon membrane; the fragment(s) containing the specific sequence (loci) are then detected using a labelled piece of homologous DNA as a probe. Under the appropriate conditions, the probe 'binds' (hybridises) by complementary base pair matching only to those fragments containing identical or nearly identical nucleotide sequences. Variations in the number and size of the fragments detected are referred to as Restriction Fragment Length Polymorphisms (RFLPs). These reflect variations in both the number of loci that are homologous to the probe and the location of the restriction sites within or flanking those loci.

2.3.1 Plasmid Analysis

Bacterial plasmids are autonomously replicating extra-chromosomal genetic elements that are normally quite distinct from the chromosomal genotype that defines the host strain[1813;21]. Plasmids can be acquired by a variety of mechanisms and although often inherited as the host strain replicates, they can be lost spontaneously, as they are not generally essential for the survival of the host cell. Plasmids often carry antibiotic resistance determinants contained within mobile genetic elements (transposons). Such plasmids may spread rapidly among different strains and may persist for long periods. Transposons can be readily acquired or deleted, thereby altering the DNA composition of the plasmid. Thus, both plasmid number and structure can vary among epidemiologically related isolates.

Plasmid profile analysis is technically simple and represents the first DNA-based typing method applied to *S. aureus* (Weller, 2000). The number and size of the plasmids carried by an isolate are determined by preparing a plasmid extract and subjecting it to routine gel electrophoresis. The technical reproducibility of plasmid profile is hampered by the fact that plasmids can exist in different forms: supercoiled (closed circle), nicked (open circle), linear and oligomeric, each of which migrates differently during agarose gel electrophoresis. Thus, in different preparations, there can be variation in the number and relative intensity of bands representing each individual plasmid. Only the overall size of the plasmids is being assessed, therefore biologically distinct plasmids of the same size cannot be differentiated.

Plasmid analysis can be improved by digestion with restriction enzymes and analysing the number and size of the resulting restriction fragments. Epidemiologically related isolates of the same chromosomal genotype may have substantially different plasmid content, including the gain and loss of entire plasmids, or substantial rearrangement within the plasmid. Different host strains may have the same plasmid content.

2.3.2 PCR Typing Systems

The essential feature of PCR is the ability to replicate ('amplify') rapidly and exponentially a particular DNA sequence (the 'template'). PCR can be readily performed using commercially available reagents and thermocyclers.

Epidemiological studies and molecular typing of MRSA exploit the presence of a hypervariable segment of DNA between *mec*A and IS431*mec*. Strain typing requires additional information beyond the presence or absence of the target sequences (Williams *et al.*, 1999; Weller, 2000; Weber and Pfaller, 1997).

Repetitive Chromosomal Sequence (rep-PCR) based PCR uses primers which target short extragenic repetitive sequences. These sequences are typically present at many sites around the bacterial chromosome. When two sequences are located near enough to each other, the DNA fragment between those sites (referred to as inter-repeat fragment) is effectively amplified. Since the number and location of the repetitive sequences are quite variable, the number and size of the inter-repeat fragments generated can similarly vary from strain to strain. The target repetitive sequences may be transposons, insertion sequences, or areas within gene sequences.

The primers used by Cuny and Witte (Cuny and Witte, 1996) detect length polymorphisms of DNA sequences flanked by a multilocus transposon target site (tar 916) and the ribosomal binding site (Shine-Dalgarno sequence (Shida)). Similarly, del Vicchio *et al.* (del Vicchio *et al.*, 1995) selected IS256 elements as the target element. IS256 elements are present in *S. aureus* either independently or as part of the composite transposon Tn4001. It is thought that IS256 insertion sequences are strain specific.

The intergenic spacer region between the 16S and 23S gene in the rRNA operon is characterised by extensive sequence and length variations. PCR amplification of the intergenic spacer region, as in ribosomal spacer PCR (RS-PCR), using primers complimentary to the conserved regions of the 16S and 23S genes, produces multiple fragments for each isolate, with appreciable diversity in the pattern of fragments.

Primers derived from repetitive sequences from other organisms such as *Mycoplasma peumoniae* have also been used (del Vicchio *et al.*, 1995). It is assumed that because *M. pneumoniae* evolved from Gram-positive bacteria, that repetitive sequences found in *M. pneumoniae* could be used as primers for rep-PCR. Rep-MP3 sequences vary in number and position on the MRSA chromosome.

The variation known as **arbitrarily primed PCR** (AP-PCR), also referred to as randomly amplified polymorphic DNA (RAPD), is based on the observation that short primers (typically 10 bp) whose sequence is not directed to any known genetic locus will hybridise with sufficient affinity at random chromosomal sites to permit initiation of polymerisation. If two such sites are located within a few kilo-bases of each other, on opposite DNA strands and in the proper orientation, amplification of the intervening fragment will occur. The number and location of these sites will vary among different strains; thus, the number and size of the fragments detected by electrophoresis of the amplicon will also vary.

2.3.3 Restriction Endonuclease Analysis of Chromosomal DNA

2.3.3.1 Pulsed-Field Gel Electrophoresis of Chromosomal DNA

Pulsed field gel electrophoresis (PFGE) was developed by Schwartz and Cantor in 1984. It is a variation of agarose gel electrophoresis, in which the orientation of the electric field across the gel is changed periodically ('pulsed'), rather than being kept constant, as in the case of conventional agarose gel electrophoresis used in REA and Southern blot studies (Weber and Pfaller, 1997; Arbeit, 2000). Alternating the electric field to occur at just two bearings is known as field inversion gel electrophoresis (FIGE). The most commonly used variation; contour clamped homogeneous electrophoresis (CHEF) uses an electrophoresis chamber consisting of six electrodes arranged in a hexagonal pattern. The current is applied in each of three directions, 120° apart, in turn, for short intervals (Weller, 2000). This modification enables DNA fragments as large as megabases to be separated effectively by size.

Suitable un-sheared DNA is obtained by embedding intact organisms in agarose plugs ('inserts'), followed by enzymatic lysis of the cell wall and digestion of cellular proteins. The

isolated genomes are then digested, *in situ*, with restriction enzymes that have few recognition sites. Rare cutters have longer recognition sequences that occur less often within the chromosome. Thus, instead of hundreds of fragments ranging from 0.5 to 50kb in length, a simpler pattern consisting of between 10 to 30 fragments, 10-800kb in length are produced. Various restriction enzymes have been used for *S. aureus*, but none have been found to be better than *sma*I.

PFGE has two limitations. Firstly, because of the need to diffuse all buffers and enzymes into the agarose inserts, the preparation of suitable DNA involves several extended incubations and takes 2 to 4 days. Secondly, PFGE requires relatively expensive specialized equipment.

2.3.3.2 Southern Blotting

This method uses probes (genes, IS elements, rRNA), in combination with REA. The probes hybridise to endonuclease restriction digests of genomic DNA (Weller, 2000; Arbeit, 2000) separated by conventional electrophoresis and transferred to a nitrocellulose membrane (Southern blotting).

2.3.3.3.1 Ribotyping

This method characterises bacterial strains based on RFLPs associated with the ribosomal operons. Operons are clusters of genes that share related functions, transcribed together on a single piece of messenger RNA (mRNA). The ribosomal operon is compromised of nucleotide sequences coding for 16S ribosomal RNA (rRNA), 23S rRNA, as well as one or more transfer RNA (tRNA).

Probes prepared from isolated *Eschericia coli* rRNA or cloned ribosomal operon (*rrn*), hybridise to the chromosomal ribosomal operons of staphylococci, as well as many other bacterial species.

Ribosomal sequences are highly conserved (Weller, 2000; Weber and Pfaller, 1997; Arbeit, 2000), however their number and position vary. All staphylococci carry multiple (five to seven) ribosomal operons and are therefore typable.

Chromosomal DNA is digested using one or more, frequently cutting restriction endonuclease, followed by agarose gel electrophoresis. Ribotyping of MRSA has been carried out extensively with different restriction enzymes (*Eco*RI, *Cla*I and *Hind*III) and probes from different species (*E. coli*, *Bacillus subtilis,* and *S. aureus).*

This generates hundreds of small DNA fragments of various sizes. These are transferred onto a nitrocellulose or nylon membrane (Southern blotting). The DNA on the membrane is then hybridised with a RNA probe, labelled either chemically or radioactively, which binds to complementary nucleic acid fragments on the membrane.

Ribotypes are biologically very stable and reproducible and isolates from an outbreak typically have the same ribotype. However, epidemiologically unrelated isolates often demonstrate the same pattern; many patterns differ only by one or two fragments.

Southern blot analysis is technically demanding and time consuming, however recent developments, technical refinements and commercially available materials, have simplified the technique, although the procedure still requires expertise and specialized equipment. Automated ribotyping systems are available (The RiboPrinter System by Qualicon), however, the equipment is expensive and also requires a sophisticated computerised imaging system to analyse the results.

2.3.3.3.2 Insertion sequence (IS) and transposons

These mobile genetic elements are used as probes for typing staphylococci, as they are present in multiple copies and positioned at different chromosomal loci. The number of these elements, chromosomal location, and consequently, the RFLP(s) detected, can vary appreciably among different bacterial strains (Weller, 2000; Weber and Pfaller, 1997). One potential complication of a typing system based on mobile elements is that they may be present on plasmids as well as on the bacterial chromosome. No single element has been established as sufficiently discriminatory for wide use in the typing *S. aureus*. Tn554, IS256, and IS257/431 are prevalent among MRSA isolates.

2.3.3.3.3 Binary Typing

This is a novel technique based on reverse hybridisation of Digoxigenin Universal Linkage System (DIG-ULS) labelled whole genomic bacterial DNA extracts onto strip-immobilised probe DNA (van Leeuwen *et al.*, 1999). After hybridisation, detection is carried out by chemillumiescence or chromogenic methods. The degree of hybridisation of the labelled DNA to 8-15 different target areas in the *S. aureus* genome is scored with a 1 or a 0 according to the presence or absence of a hybridisation signal. The resulting binary code is translated into a decimal number.

The DNA probes were generated as a result of previous work involving RAPD analysis of a number of staphylococcal strains (van Leeuwen *et al* 1996).

Binary typing claims to offer a simple and fast probe-based molecular typing strategy generating easily interpretable results (Weller, 2000; Shopsin, 2001). Binary typing however

is in its early stages of development with limited work having been carried out to establish its usefulness in clinical practice.

2.3.4 Multi Locus Sequence Typing

Multilocus sequence typing has been developed for a limited number of organisms, including *Neisseria gonorrhoeae, Streptococcus pneumoniae* and *S. aureus* (Enright *et al.*, 1999, Maiden *et al.*, 1998). MLST offers the ultimate in molecular typing in that the results obtained are at the nucleotide level, are unambiguous and highly discriminatory. There is the added advantage that results are portable between laboratories.

The majority of molecular typing methods are based on variations in one target region of the bacterial genome. MLST compares sequence variation in 5-7 housekeeping gene targets. MLST is based on the same principles as MLEE but used to study genetic variability rather than enzymes. MLST differs in that it assigns alleles at multiple housekeeping loci directly by DNA sequencing, rather than indirectly via the electrophoretic mobility of their gene products.

Allelic profiles of *S. aureus* are obtained by sequencing internal fragments, approximately 450-500bp, of seven housekeeping genes, using an automated DNA sequencer.

For each housekeeping gene the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST). The number of nucleotide differences between alleles are ignored and sequences are given different allele numbers weather they differ at a single site or at many sites.

Sequence analysis of these genes provides a database from which relationships can be inferred (Shopsin, 2001; Enright *et al.*, 2000; van Belkum *et al.*, 2000; Plantonov *et al.*, 2000).

MLST typing however, requires expensive equipment, is labour intensive, and is too time consuming to be used in a clinical setting, requiring more than 2,500bp to be compared for each isolate. Thus, it is a technique restricted to use in reference laboratories.

4.0 AIMS AND OBJECTIVES

4.1 Aims

To systematically evaluate a selection of methods for molecular typing of MRSA, for use in the routine microbiology laboratory.

4.2 Objectives

To evaluate each of the following molecular typing systems for typability, reproducibility, discriminatory ability, stability, applicability, cost effectiveness and turn around time.

1 Pulsed-field gel electrophoresis. The ultimate 'gold standard' would be full nucleotide sequencing of the whole bacterial genome, however this is impractical due to cost and time constraints in the routine microbiology laboratory. PFGE will be used as the 'gold standard' against which all other typing methods will be assessed as it involves analysis of the whole genome and has been shown to be the most reproducible and discriminatory method.

2 Repetitive element sequence based PCR

Amplification using repMP3 primer derived from *Mycoplasama pneumoniae* (del Vicchio *et al.*, 1997),

Shine-Dalgarno-transposon 916 spacer amplification (Cuny and Witte, 1996),

Inter-IS256 fragment amplification (Deplano *et al.*, 1995), and

Amplification of the 16S-23S rRNA intergenic spacer region (Kumari *et al.*, 1997).

3 Southern Hybridisation:

rRNA transcriptional sequences (ribotyping) (Blumberg, 1992)

Binary typing (van Leeuwen, 1999)

4 Multi Locus Sequence Typing.

4.0 MATERIALS AND METHODS

4.1 Materials

4.1.1 Bacterial Strains

A total of 152 MRSA isolates were tested. These comprised of two sets:

1 Out-break/connected isolates, and

2 A diverse, non-epidemiologically connected set.

The connected set comprised of 36 isolates from 6 outbreaks from hospitals in Oxford, Aberdeen and Birmingham. The remaining 116 were sporadic isolates from 29 hospitals in the UK and epidemic MRSA stains (EMRSA 1-16). See appendix A.

All isolates were recovered onto Columbia blood agar plates (Biomerieux, Basingstoke, UK), either from frozen beads stored at –70°C, or from agar slopes. These were then stored as gelatine discs at 4°C until required for molecular typing.

4.1.2 Organism Storage

All organisms were stored as gelatine discs. The gelatine storage mixture was prepared by mixing 12.5g nutrient gelatin (Sigma, Dorset, UK), 0.3g charcoal (Sigma), 1.5g purified casein powder (Sigma) and 5g inositol (Sigma), in 100mLs distilled, deionised water. This was dissolved on a hotplate stirrer and dispensed into 3mL bijoux bottles before autoclaving at 121°C for 15mins.

At least one loopful of the culture organism, from a purity plate was emulsified in a warmed bijoux of gelatin solution. This was mixed well and, using a sterile pastette dispensed as single drops over the surface of a sterile petri dish. The petri dish was then freeze-dried

(Modulyo, Edwards) at –20°C for 18hrs. The freeze-dried discs were then transferred into sterile bottles containing silica gel (Sigma), and stored at 4°C.

One gelatin disc was cultured on Columbia blood agar to check purity and viability of the stored organism.

4.2 Methods

4.2.1 Antimicrobial Sensitivity Testing

Antimicrobial sensitivity testing was carried out using a modified BSAC method. A 3ml volume of nutrient broth was inoculated with one colony of the test isolate. A sterile swab was dipped into the suspension and evenly applied onto the surface of an Iso-sens agar plate (Biomerieux), ensuring semi-confluent growth. A multi-antibiotic disc was then placed onto the surface and pressed gently to ensure contact with the agar surface. The plates were then incubated at 37°C for 18-24hrs. The results were determined by comparing the zone sizes as indicated on a template. The antibiotics to which the isolate were resistant was recorded.

Table 4.2.1.1 Antibiotics tested:

Antibiotic	**Concentration** (μg/mL)	**Cut off Zone Size (mm)**
Penicillin G (P)	1	32
Tetracyline (T)	10	23
Gentamicin (G)	10	26
Erythromycin (E)	5	31
Fusidic acid (F)	10	35
Clindamycin (L)	2	34
Kanamycin (K)	30	29
Methicillin (M)	1	32
Mupirocin (Mu)	5	30

4.2.2 DNA Extraction Methods

4.2.2.1 PCR

A 5mL volume of Brain Heart Infusion Broth (Oxoid, UK) was inoculated with one colony of test organism (from previously inoculated Columbia blood agar plates) and incubated overnight at 37°C. A 1ml volume of the bacterial suspension was centrifuged at high speed for 3mins, washed and re-suspended in 400µL of lysis solution (50mM Tris-HCl pH8 (Gibco, Paisley, UK), 5mM EDTA (Sigma) and 50mM NaCl (Merck, Dorset, UK) supplemented with 20µg/mL of lysostaphin (Sigma). Post incubation at 37°C for 2hrs, 80µL of proteinase K solution (0.5% sodium dodecyl sulphate (Sigma), 50mM Tris-HCl pH8, 0.4M EDTA, 1mg/mL Proteinase K (Sigma)) was added, and incubated at 50°C for a further 2hrs.

An equal volume of phenol: chloroform (Merck) was added. Post centrifugation at high speed for 30mins, the upper layer was carefully transferred to a fresh micro-centrifuge tube. 2.5 X volume ice-cold 100% ethanol (Merck) was added. This was gently mixed and centrifuged at high speed for 5mins. The ethanol was removed and discarded, and the pellet allowed to air dry. The DNA was re-suspended in 20 µL of TE buffer (10mM Tris-HCl pH8 and 1mM EDTA pH8) and stored at –20°C.

4.2.2.2 DNA Extraction using cetrimide

A 5mL volume of an overnight nutrient broth culture of the test organism was centrifuged and re-suspend in 223µL TE buffer supplemented with 20µL/mL lysostaphin and 1mg/mL lysozyme (Merck). After incubation at 37°C for 2 hr 10% sodium dodecyl sulphate (SDS) (Sigma) and 10mg/mL of proteinase K was added and incubated for a further 20mins at 37°C,

81µL of pre-warmed CTAB solution (10% cetrimide (Merck) in 0.7% NaCl) and 2M NaCl was added. Following incubation at 65°C for 30mins an equal volume of 24:1 chloroform: isoamyl alcohol (Merck) was added and centrifuged at 13,000rpm for 15mins. The upper layer was transferred to a fresh micro-centrifuge tube and 500µL of 25:24:1 phenol: chloroform: isoamyl alcohol (Merck) was added and centrifuged at 13,000rpm for 15 mins. The upper layer was carefully transferred into a fresh micro-centrifuge tube and $1/10^{th}$ volume of 3M sodium acetate buffer solution (pH 5.2) (Sigma) was added. 2.5X volume of ice cold 100% ethanol was then added and mixed by gentle inversion and placed in -20°C freezer for at least 1hr. The DNA was pelleted by centrifugation at 13,000rpm for 30mins, washed once with 70% ethanol and allowed to air dry. The DNA was re-hydrated in 20µL sterile distilled water and stored at -20°C.

4.2.2.3 DNA extraction for Ribotyping

The above method was modified to include 0.1mg/mL of RNase A (Sigma) before the addition of proteinase K.

4.2.2.4 DNA Quantification

A volume of 5µl of the extracted DNA was diluted into 995µl of sterile distilled water and the optical density measured at A_{260} and A_{280}.

Assuming the ratio of the two values to be 1.8 for pure DNA and an O.D. of 1 is equivalent to 50μg/ml double stranded DNA the concentration of the extracted DNA was estimated using the following equation:

$$\text{Concentration} = (A_{260} \times 200) \times 50 \ (\mu g/ml)$$

4.2.3 PCR Methods

4.2.3.1 Detection of PCR Products

A 1kb marker (Gibco) was used for estimation of fragment size, for all PCR gels, unless otherwise stated. All gels were stained with 0.5µg/mL ethidium bromide (Sigma) for 30mins and viewed with a UV transilluminator.

4.2.3.2 *mec*A Gene Amplification

The PCR mixture of 20µL, consisted of approximately 200ng of previously extracted template DNA, 200pmol of primers *mec*A1 and *mec*A2 primers (Nimmo *et al.*, 2001)(Alta Biosciences, Birmingham, UK), 5mM dNTP mix, 2mM $MgCl_2$ (Bioline, London, UK), and 0.5U of *Taq* polymerase (Bioline, London, UK). The amplification cycle consisted of 1 cycle at 95°C for 5mins; 95°C for 30secs, 55°C for 30secs and 72°C for 2min, for 30 cycles. The PCR products were detected by electrophoresis through a 1% gel in 0.5X TBE buffer at 150V for 1hrs.

4.2.3.3 Shine-Delgarno/ Transposon 916 Amplification

The PCR mixture of 20µL, consisted of approximately 200ng of template DNA, 100pmol of each primer; (primers Tn916 and Shida, see Table 4.2.3.6), 5mM dNTP mix, 2mM $MgCl_2$, and 0.5U of *Taq* polymerase. The amplification cycle consisted of 94°C for 2mins, 30°C for 1min, and 72°C for 1min; for 1 cylce, 94°C for 30secs, 30°C for 30secs and 72°C for 30secs, for 29 cycles; and 94°C for 30secs, 30°C for 30secs and 72°C for 4mins, for the final cycle. The PCR products were detected by electrophoresis through a 1.5% gel in 0.5X TBE buffer at 150V for 2hrs.

4.2.3.4 Inter-IS256 Amplification

The PCR mixture of 20μL, consisted of approximately 200ng of template DNA, 100pmol of each primers; (P1 and P2, see Table 4.2.3.6), 5mM dNTP mix, 3.5mM $MgCl_2$, and 0.5U of *Taq* polymerase. The amplification cycle consisted of 94°C for 2mins; 94°C for 30secs, 45°C for 1min and 72°C for 1min, for 40 cycles. The PCR products were detected by electrophoresis through a 1.5% gel in 0.5X TBE buffer at 140V for 2hrs.

4.2.3.5 RepMP3 sequence Amplification

The PCR mixture of 20μL consisted of approximately 200ng of template DNA, 50pmol of primer, (primer RW3A, see Table 4.2.3.6). 5mM dNTP mix, 5mM $MgCl_2$, and 0.5U of *Taq* polymerase. The amplification cycle consisted of 94°C for 2mins; 94°C for 30secs, 54°C for 1min and 72°C for 1min, for 40 cycles. The PCR products were detected by electrophoresis through a 2% gel in 0.5X TBE buffer at 140V for 2hrs.

4.2.3.6 RS-PCR (Ribosomal Spacer PCR)

PCR amplification was carried out in 25uL of reaction mixture containing 200ng of template DNA, 1mM dNTP mix, 3mM $MgCl_2$, 50pmol of each primer (RS-PCR-N1 and RS-PCR-G1, see Table 4.2.3.6), and 0.5units of *Taq* polymerase. Amplification conditions consisted of 94°C for 1min, 55°C for 1min and 72°C for 1min for 34 cycles; and 72°C for 1min.

PCR products were diluted 1:2 prior to seperation by electrophoresis through 2% agarose gel in 0.5X TBE buffer at 50V for 16hrs. A 100bp ladder (Roche Diagnostics, East Sussex, UK) was used for estimation of fragment size.

Table 4.2.3.6 Primers used for repetitive sequence PCR

Target	Primer Name	Primer Sequence	Nucleotide Position
*mec*A	*mec*A1	GTGGAATTGGCCAATACAGG	478-497
	*mec*A2	TGAGTTCTGCAGTACCGGAT	1816-1797
Transposon 916	Tn916	AGAGAGCTATTTTA	
Shine-Dalgarno	Shida	AAAGGAGGAATTA	
Transposon gene	P1	GGACTGTTATATGGCCTTTT	50-30 in IS256 L element
Insertion Sequence 256 L	P2	GAGCCGTTCTTATGGACCT	1204-1222 in IS256 L element
RepMP3	RW3A	TCGCTCAAAACAACGACACC	
16s rRNA- 23s	RS-PCR-N1	CAAGGCATCCACCGT	
rRNA spacer region	RS-PCR-G1	GAAGTCGTAACACGG	

4.2.4 Pulsed Field Gel Electrophoresis

4.2.4.1 Agarose Plug Preparation

The test organism was recovered from gelatine disc by inoculating onto a Columbia blood agar plate and incubated at 37°C for 18-24hrs.

The test organism was inoculated into 5mL of Brain Heart Infusion Broth and incubated overnight at 37°C. A 1mL volume of the overnight suspension of test organism was centrifuged at high speed (13,000rpm) for 3mins. The supernatant was removed and the cells re-suspended in 0.5ml NET-100 (0.1M NaCl, 0.1M EDTA, 100mM Tris-HCl pH8). The suspension was centrifuged, the supernatant removed and re-suspended in 0.5ml -100. The cells were then placed in a 50°C water bath.

A volume of 0.5ml of the warmed cell suspension was mixed with 0.5mL of 1% molten Chromosomal Grade Agarose (BioRad, Hampstead, UK) and dispensed into Perspex moulds. When set, the plugs were removed into 3mL lysis solution (6mM Tris HCl pH7.6, 1M NaCl, 100mM EDTA pH8) and incubated at 37°C for 24hrs. The lysis solution was then replaced by 3mL ESP (0.5M EDTA pH9 containing 1% sarcosyl (Merck) and 1.5mg/mL proteinase K solution and further incubated at 50°C for 48hrs. The plugs were then stored at 4°C.

4.2.4.2 Pre-digestion Treatment

The ESP solution was removed and replaced by 3mL TE buffer (10mM Tris-HCl pH8.0, 1mM EDT pH8.0) containing 30µL of 0.1M PMSF (phenylmethysulfonyl floride, Sigma) and placed on a slow roller for 2hrs. The TE buffer/PMSF was discarded and replaced with fresh

TE buffer and PMSF and the washing step repeated. This was followed by three further washes for 1hr each, with TE buffer only. The plugs were stored at 4°C.

4.2.4.3 Digestion of Plugs

A 2mm sliver of plugs was cut using a coverslip and transferred to a labelled micro-centrifuge tube. Restriction enzyme buffer (Tango Y, Helena Biosciences Ltd, UK) was added at the concentration recommended by the manufacturer and incubated on ice for 15mins. This was removed and replaced with fresh buffer containing 20U of *Sma*I enzyme (Helena Biosciences). After incubation at 30°C overnight, the enzyme solution was replaced with 200µL ES (0.5M EDTA pH9 containing 1% sarcosyl) and incubated at 50°C for 15mins. The ES was replaced with 1mL TE buffer and left at room temperature for 15mins prior to loading onto a 1% Molecular Biology Grade Agarose gel (BioRad). After loading all the samples onto the gel, the wells were filled with cooled molten agarose and allowed to solidify.

4.2.4.4 Electrophoresis was carried out on a CHEF-DRII system (Bio-Rad) at 6V, switch times of 5-40s for 22hrs. The gel was then removed and stained in 0.5µL/mL ethidium bromide for 30mins and viewed with a UV transilluminator.

In addition to the test isolates, plugs were also prepared for the strain *S. aureus* NCTC 8325 and digested with *Sma*I as described. This was used as the reference strain on the two outside and the middle lanes of each gel.

4.2.5 Ribotyping Method

4.2.5.1 Chromosomal DNA Extraction

DNA was extracted using the cetrimide method (see section 4.2.2.2)

4.2.5.2 Digestion of Chromosomal DNA

Chromosomal DNA was digested using the restriction endonuclease, *Eco*RI (Bioline) at a ratio of 10U/μg of DNA where the concentration of DNA was 1-5μg in a reaction volume of 10μL. Incubation was carried out at 37°C for time intervals varying from 2hrs to overnight. The reaction was stopped by the addition of EDTA to a concentration of 50mM.

4.2.5.3 Gel electrophoresis

Confirmation of complete digestion was carried by electrophoresis on a 0.8% agarose gel at 35V for 16hrs. The gel was stained with 0.5mg/mL ethidium bromide for 30mins and visualised using an ultraviolet light transilluminator.

4.2.5.4 Southern Blotting by Capillary Transfer

The agarose gel was soaked for 10mins in depurination solution (0.25M HCl) with gentle shaking. This was then discarded, washed with distilled water, and replaced with denaturation solution (1.5M NaCl; 0.5M NaOH) and soaked for 20mins with gentle shaking. The

denaturation solution was removed, the gel washed with distilled water and then soaked in neutralization solution (1.5M NaCl; 0.5M Tris-HCl pH7.5) for 20mins.

A support larger than the gel was placed in a tray and covered with a glass plate. Two lengths of filter paper long enough to fit under the gel and reach the bottom of the tray on either side were placed in the tray containing a reservoir of 20 X SSC (0.3M sodium citrate; 3M NaCl). Approximately 8-10 filter papers, larger than the gel soaked in 20X SSC were then placed on glass plate. The gel is then placed on top of these filter papers, with the wells of the gel facing upper most. A piece of nylon membrane (Hybond-N, Amersham) the same size as the gel, is placed on top of the gel. Four pieces of filter paper, the same size as the gel, pre-soaked in 20 X SSC are then placed on top of the nylon membrane followed by 4 dry filter papers. A 2.5cm layer of paper towels was then placed on top, weighed down with a bottle, and left to blot overnight.

4.2.5.5 UV cross-linking

The DNA on the nylon membranes was then immobilized with ultraviolet irradiation (254nm) using an UV Stratalinker 1800 ultraviolet cross-linker (Stratagene Europe, Amsterdam, The Netherlands) set at 1200μJ for 45 seconds.

4.2.5.6 PCR Amplification

The 16s rRNA and *mec*A genes were amplified and used as labelling and hybridisation controls.

Amplification was carried out in a 20μL reaction mixture consisted of approximately 200ng of previously extracted template DNA, 5mM dNTP mix, 2mM $MgCl_2$, and 0.5U of *Taq* polymerase (Bioline, London, UK). A concentration of 200pmol of each primer (*mec*A1 and *mec*A2 primers (see Table 4.2.3.6) for *mec*A amplification and the primers RW01 (5'-AAC TGG AGG AAG GTG GGG AT-3') and DG74 (5'-AGG AGG TGA TCC AAC CGC A-3') (Alta Biosciences, Birmingham, UK) for 16s gene amplification were used (Greisen *et al.*, 1994). The amplification cycle for 16S consisted of 95°C for 5mins; 95°C for 30secs, 55°C for 30secs and 72°C for 2min, for 30 cycles.

4.2.5.7 Hybridisation

The nucleic acid probe (RNA, 16s PCR product or *mec*A PCR product) was labelled using the AlkPhos Direct Labelling kit (Amersham, Lifesciences, UK). By following the manufacturer's instructions, a thermostable alkaline phosphatase was cross-linked to the nucleic acid.

Pre-hybridisation was carried out in a Hybaid MKII mini-oven. The nylon membrane was placed inside a hybridisation bottle with 0.125 mL/cm^2 of hybridisation buffer pre-heated. The membrane was allowed to pre-hybridise for 30mins. After pre-hybridisation, the required labelled probe was added to the hybridisation buffer to a concentration of 2.5 ng/mL. The membrane was then allowed to hybridise overnight.

4.2.5.7 Washes and Detection

Following hybridisation, excess probe was removed by washing twice in primary wash buffer (2M urea; 0.1% SDS; 50mM sodium dihydrogen phosphate (Merck); 150mM sodium

chloride; 1mM magnesium chloride (Merck); 0.2% blocking reagent (included in kit)) at 65°C for 10 minutes followed by two 5 minute washes in secondary buffer (50mM Tris; 100mM sodium chloride; 0.2mM magnesium chloride adjusted to pH 10) at room temperature.

Detection for hybridisation of the probe was achieved using a chemiluminescent detection reagent that emits light when digested by the probe bound alkaline phosphatase. The membrane was soaked for four minutes in the detection reagent, (a solution of disodium2-chloro-5-(4-methoxyspiro[1.2-dioxetane-3,2'-(5'-chloro)-tricyclo(3,3,1,1]decan]-4yl)phenyl phosphate) as supplied in the AlkPhos Direct kit and sealed in saran wrap. The membrane was put in a cassette and a sheet of Hyperfilm™ECL™(Amersham Pharmacia Biotech Ltd) placed on top. The film was developed after 1hr by immersion for four minutes in developing solution (Kodak, Sigma), rinsing briefly in distilled water and then immersion in a fixative solution (Kodak, Sigma,), for a further four minutes. The film was then rinsed thoroughly in distilled water and allowed to air dry.

4.2.6 Binary Typing

4.2.6.1 DNA Extraction

The test DNA was initially isolated using the extraction method as described in section 4.2.2.2. This was later replaced with the following method using the QIAamp DNA mini kit (QIAGEN, West Sussex, UK). The test strain were inoculated onto Columbia blood agar plates and incubated overnight at 37°C. A loopful of the overnight growth was then suspended in 125μL of TEG (50mM glucose (Merck), 10mM EDTA, and 25mM Tris adjusted to pH8.0) buffer and supplemented with 100mg/L lysostaphin and incubated at 37°C for 1hr. A volume of 20μL of proteinase K solution and 200μL of AL buffer, as supplied by the kit manufacturer, were added, vortex mixed and incubated for 30mins at 56°C followed by 15mins at 95°C. The protocol set by the manufacturer was then followed to elute the DNA. The DNA was quantified by U.V. spectroscopy and stored at –20°C.

4.2.6.2 Probe Preparation

Plasmid DNA was provided by Dr. W. van Leeuwen, at a concentration of 10ng/μL.

4.2.6.2.1 PCR amplification of probes

Each of the probes were PCR amplified from the plasmid DNA. This was carried out in a 100uL reaction mixture containing 20ng of template plasmid DNA, 1mM dNTP mix, 2mM $MgCl_2$, 50pmol of each primer (T7 primer:5'-TAA TAC GAC TCA CTA TAG GG-3' and M13 reverse primer: 5'-AAC AGC TAT GAC CAT G-3') (Alta Biosciences), and 0.5units of *Taq* polymerase. Amplification was carried out by 4 mins pre-denaturation at 94°C, followed

by 25 cycles of 1 min at 94°C, 1min at 25°C and 1 min at 72°C and a post extension of 15mins at 72°C.

Nuclease gene amplification was carried out in 50uL reaction mixture containing 200ng of template DNA, 1mM dNTP mix, 2mM $MgCl_2$, 100pmol of each primer (Alto Biosciences) and 0.5units of *Taq* polymerase. The primers *nuc*1 (5'-GCG ATT GAT GGT GAT ACG GTT-3') and *nuc*2 (5'-AGC CAA GCC TTG ACG AAC TAA AGC-3') were used (based on a method used by Ian Kay, Royal Perth Hospital, Australia). The amplification program consisted of 1 cycle of 5mins at 95°C followed by 30 cycles of 55°C for 90secs, 72°C for 90secs and 94°C for 1min, and a final stage of 72°C for 10mins.

All PCR products were purified with QIAquick PCR purification kit (QIAGEN), according to manufacturer's protocol and eluted with 40uL of EB buffer (included in kit). The size of the PCR products were checked on a 1% agarose gel in 0.5x TBE at 150V for 1.5hrs and the concentration was estimated by comparison with GeneRuler™ DNA ladder mix (MBI Fermentas, Helena Biosciences, UK).

In addition to the 12 probes and nuclease gene (positive control), fish sperm DNA (Roche Diagnostics, Germany) (approx fragment size 200bp) was used as a negative control.

Table 4.2.6.2.1.1 PCR product length

DNA Probes	Length of PCR product M13/T7 (bp)
AW1	1031
AW2	655
AW3	756
AW4	971
AW5	1500
AW6	567
AW7	1182
AW8	501
AW9	1700
AW11	1181
AW14	881
AW15	595
Nuclease gene	267

4.2.6.2.2 Application of Probe to Membrane

The nylon membrane was divided into a strip of 14 squares 0.5cm^2.The DNA probes were diluted to a concentration of 5ng/μL. A volume of 1μL of each of the DNA probes was then applied in to each corresponding square.

4.2.6.2.2.1 Diagram to represent order of application of probe onto membrane

	AW1	AW2	AW3	AW4	AW5	AW6	AW7	AW8	AW9	AW11	AW14	AW15	PC	NC
Date	•	•	•	•	•	•	•	•	•	•	•	•	•	•

The DNA probes were immobilized onto the membrane by baking at 80°C for 30mins and then stored at 4°C until required.

4.2.6.3 Quality Control of Binary Filterstrips

The following procedure was carried out to establish the presence of all the probes on the membrane. A 1μL volume of each of the probes was mixed together. The total volume was then made up to 18.6μL with sterile distilled water, and 1.4μL of DIG-ULS label was added. This mixture was incubated at 85°C for 30mins, cooled on ice and centrifuged briefly to collect the condensation. A 5mL volume of stop solution was added and incubated for 10mins at room temperature. The labelled DNA sample was then diluted 1:100 in sterile distilled water. A 20μL sample was denatured for 5mins at 96°C followed by incubation on ice for 5mins, and centrifuged briefly to collect the condensation. A 6.25μL volume of the denatured DNA mix was then hybridised in 2.5mL DIG Easy Hybridisation buffer for 18hr at 42°C.
See hybridisation method section 4.2.6.5.

4.2.6.4 Labelling of Test DNA

A 1μg volume of the test DNA was labelled with 1U DIG-ULS and the volume adjusted to 20μL in sterile distilled water. The mixture was incubated for 30mins at 85°C cooled down on ice and centrifuged briefly to collect the condensation. A 5μL volume of stop solution was added and incubated for 10mins at room temperature. The labelled DNA was then either denatured at 96°C for 5mins prior to hybridisation or stored at –20°C until required.

4.2.6.5 Hybridisation

The BT filterstrips were dated and coded, and inserted into a 15mL sterile centrifuge tube (one BT filterstrip per tube) and 2.5mL of pre-warmed hybridisation buffer was added. These were allowed to pre-hybridise for 30mins at 42°C. The labelled test DNA sample was

denatured at 96°C for 5mins followed by incubation on ice for 5mins. 12.5μL of the labelled DNA was then added to the hybridisation buffer in the tubes and allowed to hybridise for 18hr at 42°C in a Micro-4 hybridisation oven (Hybaid Ltd, Middlesex, UK).

4.2.6.6 Washes and Detection

The filterstrips were removed into a container of 50-100mL 2X SSC/0.1% SDS buffer washed twice for 5mins each at room temperature on an orbital shaker. The BT filterstrips were then washed twice for 15mins in 50-100mL pre-warmed 0.5X SSC/0.1% SDS buffer at 60°C in the hybridisation oven. This was followed by a single wash in 20mL 1X Maleic acid buffer for 1min followed by 30mins at room temperature, in 1X blocking buffer in 1X Maleic acid buffer. The BT filterstrips were then transferred into 20mL of fresh 1X blocking buffer in 1X Maleic acid buffer containing 4μL anti DIG-AP and incubated for 30mins at room temperature on an orbital shaker. Finally, two washes for 5mins in 1X washing buffer and rinsed in 20mL detection buffer for 2mins were carried out. The strips were then placed face up on Saran wrap. 2mL of CDP-star detection reagent was then applied to the surface of the membranes and incubated for 5mins at room temperature. Excess CDP-Star reagent was removed by blotting with tissue paper.

The strips were sealed in saran wrap and placed inside a Hyperfilm cassette and a sheet of Hyperfilm™ECL™ (Amersham Pharmacia Biotech Ltd) placed on top. The film was developed after 1hr by immersion for four minutes in developing solution (Kodak, Sigma), rinsing briefly in distilled water and then immersion in a fixative solution (Kodak, Sigma,), for a further four minutes. The film was then rinsed thoroughly in distilled water and allowed to air dry.

4.2.7 Multilocus Sequence Typing

MLST was carried out by Dr M. Enright at the University of Bath.

The method used is outlined on the MLST web site; www.mlst.net.

The *S. aureus* MLST scheme uses internal fragments of the following seven house-keeping genes:-

arc (Carbamate kinase)

aro (Shikinate dehyrogenase)

glp (Glycerol kinase)

gmk (Guanylate kinase)

pta (Phosphate acetyltransferase)

tpi (Triosephosphate isomerase)

ypi (Acetyle coenzyme A acetyltransferase)

PCR amplification was carried out on chromosomal DNA of the test strains using an extension time of 30 seconds, and an annealing temperature of 55°C with Qiagen *Taq* polymerase. The following primers were used:-

arc up – 5' TTC ATT CAC CAG CGV GTA TTG TC –3'

arc dn – 5' AGG TAT CTG CTT CAA TCA GCG –3'

aro up – 5' ATC GGA AAT CCT ATT TCA CAT TC –3'

aro dn – 5' GGT GTT GTA TTA ATA ACG ATA TC –3'

glp up – 5' CTA GGA ACT GCA ATC TTA ATC C –3'

glp dn – 5' TGG TAA AAT CGC ATG TCC AAT TC –3'

gmk up - 5' ATC GTT TTA TCG GGA CCA TC –3'

gmk dn – 5' TCA TTA ACT ACA ACG TAA TCG TA –3'

pta up – 5' GTT AAA ATC GTA TTA CCT GAA GG –3'

pta dn – 5' GAC CCT TTT GTT GAA AAG CTT AA –3'

tpi up – 5' TCG TTC ATT CTG AAC GTC GTG AA –3'

tpi dn – 5' TTT GCA CCT TCT AAC AAT TGT AC –3'

yqi up – 5' CAG CAT ACA GGA CAC CTA TTG GC –3'

yqi dn – 5' CGT TGA GGA ATC GAT ACT GGA AC – 3'

The amplified products were precipitated, and both strands were sequenced by using BigDye fluorescent terminators and the primers used in the initial PCR amplification. The sequences obtained were assigned allele numbers following comparison of the DNA sequence with the sequences of previously typed strains by using the MLST website. For each isolate, the allele numbers at each of the seven loci were defined, the allelic profile or sequence type (ST). Both strands of all PCR products were fully sequenced, and novel alleles and STs not found on the MLST database were confirmed by repeating both the PCR and the sequencing.

4.2.8 Stability Testing

A sample 16 isolates were selected for stability testing.10 colonies were touched with a flame sterilised straight wire and inoculated on to a fresh Columbia blood agar plate, and cultured overnight at 37°C. Passage was repeated daily for 50 days. The strains were then stored as gelatine discs at 4°C until required for stability testing.

4.2.9 Reproducibility Testing

A sample 21 isolates were selected to assess reproducibility of each of the typing methods. The isolates were recovered from frozen stocks at –70°C, onto Columbia blood agar plates and incubated aerobically at 37°C overnight. Repeat testing using each of the typing methods was then carried out on these isolates. The research worker was blind to the identity of the chosen isolates.

4.3 Interpretation of Results

Results obtained for all PCR methods and PFGE were in the form of digital photographs. These were analysed using the program Gel Compar II (Applied Maths, Belgium). The degree of homology was determined by DICE coefficient and clustering correlation by UPGMA, with a 1.2% position tolerance.

As there are no standard guidelines for the interpretation of molecular typing generated profiles except those proposed by Tenover *et al.* for PFGE. It was decided that isolates would be assigned groups according to two sets of criteria;

1 a one band difference or

2 a difference of 7 or more bands according to Tenover's criteria.

The resulting groups were used to determine reproducibility, epidemiological concordance and Simpson's Index of diversity (Hunter, 1988), using the formulae stated on p58. Simpson's Index of diversity was determined with the StatView package (SAS Institute Inc., USA).

4.3.1 Formulae:

Typability	$T = Nt \div N$
Reproducibility	$R = Nr \div N$
Epidemiological concordance	$E = Ne \div N$
Discriminatory Ability	$D = 1 - \frac{1}{N-(N-1)} \cdot \sum_{j=1}^{s} n_j(n_j - 1)$

N is the number of isolates tested.

Nt is the number of isolates assigned a type.

Nr is the number of isolates assigned to the same type on repeated testing.

Ne is the number of isolates assigned to epidemiologically linked clones.

s is the total number of types described.

N_j is the number of isolates belonging to the jth type.

5.0 RESULTS

5.1 Antimicrobial Sensitivity Testing

Table 5.1.1: Range and frequency of resistance antibiograms detected

Antibiogram	N° of isolates detected
P	2
PM	30
PEM	31
PTM	2
PFM	1
PKM	2
PEKM	3
PELKM	7
PELKM Mu	5
PEFM	2
PELM Mu	2
PTEM	7
PTEFM	2
PTEKM	3
PTEL	1
PTELM	1
PTELM Mu	1
PTELKM	1
PTEFKM	2
PTEFKM Mu	2
PTGFKM	1
PTGKM	1
PTGEKM	9
PTGEKM Mu	3
PTGEKLM	5
PTGEFLKM	1
PGEK	1
PGKM	3
PGEKM Mu	7
PGELKM	1
PGELKM Mu	2
T	1
TGEK	1
TGEFK	1

P: Penicillin G; T: Tetracycline; G: Gentamicin; E: Erythromycin; F: Fusidic acid;

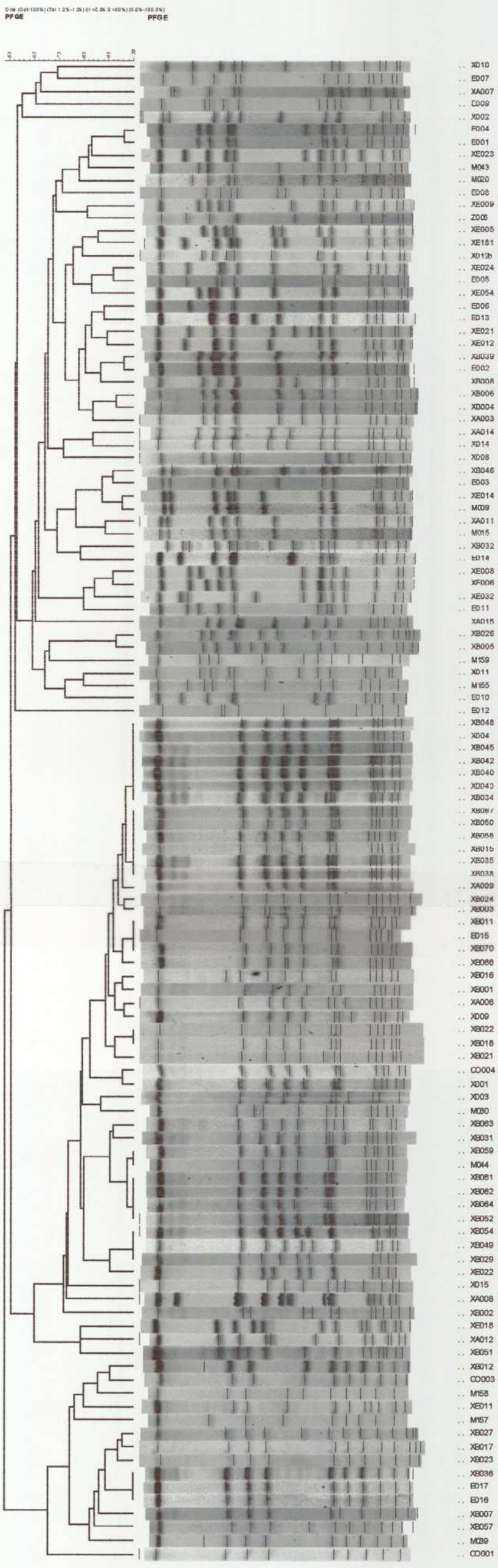
L: Clindamycin; K: Kanamycin; M: Methicillin; Mu: Mupirocin.

Table 5.1.1 shows two dominant groups; PM (30 isolates) and PEM (31 isolates). There are also four smaller major groups; PELKM (7 isolates), PGEKM Mu (7 isolates) PTEM (7 isolates) and PTGEKM (9 isolates) with resistance to multiple antibiotics.

Of the 152 isolates assessed, 7 isolates were found to be methicillin sensitive by disc diffusion.

5.2 PFGE investigation of diverse isolates

Figure 5.2.1 Dendrograms representing PFGE investigation of diverse isolates

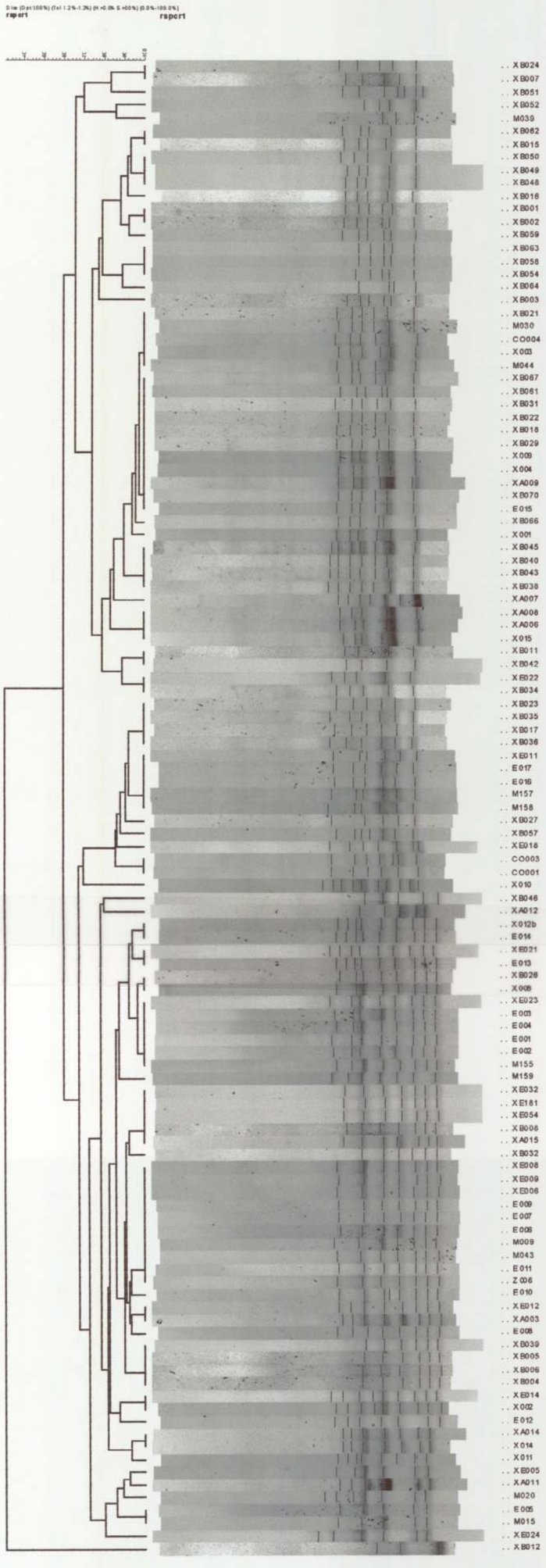


The profiles obtained by PFGE, were analysed visually using two sets of criteria. Application of these criteria resulted in 79 groups by the one-band rule and 27 groups were obtained by application of Tenover's 7-band rule. The largest group comprised of 41 isolates, which included the EMRSA 15 isolate. The isolate EMRSA16 also formed a clearly defined group of 11 isolates.

These groups were then used to calculate typability, reproducibility, stability, and Simpson's Index of diversity

Ribosomal Spacer-PCR investigation of diverse isolates

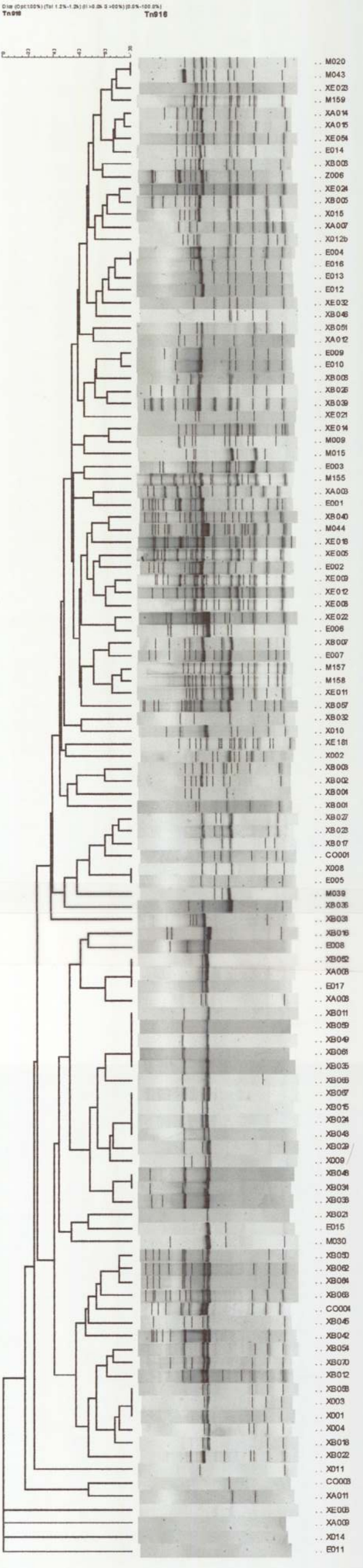
Figure 5.3.1 Dendrograms representing RS-PCR investigation of diverse isolates



Profiles for RS-PCR comprised of 3-10 bands were obtained. Application of the one-band rule resulted in 57 groups. The 7-band rule gave only 5 groups. The largest group obtained with the one-band rule was of 16 isolates, which included EMRSA 15. Tenover's 7-band rule resulted in two major groups of 60isolates, which included EMRSA15 and 50 isolates which included EMRSA16.

5.1 Tn916/Shida PCR investigation of diverse isolates

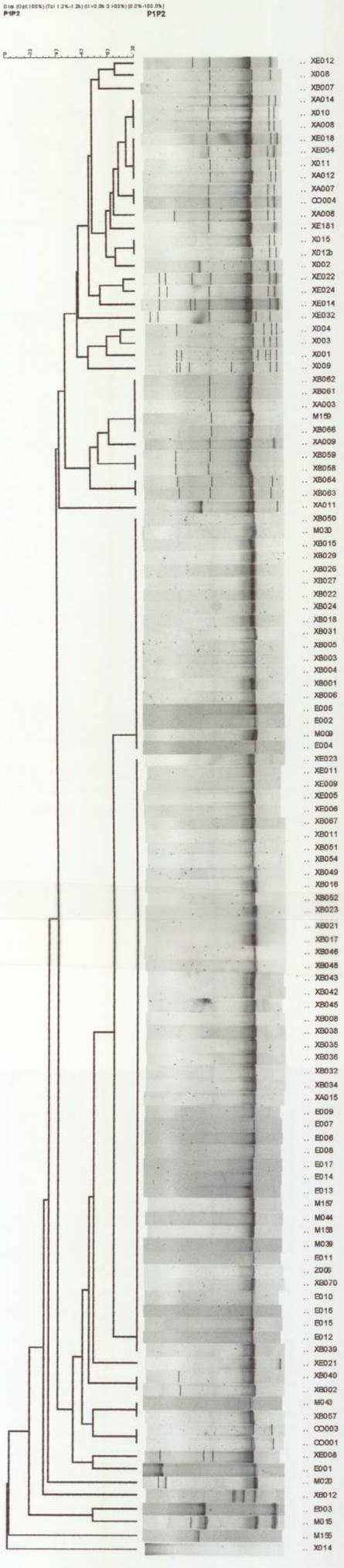
Figure 5.4.1 Dendrograms representing Tn916/Shida investigation of diverse isolates



The profiles of Tn916/Shida PCR analysis comprised of 0-22 bands. Application of the one-band rule resulted in 95 groups, the largest group was one of 5 isolates. Application of the 7-band rule gave 39 groups; the largest group of 24 isolates includes the EMRSA 15 isolate.

5.2 Inter-IS256 PCR investigation of diverse isolates

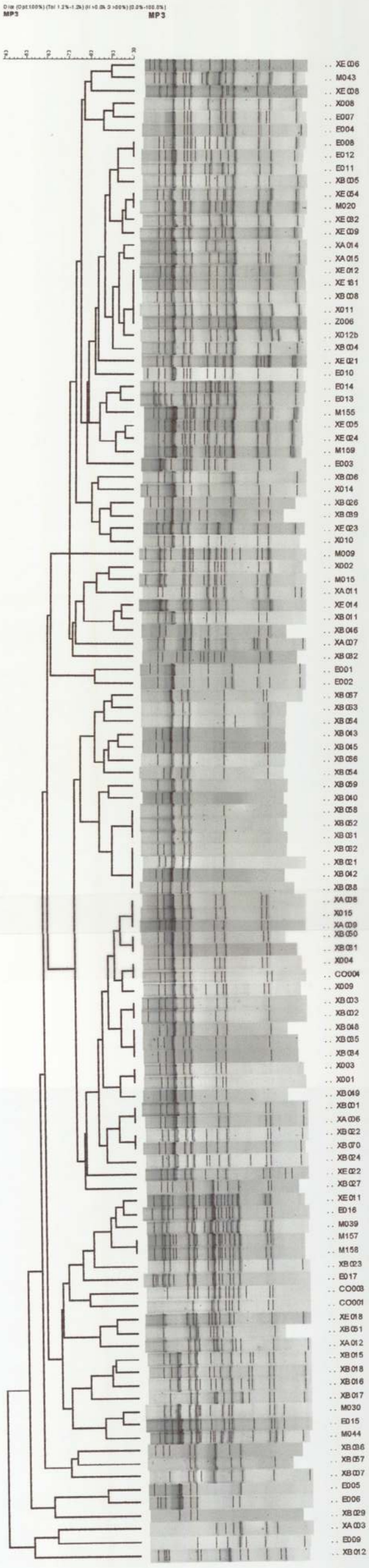
Figure 5.5.1 Dendrograms representing Inter-IS256 investigation of diverse isolates



Profiles of Inter IS256 PCR analysis were made up of only 0-6 bands. On application of the one-band rule to the results 33 groups were obtained, and only one group on application of Tenovers' 7-band rule. The largest group had a profile of only one band and included 65 isolates, the EMRSA 15 and EMRSA 16 isolates.

5.3 Dendrograms representing MP3 investigation of diverse isolates

Figure 5.6.1 Dendrograms representing MP3 investigation of diverse isolates

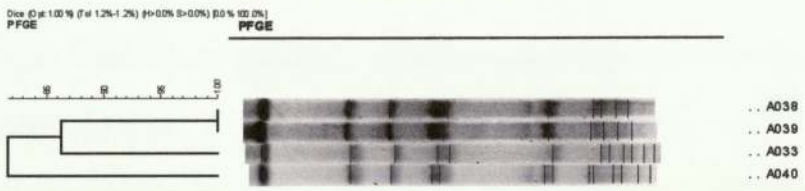


Profiles of MP3 PCR analysis comprised of 5-20 bands. Application of the two sets of criteria on the MP3 PCR results, showed 91 groups and 19 groups for the one-band rule and the 7-band rule respectively. The largest group obtained by Tenover's 7-band rule comprised of 36 isolates. EMRSA 15 was not part of this group.

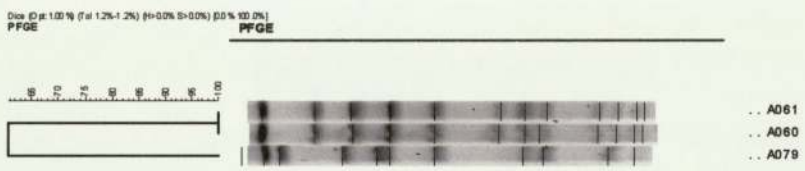
5.7 PFGE investigation of outbreak isolates

Figure 5.7.1 Dendrograms representing PFGE investigation of outbreak isolates

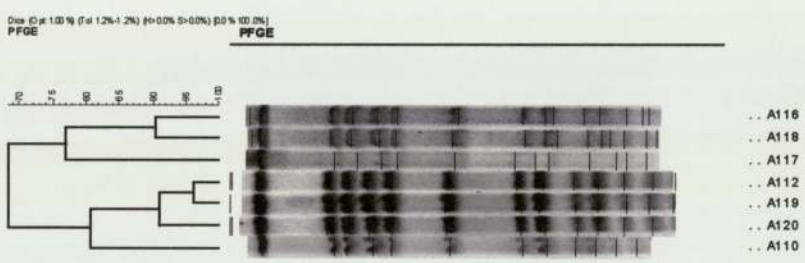
ARI26



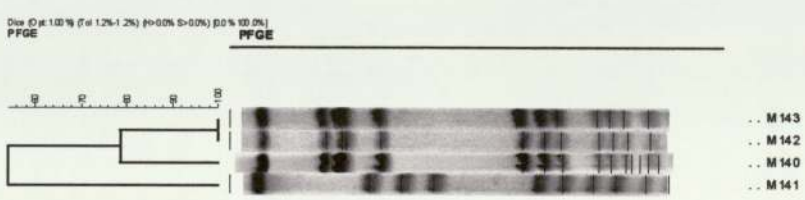
Grays Family



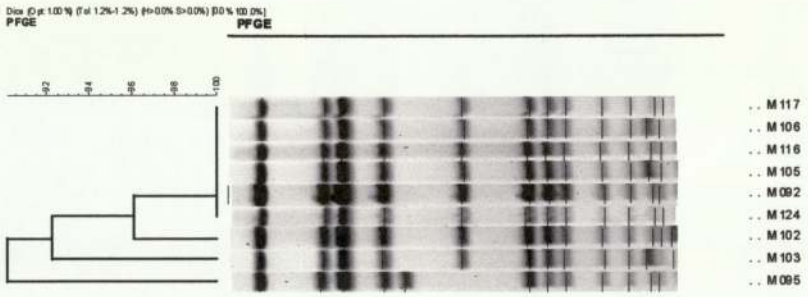
Glenodee



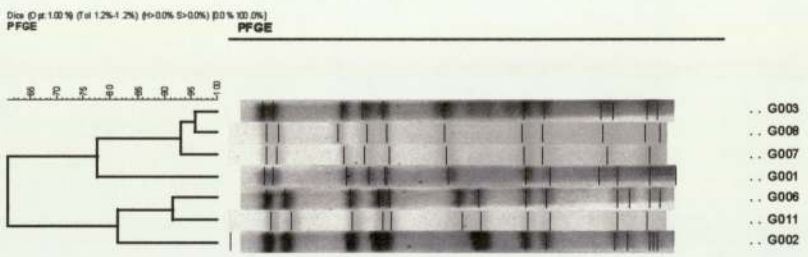
Oxcom



Kenya



Glasgow

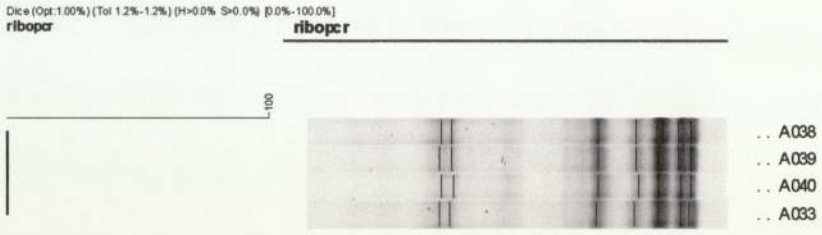


PFGE was found to group all of the isolates into the outbreaks according to Tenover's criteria. The Glasgow outbreak seems to form two clusters, however application of the 7-band rule, and for epidemiological concordance this was viewed as one outbreak. In outbreak ARI26 and outbreak Oxcom, one of the isolates in each of the outbreaks was found not to fit the outbreak profile.

5.8 Ribosomal Spacer-PCR investigation of outbreak isolates

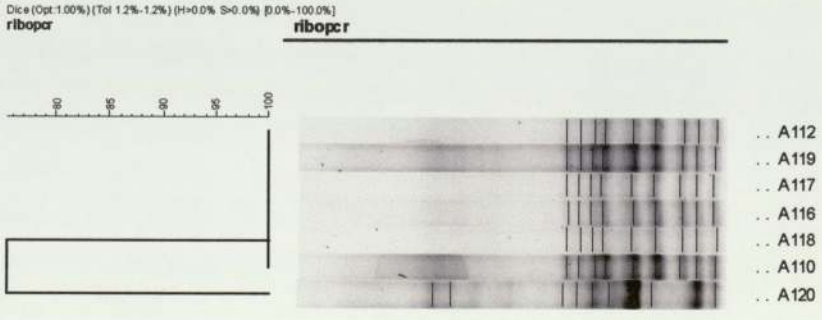
Figure 5.8.1 Dendrograms representing RS-PCR investigation of outbreak isolates

ARI26

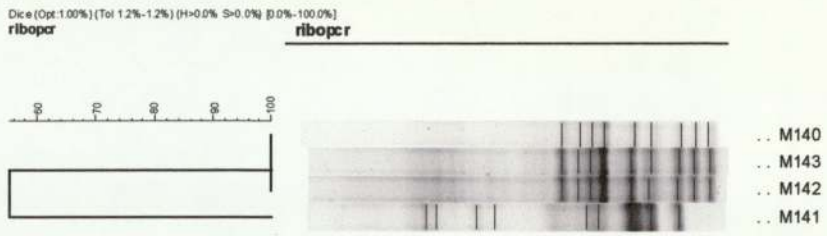


Grays Family

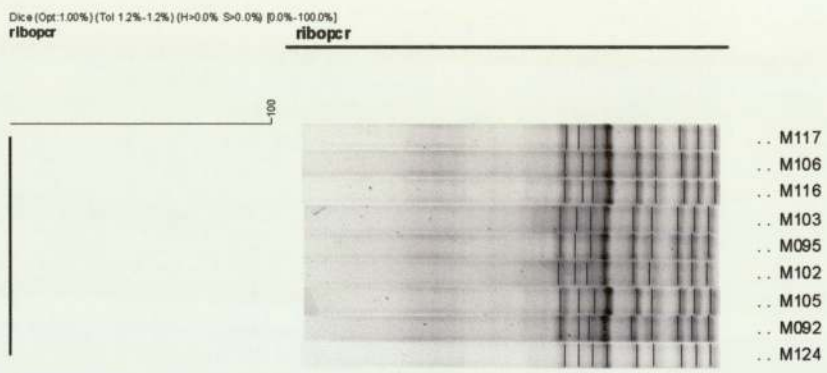
Glenodee



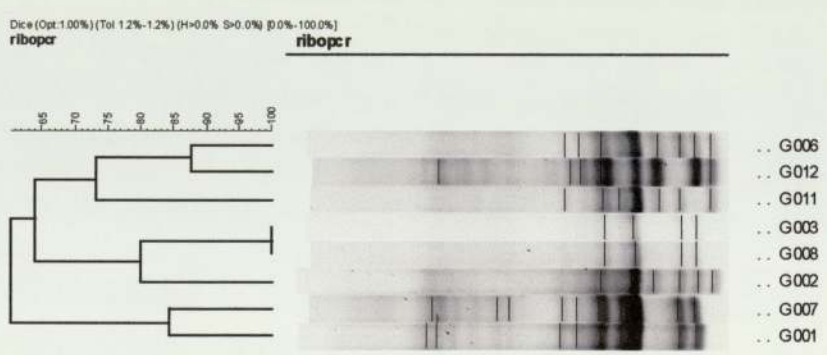
Oxcom



Kenya



Glasgow

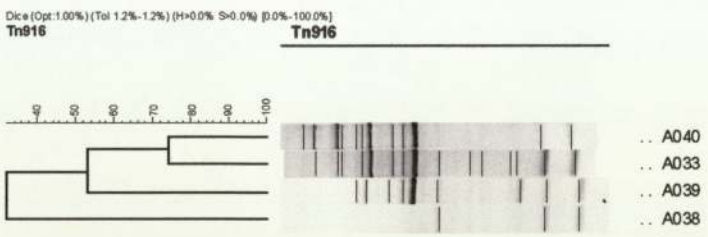


Application of Tenover's criteria correctly grouped all of the outbreaks. Only one isolate in the Oxcom outbreak was found not to fit the outbreak profile.

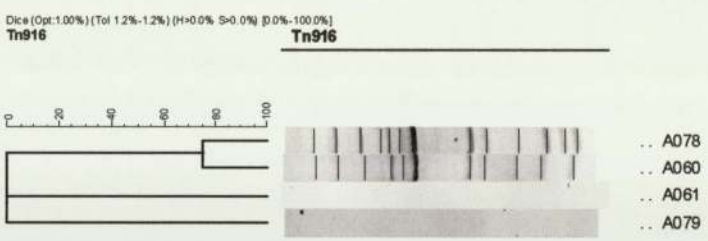
5.9 Tn916/Shida PCR investigation of outbreak isolates

Figure 5.9.1 Dendrograms representing Tn916/Shida investigation of outbreak isolates

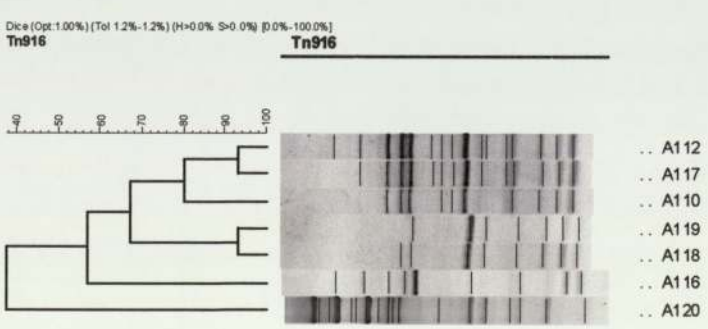
ARI26



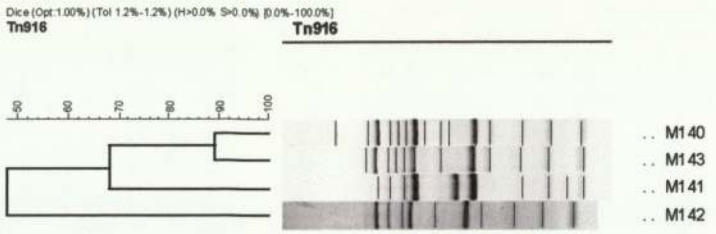
Grays family



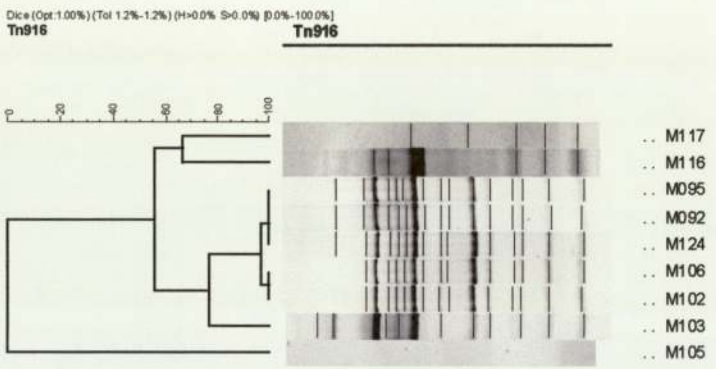
Glenodee



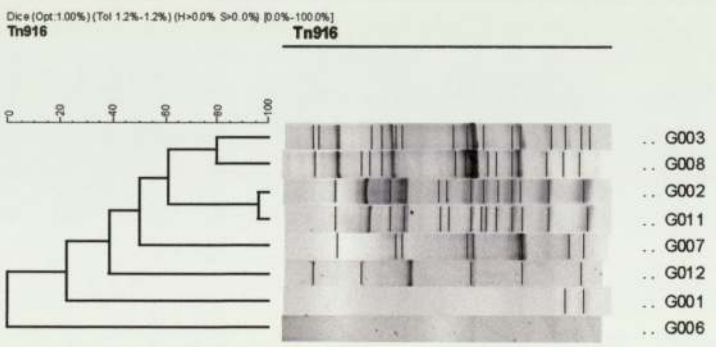
Oxcom



Kenya



Glasgow

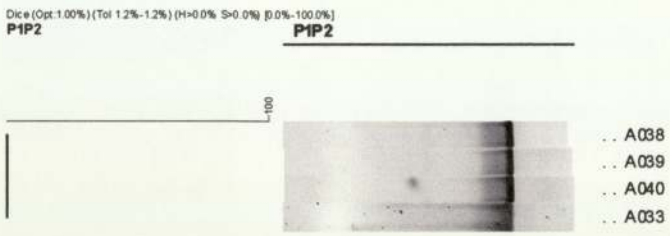


There is much variability of profiles of the isolates within all outbreaks. Many isolates do not fit the outbreak profiles and 4 of the isolates were not typable.

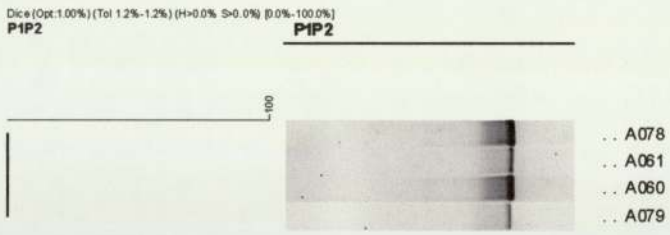
5.10 Inter-IS256 PCR investigation of outbreak isolates

Figure 5.10.1 Dendrograms representing Inter-IS256 investigation of outbreak isolates

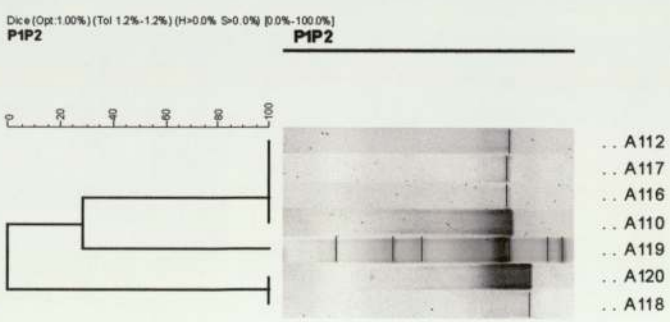
ARI26



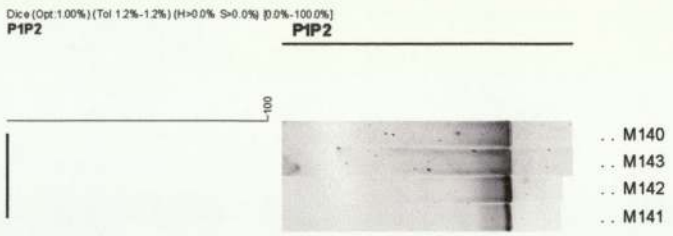
Grays Family



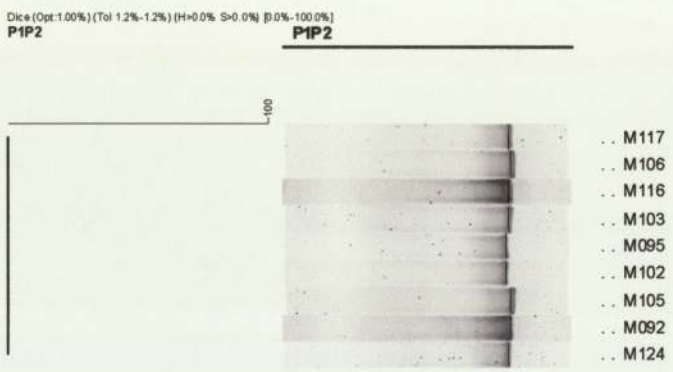
Glenodee



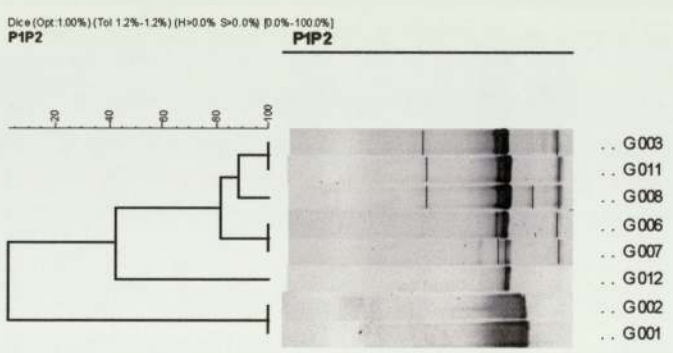
Oxcom



Kenya



Glasgow

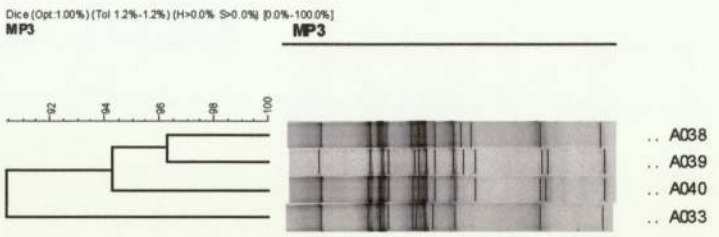


All isolates were found to fit into the outbreak groups according to Tenover's 7-band rule.

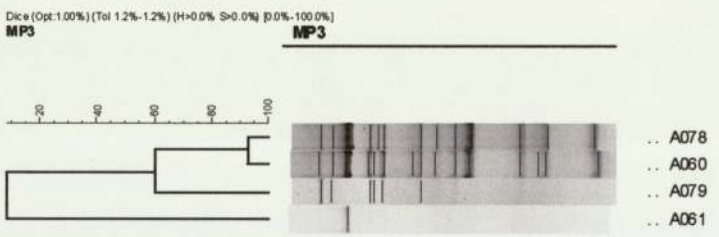
5.11 MP3 PCR investigation of outbreak isolates

Figure 5.11.1 Dendrograms representing MP3 investigation of outbreak isolates

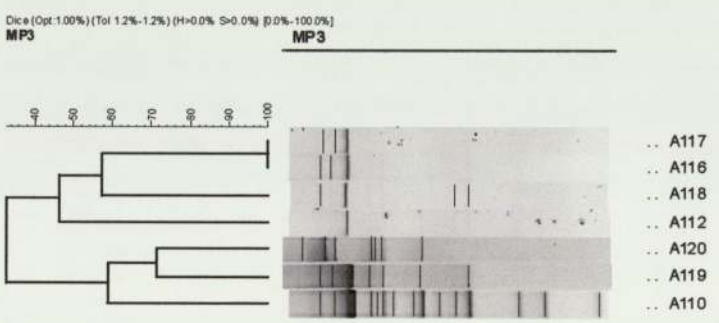
ARI26



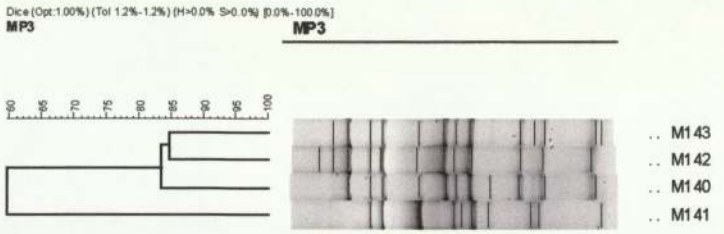
Grays Family



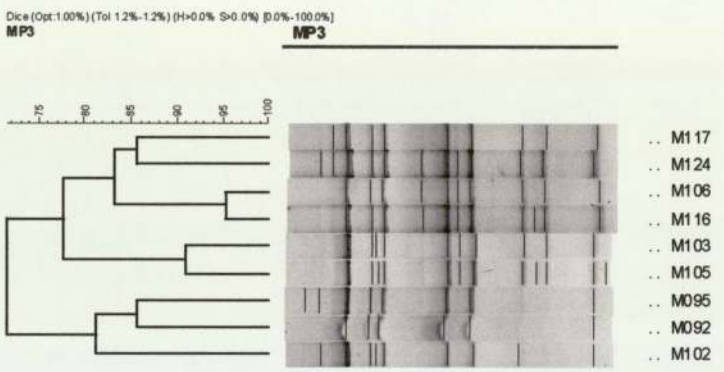
Glenodee



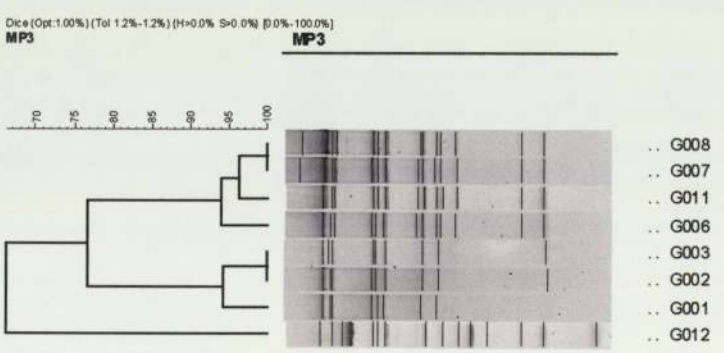
Oxcom



Kenya



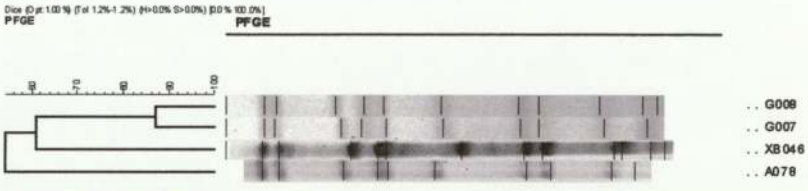
Glasgow



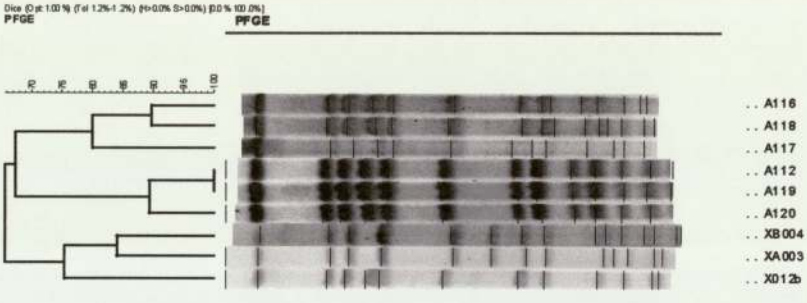
All profiles of the isolates in the ARI26 and Kenya outbreaks group well. The Glasgow outbreak seems to form two clusters, however according to the 7-band rule they were found to form the same group. Outbreaks Grays family and Genodee also seem to form two clusters.

5.12 Comparison of MLST Sequence Types and PFGE profiles

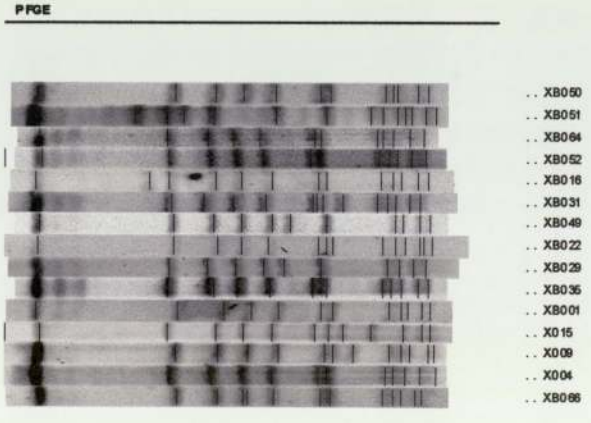
Group1 ST5



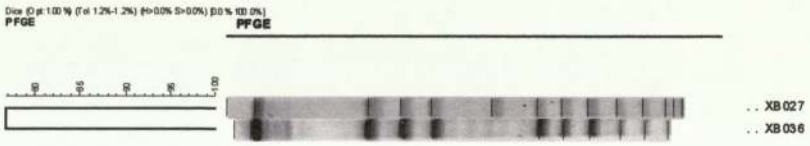
Group2 ST8



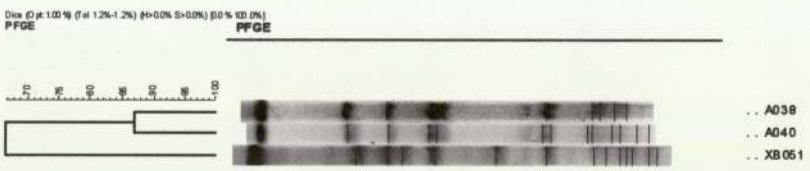
Group3 ST22



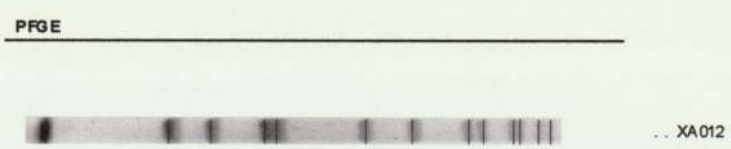
Group4 ST36



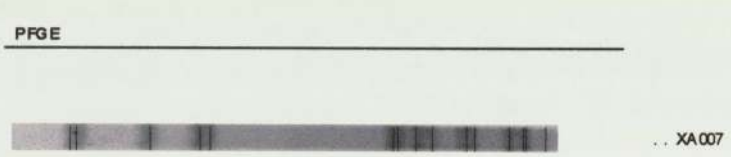
Group5 ST45



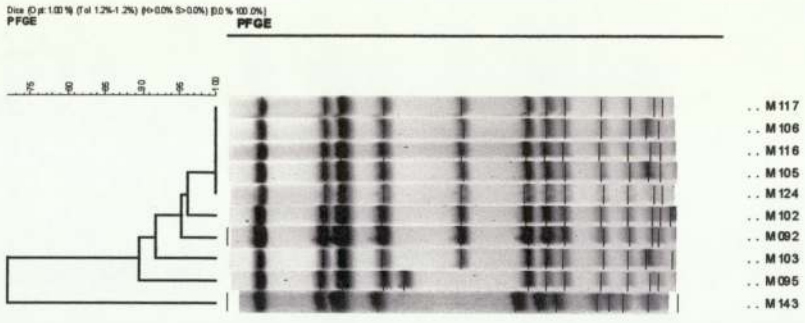
Group6 ST 47



Group7 ST59



Group8 ST241



Group9

MLST analysis resulted in 9 sequence types. PFGE profiles of the corresponding isolates were collated into these groups. The one-band rule and Tenover's 7-band rule were applied to establish PFGE/MLST concordance.

Group8 (ST 241) is the Kenya outbreak isolates.

Typing Technique	Typability	Reproducibility		Stability		Epidemiological Concordance		N° of Groups		Simpson's Index of Diversity (95% Confidence levels)	
		1 band	≥ bands	1 band	≥ bands	1 band	≥ bands	1 band	≥ bands	1 band	≥ bands
PFGE	1.0	0.9	1.0	0.15	0.92	0.69	1.0	79	27	0.949 (0.915-0.983)	0.85 (0.797-0.908)
RS-PCR	1.0	0.05	0.86	0.12	0.87	0.78	1.0	57	5	0.967 (0.965-0.970)	0.554 (0.553-0.556)
Tn916/Shida	0.97	0	0.33	0	0.19	0.14	0.56	95	39	0.995 (0.990-1.0)	0.933 (0.924-0.941)
IS256	0.99	0	0.95	0	1.0	0.75	1.0	33	1	0.977 (0.960-0.993)	0.009 (0.009-0.009)
MP3	1.0	0	0.39	0	0.44	0.11	0.72	91	19	0.994 (0.989-0.999)	0.868 (0.821-0.914)

Table 5.13: Summary of results

The degree of typability, reproducibility, stability, epidemiological concordance and discriminatory ability using PFGE and Rep-PCR methods were calculated using the standard formulae (section 4.3.1).

All methods exhibit 97-100% typability. Application of Tenover's criteria resulted in reproducibility of 33%-86% for Rep-PCR methods and 100% for PFGE; stability ranging from 19% to 87% for the Rep-PCR methods and 92% for PFGE. Epidemiological concordance of 100% was obtained for PFGE, RS-PCR and IS256. Diversity ranged from 85% to 93% for all method except for IS256 (9%).

6.0 DISCUSSION

This study was carried out to assess the suitability of a range of available molecular MRSA typing methods for their suitability for use as a typing method in the routine microbiology laboratory. When selecting a molecular typing method, characteristics such as typability, reproducibility, stability and discriminatory ability, are the main considerations. Additional considerations include, cost, technical staff training, turn around time, ease of use and data processing and interpretation.

Epidemiological surveillance studies are carried out to determine the relationship of strains isolated in a short period of time. In an outbreak investigation, comparative typing may be carried out to establish the extent of epidemiological spread of microbial clones, the number of clones and monitoring of reservoirs of clones or for the evaluation of the efficacy of infection control measures.

The first indication of an outbreak is at the microbiology bench where culture morphology, biotyping, and antibiotic resistance patterns, are often the first indicators that something unusual is taking place. In fact, it is the results of antibiotic susceptibility testing of *S. aureus* and the isolation of MRSA strains, which determines further investigation. Additional phenotypic investigations such as phage typing may be carried out depending on the resources available to the laboratory.

Phenotypic characteristics however are unstable and are subject to change in response to both environmental and genetic changes.

Typing methods that are currently in use are either phenotypic or genotypic in principle. Antibiotic susceptibility testing is one of the most useful methods as it is cheap, rapid and

readily accessible, however until the recent introduction and standardisation by the British Society for Antimicrobial Chemotherapy (BSAC), inter-centre reproducibility varied due to the variety of methods in use. Selective environmental pressures as well as genetic mechanisms can influence variations in antibiotic susceptibility

Resistance due to genetic mutation is relatively stable, however transient acquisition and loss of plasmids and transposons, results in poorly reproducible results. Antibiotic resistance testing is of limited epidemiological use due to reduced reproducibility and poor discrimination. . The results obtained for antimicrobial testing showed a variety of patterns. A number of isolates were found to methicillin sensitive, however these were positive on testing for *mec*A.

Phage typing has been used for many years however it requires the maintenance of stock phages and propagating strains. The increasing incidence of non-typable strains with the approved set of phages resulted in the introduction of additional experimental phages, which are only available to larger laboratories and reference centre. The procedure is fairly laborious and requires a certain degree of skill. The lack of uniformity of practice and interpretation as well as poor reproducibility are the main limiting factors.

There is currently no definitive typing technique for MRSA, however Pulsed Field Gel Electrophoresis is generally considered the 'gold standard' of molecular typing. It has been applied to broad range of Gram-positive and Gram-negative bacteria and has been used successfully for epidemiological purposes in outbreak situations. It is the general opinion of multiple investigators that PFGE is *the* most useful and reliable typing method available (Saulnier,P *et al.* 1993; Tenover, F *et al.* 1994; Senna *et al.* 2002;Bannerman, T *et al.* 1995). It has been found to be highly discriminatory and reproducible. This is in concordance with

the result obtained in this study. PFGE has two major limitations, namely the cost and the length of time taken to carry out the procedure. PFGE requires relatively expensive specialized equipment. Agarose embedded DNA requires extended incubation times to facilitate adequate diffusion of buffers and enzymes. Hence PFGE can take between 4 to 6 days. Methods requiring shorter periods of incubation have been proposed (Matushek, 1996) where the whole procedure could be completed within 3 days. PFGE is also the only method for which interpretation guidelines of the profiles produced have been proposed (Tenover, 1997).

The methods used in this study ranged from PCR, the most likely candidate for use in the microbiology laboratory, to more complicated and demanding method such as ribotyping. All the techniques used are well documented and show varying results, however all the methods used in this study have not previously been investigated together.

The techniques were assessed for typability, reproducibility, discriminatory ability, stability, applicability, epidemiological concordance, and turn around time.

6.1 Typability

All methods assessed exhibited good typability with PFGE, RS-PCR and MP3 were able to type all isolates. Of the 116 isolates under investigation, 1 isolate investigated with IS256 and four isolates investigated with Tn916/Shida, were found to be non-typable. These isolates may have been typable if repeated on more than on occasion, under different conditions or a different concentration of template DNA.

6.2 Reproducibility

The degree of reproducibility is measured by the proportion of isolates for which the same profile and the same interpretation of the profile is obtained on repeat testing. Good reproducibility was achieved with PFGE when apply the one band difference rule and Tenover's, more then 7 bands difference rule was applied. Bands smaller than 80bp were difficult to distinguish and contributed to a decrease in the reproducibility of interpretation. Some of the larger fragments may also have comprised of more than one band which separated on one electrophoresis gel but not on others.

Of the Rep-PCR methods RS-PCR also shows good reproducibility, but only when applying the greater than 7 band rule. IS256 seems to show good reproducibility, however results for IS256 typing are misleading as only one band was obtained in the majority of cases with a maximum of four bands.

6.3 Stability

The degree of stability is determined by stability of the genetic feature under investigation over a period of time although it can also be influenced by the reproducibility of the profile generated.

Stability was found to be good both for PFGE and RS-PCR when applying the seven-band rule. Results are again misleading for IS256 due to reduced number of bands in the profiles generated.

6.4 Epidemiological concordance

For both PFGE and RS-PCR epidemiological concordance was very good with both the 1 band difference rule and the 7-band difference rule, although RS-PCR comes out slightly better when applying the 1-band difference criteria.

There are a few isolates that do not fall into the outbreak groups when analysed by any method. It is concluded that although these isolates appeared to be epidemiologically linked they were not part of the outbreak investigated.

6.5 Discriminatory Ability

Discrimination was found to be very good for all the methods applying the single band difference rule, with Simpson's Index values of 0.95 and greater. However, application of the seven-band difference rule shows Tn916/Shida to have the greatest discriminatory ability followed by PFGE and MP3. This is misleading as Tn916/Shida exhibits poor reproducibility.

6.6 Turn around time

Rep-PCR gave results in the shortest time of 18hrs. Many of the steps such as preparation of the gel whilst PCR amplification is taking place, can be overlapped so that results may be obtained the same day. RS-PCR was the longest of the PCR methods taking up to 43.5hrs, as electrophoresis of the PCR products is carried out over 16hrs. However, as this is carried out overnight, and the gel stained the following morning, relatively little time is lost.

PFGE is the longest of the methods investigated taking 4-5 days. Long incubation times of 24hrs with lysozyme and lysostaphin and 48hrs with proteinase K, are required to allow

penetration of enzymes into the agarose embedded DNA. Prolonged washing steps also increase the turn around time. These are necessary to ensure sufficient removal of proteinase K and PMSF, which may interfere in adequate resolution of the PFGE profile such as lane smearing. Methods requiring shorter periods of incubation have been proposed (Matushek, 1996) where the whole procedure could be completed in 3 days. The quality of the gel profiles obtained may, however, be compromised.

Ribotyping and Binary Typing in take 3-4 days and 26hrs respectively. The longest steps are enzyme digestion, Southern blotting and hybridisation, which are all carried out overnight for a minimum of 16hrs.

6.7 Ease of Use

All the Rep-PCR methods were found to be fairly easy. Some care was needed whilst carrying out DNA extraction to avoid contamination and ensure good yields of DNA. PFGE was the easiest of the techniques. Manipulation of the agarose plugs for digestion is the only area requiring care and gentle handling to avoid damage to the plugs.

As a technician with no experience of Southern blotting and hybridisation, ribotyping would be difficult to establish as a molecular typing method in the routine microbiology laboratory. Ribotyping was found to be a technically complicated, time consuming and a frustrating method. The proposed method when carried out successfully would take at least three days. There are many variables, which can influence the results obtained. To standardise this method, further work would be required for example establishing the ideal sodium chloride concentration, varying the incubation temperature when labelling the probe and establishing the ideal hybridisation temperature.

Binary Typing is fairly straightforward - *if* carried out as instructed. Some technical skill is required. Problems that were encountered were as a result of poor quality DNA and inadequate labelling of the test DNA. These have been resolved as a result of changing the DNA extraction method.

6.8 Ease of Interpretation

There are no standard guidelines for the interpretation of any molecular typing generated profiles. Guidelines for PFGE have been proposed by Tenover (Tenover *et al.,* 1997). The application of these guidelines however are not applicable to all organisms and all typing techniques. For any species, the degree of PFGE variability has to be matched with the degree of inherent genetic variability of that species (van Belkum, 2000).

The establishment of a suitable guidelines for the interpretation of profiles is very important. It has been shown that Tenover's criteria, proposed for PFGE, are not suitable for all of the methods used in this study.

PCR Methods – Both Tn916/Shida and MP3 gave profiles of 15-20 bands. IS256 amplification however resulted, in majority of cases, in only one band. This made the application of Tenover's guidelines impossible. RS-PCR gave only 7-12 poorly resolved bands. Profiles generated for RS-PCR often comprised of a mixture of faint and intense staining bands, which made comparisons of isolates between different gels difficult, although an overall pattern could be discerned. PFGE resulted in profiles of 12–15 clearly defined bands, although interpretation of bands smaller then 80bp was subjective to a certain extent.

6.9 Multilocus Sequence Typing

MLST was carried out on a selection of strains to validate the reputation of PFGE as the gold standard. A sample of 48 isolates were selected and subjected to MLS Typing. The results showed that all isolates were typable. Comparison with PFGE profiles showed 100% concordance when Tenover's criteria are applied. The cost of the specialised equipment required and the technically demanding nature of method makes this an unsuitable method for use in the routine lab.

7.0 CONCLUSION

In conclusion, PFGE was found to be the best method in terms of exhibiting excellent typability, reproducibility and epidemiological concordance, good stability and discriminatory ability, when Tenover's criteria were applied. However it requires expensive equipment and has a long turn around time. Of the Rep-PCR methods, RS-PCR found to be the best PCR method. It was found to show excellent typability and epidemiological concordance and good reproducibility and stability, however discriminatory ability was only moderate. This is in concordance with the findings of Kumari *et al.* (1997) who found this method to be highly reproducible and good discriminatory ability, although not as good as PFGE.

Although typability was good for Tn916/Shida, IS256 and MP3 methods, poor results obtained for the remaining characteristics means that these methods would only be suitable for obtaining quick comparative results on a small number of isolates. Results of previous work (Cuny and Witte, 1996; Deplano et al. 1997 and Del Vicchio, 1997) suggests that these methods would provide a rapid and discriminatory method typing of MRSA. The results of the study however showed that reproducibility was poor and discriminatory ability was moderate to poor.

The best methods are PFGE and MLST however the cost implications and time involved restricts their use to reference and research institutions. PFGE would be a suitable choice for use in the routine microbiology laboratory depending on the availability of resources. If rapid results are required RS-PCR is an acceptable alternative method provided reference strains are loaded onto the gel for comparison purposes, and all isolates under investigation are compared together on the same gel.

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APPENDIX A

Description of Bacterial Isolates and Epidemiology:

A total of 152 MRSA isolates were tested. These comprised of two sets:

3 Out-break/connected isolates, and

4 A diverse, non-epidemiologically connected set.

Outbreak Isolates:

The connected set comprised of 36 isolates from 6 outbreaks from hospitals in the UK.

Kenya Outbreak – isolates M92 – M124. This outbreak comprises of 9 isolates from three hospitals in Oxford during 1992. The outbreak source was a patient who had returned to the UK from Kenya. Exact dates of isolation are not available.

Oxcom Outbreak – is comprised of 4 isolates (M140-M143) from patients at the Oxford Community hospital.

ARI26 – This outbreak was located in ward 26 at the Aberdeen Royal Infirmary hospital, comprising 4 isolates (A33-A40).

Grays family Outbreak – This is made up of two sets of two connected isolates. A60 and A61 are strains isolated from husband and wife, where one was admitted to hospital. The isolates A78 and A79 are from Dr Gray's Districr General Hospital in Elgin.

Isolates G1- G12 (9 isolates) are from Scottish Reference Laboratory at Glasgow. Isolate G12 was not included in the results analysis as it did not survive storage and could not be used in all of the tests carried out.

Isolates A110 – A120 (7 isolates) are from an outbreak in Glenodee Hospital.

Diverse Set:

The remaining 116 were sporadic isolates from 29 hospitals in the UK.

Epidemic MRSA stains (EMRSA 1-16) were kindly supplied by Dr Mark Enright at the University of Bath.

Ref No	Lab No	Isolation date	Location	Sensitivity	*mec*A	Outbreak/Sporadic
C01949	CO 1	8-Dec-97	CHT	PELKM Mu	+	Sporadic
C02056	CO 4	19-Aug-97	Leeds	PEM	+	Sporadic
X00025	XO 1	9-Oct-96	GR Micro	PM	+	Sporadic
X00584	XO 2	31-Oct-96	St Thom	PEKM(G)	+	Sporadic
X01106	XO 3	1-Oct-96	Bristol	PEM	+	Sporadic
X01441	XO 4	4-Oct-96	Edinburgh	PEM	+	Sporadic
X02038	XO 8	23-Nov-96	Cardiff	PTELM Mu	+	Sporadic
X02164	XO 9	11-Oct-96	CHT	PM	+	Sporadic
X02370	XO 10	12-Oct-96	Derry	P	+	Sporadic
X02392	XO 11	13-Dec-96	Derry	PEKM(G)	+	Sporadic
X02547	XO 12	7-Oct-96	Plymouth	PKM (2T)	+	Sporadic
X04368	XO 15	25-Oct-96	Trafford	PM	+	Sporadic
XA00401	XA 3	31-Oct-97	Belfast	PGEK	+	Sporadic
XA00776	XA 6	20-Oct-97	Leicester	PEM	+	Sporadic
XA00951	XA 7	22-Oct-97	Leeds	PM	+	Sporadic
XA01127	XA 8	1-Oct-97	Bristol	PM	+	Sporadic
XA01494	XA 9	15-Oct-97	Edinburgh	PE(M)	+	Sporadic
XA01810	XA 11	7-Oct-97	North Mid	PGEKM Mu	+	Sporadic
XA02030	XA 12	13-Oct-97	Cardiff	P(M)	+	Sporadic
XA02378	XA 14	13-Oct-97	Derry	PTEKM	+	Sporadic
XA02382	XA 15	13-Oct-97	Derry	PEK(M)	+	Sporadic
XB00057	XB 1	6-Oct-98	GR Micro	PEM	+	Sporadic
XB00220	XB 2	6-Oct-98	Sheffield	PTEM	+	Sporadic
XB00230	XB 3	7-Oct-98	Sheffield	PM	+	Sporadic
XB00379	XB 4	15-Oct-98	Belfast	PEKM	+	Sporadic
XB00386	XB 5	19-Oct-98	Belfast	PGELKM Mu	+	Sporadic
XB00413	XB 6	10-Nov-98	Belfast	PEKM	+	Sporadic
XB00584	XB 7	22-Oct-98	St Thom	PELKM Mu	+	Sporadic
XB00585	XB 8	19-Oct-98	St Thom	PTEFKM Mu	+	Sporadic
XB00933	XB 11	22-Oct-98	Leeds	PEM	+	Sporadic
XB00957	XB 12	17-Oct-98	Leeds	PELKM	+	Sporadic
XB01278	XB 15	2-Oct-98	Camb	PEM	+	Sporadic
XB01289	XB 16	6-Oct-98	Camb	PM	+	Sporadic
XB01313	XB 17	9-Oct-98	Camb	PELKM Mu	+	Sporadic
XB01650	XB 18	1-Nov-98	S'hants	PEM	+	Sporadic
XB01843	XB 21	6-Oct-98	North Mid	PM	+	Sporadic
XB01858	XB 22	9-Oct-98	North Mid	PEM	+	Sporadic
XB01859	XB 23	12-Oct-98	North Mid	PELKM Mu	+	Sporadic
XB02214	XB 24	8-Oct-98	CHT	PEM	+	Sporadic
XB02370	XB 26	16-Dec-98	Derry	PGELKM Mu	+	Sporadic
XB02560	XB 27	12-Oct-98	Plymouth	PGEKM Mu	+	Sporadic
XB02568	XB 29	13-Oct-98	Plymouth	PEM	+	Sporadic
XB02759	XB 31	3-Nov-98	Withing	PEM	+	Sporadic
XB02760	XB 32	3-Nov-98	Withing	P(M)	+	Sporadic
XB02940	XB 34	19-Oct-98	Salford	PM	+	Sporadic
XB03117	XB 35	6-Oct-98	Frenchay	PEM	+	Sporadic
XB03291	XB 36	8-Oct-98	Exeter	PGEKM Mu	+	Sporadic
XB03467	XB 38	6-Oct-98	Lewisham	PEM	+	Sporadic
XB03477	XB 39	7-Oct-98	Lewisham	PEM	+	Sporadic

Ref No	Lab No	Isolation date	Location	Sensitivity	*mec*A	Outbreak/Sporadic
XB03478	XB 40	17-Oct-98	Lewisham	PEM	+	Sporadic
XB03633	XB 42	6-Oct-98	Barnstab	PEM	+	Sporadic
XB03801	XB 43	5-Oct-98	Borders	PFM	+	Sporadic
XB03827	XB 45	14-Oct-98	Borders	PEM	+	Sporadic
XB03976	XB 46	13-Oct-98	Whiston	PEFM	+	Sporadic
XB03986	XB 48	13-Oct-98	Whiston	PM	+	Sporadic
XB03997	XB 49	25-Oct-98	Whiston	PEFM	+	Sporadic
XB04015	XB 50	6-Nov-98	Whiston	PEM	+	Sporadic
XB04326	XB 51	6-Oct-98	Trafford	PELKM	+	Sporadic
XB04365	XB 52	21-Oct-98	Trafford	PEM	+	Sporadic
XB04507	XB 54	2-Oct-98	Newport	PEM	+	Sporadic
XB04557	XB 57	4-Nov-98	Newport	PELKM	+	Sporadic
XB04681	XB 58	2-Oct-98	Gloucs	PEM	+	Sporadic
XB04717	XB 59	8-Oct-98	Gloucs	PEM	+	Sporadic
XB04865	XB 61	5-Oct-98	Dudley	PEM	+	Sporadic
XB04905	XB 62	12-Oct-98	Dudley	PEM	+	Sporadic
XB04908	XB 63	8-Oct-98	Dudley	PM	+	Sporadic
XB04910	XB 64	13-Oct-98	Dudley	PM	+	Sporadic
XB05079	XB 66	7-Nov-98	Wolves	PM	+	Sporadic
XB05090	XB 67	10-Nov-98	Wolves	PEM	+	Sporadic
XB05263	XB 70	14-Oct-98	Alexandr	PEM	+	Sporadic
Z00956	Z 6	17-Jun-99	Oxford	PTEFKM	+	Sporadic
	M9	1990-1994	Oxford	PELKM	+	Sporadic
	M15	1990-1994	Oxford	PGEKM	+	Sporadic
	M20	1990-1994	Oxford	PTELKM	+	Sporadic
	M30	1990-1994	Oxford	PM	+	Sporadic
	M39	1990-1994	Oxford	PGELKM	+	Sporadic
	M43	1990-1994	Oxford	PTGEKM	+	Sporadic
	M44	1990-1994	Oxford	PEM	+	Sporadic
	M92		Oxford	PTGEKM	+	Outbreak - Kenya
	M 95		Oxford	PTGEKM	+	Outbreak - Kenya
	M 102		Oxford	PTGEKM	+	Outbreak - Kenya
	M 103		Oxford	PTGEKM Mu	+	Outbreak - Kenya
	M 105		Oxford	PTGEKM Mu	+	Outbreak - Kenya
	M 106		Oxford	PTGEKM Mu	+	Outbreak - Kenya
	M 116		Oxford	PTGEFLKM Mu	+	Outbreak - Kenya
	M 117		Oxford	PTGEKM Mu	+	Outbreak - Kenya
	M 124		Oxford	PTGEKM	+	Outbreak - Kenya
	M 140		Oxford	PTEM	+	Outbreak - Oxcom
	M 141		Oxford	PELKM	+	Outbreak - Oxcom
	M 142		Oxford	PTEM	+	Outbreak - Oxcom
	M 143		Oxford	PTEM	+	Outbreak - Oxcom
	M155	Post 1994	Oxford	PTGELKM	+	Sporadic
	M157		Oxford	PM	+	Sporadic
	M158		Oxford	PM	+	Sporadic
	M159		Oxford	PTGELKM	+	Sporadic
	A 33	14-Mar-95	Aberdeen	PM	+	Outbreak - ARI26
	A 38	25-May-95	Aberdeen	PM	+	Outbreak - ARI26

Ref No	Lab No	Isolation date	Location	Sensitivity	*mec*A	Outbreak/Sporadic
	A 39	30-May-95	Aberdeen	PM	+	Outbreak - ARI26
	A 40	5-Jun-95	Aberdeen	PM	+	Outbreak - ARI26
	A 60	2-Aug-96	Aberdeen	P(M)	+	Outbreak - Grays family
	A 61	23-Aug-96	Aberdeen	PTEFM	+	Outbreak - Grays family
	A 78	24-Jan-97	Aberdeen	PTEFM	+	Outbreak - Grays family
	A 79	27-Jan-97	Aberdeen	PELM Mu	+	Outbreak - Grays family
	A 110		Aberdeen	PELM Mu	+	Outbreak - Glenodee
	A 112		Aberdeen	PM	+	Outbreak - Glenodee
	A 116		Aberdeen	PM	+	Outbreak - Glenodee
	A 117		Aberdeen	PM	+	Outbreak - Glenodee
	A 118		Aberdeen	PM	+	Outbreak - Glenodee
	A 119		Aberdeen	PM	+	Outbreak - Glenodee
	A 120		Aberdeen	PM	+	Outbreak - Glenodee
RU0,2504.N	G 1		Glasgow	PGEK(M) Mu	+	Outbreak - Glasgow
00,2839.S	G 2		Glasgow	PGK(M)	+	Outbreak - Glasgow
00,2869.T	G 3		Glasgow	PGEK(M) Mu	+	Outbreak - Glasgow
98,3477.K	G 4		Glasgow	PTEF(K)M Mu	+	Outbreak - Glasgow
00,4950.S	G 6		Glasgow	PGK (M)	+	Outbreak - Glasgow
00,5916.R	G 7		Glasgow	PGEK(M) Mu	+	Outbreak - Glasgow
00,6919.F	G 8		Glasgow	PGEK(M) Mu	+	Outbreak - Glasgow
R00,5474.S	G 11		Glasgow	PGKM	+	Outbreak - Glasgow
00,3872.X	G 12		Glasgow	PTEFKM Mu	+	Outbreak - Glasgow
EMRSA 1	E 1		Bath Uni	PTGEKLM	+	English EMRSA
EMRSA 2	E 2		Bath Uni	PEM	+	English EMRSA
EMRSA 3	E 3		Bath Uni	PGEKM	+	English EMRSA
EMRSA 4	E 4		Bath Uni	PTEM	+	English EMRSA
EMRSA 5	E 5		Bath Uni	PTGKM	+	English EMRSA
EMRSA 6	E 6		Bath Uni	PTEKM	+	English EMRSA
EMRSA 7	E 7		Bath Uni	PTEM	+	English EMRSA
EMRSA 8	E 8		Bath Uni	PTM	+	English EMRSA
EMRSA 9	E 9		Bath Uni	PTGEKM	+	English EMRSA
EMRSA 10	E 10		Bath Uni	PTGEKM	+	English EMRSA
EMRSA 11	E 11		Bath Uni	PTGEKM	+	English EMRSA
EMRSA 12	E 12		Bath Uni	PTEFKM	+	English EMRSA
EMRSA 13	E 13		Bath Uni	PTGFKM	+	English EMRSA
EMRSA 14	E 14		Bath Uni	TGEK	+	English EMRSA
EMRSA 15	E 15		Bath Uni	PM	+	English EMRSA
EMRSA 16	E 16		Bath Uni	PELKM	+	English EMRSA
EMRSA 17	E 17		Bath Uni	PELKM Mu	+	English EMRSA
	XE5	Pre 1990	Oxford	T	+	Sporadic
	XE6	Pre 1990	Oxford	PTGEK(M)	+	Sporadic
	XE8	Pre 1990	Oxford	PTGEK(M)	+	Sporadic
	XE9	Pre 1990	Oxford	PTGELK(M)	+	Sporadic
	XE11	Pre 1990	Oxford	PTGELK(M)	+	Sporadic
	XE12	Pre 1990	Oxford	PTEL(M)	+	Sporadic
	XE14	Pre 1990	Oxford	PELK(M)	+	Sporadic
	XE18	Pre 1990	Oxford	PK(M)	+	Sporadic
	XE21	Pre 1990	Oxford	TGEFK	+	Sporadic
	XE22	Pre 1990	Oxford	PE(M)	+	Sporadic

Ref No	Lab No	Isolation date	Location	Sensitivity	*mec*A	Outbreak/Sporadic
	XE23	Pre 1990	Oxford	PTGEFLKM	+	Sporadic
	XE24	Pre 1990	Oxford	PTELM	+	Sporadic
	XE32	Pre 1990	Oxford	PTGEK(M)	+	Sporadic
	XE54	Pre 1990	Oxford	PT(M)	+	Sporadic
	XE181	Pre 1990	Oxford	PTE(M)	+	Sporadic

APPENDIX B

MLST Results From University of Bath:

Isolate	ST	SCCmec	Multi	arcC	aroE	glpF	gmk	pta	tpi	yqil
G7	5	1	1	1	4	1	4	12	1	10
G8	5	1	1	1	4	1	4	12	1	10
M78	5	2	2	1	4	1	4	12	1	10
XB46	5	4	4	1	4	1	4	12	1	10
X12	8	4	4A	3	3	1	1	4	4	3
XB39	8	4	4	3	3	1	1	4	4	3
A117	8	4	4	3	3	1	1	4	4	3
XB4	8		3	3	3	1	1	4	4	3
A116	8	4	4	3	3	1	1	4	4	3
A118	8	4	4	3	3	1	1	4	4	3
A119	8	4	4	3	3	1	1	4	4	3
X43	8	4		3	3	1	1	4	4	3
A112	8	4	4	3	3	1	1	4	4	3
A120	8	4	4	3	3	1	1	4	4	3
XB66	22	4	4	7	6	1	5	8	8	6
X15	22			7	6	1	5	8	8	6
XB64	22	4	4	7	6	1	5	8	8	7
XB35	22	4	4	7	6	1	5	8	8	6
X49	22	4	4	7	6	1	5	8	8	6
XB22	22			7	6	1	5	8	8	6
XB31	22	4	4	7	6	1	5	8	8	6
XO4	22	4	4	7	6	1	5	8	8	6
XB50	22	4	4	7	6	1	5	8	8	6
XB1	22		4	7	6	1	5	8	8	6
XB29	22	4	4	7	6	1	5	8	8	6
XB51	22	4	4	7	6	1	5	8	8	6
XB52	22	4	4	7	6	1	5	8	8	6
XB16	22	4	4	7	6	1	5	8	8	6
XO9	22	4	4	7	6	1	5	8	8	6
XB23	36	2	2	2	2	2	2	3	3	2
XB27	36	2	2	2	2	2	2	3	3	2
XB36	36	2	2	2	2	2	2	3	3	2
A40	45	4	4	10	14	8	6	10	3	2
A38	45	2	4	10	14	8	6	10	3	2
XB51	45	4		10	14	8		10	3	2
XA12	47	4	4	10	11	8	6	10	3	2
X47	59		4	19	23	15	2	19	20	15
M116	241	3	3	2	3	1	1	4	4	30
M117	241	3	3	2	3	1	1	4	4	30
M143	241	3	3	2	3	1	1	4	4	30
M106	241	3	3	2	3	1	1	4	4	30
M124	241	3	3	2	3	1	1	4	4	30
M102	241	3	3	2	3	1	1	4	4	30
M92	241		4b	2	3	1	1	4	4	30
M95	241	3	3	2	3	1	1	4	4	30
M103	241	3	3	2	3	1	1	4	4	30
M105	241	3	3	2	3	1		4	4	30
G2		4	4	1	4	1	1	12	41	10

APPENDIX C

Poster Presentation : Typing of methicillin resistant *Staphylococcus aureus* (MRSA) by repetitive sequence PCR

Typing of Methicillin-resistant *Staphylococcus aureus* by repetitive sequence PCR

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Introduction

- Methicillin resistant *Staphylococcus aureus* (MRSA) is a frequent cause of nosocomial infection worldwide. It is endemic in many hospitals and also causes defined outbreaks.
- An efficient, accurate and rapid epidemiological typing system can help to monitor and limit its spread but the best method to use routinely has not yet been determined. PCR-based typing methods are rapid and simple to perform and, therefore, may be suitable.

Repetitive element sequence based PCR (Rep PCR)

- Amplification of repetitive element sequences is one PCR method that has been used for fingerprinting unrelated and outbreak-related isolates of MRSA.
- Primers target short DNA segments, which are conserved, genetically stable and distributed randomly on the chromosome. The space between primers is amplified generating DNA fragment patterns that are specific for individual strains.

Objectives

We examined three different primers sets previously used for Rep PCR to identify which target sequences were most useful for MRSA typing. Each was also compared to pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Bacterial strains

- 152 MRSA were included.
- 36 from 7 outbreaks.
- 116 sporadic isolates from 29 hospitals in the UK. These also included all epidemic (EMRSA E1-17) strains.
- Blinded repeat testing of 23 MRSA was performed by a second person to assess reproducibility of the results.

Rep-PCR

- DNA was extracted from all the isolates using lysostaphin, proteinase K, phenol and chloroform.
- All isolates were confirmed as MRSA by amplification of *mecA*.
- Isolates were analysed by PCR with three different sets of primers:
- Transposon916-Shing-Delagarno sequence (Tn916-Shida)[1]
- Inter-IS256 PCR spacer length primers (IS256)[2]
- RW3A derived from *Mycoplasma pneumoniae* repetitive sequence (RepMP3)[3]

Electrophoresis of TN916-shida and IS256 PCR products was performed on 1.5% agarose gel and RepMP3 on 2% agarose gel, run at 140 volts for 130 minutes. A 1Kb marker was included in three lanes on all gels as molecular size markers.

Pulsed Field Gel Electrophoresis

- PFGE was performed using a modification of the method proposed by Banneman *et al.* [4]
- DNA was digested by restriction enzyme *sma*I.
- Running parameters were: 6V/cm, initial pulse time 5s, final pulse time 40s, for 22 hours.
- Control strain NCTC 8325 was run as a size marker on all gels.

Analysis

- Gels were analysed with GelCompar (Applied Maths, Belgium) software.
- Degrees of homology were determined by DICE comparisons, using a 3% position tolerance, and clustering correlation was by UPGMA.
- Isolates exhibiting ≥80% homology were classified as related in order to enable the calculation of reproducibility, epidemiological concordance and discriminatory ability.

Formulae

- Typeability T = Nt ÷ N
- Reproducibility R = Nr ÷ N
- Epidemiologic concordance E = Ne ÷ N
- Discriminatory ability Simpson's index of diversity[5]

N is number of isolates tested
Nt is number of isolates assigned a type
Nr is number of isolates assigned to the same type on repeat testing
Ne is number of isolates assigned to epidemiologically linked clones

Results

Table: Comparison of Rep-PCR methods and PFGE

	Typeability	Reproducibility	Epidemiologic concordance	Discriminatory ability (95% CI)
PFGE	1.0	0.91	0.87	0.74 (0.69 to 0.79)
Tn916-Shida	0.93	0.21	0.55	0.07 (0.04 to 0.10)
Inter-IS256	0.99	0.21	0.56	0.05 (0.02 to 0.08)
MRSA	1.0	0.30	0.63	0.41 (0.33 to 0.49)

Interpretation of results

- Rep-PCR by all three primers showed good typeability (T ≥0.95)
- Poor reproducibility was observed with all three primer sets
- Epidemiologic concordance of outbreak isolates was also found to be low with all three-primer sets. Figure 3 shows band patterns and resulting dendrograms obtained by Rep-PCR and PFGE for one outbreak.
- Tn916-shida PCR was found to be the most discriminatory when applied to the sporadic isolates. However, the poor reproducibility of results obtained by all three primer sets makes this measure unreliable.

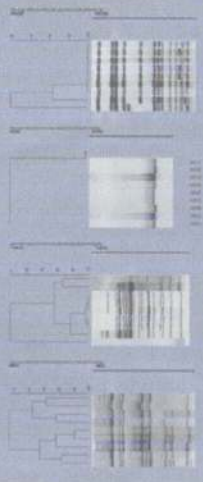
Conclusion

- Repetitive-element PCR based typing methods offer the advantage of rapidity. Low reproducibility and poor epidemiologic concordance limits the usefulness of these techniques for long term monitoring of MRSA. However, they could be useful for the rapid fingerprinting of a small number of epidemiologically linked isolates.

Fig. 1. PFGE patterns of MRSA isolates

Fig. 2. PCR generated DNA fingerprints of MRSA isolates

Fig. 3. Dendrogram of similarity of MRSA isolates from an outbreak



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