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Molecular investigation of an epidemic MRSA: a comparative study of its recognition, introduction and spread in the West of Scotland

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A thesis submitted for the Degree of Master of Science
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December 1998

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DECLARATION

This thesis is the original work of the author and the information, ideas and opinions expressed herein are my own unless otherwise stated.

Alistair W. Brown

ACKNOWLEDGEMENTS

I would like to thank Dr David J. Platt, Dr Dugald R. Baird and Dr Giles F.S. Edwards for their advice and patience, which must have been stretched on several occasions during the course of this study. Thanks also to Dr Tony Girdwood for allowing me the opportunity to undertake this course of study.

Special thanks to Mr George Donnelly for extraordinary computing skills used in the presentation of this thesis.

Thank you also to staff at Microbiology, Stobhill NHS Trust and particularly to Shelley and Linda for help, advice and shoulders to moan on when the going got tough.

Thanks to all family and friends for encouragement and for having faith and finally, very special thanks to my wife Heather for continued support and incredible amounts of patience during some very difficult times.

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ABSTRACT

The incidence of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has increased world-wide over the past 30 years.

A strain that was introduced into Scotland in 1990 via a patient recently returned from Lisbon, Portugal had an unusually resistant phenotype. Ninety-three isolates were selected and investigated by molecular methods. The principal method chosen was agarose gel electrophoresis following digestion of whole cell genomic DNA with the restriction enzymes *Hha*I and *Sau3A*I. These enzymes recognise 4-base DNA sequences and produced an analytical window at the top of an agarose gel, which allowed the recognition of plasmid DNA fragments and partial digest products. The final result of electrophoresis by this method was a considerable improvement over previous methods employing enzymes that are 6-base cutters.

The strain was studied in parallel with control groups of *Staph. aureus* that consisted of methicillin-sensitive *Staph. aureus*, sporadic isolates of MRSA and the epidemic strains EMRSA-1, EMRSA-15 and EMRSA-16. Analysis of the *Hha*I restriction enzyme fragmentation patterns (REFP) of the "new" strain and control groups by Dice coefficients of similarity validated the technique with respect to discrimination; it was demonstrated that REFP's of epidemiologically unrelated MSSA isolates had low Dice coefficient values (mean S_D value = 66%) and that REFP's of known epidemiologically related isolates such as EMRSA-15 had high coefficients of similarity (mean S_D value = 99%).

The technique showed that all isolates of the new strain were clonal in origin (mean S_D value = 95%) and in addition, highlighted the existence of a

number of clonal variants (subtypes) to the major REFP type. Sixty-eight isolates (73%) gave a genomic fingerprint identical to the index case and were designated *Hha*I type H1. Twenty-five isolates were variants of this type and were designated type LH2 (7 isolates), LH3 (6 isolates), LH10 (2 isolates) and fifteen of the twenty-five were unique variants designated LH4 – LH9 and LH11 – LH14. Nine isolates of another strain, imported from France and phenotypically similar to the study strain were shown to be genetically closely related to it.

Inter-group matching of REFP's showed each control group to be genetically distinct to each other and to the "new" MRSA strain.

In a collaborative study, this new strain which has been trivially termed the "Lisbon strain" was shown to be closely related to the now well characterised Iberian clone MRSA. Variants detected using *HhaI*/*Sau3A*I typing also showed parallel variation in PFGE.

A small number of genomic variants were also found within the EMRSA-1, 15 and 16 control groups, highlighting the capacity of the technique to detect minor genetic change.

Restriction enzyme fingerprinting of whole cell genomic DNA using the restriction enzymes *Hha*I and *Sau3A*I proved to be a simple, economic and highly discriminatory method of typing *Staph. aureus* strains requiring no expensive apparatus.

1. INTRODUCTION

1. 1 The genus *Staphylococcus*

Bacteria of the genus *Staphylococcus* are non-motile Gram positive cocci 0.5 - 1.5μm in diameter, which may occur singly, in pairs, in short chains, or most often in grape-like clusters. Medically important members of the genus are divided by their ability to produce the enzyme coagulase, a virulence factor. *Staphylococcus aureus* is the major coagulase positive species found in human infections although *Staph. intermedius* and *Staph. hyicus* are common veterinary pathogens. Currently the genus comprises 32 species (Kloos, 1998) which are widespread in nature, and are found mainly on skin and mucous membranes of birds and mammals.

Other than *Staph. aureus*, species of staphylococci frequently implicated as the aetiologic agents of human infections include *Staph. epidermidis, Staph. saprophyticus*, *Staph. haemolyticus*, and *Staph. lugdunensis*.

Staph. aureus may cause a toxaemic disease in which toxins released by multiplying organisms are absorbed by the body. These include epidermolytic toxins, which give rise to scalded skin syndrome, enterotoxins - found in staphylococcal food poisoning, and toxic shock associated toxin (TSST-1) - associated with use of tampons. Most commonly however, *Staph. aureus* gives rise to infections which include boils, carbuncles, cellulitis, impetigo, wound infection, endocarditis and septicaemia.

Most infections arise from endogenous sources, with the infecting strain identical to the organism isolated from the patients nose swabs (Hobbs *et al.*,

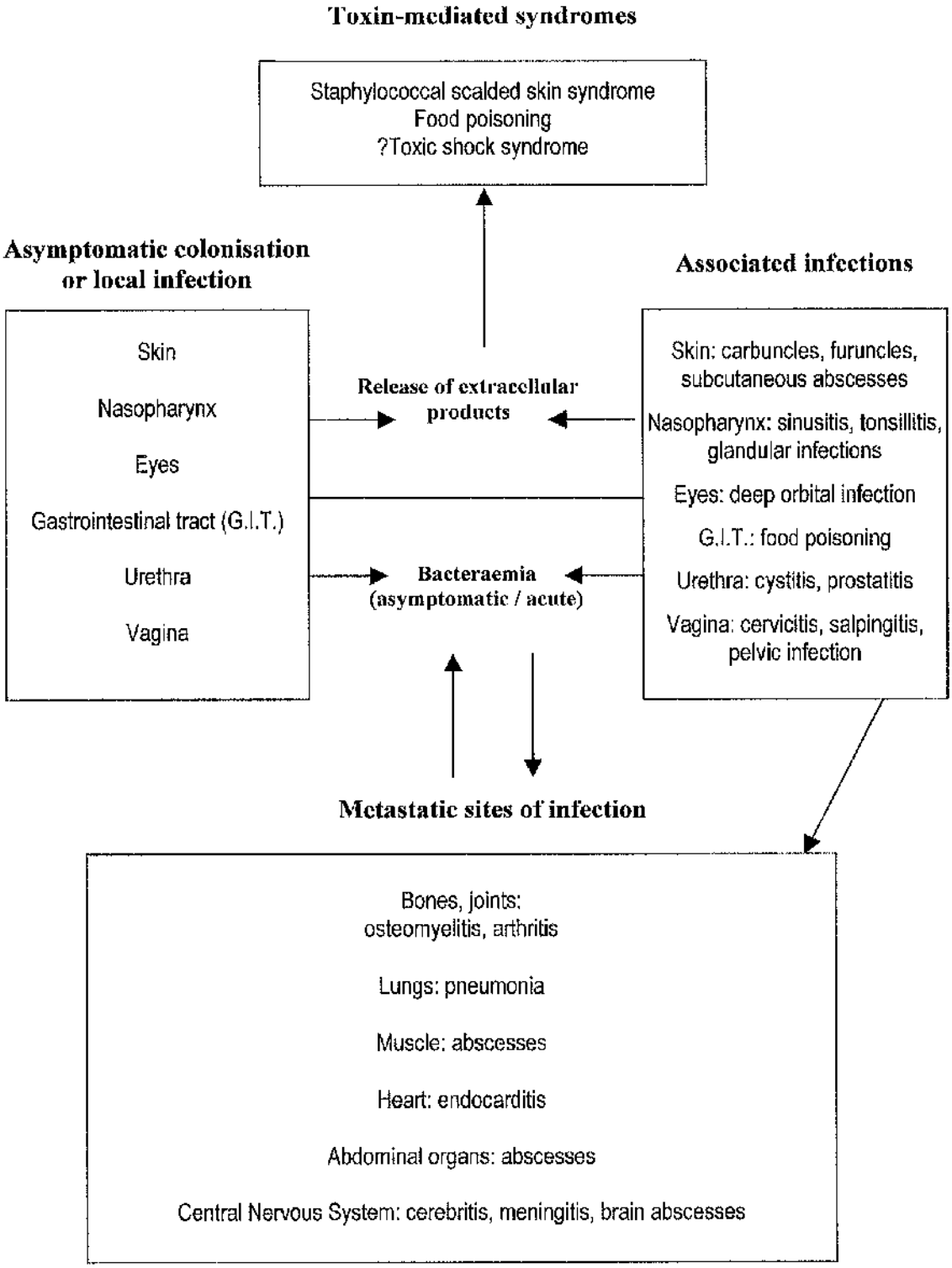
1947, Valentine and Hall-Smith, 1952, Tulloch, 1954). This is especially the case when lesions occur on the face as with *sycosis barbae* or styes (Kay, 1962).

1.2 Virulence of *Staph. aureus*

Staphylococcal disease is clinically diverse as illustrated in Figure 1.1. The outcome of the relationship of *Staph. aureus* with its host is dependent on a number of factors including the properties of the particular strain, the site of infection and the competency of the hosts' defences. *Staph. aureus* produces a wide range of virulence factors which play various roles in the different disease processes. Some of these factors are associated with the cell surface such as a protein A (Petersen *et al.*, 1977), fibronectin binding protein (Wadstrom, 1991) and collagen binding protein (Holderbaum *et al.*, 1987). In addition to cell surface proteins, some strains of *Staph. aureus* also produce a range of extra cellular virulence factors including five membrane-damaging toxins, six enterotoxins, epidermolytic toxin, toxic shock syndrome toxin (TSST-1), and a pyrogenic exotoxin. Exoenzymes which act as virulence factors include coagulase, DNAase, staphylokinase, proteases, lipase, hyaluronidase, phosphatase and phospholipase (Arbuthnott *et al.*, 1990, Arvidson, 1983).

Figure 1.1 (From Arbuthnott *et al.*, 1990)

***Staphylococcus aureus*: its host / pathogen relationship in man**



The virulence of MRSA has been the subject of much debate. There appear to be two points of view. Most authorities agree that MRSA are potentially as pathogenic as methicillin sensitive *Staph. aureus* (MSSA), though some believe that MRSA are not as virulent, and cause infection only in certain high risk groups (Lacey, 1987) whereas others believe them to be true pathogens (French *et al.*, 1990, Keane and Cafferkey, 1984, Thompson *et al.*, 1982, Peacock *et al.*, 1981). Thompson and colleagues (1982) highlighted three studies in which the overall mortality rate of nosocomial outbreaks of MRSA infection had been compared to case matched controls of outbreaks of MSSA infection (Crossley *et al.*, 1979a, 1979b, Peacock *et al.*, 1980, Boyce *et al.*, 1981). All three studies found no significant difference in overall mortality, suggesting that MRSA and MSSA are equally virulent.

In the study by French (1990) above, more than 5000 Hong Kong MRSA isolates were shown to be equally as virulent as MSSA. Both groups of organisms were isolated in similar proportions from sites associated with serious infection and sites associated with colonisation indicating an equal ability of both groups to produce invasive infection. In patients with hospital acquired bacteraemia, mortality rates were found to be similar in both groups of organisms when adjusted for clinical factors.

Transfer of genetic information between different strains of MRSA (and MSSA) by plasmids, transposons and bacteriophages leads to evolutionary changes *i.e.* divergence of strains. These changes may result in strains with altered virulence potential (Coleman *et al.*, 1989). Thus, it can also be argued that both MRSA and MSSA are heterogeneous with respect to virulence.

1.3 Development of resistance

Before the antibiotic era severe staphylococcal infection was associated with a high mortality. A limited success was achieved with the introduction of sulphonamide in the 1930's but this was short lived, as many strains soon became resistant.

In the early 1940's the mortality rate declined sharply but temporarily following the introduction of penicillin into clinical use. However, the widespread use of penicillin resulted in the selection of penicillinase-producing resistant strains such that by the late 1940's virtually all nosocomially acquired strains were resistant (Barber, 1948). New antimicrobials continued to appear during the 1940's and 50's including streptomycin, chloramphenicol and erythromycin. Strains resistant to all available systemic antibiotics had appeared by the end of the 1950's. A major breakthrough in anti-staphylococcal therapy came in 1960 with the advent of the semi-synthetic penicillinase resistant penicillins, methicillin and cloxacillin (BMJ editorial, 1960). Initially, this appeared to provide a solution to the problem of drug resistance, however, as with previous antimicrobials, resistant strains were soon detected (Jevons, 1961, Knox, 1961, Cetin and Ang, 1962, Borowski *et al.*, 1964). The incidence of infection caused by these strains remained low until the late 1960's when invasive infection became more prominent. This increase was "controlled" somewhat during the 1970's by the use of gentamicin for severe infection. Gentamicin had been in use for 10 years before the first resistant strains were recorded. This resistance was plasmid mediated and probably developed as a result of the widespread topical use of the agent in

dermatology where patients shed large numbers of organisms in skin scales, aiding the dissemination of resistance in different strains of *Staph. aureus* (Porthouse *et al.*, 1976, Speller *et al.*, 1976, Wyatt *et al.*, 1977, Warren and Roberts, 1976). Almost inevitably, infections due to both gentamicin and methicillin-resistant *Staph. aureus* were recorded (Shanson *et al.* 1976, Cafferkey *et al.*, 1983, Selkon *et al.*, 1980). Strains resistant to multiple antibiotics including methicillin and gentamicin began to appear in Australia (Pavillard *et al.*, 1982) and London (Shanson *et al.*, 1976). By the late seventies, strains of *Staph. aureus* causing nosocomial infections which were resistant to both these antibiotics, had become very difficult to treat. Whereas previously, strains of *Staph. aureus* resistant to methicillin and other β-lactams had not caused major problems, these multiply resistant strains have now been responsible for numerous endemic and epidemic outbreaks of infection world-wide. They have also become extremely difficult to control or eradicate and pose serious problems for patients and healthcare workers alike and many of these infections are effectively treated only with the glycopeptide antibiotic vancomycin.

1.4 Prevalence of MRSA infection

The incidence of infections caused by MRSA in the UK throughout the 1960's was generally very low. Reports of infection began to rise towards the end of the decade and these may have been due, in part, to improved methods of detection and the understanding of resistance mechanisms. Infection rates throughout Europe around this time were also on the increase. In the USA

however, numbers of infections due to MRSA remained at less than 1% (Barrett *et al.*, 1968, Sabath *et al.*, 1968), until the early 1970's when the incidence showed a steady increase (Klimek *et al.*, 1976, Crossley *et al.*, 1979a, Locksley *et al.*, 1982, Schaefler *et al.*, 1984). By the middle of the decade the number of infections was falling once again both in the USA and Europe (Plorde and Sherris, 1974, Rosendal *et al.*, 1977, Kayser, 1975). Because of the general reduction of MRSA infection together with an increasing number of effective anti-staphylococcal antibiotics by the end of the seventies, the period was later termed as "the decade of complacency" (Shanson, 1981).

MRSA infections have since risen steadily world-wide and many questions still remain unanswered, not least why certain strains (epidemic or EMRSA) seem to have a remarkable propensity for spreading and causing disease whereas others do not.

To date, 16 epidemic MRSA strains (termed EMRSA-1, 2 etc.) have been typed and characterised by The National Staphylococcal Reference Laboratory at Colindale, London. These strains have been responsible for widespread outbreaks of infection in the UK. The first of these epidemic strains, EMRSA-1, was responsible for many outbreaks in the London area and the same strain has also been shown to be responsible for similar outbreaks in Australia (Bradley *et al.*, 1985, Cookson and Phillips, 1988, Duckworth *et al.* 1988). Since the characterisation of EMRSA-1 the prevalence of major EMRSA types has changed in the UK. In 1990 and prior to this, EMRSA-1 was the major type sent by laboratories in England and Wales to the Reference Laboratory at Colindale. EMRSA-2 was also seen but to a lesser extent. In

contrast to this, by 1995 isolates of EMRSA-1 had declined and the predominant strains were now EMRSA-3, EMRSA-15 and EMRSA-16. The number of hospitals affected with EMRSA-3 have remained steady since 1993 whereas numbers of hospitals affected with EMRSA-15 and 16 have been rising together, affecting between 80 - 100 hospitals by the middle of 1995 (CDR weekly, September 1995).

1.5 Epidemiology of MRSA infection

(a) Sources and transmission of MRSA

Within the hospital environment there are certain high-risk units in which patients are at greater risk of infection. These tend to be critical care areas (Thompson *et al.*, 1982) such as ITU and burns units. Patients in these areas are more susceptible to infection having had major surgery, traumatic injury or severe burns (Peacock *et al.*, 1980, Crossley *et al.*, 1979a, 1979b, Boyce *et al.*, 1981, Saraglou *et al.*, 1980). Factors associated with MRSA acquisition include prolonged hospitalisation, long term and previous antibiotic therapy (especially multiple antibiotic therapy), and instrumentation.

The most important mechanism for introduction of MRSA into a hospital is probably by transfer of a patient who is already colonised or infected with MRSA (Peacock *et al.*, 1980, Price *et al.*, 1980, Saraglou *et al.*, 1980). It is perhaps for this reason that large tertiary care facilities experience greater problems with MRSA than smaller hospitals, providing a mechanism for the transfer of epidemic strains of MRSA over great distances. Saraglou *et al.* (1980) highlighted the case of a burns patient who was transferred to another

hospital 500 miles away. Following this, six patients became colonised with the same strain of MRSA, which later developed resistance to gentamicin and gave rise to infection in three of the six.

Other than the patients themselves, hospital personnel and the environment may be important reservoirs of MRSA (Thompson *et al.*, 1982), and hospital staff working in areas where such strains are a recognised problem are frequently screened for MRSA. However, the overall carriage rate by hospital personnel is generally low (Saraglou *et al.*, 1980) and outbreaks of MRSA often occur with no obvious link to hospital personnel.

Whereas in otherwise healthy individuals nasal carriage of MSSA is common, nasal carriage of MRSA occurs only infrequently (Crossley et al., 1979b, Klimek et al., 1976). Reasons for low nasal carriage of MRSA are unknown but it has been suggested that factors influencing adherence of MSSA to nasal epithelia may differ in MRSA (Aly et al., 1981).

Nasal carriage of small numbers of MRSA may not be important in the transmission of MRSA, as normal breathing does not result in widespread dispersal of *Staph. aureus* into the atmosphere. Of greater importance is the potential for nasal carriage to act as a source for the transient carriage of MRSA on the hands of hospital personnel. This route of transmission is well-documented (Peacock *et al.*, 1980). Although this is a major route of transmission it may be interrupted simply by hand washing precautions by staff handling MRSA patients (Thompson *et al.*, 1982).

The role of the third reservoir, the environment, has been more difficult to assess, since there have been fewer detailed investigations. Crossley *et al.*

(1979b), isolated MRSA from 33 of 145 environmental surfaces during an MRSA outbreak in a burns unit. Thompson and co-workers (1982) found similar high rates of contamination in a burns unit. Thus environmental contamination may be an important factor in maintaining outbreaks in burns units. Other areas where environmental contamination may be high include dermatology wards where patients shed large amounts of skin scales.

MRSA although primarily hospital pathogens do cause outbreaks of community acquired infection. Intravenous drug abusers are a well-documented group who commonly develop MRSA septicaemia and endocarditis (Levine *et al.*, 1982). Saravolatz *et al.* (1982a, 1982b) list drug abuse, serious underlying disease, previous antibiotic therapy or previous hospital admission as major factors associated with community acquired MRSA. Hospital admission of patients with community acquired infections is an important source of nosocomial epidemics.

Although hospital personnel and the environment may be responsible for the transmission of MRSA, the ultimate source is usually the infected or colonised patient.

(b) Control of MRSA

The most important factors in the control of MRSA in the hospital environment include awareness of the patient and institution of excellent hygiene (e.g. hand washing). Once introduced into the hospital, eradication of MRSA has usually proved to be very difficult. In a study of 104 hospital outbreaks of MRSA since 1975, Boyce (1981) reported that over 85%

continued to have problems with nosocomial infection due to these strains. Most control programmes involve regular screening of high-risk patients and sometimes of staff who have direct patient contact. Although often overlooked in many hospitals, hand-washing procedures can be a major factor in control of MRSA spread by breaking the chain of transmission. Construction of purpose built isolation units to deal with MRSA patients can be a very costly project. A more convenient and more cost effective method of isolation of carriers is to discharge them as soon as possible.

As a useful control measure Thompson *et al.* (1982) recommended specific precautions appropriate for the site of colonisation or infection, i.e. patients with colonised or infected wounds in whom direct contact transmission was the most likely mode of spread were managed with wound and skin precautions. Patients with extensive burns or respiratory infection were confined using strict isolation procedures because of the potential for airborne transmission. Patients with colonisation or infection of mucosal surfaces or the urinary tract were managed with strict hand-washing precautions after direct contact. These precautions were maintained for the duration of hospitalisation.

In the UK, a combined working party was set up to devise measures for the control of epidemic strains of MRSA within the hospital environment. As a result, guidelines were drawn up and published in 1986 (Working party report, Ayliffe *et al.*, 1986). Since this date these guidelines have been revised twice (Ayliffe *et al.*, 1990, 1998). In addition the working party also published guidelines on control of MRSA in the community (Ayliffe *et al.*, 1995).

The working party guidelines highlighted the need for screening of patients and of staff in particular situations. For example, on admission a patient known to be a carrier or infected with MRSA or a patient admitted from another hospital or ward where there is currently MRSA present, should initially be admitted to an isolation ward or side room. Screening swabs should be taken from sites including nose, throat, perineum, wounds and areas of abnormal skin. In addition, patients from abroad should also be screened as many other countries have major EMRSA problems. Appropriate measures should be taken by staff to prevent spread of MRSA by improving hand-washing procedures using antiseptic disinfectants or 70% alcohol. Where patients or staff are found to be infected with or carrying MRSA, prompt and appropriate measures should be taken. Nasal carriage may sometimes be eradicated by treatment with mupirocin ointment three times daily for five days. In general systemic therapy to eliminate colonisation is not recommended as resistance may develop. Where a member of staff is colonised a short systemic course of rifampicin may be considered if the isolate has been shown to be susceptible, preferably in combination with another agent such as ciprofloxacin or fusidic acid.

In cases of serious clinical infection vancomycin is the preferred option. Teicoplanin, another glycopeptide antibiotic may also be effective. Although a more expensive option, it is less toxic and easier to administer.

The guidelines also recommended regular sampling of previously positive patients. A set of screening swabs as previously mentioned should be

taken on a weekly basis and three sets of negative swabs must be obtained before the patient be considered clear of MRSA.

Other areas covered by the working party report include implications to health care staff and their families infected or colonised with MRSA, transfer of colonised / infected patients within hospital and between other hospitals. Finally, the guidelines outline basic microbiological procedures involving sampling and processing of screening swabs and characterisation of EMRSA strains.

The cost of controlling MRSA can be high although there is general agreement that ignoring the problem can be even greater particularly when the possibility of litigation from an infected patient is considered. In their Hong Kong study, Cheng and French (1988) showed that the average cost of antimicrobial therapy per patient with MRSA bacteraemia was £440 compared to £60 for patients with MSSA bacteraemia. The greater expense was due to more costly antimicrobials and longer treatment.

1.6 Mechanism of methicillin resistance

(a) mecA and mec associated DNA

MRSA contain approximately 30 - 50 kb of additional chromosomal DNA known as the *mec* region which is not found in methicillin susceptible strains (Beck *et al.*, 1996). It is located close to the *pur-nov-his* gene cluster on the *Sma*I-G fragment of the NCTC 8325 *Staph. aureus* chromosome (Pattee *et al.*, 1990). Within the *mec* region is contained *mecA*, a structural gene for PBP

2a, a penicillin-binding protein with low affinities to practically all β-lactam antibiotics; *mec*I and *mec*R1, regulatory elements controlling *mecA* transcription; and 20 - 45 kb of *mec*-associated DNA.

The *mecA* promoter region, which is the first 300 nucleotides of *mecA* and its regulatory genes *mec*I and *mec*R1, is similar in sequence to the staphylococcal β-lactamase gene (Matsuhashi *et al.*, 1986; Song *et al.*, 1987). Introduction of *mec*A confers methicillin resistance on MSSA isolates and transposon mutagenesis renders highly resistant *Staph. aureus* strains susceptible to methicillin, therefore the principal role of the *mec*A gene in expression of methicillin resistance has been well established (Matthews and Tomasz, 1990; de Lencastre *et al.*, 1994). As the *mec*A gene is found in > 90% of clinical MRSA and is absent in MSSA strains, the presence of the *mec*A gene is considered to be the hallmark for identification of MRSA strains and many laboratories now use various PCR protocols for the detection of the *mec*A gene (Tokue *et al.*, 1992; Unal *et al.*, 1994). In addition to MRSA, the *mec*A gene is widely distributed among other species of staphylococci but has not been found in any other genus of bacteria. At present the origins of *mec*A in *Staph. aureus* are uncertain but it may have arisen in a coagulase-negative strain, possibly *Staph. sciuri* (Wu *et al.*, 1996; Wu *et al.*, 1998). It is unknown exactly how *mec*A was acquired by MRSA but transposition seems likely since *mec*A contains one or more copies of IS*257*, inverted repeats at its ends, and two open reading frames that may encode recombinases.

Two structurally different types of *mec* region DNA are known. When the *mec* region DNA of Jevons' first reported MRSA from 1961 (NCTC10442)

was compared with a Japanese strain from 1982 (N315), it was found that the MRSA from 1961 contained a *mec* region of about 32kb as opposed to a *mec* region of about 51kb in the Japanese strain. The *mec* region from NCTC 10442 was found to differ from N315 due to (1) absence of the *mec* regulator gene *mec*I, (2) a truncated version of *mec*R1 and (3) presence of part of a presumptive mobile genetic element (Hiramatsu, 1995). These are representative of the two distinct types of *mec* region DNA carried by MRSA all over the world. In addition most modern strains of MRSA carry a secondary insertion of the transposon Tn*554* integrated into their *mec* DNA which harbours genes for resistance to macrolides, lincosamides and streptogramin B (MLS).

Although the two *mec* regions have a different genetic organisation, the nucleotide sequences outside the boundaries of these regions are common to both types indicating that they integrated at exactly the same site of the *Staph. aureus* chromosome. This is highly suggestive of the acquisition of *mec* as a single primary event and subsequent divergence and modification.

(b) Expression of methicillin resistance

It is now well known that expression of methicillin resistance in *mec*A - carrying clinical strains of *Staph. aureus* is typically heterogeneous and MIC's range from susceptible (<16mg/L) to highly resistant (MIC>2000mg/L). Such a wide variation in MIC's indicates that the acquisition of the *mec*A gene alone is not sufficient to render the cell fully resistant to methicillin. By insertional inactivation of genes using the transposon Tn*551*, Berger-Bachi and co-workers highlighted the role of at least six additional *aux* or *fem* genes (Berger-Bachi,

1983; Berger-Bachi *et al.*, 1989; de Lencastre and Tomaz, 1994). These gene names were abbreviated from auxiliary or factors essential for methicillin resistance and include *fem*A - E, which are necessary for full expression of methicillin resistance. These *fem* factors are chromosomal genes located distantly to *mec* and have been shown to be present in both resistant and susceptible strains. With the exception of *fem*E whose exact function is as yet unclear, all *fem* factors are involved in peptidoglycan synthesis.

1.7 Strain identification and epidemiological typing of MRSA

Several authors have expressed different views on the evolution of MRSA. Some have argued for the evolution of MRSA from a single clone suggesting that the acquisition of the *mec*A gene occurred as a singular event, all later strains being descendants of this original single strain (Lacey and Grinstead, 1973; Kreiswirth *et al.*, 1993). Others suggest horizontal transmission of the *mec*A gene, giving rise to a number of unrelated clones (Musser and Kapur, 1992).

Irrespective of whether mono or polyclonal, MRSA have evolved into a heterogeneous group of organisms and it is now necessary to use epidemiological typing schemes to identify individual strains responsible for outbreaks of infection, to trace the sources and monitor the spread of outbreaks. Many of the genetic techniques used to type or "fingerprint" strains of MRSA can also yield valuable information about the organisms' evolution and the degree of diversity amongst strains. In the early MRSA encountered by Jevons, Cetin, Knox and others in the early 1960's, resistance to methicillin,

erythromycin (inducible) and streptomycin (high level) was typically chromosomal whereas resistance to tetracycline, β-lactamase and heavy metals was plasmid encoded. In contrast, EMRSA encountered today in the UK, Australia and Europe contain many more chromosomal resistance determinants including β-lactamase, various heavy metals, sulphonamide, trimethoprim, fusidic acid, rifampicin and gentamicin.

Epidemiological typing can be both costly and labour intensive, and should only be performed with clear objectives in mind. These include the determination of the extent of an outbreak of infection, determination of ways in which infection is spread and the evaluation of preventative measures and monitoring of infection in specific areas where infection is a particular hazard.

Numerous typing schemes for the epidemiological investigation of many bacterial species have been developed. Traditional phenotypic methods include antibiogram typing (Hartstein *et al.* 1987, Holmberg *et al.* 1984, Parisi 1985, Pfaller and Herwaldt, 1988), biotyping (Grimont and Grimont 1978, Parisi 1985, Rennie *et al.* 1978, Granato *et al.* 1983), serotyping (Crichton and Old 1980, Delmer *et al.* 1986, Poh *et al.* 1988, Joly *et al.* 1986) and phage typing (Holmberg *et al.* 1984, Parisi 1985). Molecular phenotypic techniques include immuno-blotting (Lee and Burnie 1988, Mulligan *et al.* 1988, Coia *et al.* 1990) and multi-locus enzyme electrophoresis (MLEE) (Caugant *et al.* 1986, Selander *et al.* 1986).

Genotypic techniques have evolved due to the advances in molecular biology and most utilise differences in nucleotide sequence between the organisms' genomic or extra-chromosomal DNA. These include the

determination of plasmid profiles (Schaberg *et al.* 1981, Holmberg *et al.* 1984, Hartstein *et al.* 1987, Parisi 1985, Coia *et al.* 1988, Mayer 1988, Poh *et al.* 1988) and restriction endonuclease (RE) analysis of plasmid and genomic DNA (Mickelsen *et al.* 1985, Parisi 1985, Tenover 1985, Hawkey 1987, Coia *et al.* 1988, Grothues *et al.* 1988, Mayer 1988, Renaud *et al.* 1988, Jordens and Hall 1988, Patterson *et al.* 1989). Newer techniques have been developed which avoid interpretation of large numbers of fragments. These include pulse-field gel electrophoresis (PFGE), (Ichiyama *et al.* 1991, Prevost *et al.* 1992, Struelens *et al.* 1992, Wei and Grubb 1992) and Southern hybridisation techniques using a variety of DNA and RNA probes (Kreiswirth *et al.* 1990, Goering and Duensing 1990, Hadorn *et al.* 1990, Schwarzkopf and Karch 1994).

All typing methods have advantages and disadvantages in any given situation dependent upon reasons for typing and the degree of discrimination required.

Phenotypic typing methods depend on the expression of markers, for example antibiotic resistance or the production of a particular enzyme. A major limitation of this approach is that phenotypic markers are not always stably expressed (e.g. antibiotic resistance mediated by mobile genetic elements) under different cultural or environmental conditions. The genetic basis of the phenotypic variability is usually unknown and the observed phenotypic variations can often be caused by more than one type of genetic event, as is the case with staphylococcal bacteriophage typing (Kreiswirth *et al.*,1993). Not all phenotypic methods can assign an isolate to a definite type.

This becomes a limitation when phage typing MRSA, as many strains are non-typeable. Another major disadvantage of phenotypic typing methods is that they do not demonstrate relationships between types.

In addition, techniques such as phage typing require careful standardisation and are therefore generally only performed by reference laboratories.

In contrast, systems based on DNA analysis will always place an isolate into a "type", and the techniques are not limited to specific organisms or groups of organisms. For each technique the method is virtually identical and uses the same reagents with only minor changes regardless of the source of the DNA.

The principle involved in genomic fingerprinting is that the chromosome contains regions that are highly conserved, (generally containing sequences for proteins vital for cell function or sequences for rRNA) and other regions in which the DNA is subject to rearrangements and mutations. When a mutation occurs in a restriction site the DNA is not cleaved and differences in fragment size and number can be demonstrated between isolates with non-identical chromosomes. The degree of difference between a set of isolates' DNA fingerprints gives an indication as to whether the isolates are related, identical or different. Although mutations can occur in highly conserved regions of the chromosome these tend to be lethal and are therefore not passed on. These differences in fragment sizes between a group of related organisms are known as restriction fragment length polymorphisms or RFLP's.

Natural mutations occur in bacteria over time and may result in formation of RFLP's within a single strain. It is therefore important to bear this

in mind when examining isolates recovered from outbreaks spanning many months or years, as is the case with the "Lisbon" strain which has persisted in Scotland since its introduction in 1990.

All of the techniques mentioned thus far have been extensively applied and evaluated either alone or in various combinations in studying the clinical epidemiology of a wide range of organisms causing nosocomial infection. For example, Archer and Mayhall (1983) used antibiogram, phage type, aminoglycoside inactivating enzymes and plasmid profiles to trace an outbreak of nosocomial MRSA infection. They found 17 patients, 12 environmental sites and three hospital personnel to be infected or colonised with the epidemic strain. The outbreak strain was rifampicin resistant and all indigenous strains were sensitive to rifampicin thus making the antibiogram a very useful marker with which to initially screen large numbers of specimens. They found phage typing to be poorly reproducible within local laboratories and results too slow to be of any immediate epidemiological value. These findings were consistent with the general view that phage typing of staphylococci is a skilled technique, requires specialist knowledge and expertise, does not always provide discrimination and as such is best performed by reference centres. Plasmid pattern analysis revealed all isolates of the epidemic strain to contain three plasmids of 34, 1.8, and 1.5 megadaltons. No other strains examined exhibited this profile. They concluded that plasmid analysis was of greatest value in this study and recommended its use in future epidemiological investigations.

Coia *et al.* (1990) used the greater discriminatory powers of restriction enzyme fingerprinting of plasmid DNA in conjunction with simple biotyping,

phage typing and immunoblotting of exported proteins to characterise a collection of 45 MRSA isolates from Glasgow Royal Infirmary between 1985 - 1986. Using *Hae* III to digest the plasmid DNA they were able to group 43 of the 45 isolates into two major groups that correlated strongly with the two major immunoblot groups found. The techniques used were able to provide a breadth of epidemiological information, confirming the existence of two major clones within the hospital, which however was evident from antibiogram and biotype analysis.

In a multi-institutional study to determine the strengths and weaknesses of 12 epidemiological typing systems, Tenover and co-workers (1994) compared traditional methods such as phage typing, biotyping and antibiogram typing with more modern molecular typing including RFLP typing with gene probes, IS probe typing, FIGE, PFGE, immunoblotting, MLEE, restriction enzyme (RE) analysis of PCR products (coagulase genes), ribotyping and RE analysis of plasmid DNA. Although the molecular techniques were highly successful in identifying the outbreak strains, as stated previously they are for the most part difficult to perform, expensive and require a considerable amount of expertise in the interpretation of results. Overall, no single method was found to be obviously superior and as other workers have also found (Parisi, 1985) a carefully selected combination of techniques dependent on the organism to be typed is often the most useful approach to epidemiological typing.

With the range of typing techniques now available, a very detailed picture of the epidemiology of infectious agents is often possible allowing

identification of individual strains or clones to be made and their relationships with other strains and clones of an individual species to be ascertained.

1.8 MRSA in the West of Scotland

Outbreaks of infection due to epidemic strains of methicillin-resistant *Staph. aureus* have been well documented in other parts of the UK, however in Scotland less MRSA data has been published and the picture has been less clear. Figures obtained from the Scottish Centre for Infection and Environmental Health (SCIEH) suggest that prior to 1990 there were relatively few reports of MRSA, and of these the majority were from Greater Glasgow Health Board (Figures 1.2 and 1.3). In 1991 there was a sudden increase in notifications which corresponded to the arrival of the Lisbon strain in Glasgow. Following this increase the numbers remained steady until 1995 after which numbers rose sharply to more than 4000 in 1997 and this figure is still rising in 1998. The sudden increase in MRSA from 1995 onward represented the arrival in Scotland of EMRSA-15 and 16.

Preliminary phenotyping (simple biotyping, antibiogram and phage typing) of MRSA strains sent from laboratories in the West of Scotland to Dr Dugald Baird at Glasgow Royal Infirmary led to recognition of an unusual strain of MRSA. The strain was urease positive, Tween 80 hydrolysis negative, resistant by disc diffusion test to erythromycin, clindamycin, tetracycline, ciprofloxacin, rifampicin and all aminoglycosides in addition to β-lactam antibiotics. It was sensitive to trimethoprim, fusidic acid, mupirocin and

chloramphenicol. Most strains were phage type 29/77/84/85 or 54/77/84/85. A few strains were untypable by phage.

Figure 1.2

Number of MRSA notifications to SCIEH during the period 1986 - 1997

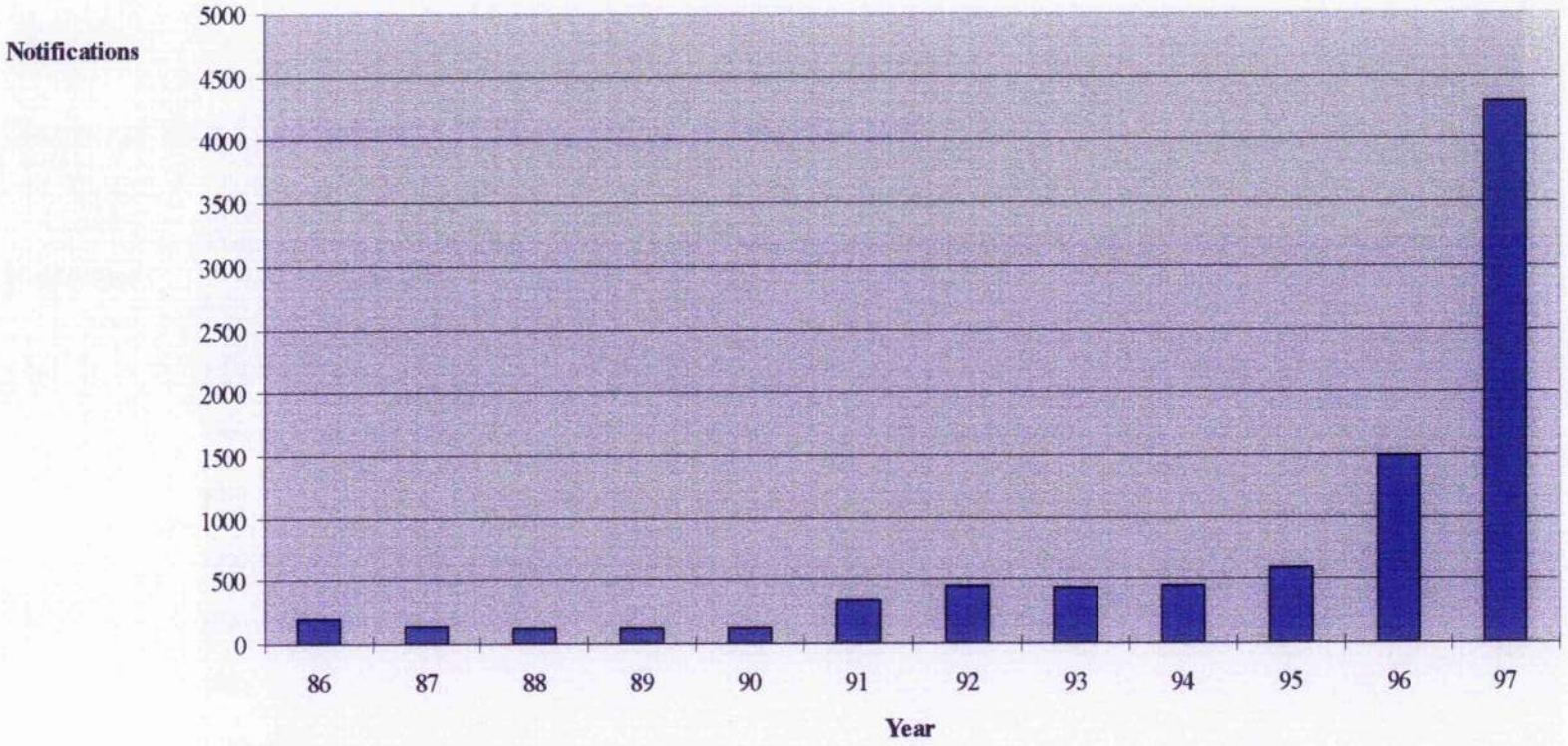
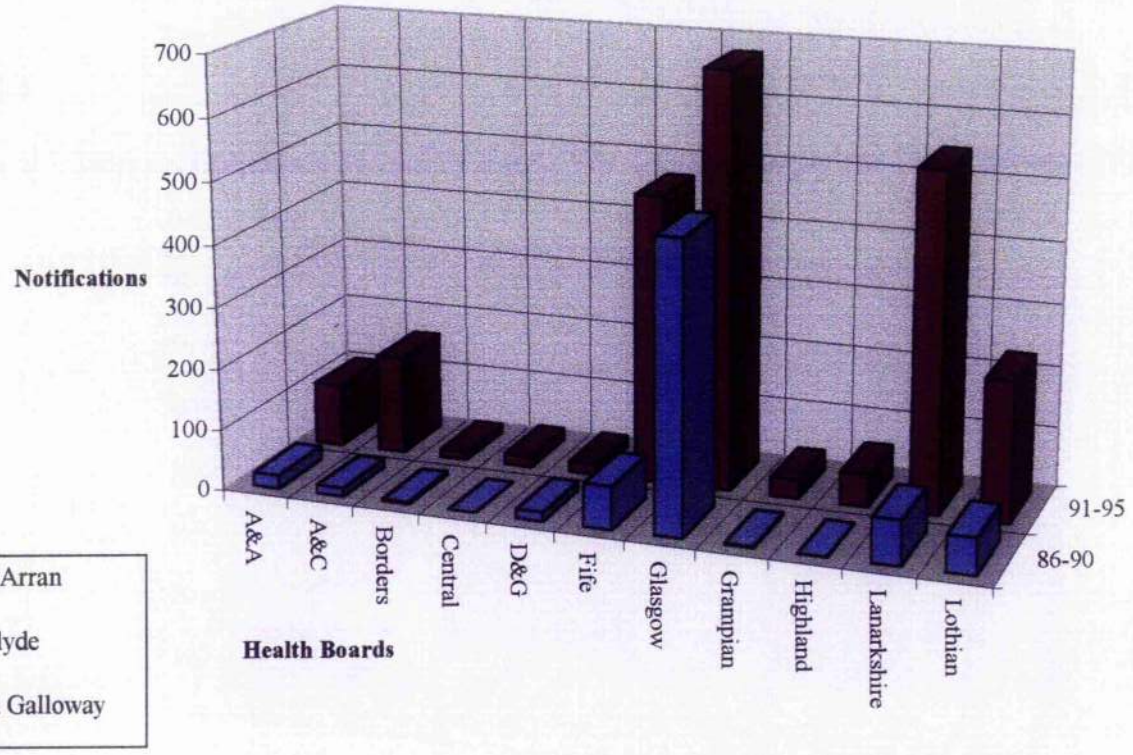


Figure 1.3

Number of MRSA notifications to SCIEH by Health Board during the periods 1986 - 1990 and 1991 - 1995



A&A: Ayrshire and Arran

A&C: Argyle and Clyde

D&G: Dumfries and Galloway

Subsequent investigations suggested that the earliest recognisable isolate in Scotland had been obtained in late 1990 from a patient admitted to Gartnavel General Hospital in Glasgow, from Lisbon, Portugal. Soon after this initial isolation the strain was also detected in the nearby Glasgow Western Infirmary, and over the next few years it was isolated from patients in most acute hospitals in the West of Scotland. It resulted in a wide range of staphylococcal infections as well as much colonisation and it produced infection control problems similar to those seen in England with other epidemic MRSA strains. The strain was referred to locally as the " Lisbon" strain.

1.9 Aims of the investigation and study design

(a) Aims of the investigation

The aims of the investigation were as follows:

1. To establish whether epidemiologically unrelated isolates of MRSA were diverse on the basis of *Hha*I and *Sau3A*I REFP's.
2. To assess the diversity of *Staph. aureus* from different epidemiological groups.
3. To determine whether those isolates phenotypically similar and designated the Lisbon strain were genotypically consistent with the expansion of a single clone.
4. To determine whether *Hha*I REFP's were sufficiently discriminating to allow recognition of variants.

5. To determine whether this strain was closely related to other MRSA, EMRSA or MSSA

By examination of any REFP's produced following digestion and fragment separation in agarose gels, it was hoped to gain an insight into the strains' evolution since its first appearance in the West of Scotland, and by study of all of the data generated, gain a greater understanding of the epidemiology of infections caused by "Lisbon strain" MRSA.

(b) Techniques used to characterise the "Lisbon strain"

The isolates investigated in this study comprised a representative number (93) of Lisbon strain MRSA from a collection gathered by Dr D.R. Baird. Temporally, the isolates spanned a period from 1990 to 1995. The sources of the isolates covered an area that encompassed five Health Boards. Prior to their inclusion in the study, extensive phenotyping of the isolates was carried out at Glasgow Royal Infirmary and Hairmyres Hospitals with phage typing being performed initially at Gartnavel Hospital and latterly at the Victoria Infirmary, Glasgow. The isolates were tested by disc diffusion method for sensitivity to penicillin, methicillin, erythromycin, clindamycin, neomycin, kanamycin, streptomycin, gentamicin, netilmicin amikacin, tetracycline, sulphonamide, trimethoprim, fusidic acid, chloramphenicol, rifampicin, vancomycin, mupirocin and ciprofloxacin. Tests performed to obtain a simple biotype included Tween 80 hydrolysis, urease production, tube coagulase, latex slide coagulase (Staphaurex™, Murex diagnostics Ltd.), latex / RBC

haemagglutination slide coagulase (Slidex™, Biomerieux) and DNase production in agar (Oxoid, Unipath) following overnight incubation.

The results of initial phenotypic typing had shown all isolates to be closely related. Isolates that conformed to the "Lisbon" strain phenotype were included in the study, namely, rifampicin resistant, aminoglycoside resistant, urease positive and Tween 80 negative. All phenotypic tests were repeated as part of the study.

On the basis that genotypic typing can provide a greater degree of discrimination and can give insights into the evolution and divergence of clonal populations, a molecular approach was undertaken to study these isolates using RE analysis of genomic DNA. The restriction enzymes used for this analysis were the four-base cutters *Hha*I (recognition site: GCG' C) and *Sau3A*I ('GATC), as opposed to the six-base cutters used by other authors (Jordens and Hall, 1988; Hall *et al*., 1989; Matthews *et al*., 1992). The four base cutters result in a number of large DNA fragments (approximately 4-20kb) which can be clearly resolved on an agarose gel.

A number of restriction enzymes had been screened previously for their suitability to type *Staph. aureus* and on this basis *Hha*I and *Sau3A*I were selected for use in this study (D. Platt, personal communication).

To evaluate the usefulness of this technique with the chosen enzymes for detecting RFLP's, the genetic relatedness of "Lisbon" MRSA to other MRSA (OMRSA) strains and to MSSA strains from a variety of sources was also examined. If the restriction enzyme fragmentation patterns (REFP) of

"Lisbon strain" isolates appeared similar to unrelated strains, then the inference was that the region of the genome optimally separated by a particular RE under specific gel running conditions was too highly conserved and therefore the technique as an epidemiological typing tool was not discriminatory enough when used with these enzymes. Restriction enzyme analysis of plasmid DNA using the same enzyme was also performed on selected isolates in order to ascertain to what degree plasmid DNA contributed to these REFP's or indeed the overall genomic fingerprint.

2. MATERIALS & METHODS

2.1 Collection of isolates

(a) Lisbon isolates

One hundred isolates of MRSA previously categorised as Lisbon strains on the basis of phenotypic typing methods (Table 2.1) were obtained from the collection of Dr. D. Baird at Hairmyres Hospital in East Kilbride for inclusion in the study. All isolates were subcultured to horse blood agar to confirm purity and identity as *Staph. aureus* isolates by Gram stain and Slidex test (Biomerieux).

The Lisbon isolates were originally isolated from a wide area of the West of Scotland; from Dumfries in the south-west to Oban in the north-west and included both community and hospital acquired isolates. The majority of these isolates were collected between October 1992 and October 1993, although a number of early isolates from the Western Infirmary in Glasgow (1990) were also included, as were some later isolates of the strain from 1995.

They represented isolates from a wide range of clinical conditions; from nasal carriage and colonisation through localised infection to septicaemia.

Table 2.1

Phenotypic characteristics of Lisbon strain MRSA

Tween 80 Hydrolysis	*Urease*	*Staphaurex*	*Phage type*	*Resistant to*
NEGATIVE	POSITIVE	MOSTLY NEGATIVE (60%)	54/77/84/85 29/77/84/85	MET,ER,CD TET.CIP,RIF AMINO SU

MET: methicillin, ER: erythromycin, CD: clindamycin, TET: tetracycline, CIP: ciprofloxacin RIF: rifampicin, AMINO: all classes of aminoglycosides, SU: sulphamethoxazole

(b) Control groups

A number of control groups were included in the study to enable comparisons to be made between the Lisbon strain and other groups of both related and unrelated strains of *Staph. aureus*. This included isolates of epidemiologically unrelated MSSA. These were included in order to show that the enzymes chosen had sufficient resolving power to type known unrelated isolates as different strains and in so doing obtain an estimate of the expected breadth of diversity among unrelated isolates of the same species. Isolates of EMRSA (EMRSA-1, 15 and 16) were included to determine the sensitivity of the typing systems in identifying them as belonging to these types. Isolates of sporadic MRSA were included in order to determine relationships (if any) between these and the other MRSA clonal groups. Each of these MRSA groups was also chosen to provide some insight to the diversity of MRSA when compared within their groups, between the groups and with MSSA.

A strain of *Staph. hyicus* was also included to illustrate the genetic distance between different strains of *Staph. aureus* and a different species of coagulase positive *Staphylococcus* and to provide an outlier for the construction of an evolutionary tree (Saitou and Nei, 1987).

These strains were collected from various sources as shown in Table 2.2, and their identity was confirmed as described above.

2.2 Storage of isolates

Three to four well isolated colonies from each isolate grown in air overnight at 37°C on Columbia horse blood agar were inoculated into a Protect™ vial (Technical Service Consultants) as per manufacturer's instructions and stored at -70°C. When required, isolates were revived from Protect™ by sub culture of a bead onto horse blood agar. Each isolate was kept on this medium at 4°C for short-term maintenance prior to all tests.

2.3 Antimicrobial susceptibility testing

Susceptibility tests were performed by disc diffusion on lysed blood DST agar (Stokes and Ridgway, 1980) against the following agents: methicillin 5μg (MET), penicillin 1U (PEN), erythromycin 5μg (ERY), clindamycin 10μg (CD), fusidic acid 10μg (FUS), ciprofloxacin 1μg (CIP), mupirocin 5μg (MUP), tetracycline 10μg (TET), chloramphenicol 10μg (CM), rifampicin 2μg (RIF), sulphamethoxazole 25μg (SU), trimethoprim 1.25μg (TM), neomycin 10μg (NM), kanamycin 30μg (KM), streptomycin 10μg (SM), gentamicin 10μg (GM), netilmicin10μg (NET), and amikacin 30μg (AK).

Table 2.2

Origin of isolates selected for study

STRAIN No.	STUDY TYPE	HOSPITAL	SITE	DATE
AB 01	LISBON	CLYDEBANK HEALTH CENTRE	PUS	09-Nov-92
AB 02	LISBON	NK[1]	NK	NK
AB 03	LISBON	NK	NK	NK
AB 04	LISBON	NK	NK	NK
AB 05	LISBON	BALLOCHMYLE HOSPITAL	GROIN WOUND	23-Oct-92
AB 06	LISBON	BALLOCHMYLE HOSPITAL	SPUTUM	22-Jul-93
AB 07	LISBON	CROSSHOUSE HOSPITAL	HIP WOUND	11-May-93
AB 08	LISBON	CROSSHOUSE HOSPITAL	PERINEUM	17-May-93
AB 09	LISBON	CROSSHOUSE HOSPITAL	EYE	26-May-93
AB 10	LISBON	CROSSHOUSE HOSPITAL	PERINEUM	31-May-93
AB 11	LISBON	CROSSHOUSE HOSPITAL	HIP WOUND	31-May-93
AB 12	LISBON	CROSSHOUSE HOSPITAL	NASAL	01-Jun-93
AB 13	LISBON	CROSSHOUSE HOSPITAL	VARICOSE ULCER	31-May-93
AB 14	LISBON	LARGS HEALTH CENTRE	VARICOSE ULCER	03-Jun-93
AB 15	LISBON	CROSSHOUSE HOSPITAL	CENTRAL LINE EXIT	NK
AB 16	LISBON	CROSSHOUSE HOSPITAL	NK	NK
AB 17	LISBON	CROSSHOUSE HOSPITAL	NK	NK
AB 18	LISBON	CROSSHOUSE HOSPITAL	NK	
AB 19	LISBON	DUMFRIES ROYAL INFIRMARY	NK	
AB 20	LISBON	DUMFRIES ROYAL INFIRMARY	NK	
AB 21	LISBON	GLASGOW ROYAL INFIRMARY	GASTROSTOMY	17-Nov-93
AB 22	LISBON	GLASGOW ROYAL INFIRMARY	WOUND DRAIN	10-Mar-93
AB 23	LISBON	GLASGOW ROYAL INFIRMARY	ABDOMEN WOUND	11-May-93
AB 24	LISBON	GLASGOW ROYAL INFIRMARY	BREAST ULCER	27-May-93
AB 25	LISBON	GLASGOW ROYAL INFIRMARY	URINE	07-Jun-93
AB 26	LISBON	GLASGOW ROYAL INFIRMARY	URINE	12-Jul-93
AB 27	LISBON	GLASGOW ROYAL INFIRMARY	EYE	26-Jul-93
AB 28	LISBON	GLASGOW ROYAL INFIRMARY	URINE	26-Jul-93
AB 29	LISBON	GLASGOW ROYAL INFIRMARY	BLOOD	02-Aug-95
AB 30	LISBON	GLASGOW ROYAL INFIRMARY	URINE	01-Aug-93

Table 2.2 Cont.

STRAIN No.	STUDY TYPE	HOSPITAL	SITE	DATE
AB 31	LISBON	GLASGOW ROYAL INFIRMARY	ABDOMEN DRAIN	11-Aug-93
AB 32	LISBON	GLASGOW ROYAL INFIRMARY	URINE	12-Sep-92
AB 33	LISBON	GLASGOW ROYAL INFIRMARY	NK	26-Nov-92
AB 34	LISBON	GLASGOW ROYAL INFIRMARY	BLOOD	14-Feb-93
AB 35	LISBON	INVERCLYDE HOSPITAL	URINE	
AB 36	LISBON	INVERCLYDE HOSPITAL	NK	
AB 37	LISBON	LAW HOSPITAL	URINE	05-Nov-92
AB 38	LISBON	LIGHTBURN HOSPITAL	EYE	15-Dec-92
AB 39	LISBON	CUMBERNAULD HEALTH CENTRE	WOUND	05-May-93
AB 40	LISBON	HAMILTON HEALTH CENTRE	ABDOMEN WOUND	21-Jul-93
AB 41	LISBON	MONKLANDS HOSPITAL	WOUND	14-Sep-93
AB 42	LISBON	ROYAL ALEXANDRIA HOSPITAL	GROIN	09-Aug-93
AB 43	LISBON	ROYAL ALEXANDRIA HOSPITAL	WOUND	21-Sep-93
AB 44	LISBON	BARRHEAD HEALTH CENTRE	WOUND	21-Sep-93
AB 45	LISBON	ALEXANDRIA HEALTH CENTRE	FOOT ULCER	04-May-93
AB 46	LISBON	VALE OF LEVEN HOSPITAL	TRACHEOSTOMY	07-May-93
AB 47	LISBON	VALE OF LEVEN HOSPITAL	URINE	10-May-93
AB 48	LISBON	VALE OF LEVEN HOSPITAL	WOUND DRAIN	09-Jul-93
AB 49	LISBON	VALE OF LEVEN HOSPITAL	GROIN	16-Aug-93
AB 50	LISBON	VALE OF LEVEN HOSPITAL	NK	
AB 51	LISBON	HELENSBURGH HEALTH CENTRE	WOUND	06-Aug-93
AB 52	LISBON	STOBHILL HOSPITAL	PERM CATHETER	03-Aug-93
AB 53	LISBON	STOBHILL HOSPITAL	STUMP WOUND	29-Sep-93
AB 54	LISBON	STOBHILL HOSPITAL	BLOOD	29-Sep-93
AB 55	LISBON	GLASGOW ROYAL INFIRMARY	PENIS	07-Nov-94
AB 56	LISBON	GLASGOW ROYAL INFIRMARY	PERISPLENIC ABSCESS	19-Jul-94
AB 57	LISBON	GLASGOW ROYAL INFIRMARY	NEPHROSTOMY URINE	06-Jul-94
AB 58	LISBON	STOBHILL HOSPITAL	WOUND	23-Aug-93
AB 59	LISBON	GLASGOW WESTERN INFIRMARY	BLOOD	01-Dec-90
AB 60	LISBON	CROSSHOUSE HOSPITAL	SKIN	05-Feb-91
AB 61	LISBON	CROSSHOUSE HOSPITAL	SPUTUM	04-Mar-91

Table 2.2 Cont.

STRAIN No.	STUDY TYPE	HOSPITAL	SITE	DATE
AB 62	LISBON	GLASGOW ROYAL INFIRMARY	CATHETER EXIT SITE	17-Jun-91
AB 63	LISBON	CANNIESBURN HOSPITAL	NECK WOUND	21-Aug-91
AB 64	LISBON	GLASGOW WESTERN INFIRMARY	NK	
AB 65	LISBON	INVERCLYDE HOSPITAL	CSF DRAIN	24-Sep-91
AB 66	LISBON	STOBHILL HOSPITAL	BLOOD	26-Oct-94
AB 67	LISBON	QUEEN ELIZ. HOSPITAL	NK	
AB 68	LISBON	STOBHILL HOSPITAL	PERM CATHETER	01-Aug-95
AB 69	LISBON	CANNIESBURN HOSPITAL	FLAP	20-Jul-93
AB 70	LISBON	GLASGOW ROYAL INFIRMARY	# NECK OF FEMUR	10-Jun-93
AB 71	LISBON	GLASGOW ROYAL INFIRMARY	NK	10-Jun-93
AB 72	LISBON	GLASGOW ROYAL INFIRMARY	BLOOD	30-Jun-93
AB 73	LISBON	GLASGOW ROYAL INFIRMARY	TISSUE	10-Nov-93
AB 74	LISBON	GLASGOW ROYAL INFIRMARY	URINE	12-Jun-91
AB 75	LISBON	STOBHILL HOSPITAL	APPENDECTOMY WOUND	02-May-96
AB 76	LISBON	CROSSHOUSE HOSPITAL	NK	
AB 77	LISBON	CROSSHOUSE HOSPITAL	NK	
AB 78	LISBON	GLASGOW ROYAL INFIRMARY	BLOOD	30-Oct-92
AB 79	LISBON	ROYAL ALEXANDRIA HOSPITAL	NASAL	07-Jun-93
AB 80	LISBON	GLASGOW WESTERN INFIRMARY	TRACHEAL ASPIRATE	01-Dec-90
AB 81	LISBON	QUEEN ELIZABETH HOSPITAL	NK	
AB 82	LISBON	GARRICK (DUMFRIES)	FOOT WOUND	13-Aug-93
AB 83	LISBON	VALE OF LEVEN HOSPITAL	TRACHEOSTOMY	08-Dec-93
AB 84	LISBON	CANNIESBURN HOSPITAL	BREAST DISCHARGE	20-Oct-92
AB 85	LISBON	GLASGOW WESTERN INFIRMARY	TRACHEOSTOMY	
AB 86	LISBON	NK	NK	
AB 87	LISBON	NEWMAINS HEALTH CENTRE (LAW)	WOUND (PIN TRACT)	30-Jul-93
AB 88	LISBON	GLASGOW ROYAL INFIRMARY	NK	
AB 89	LISBON	GLASGOW ROYAL INFIRMARY	SPUTUM	21-Feb-95
AB 90	LISBON	CROSSHOUSE HOSPITAL	TOE WOUND	16-Jan-91
AB 91	LISBON	GLASGOW ROYAL INFIRMARY	TRACHEAL ASPIRATE	22-Sep-91
AB 92	LISBON	LIGHTBURN HOSPITAL	NK	16-Nov-94

Table 2.2 Cont.

STRAIN No.	STUDY TYPE	HOSPITAL	SITE	DATE
AB 93	LISBON	BALLOCHMYLE HOSPITAL	CHEST DRAIN	16-Sep-91
AB 100	EMRSA 15	DALRYMPLE HOSPITAL (DUMFRIES)	WOUND	27-Sep-93
AB 101	EMRSA 15	GLASGOW ROYAL INFIRMARY	NK	
AB 102	EMRSA 15	GLASGOW ROYAL INFIRMARY	ANAL	
AB 103	EMRSA 15	GLASGOW ROYAL INFIRMARY	CHEST WOUND	
AB 104	EMRSA 15	GLASGOW ROYAL INFIRMARY	TRACHEAL ASPIRATE	
AB 105	EMRSA 15	GLASGOW ROYAL INFIRMARY	SPUTUM	17-Jan-96
AB 106	EMRSA 15	GLASGOW ROYAL INFIRMARY	SPUTUM	
AB 107	EMRSA 15	GLASGOW ROYAL INFIRMARY	SPUTUM	
AB 108	EMRSA 15	GLASGOW ROYAL INFIRMARY	STUMP WOUND	11-Jan-96
AB 109	EMRSA 15	STOBHILL HOSPITAL	BLOOD	13-Sep-95
AB 110	EMRSA 15	STOBHILL HOSPITAL	FEMORAL LINE SITE	22-Jun-95
AB 111	EMRSA 15	STOBHILL HOSPITAL	NOSE AND FISTULA	21-Jul-95
AB 112	EMRSA 15	STOBHILL HOSPITAL	PD EXIT SITE	24-Jun-95
AB 113	EMRSA 15	STOBHILL HOSPITAL	SPUTUM	14-Jul-95
AB 114	EMRSA 15	VALE OF LEVEN HOSPITAL	WOUND	09-Sep-94
AB 115	EMRSA 15	WESTERN GENERAL HOSPITAL	NK	
AB 116	EMRSA 15	WESTERN GENERAL HOSPITAL	URINE	02-Nov-94
AB 117	EMRSA 15	WESTERN GENERAL HOSPITAL	THROAT	04-Nov-94
AB 118	EMRSA 15	YORKHILL HOSPITAL	THROAT	
AB 119	EMRSA 16	NK	NK	
AB 120	EMRSA 16	DUMFRIES ROYAL INFIRMARY	ENDO TRACHEAL TUBE	
AB 121	EMRSA 16	DUMFRIES ROYAL INFIRMARY	NASAL	
AB 122	EMRSA 16	DUMFRIES ROYAL INFIRMARY	TRACHEAL ASPIRATE	
AB 123	EMRSA 16	DUMFRIES ROYAL INFIRMARY	WOUND	
AB 124	EMRSA 16	GLASGOW ROYAL INFIRMARY	TRACHEAL SITE	
AB 125	EMRSA 16	GLASGOW ROYAL INFIRMARY	WOUND	
AB 126	EMRSA 16	HEALTH CARE INTERNATIONAL	CHEST WOUND	
AB 127	EMRSA 16	HEALTH CARE INTERNATIONAL	ORTHOPAEDIC WOUND	
AB 128	EMRSA 16	MONKLANDS HOSPITAL	BILE	
AB 129	EMRSA 16	MONKLANDS HOSPITAL	GROIN	

Table 2.2 Cont.

STRAIN No.	STUDY TYPE	HOSPITAL	SITE	DATE
AB 130	EMRSA 16	MONKLANDS HOSPITAL	PUS	
AB 131	EMRSA 16	MONKLANDS HOSPITAL	SPUTUM	
AB 132	EMRSA 16	STOBHILL HOSPITAL	SPUTUM	
AB 133	EMRSA 16	WESTERN GENERAL HOSPITAL	NASAL	
AB 134	EMRSA 16	WESTERN GENERAL HOSPITAL	SPUTUM	
AB 135	EMRSA 16	WESTERN GENERAL HOSPITAL	SPUTUM	
AB 136	EMRSA 1	BRISBANE, AUSTRALIA	NK	
AB 137	EMRSA 1	BRISBANE	NK	
AB 138	EMRSA 1	BRISBANE	NK	
AB 139	EMRSA 1	BRISBANE	NK	
AB 140	EMRSA 1	BRISBANE	NK	
AB 141	EMRSA 1	ST. BARTHOLEMEWS	NK	
AB 142	EMRSA 1	ST. BARTHOLEMEWS	NK	
AB 143	EMRSA 1	ST. THOMAS	NK	
AB 146	ENST[2]	BELVIDERE	FOOT WOUND	
AB 147	ENST	CROSSHOUSE HOSPITAL	BOIL	
AB 148	ENST	FOURHILLS NH	SACRAL	
AB 149	ENST	HARTWOOD (LAW)	ECZEMA	
AB 150	ENST	LENZIE	FOOT WOUND	
AB 151	ENST	MONKLANDS HOSPITAL	NK	
AB 152	ENST	STOBHILL HOSPITAL	ARTERIAL LINE TIP	
AB 153	ENST	STOBHILL HOSPITAL	CENTRAL LINE TIP	
AB 154	ENST	STOBHILL HOSPITAL	CENTRAL LINE TIP	
AB 155	ENST	STOBHILL HOSPITAL	LEG	
AB 156	ENST	STOBHILL HOSPITAL	LEG	
AB 157	ENST	STOBHILL HOSPITAL	LEG	
AB 158	ENST	STOBHILL HOSPITAL	PEG TUBE	
AB 159	ENST	STOBHILL HOSPITAL	RIGHT HEEL	
AB 160	ENST	STOBHILL HOSPITAL	SACRAL	
AB 161	ENST	STOBHILL HOSPITAL	SKIN	
AB 162	ENST	STOBHILL HOSPITAL	SKIN	

Table 2.2 Cont.

STRAIN No.	STUDY TYPE	HOSPITAL	SITE	DATE
AB 163	ENST	STOBHILL HOSPITAL	SKIN	
AB 164	ENST	STOBHILL HOSPITAL	SKIN	
AB 165	ENST	STOBHILL HOSPITAL	THROAT	
AB 166	ENST	STOBHILL HOSPITAL	URETHRAL	
AB 167	ENST	STOBHILL HOSPITAL	URINE	
AB 168	ENST	STOBHILL HOSPITAL	WOUND	
AB 169	ENST	WOODSIDE HEALTH CENTRE	TOE WOUND	
AB 170	ENST	YORKHILL HOSPITAL	LEG WOUND	
AB 144	SPORADIC	ROYAL ALEXANDRIA HOSPITAL	EAR	
AB 145	SPORADIC	ROYAL ALEXANDRIA HOSPITAL	WOUND	
AB 171	SPORADIC	CROSSHOUSE HOSPITAL	LEG WOUND	
AB 173	SPORADIC	CROSSHOUSE HOSPITAL	FOOT WOUND	
AB 174	SPORADIC	GLASGOW ROYAL INFIRMARY	BACK RASH	
AB 175	SPORADIC	MONKLANDS HOSPITAL	NK	
AB 176	SPORADIC	VALE OF LEVEN HOSPITAL	WOUND	
AB 177	SPORADIC	HEALTH CARE INTERNATIONAL	BLOOD	
AB 179	MSSA	BOVINE	MASTITIS	
AB 184	MSSA	BOVINE	MASTITIS	
AB 192	MSSA	COMMUNITY	NASAL	10-May-93
AB 193	MSSA	COMMUNITY	NASAL	11-May-93
AB 194	MSSA	COMMUNITY	NASAL	12-May-93
AB 187	MSSA	COMMUNITY	NASAL	13-May-93
AB 211	MSSA	STOBHILL HOSPITAL	LEFT BREAST ABSCESS	02-Dec-93
AB 212	MSSA	STOBHILL HOSPITAL	LEFT EAR	10-Dec-93
AB 205	MSSA	STOBHILL HOSPITAL	ABDOMEN WOUND	06-Dec-93
AB 215	MSSA	STOBHILL HOSPITAL	SINUS TISSUE	10-Dec-93
AB 201	MSSA	RUCHILL HOSPITAL	GROIN ABSCESS	13-Dec-93
AB 219	MSSA	WOODILEE HOSPITAL	RIGHT EYE	03-Dec-93
AB 196	MSSA	GENERAL PRACTICE (GP)	EAR	13-Feb-95
AB 197	MSSA	GP	PERIURETHRAL	13-Feb-95
AB 198	MSSA	GP	NASAL	10-Feb-95

Table 2.2 Cont.

STRAIN No.	STUDY TYPE	HOSPITAL	SITE	DATE
AB 199	MSSA	GP	LEG WOUND	11-Feb-95
AB 200	MSSA	GP	ULCER	13-Feb-95
AB 220	FRENCH STRAIN	GLASGOW ROYAL INFIRMARY	NK	30-Oct-90
AB 221	FRENCH	GLASGOW ROYAL INFIRMARY	NK	15-Nov-90
AB 222	FRENCH	GLASGOW ROYAL INFIRMARY	NK	01-Oct-90
AB 223	FRENCH	GLASGOW ROYAL INFIRMARY	NK	01-Nov-90
AB 224	FRENCH	GLASGOW ROYAL INFIRMARY	NK	27-Nov-90
AB 225	FRENCH	GLASGOW ROYAL INFIRMARY	NK	18-Oct-90
AB 226	FRENCH	GLASGOW ROYAL INFIRMARY	NK	27-Nov-90
AB 227	FRENCH	GLASGOW ROYAL INFIRMARY	NK	18-Nov-90
AB 228	FRENCH	GLASGOW ROYAL INFIRMARY	NK	15-Nov-90

[1] Not Known; [2] Endemic Stobhill MRSA

The inoculum used for performing sensitivity tests was standardised by emulsifying a portion from 2 - 3 well-isolated single colonies into a 5ml volume of sterile water. The sensitive control strain used was the Oxford *Staph.* (NCTC 6751) and the criteria used for interpretation of sensitivity were:

Sensitive - zone radius equal, wider or not more than 3 mm smaller than the control strain.

Resistant - a zone of 2 mm radius or less.

2.4 Bacteriophage typing

All "Lisbon" isolates were phage typed using the International Set of Typing Phages (Blair and Williams, 1961) for *Staph. aureus* initially at Gartnavel Hospital in Glasgow and subsequently at the Bacteriology Department of the Victoria Infirmary, Glasgow.

2.5 Biotyping

A simplified scheme was used which had been previously employed by Coia *et al.* (1990) to help define outbreaks of infection caused by local strains of MRSA at Glasgow Royal Infirmary. All Lisbon isolates were initially characterised at the bacteriology department of Glasgow Royal Infirmary or Hairmyres Hospitals.

(a) Urease production

Two to three single colonies were inoculated into 2ml volumes in bijoux of brain heart infusion (BHI) broth (Oxoid CM225) containing phenol red and 2% urea which was then incubated for 18-48 hours at 37°C (Coia *et al.*, 1990).

(b) Tween 80 hydrolysis

Hydrolysis of Tween 80 was detected on plates containing 1% Tween 80 (BDH) in nutrient agar (Nutrient broth, Oxoid CM1, containing 1% bacteriological agar, Oxoid L11). Ten isolates including a positive and negative control were spotted onto each plate and incubated for up to 3 days at 37°C (Coia *et al.*, 1990).

2.6 Genomic fingerprinting

(a) Buffers and Reagents

Details of all buffers and reagents used are given in appendix I.

(b) Centrifugation

All microcentrifugation was carried out at 13000g in a Heraeus microcentrifuge. Larger volumes (5-10ml) were centrifuged at 3000g in a Mistral 1000 centrifuge (MSE).

(c) Extraction and purification of genomic DNA

Genomic DNA was extracted and purified for restriction enzyme fingerprinting by the method of Platt et al., (1996). A well-isolated single colony from a pure overnight growth of the organism on horse blood agar was inoculated into 10ml of Todd-Hewitt broth and incubated at 37°C for 18-20 hours.

The culture was then centrifuged for 10 minutes, the supernatant fluid discarded and the pellet resuspended in 3ml of Tris-EDTA-sodium chloride buffer (TES). This suspension was divided between 3 sterile Eppendorf tubes, microcentrifuged for 30seconds and the deposit resuspended in 200µl of TESS.

To this suspension, 100µl of lysozyme (40mg/ml) and 20µl of lysostaphin (1000units/ml) was added. The suspension was vortexed and incubated at 37°C for 30 minutes. The cells were lysed by addition of 15µl of 20% w/v SDS in water followed by gentle inversion.

Cellular proteins were degraded by the addition of 50µl of proteinase K (10mg/ml). Following addition of proteinase K, the DNA was sheared by drawing the solution through a 25G-gauge hypodermic needle. Tubes were incubated for two hours at 37°C.

Cellular debris was then removed by addition of 500µl of phenol-chloroform and vortexed thoroughly. The tubes were microcentrifuged for 10 minutes and the upper aqueous layer transferred into clean sterile Eppendorf tubes.

The addition of 500µl of isopropanol either at room temperature for one hour or at 4°C for approximately 30 minutes precipitated DNA. The tubes were microcentrifuged, the supernate discarded and the pellet resuspended in 100µl of 10mM Tris-EDTA (TE_{10}). The triplicate tubes were pooled, 100µl of 7.5M ammonium acetate added and mixed followed by 600µl of ice cold absolute ethanol.

The tubes were vortexed briefly and the nucleic acid mixture precipitated overnight at -20°C.

On the following day the DNA was microcentrifuged for 10 minutes, the supernate discarded and pellet resuspended in 300µl of TE_{10}. To this was added 20µl of RNAse (10mg/ml) to degrade RNA. The tubes were incubated for one hour at 37°C.

This was followed by a second phenol-chloroform extraction, isopropanol precipitation, and overnight ethanol precipitation at -20°C as described above.

The DNA was made ready for digestion by microcentrifugation of the ethanol precipitate for 10 minutes and the dried pellet resuspended in 60µl of TE.

(d) Restriction enzyme digestion of genomic DNA

A 20µl aliquot of the purified DNA was added to a reaction mixture that contained 2µl of restriction enzyme, 5µl of appropriate reaction buffer and 23µl of sterile distilled water to give a final reaction volume of 50µl.

Fragment size calibrators were included on each gel using *Kpn*I and *Pst*I digests of phage lambda (λ) DNA. These were prepared as outlined above but with addition of only 2μl of DNA and the volume of water adjusted to 41μl.

All samples were vortexed then microcentrifuged for five seconds to ensure contact between all reactants. The tubes were incubated at 37°C for four hours to ensure complete digestion prior to electrophoresis.

(e) Horizontal gel electrophoresis

Electrophoresis was carried out in a Maxi - Plus Horizontal 20cm x 30cm Unit (Anachem) using a E321 power pack (Consort).

A 0.6% gel was prepared by adding 3g of Agarose (Sigma) to 500ml of Tris-borate-EDTA buffer (TBE). This was heated until dissolved and left to cool to approximately 45°C after which the molten gel was cast into a 20cm x 30cm gel tray and a 28 well comb placed in position.

The gel was left to set for at least one hour, placed in the electrophoresis tank and submerged in two-thirds strength running buffer (TBE).

After incubation, 5μl of tracking dye were added to all digested samples and calibrators. These were mixed and 50μl aliquots carefully loaded into the submerged wells in the gel. Samples were run at 32mA for a minimum of 22hrs.

(f) Visualisation of DNA fragments

After electrophoresis the gel was stained for 30 minutes in a solution of ethidium bromide (0.5μg/ml). Fragments were visualised under ultraviolet

light (302nm) and photographed on Polaroid Type 665 film with a Polaroid MP4 land camera.

2.7 Plasmid Profiling

(a) Preparation of cell lysates

Crude lysates were prepared for plasmid profiles by a modification of the method of Coia *et al.* (1988). Approximately 25% of the growth from an overnight culture on Oxoid nutrient agar (Unipath) was harvested into 500µl of TES in sterile Eppendorf tubes. The tubes were vortex mixed then microcentrifuged to pellet the cells. The supernate was discarded and the pellet resuspended in 400µl TESS. Addition of 100µl of lysozyme (40mg/ml) and 20µl of lysostaphin (1000units/ml) digested the cell walls. Following vortex mixing and incubation at 37°C for 30 minutes, 400µl of 10% w/v SDS was added to lyse the cells. The lysates were microcentrifuged for 10 minutes and chromosomal DNA removed with a broken swab stick.

Plasmid sizes were estimated by comparison to plasmids of known size from *E. coli* strain 39R861. This strain contains four plasmids of 151Kb, 67Kb, 38Kb and 7.4Kb (Macrina *et al.*, 1978). The *E. coli* crude lysate was prepared by harvesting 25% of overnight culture on nutrient agar into 600µl TES in Eppendorf tubes. This was vortex mixed and 400µl of 10% SDS added to lyse the cells. Subsequent steps in plasmid preparation were as per the *Staph. aureus* protocol.

(b) Vertical gel electrophoresis

One hundred microlitres of the crude lysate were mixed with 5µl of tracking dye and added to each well of a vertical agarose gel (0.7% w/v in TBE buffer). Separation of plasmids was achieved by electrophoresis in TBE buffer at 100V for 30 minutes to one hour followed by 200V for five hours (Platt and Sommerville, 1981). The gels were carefully removed from the vertical slab apparatus and stained in ethidium bromide (0.5µg/ml) for 30 minutes and photographed as described above.

2.8 Plasmid fingerprinting

(a) Extraction and purification of plasmid DNA

Cultures incubated at 37°C overnight in 10ml of BHI broth were centrifuged for 10 minutes, the supernate discarded, pellet resuspended in 2ml of TES and split equally into two Eppendorf tubes. The tubes were microcentrifuged for 30 seconds and the pellets resuspended in 200µl of TESS. 50µl of lysozyme (40mg/ml) and 20µl of lysostaphin (1mg/ml) were added followed by incubation at 37°C for 10 - 30 minutes.

Alkaline SDS was prepared freshly by adding 1ml of 10% w/v SDS to 1ml of 2M sodium hydroxide (NaOH) and making up to 10ml with sterile distilled water to give 1% SDS in 0.2M NaOH. Four hundred microlitres of this solution was added to each tube and mixed by inversion to complete cell lysis.

The tubes were then incubated on ice for 5 minutes following which 300µl of 3M sodium acetate was added and mixed gently by inversion to precipitate the chromosomal DNA.

The tubes were vortex mixed and incubated again on ice for five minutes, microcentrifuged for two minutes and the supernate transferred to clean sterile Eppendorf tubes. Five hundred microlitres of phenol-chloroform (1:1) mixture was added to each tube, vortex mixed and microcentrifuged for two minutes. The upper aqueous layers from each tube were carefully removed into clean sterile Eppendorf tubes, 500µl isopropanol added, vortex mixed and left at room temperature for 10 minutes to precipitate nucleic acids. The tubes were then microcentrifuged for five minutes, the supernate discarded and the pellets resuspended in 100µl of TE buffer (10mM Tris, 1mM EDTA, pH 8.0). Following resuspension of the nucleic acids, the tubes were vortex mixed and duplicate tubes pooled. One hundred microlitres of 7.5M ammonium acetate were then added to the DNA solution followed by 600µl of ice cold ethanol. Samples were vortex mixed and left overnight at -20°C. The samples were microcentrifuged for 2 minutes, the supernate discarded and the resulting nucleic acid pellets resuspended in 160µl of TE. The RNA fraction of the samples was digested by the addition of 18µl of RNAse (1mg/ml) followed by a 30-minute incubation at 37°C. This was followed by addition of 20µl 2.5M sodium chloride vortex mixing and a second round of phenol / chloroform extraction, isopropanol and ethanol precipitation as described above.

To prepare the DNA for digestion, the samples were microcentrifuged for two minutes, supernate discarded and the purified plasmid DNA precipitate

resuspended in 60µl of TE. The digestion of the plasmid DNA followed the same protocol as for digestion of genomic DNA.

(b) Horizontal gel electrophoresis

On completion of digestion, 5µl of tracking dye was added to each reaction tube. A horizontal 0.8% agarose gel containing ethidium bromide (0.3µl/ml) was made up in 100ml of TBE. When set, the gel was placed in a horizontal gel tank (Life Technologies Model H3) and submerged in TBE containing ethidium bromide. The samples were loaded and run at 18mA overnight. The following morning the gel was viewed and photographed as described above.

2.9 Computer aided analysis of gels

(a) Calculation of similarity coefficients

Restriction fragment mobility in ethidium bromide stained agarose gels was recorded on Polaroid film and this data transferred to computer using a Summagraphics digitiser and commercially available software (Platt and Sullivan, 1992). Each gel was calibrated with restriction fragments from both *Pst*I and *Kpn*I digests of λ DNA. The molecular weights of these fragments were fitted to a robust modified hyperbola (Plikaytis *et al.*, 1986) from which fragment sizes in adjacent tracks were estimated by interpolation. The numerical values (kb) were stored for subsequent calculation of similarity coefficients (Dice, 1945) and graphical output (logarithmic scale). A fragment size variation of 5% was set to account for small variations in the lambda

calibrators within and between gels and operator error with the use of the digitiser. The calculation of Dice coefficients of similarity was based on the formula:

$$S_D\ (\%) = [2m/(a+b)]\ x\ 100$$

Where "m" was the number of restriction fragments common to two isolates and "a" + "b" was the total number of fragments digitised from each isolate.

(b) Construction of dendrograms

Dendrograms were constructed from transformed distance matrices of REFP data using the Neighbour Joining method of Saitou and Nei (1987). Because the dendrograms depict only a small amount of the data from within the matrix, the topology of any tree contains uncertainty. One of the major determinants can be data input order. This was assessed by comparing the output from three data input orders and additionally by the calculation of the root mean squared (RMS) distance between the matrix and the generated tree.

3. RESULTS

3.1 Genotyping of *Staph. aureus* control strains with the restriction endonuclease *Hha*I

(a) Methicillin-sensitive *Staph aureus* (MSSA)

This group comprised seventeen epidemiologically unrelated isolates from healthy nasal carriers in the community (4), hospital and GP isolates from sites of infection (11) and isolates from cases of bovine mastitis (2).

The size of restriction fragments amenable to computer analysis ranged from 3.7kb to 15.6kb. The number of fragments amenable to computer analysis ranged from 8 (AB211) to 21 (AB193, AB194). When each isolate was matched with one another, Dice coefficients of similarity (S_D values) ranged between 33 - 93% with a mean S_D of 66%.

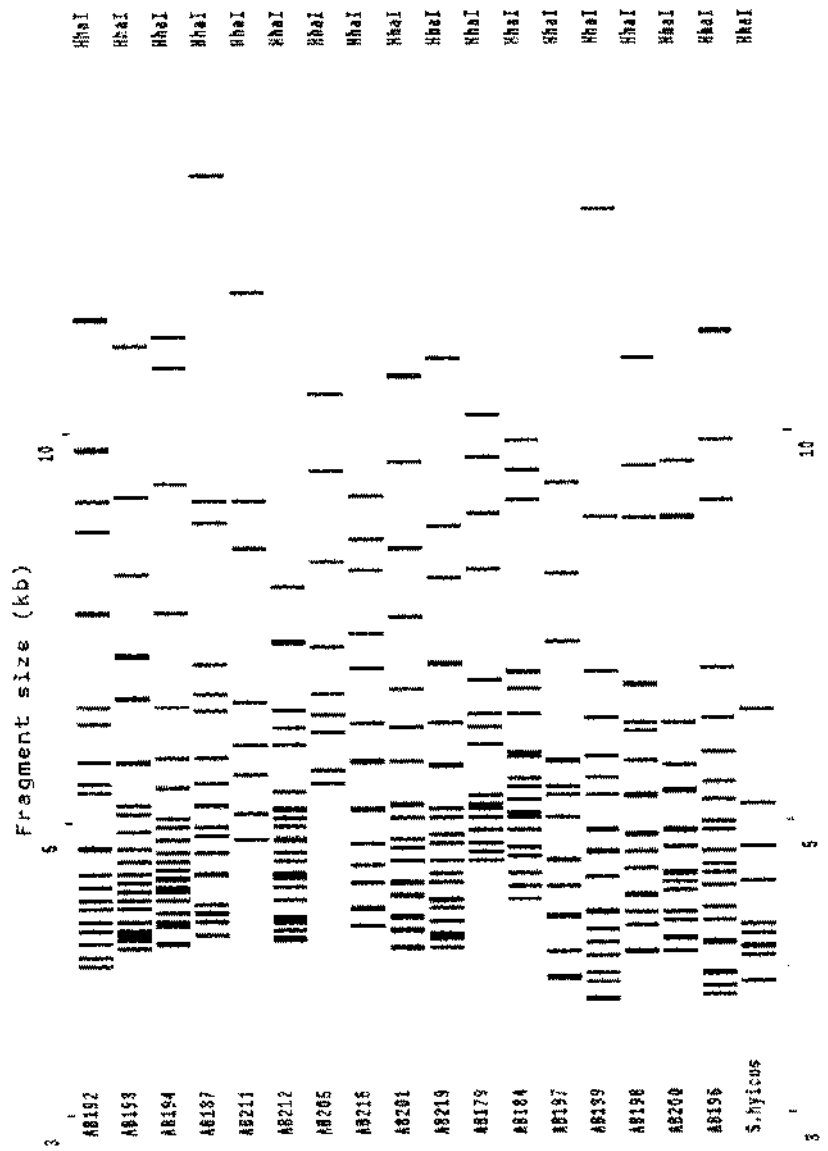
Visual comparison of the REFP's indicated considerable diversity among epidemiologically unrelated strains. Estimation of relatedness using S_D values provided a more quantitative perspective against which sub-groups of MRSA could be assessed as described below. The range indicated that some strains were very distantly related (AB194 & AB205, S_D = 33%), whereas others appeared closely related by Dice coefficient analysis, although not by visual analysis of gels or the digitised print-out as shown in Figure 3.1(AB193 & AB212, S_D = 93%). This was probably as a result of coincidental matching among smaller fragments. Matching the isolate of *Staph.. hyicus* with these MSSA isolates gave a range of S_D values from 21 - 74% with a mean S_D of 53%, indicating only distant relationships with MSSA strains as would be expected from a different staphylococcal species.

A digitised representation of the REFP's of these isolates is shown in Figure 3.1 and the range of S_D values is shown in Figure 3.2. The mean value of 66% also corresponds to the mode of the distribution. However, the distribution is asymmetric and indicates that a small number of unrelated strains showed high levels of similarity.

An example of matching pair data and fragment sizes is shown in appendix II.

Figure 3.1

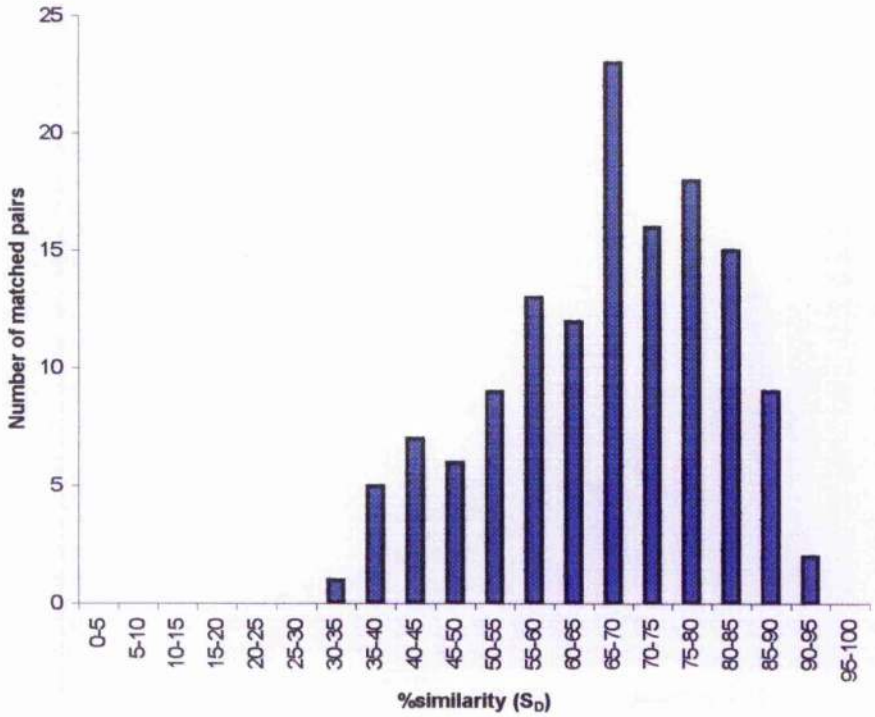
Digitised representation of *Hha*I REFP's of epidemiologically unrelated MSSA isolates



AB187, 192 - 194, community nasal isolates; AB196 - 200, GP isolates; AB201, 205, 211, 212, 215, 219, nosocomial isolates; AB179 & AB184, bovine mastitis isolates.

Figure 3.2

The range of S_D values found among the study MSSA isolates



(b) Methicillin-resistant *Staph. aureus* (MRSA)

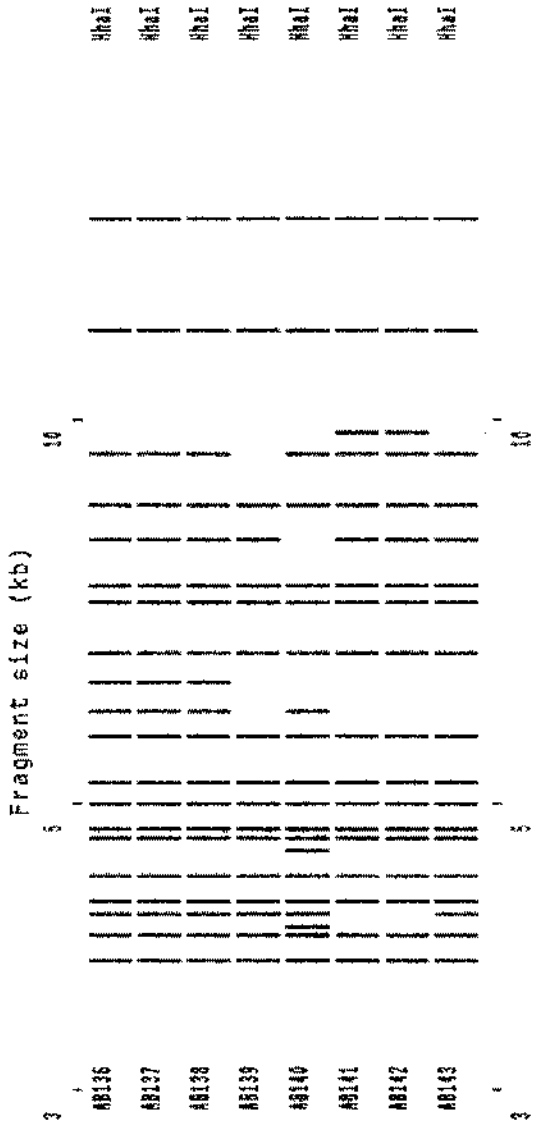
Sixty-one non-Lisbon MRSA's were genotyped on the basis of *Hha*I REFP's. This included examples of previously phenotyped EMRSA-1, EMRSA-15 and EMRSA-16. A table containing details of all study isolates and their designated strain numbers is given in the Materials and Methods section (Table 2.2).

(i) EMRSA-1

These eight isolates had previously been designated EMRSA-1 on the basis of their phenotypic characteristics. Two were from St Bartholomew's Hospital, one from St. Thomas' Hospital in London, and five from an outbreak in Brisbane, Australia. The two isolates from St Bartholomew's (AB 141 and AB 142) gave identical *Hha*I REFP's, there was a two fragment difference between them and the isolate from St. Thomas's (AB 143). The Brisbane isolates showed a greater degree of similarity to the St. Thomas's isolate than to the St. Bartholomew's although the whole group were closely related. A digitised representation of *Hha*I REFP's of these isolates is shown in Figure 3.3. Chromosomal fragments amenable to computer analysis ranged in size from approximately 3.8kb - 14.2kb. S_D analysis demonstrated the genomic variation and demonstrated that *Hha*I REFP's discriminated strains homogeneous on the basis of phenotype (range 84 - 100%; mean 92%). The range of S_D values is illustrated in Figure 3.4.

Figure 3.3

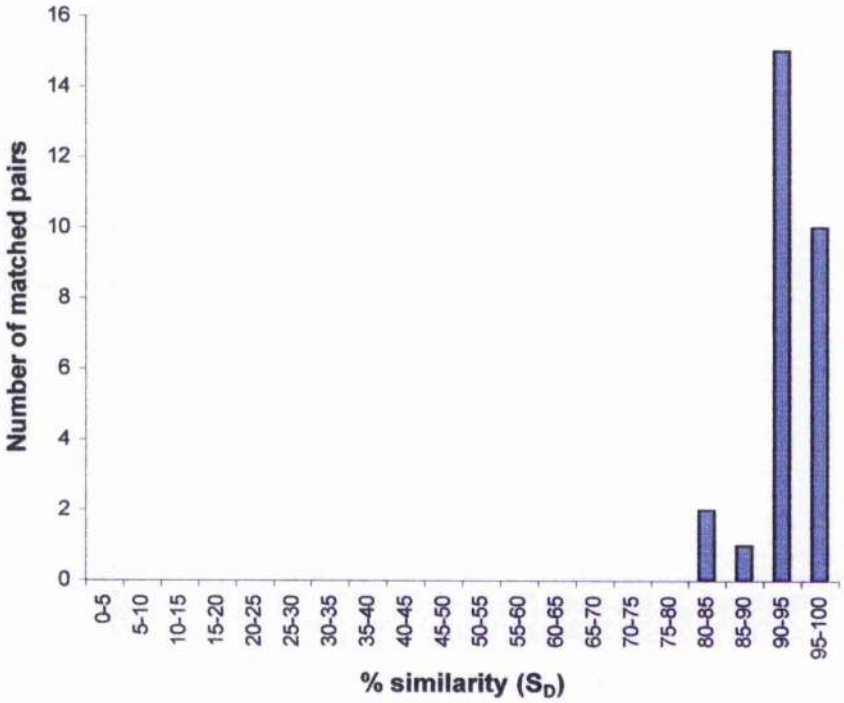
Digitised representation of *Hha*I REFP's of EMRSA-1 isolates



AB136 - 140, EMRSA-1 from Brisbane, Australia; AB141 & AB142, identical EMRSA-1 isolates from St Bartholomews Hospital, London; AB143, EMRSA-1 isolate from St. Thomas' Hospital, London.

Figure3.4

The range of S_D values found among EMRSA-1 variants

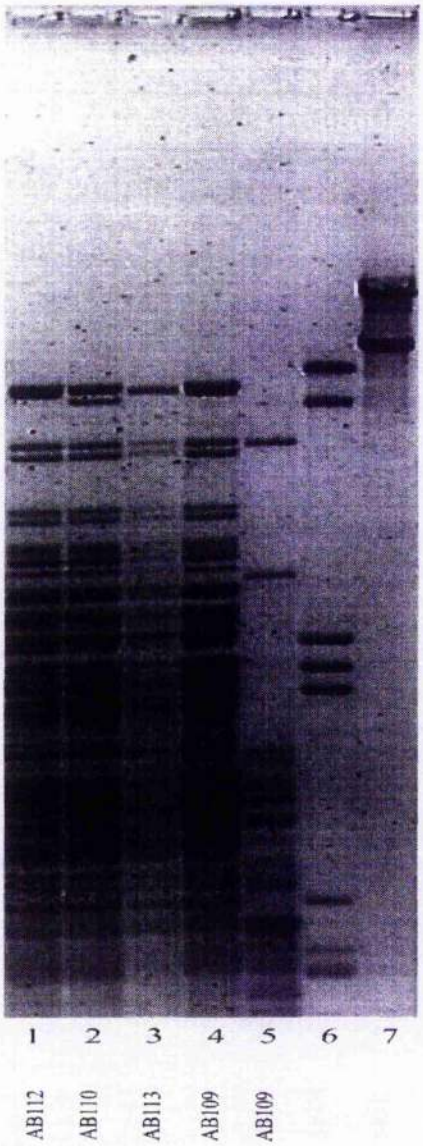


(ii) EMRSA-15

Nineteen EMRSA-15 from six different sources were typed. Three of these isolates had originally been included in the "Other" MRSA group and had not originally been recognised as EMRSA-15 isolates. Three *Hha*I genomic variants were seen among the EMRSA-15. The predominant type of which there were 16 isolates was designated type 15-H1, two isolates with a single fragment difference were designated type 15-H2 and a single isolate contained an additional fragment and was designated type 15-H3. A gel photograph showing examples of *Hha*I and *Sau3A*I REFP's of EMRSA-15's is shown in Figure 3.5. Chromosomal fragments amenable to computer analysis ranged in size from approximately 3.9kb - 11.6kb. Isolates AB109, 112 and 113 exhibited the type H1 polymorphism and AB110 exhibited the type H3 polymorphism. The largest *Hha*I fragment of these isolates is of plasmid origin. The *Sau3A*I digest shown here was typical of all EMRSA-15 study isolates. A digitised representation of all EMRSA-15 isolates illustrating the three *Hha*I variants is shown in Figure 3.6. Although a further polymorphism was evident in some strains this was later shown to be due to plasmid DNA.

Figure 3.5

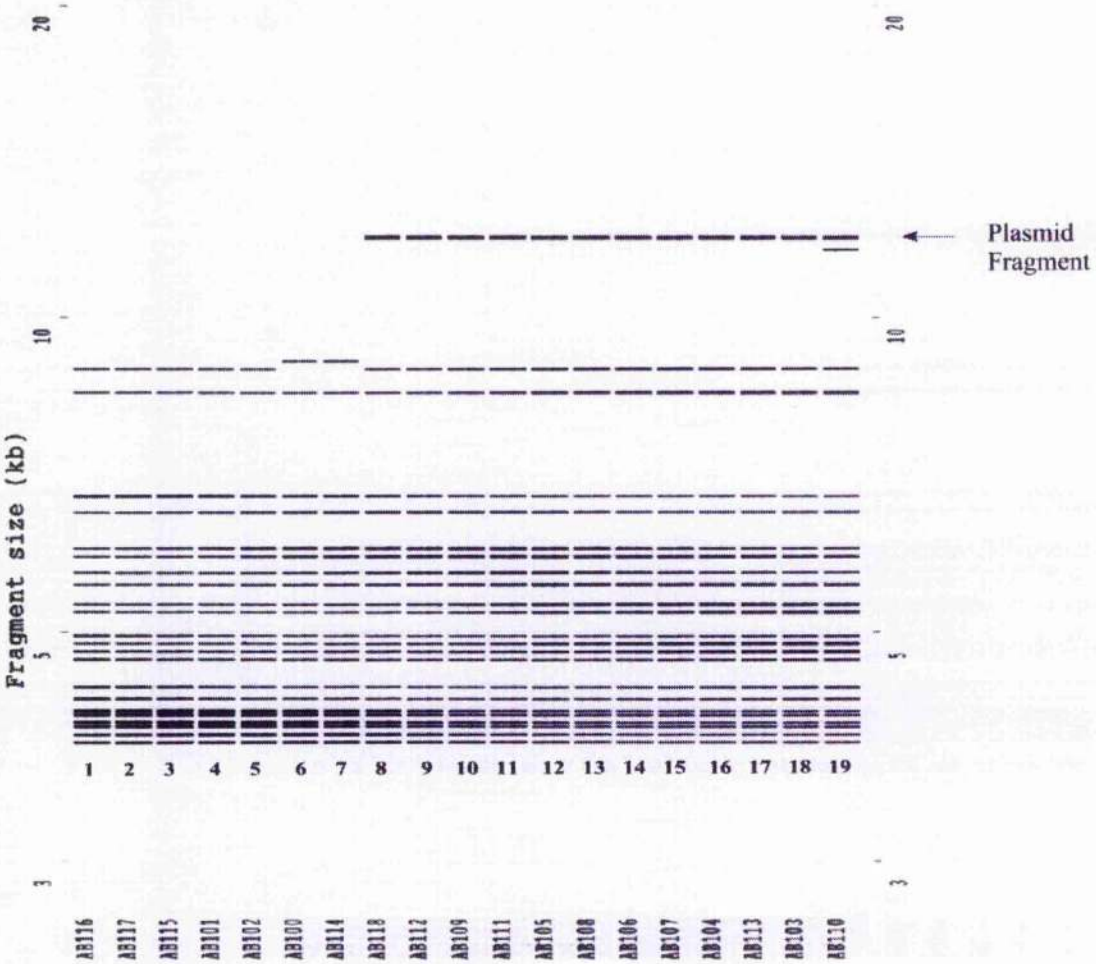
***Hha*I and *Sau3A*I REFP's of EMRSA-15 isolates.**



Lanes 1-4, *Hha*I REFP's; AB112, 113 and 109 are E15-H1 variants, and AB110 is variant E15-H3. Lane 5, *Sau*3AI REFP - no variation was seen among EMRSA-15 with this enzyme. Lanes 6 and 7 contain respectively, *Pst*I and *Kpn*I digests of phage lambda DNA. Plasmid REFP's of these isolates showed the largest densely staining fragment of these isolates to be plasmid in origin.

Figure 3.6

Digitised representation of all EMRSA-15 *Hha*I REFP's.



REFP's from isolates AB100 and AB114 exhibit the *Hha*I type, E15-H2. The REFP of isolate AB110 exhibits the *Hha*I type, E15-H3 and all other REFP's exhibit the *Hha*I type E15-H1.

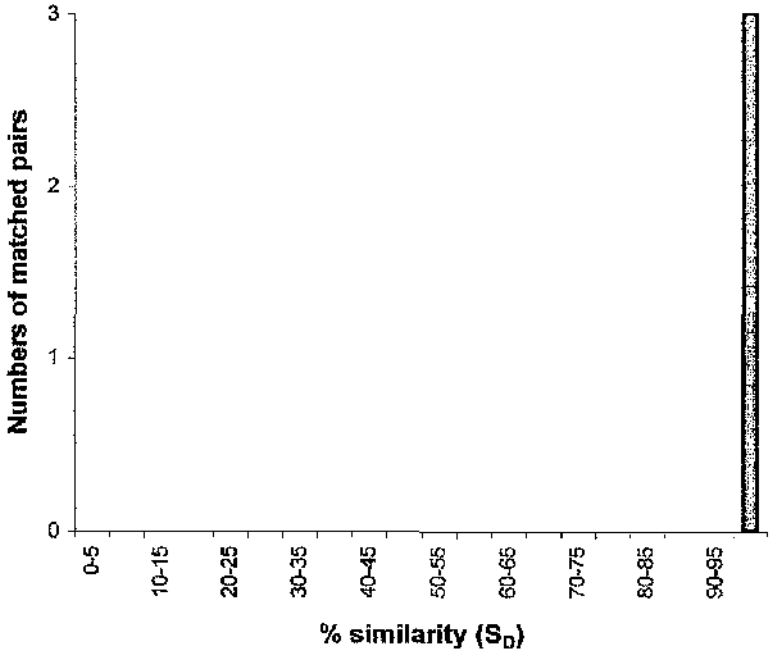
S_D analysis of these variants showed a range of 98 - 100%, with a mean S_D of 99%. Although two isolates (AB100, AB114) were visibly a variant with respect to a single fragment, they appeared to match at 100%. This was as a result of software limitations. Figure 3.7 shows the range of S_D values for the EMRSA-15 variants.

*Hha*I genotyping confirmed phenotypically identified EMRSA-15 isolates responsible for outbreaks of infection in the Intensive Care Unit at Glasgow Royal Infirmary and the Renal Unit at Stobhill Hospital.

*Hha*I REFP's and S_D analysis comparison of the variants within each control group showed EMRSA-15 to be genetically very distinct from EMRSA-1. When the groups were matched with each other, S_D values ranged from 62 - 79% with a mean of 70%. The mean of these matches was considerably less than the mean values for each group alone.

Figure 3.7

The range of S_D values found among EMRSA-15 variants

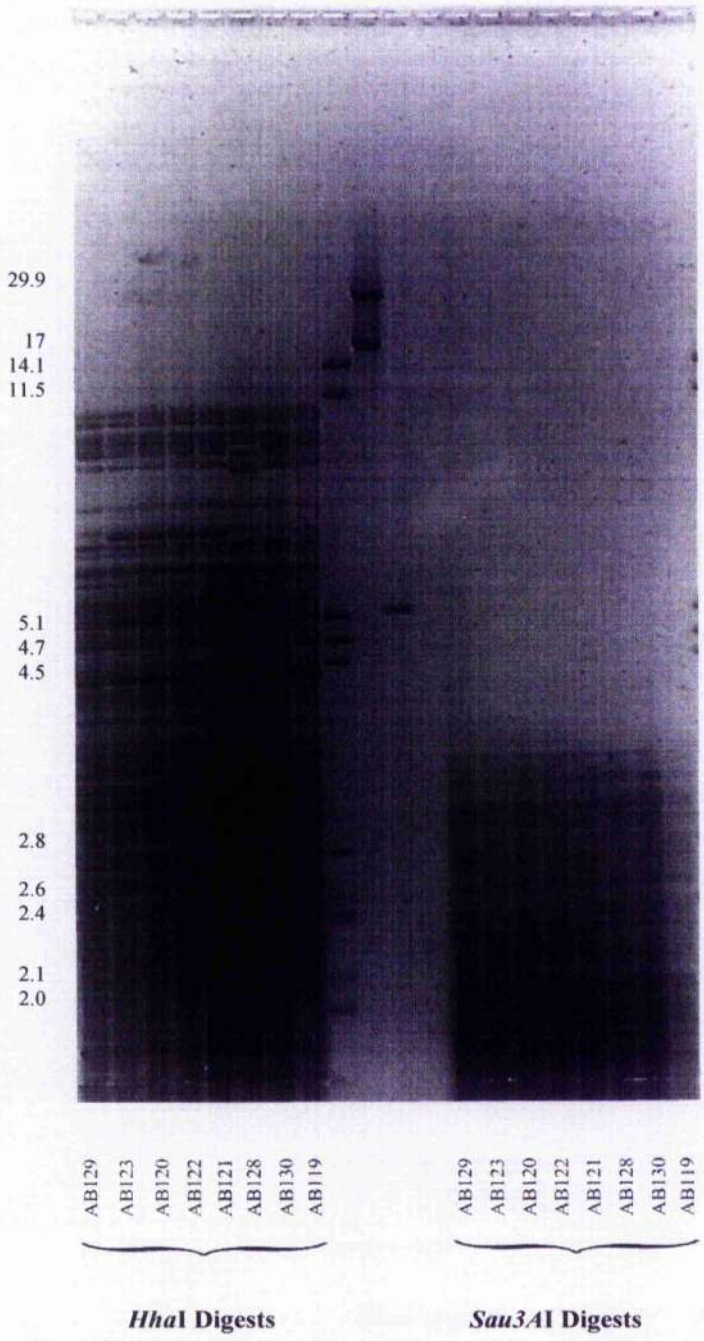


(iii) EMRSA-16

Seventeen EMRSA-16 from seven different sources were typed. On the basis of typing, four outbreaks were confirmed: at Monklands Hospital, Western General Hospital, Dumfries Royal Infirmary and Glasgow Royal Infirmary, and a further two cases were confirmed at Health Care International Hospital (HCI). All isolates were very closely related. Nine were identical and were designated type 16-H1, four sub-types were also identified, comprising 3,2,2 and a single isolate, designated 16-H2, H3, H4 and H5 respectively, each with 1 - 3 fragments different from the dominant type. A gel photograph showing examples of *Hha*I and *Sau*3AI REFP's is shown in Figure 3.8. Chromosomal fragments amenable to computer analysis ranged in size from approximately 4.2kb - 10.6kb. A digitised representation of all the EMRSA-16 *Hha*I REFP's is shown in Figure 3.9.

Figure 3.8

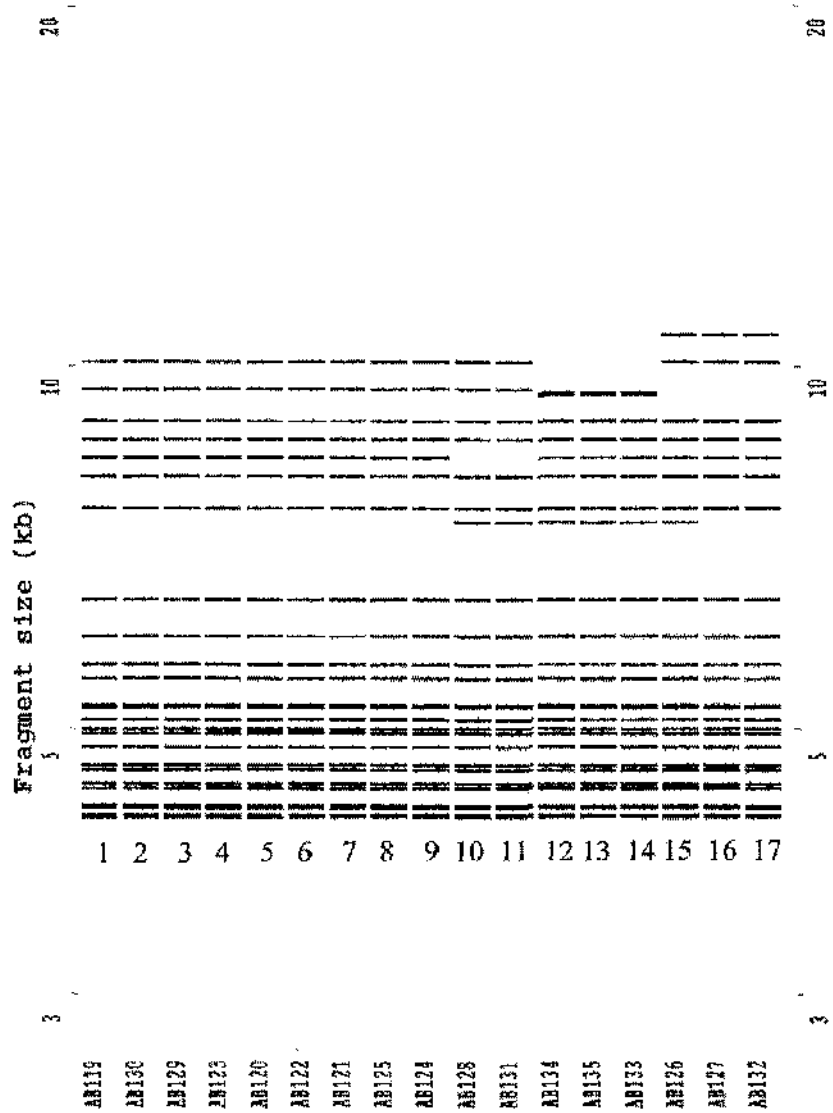
Gel photograph of *Hha*I and *Sau3A*I REFP's of EMRSA-16 isolates.



The numbers on the left of the figure indicate the fragment sizes in kb of the *Pst*I and *Kpn*I digests respectively of lambda phage DNA in the centre of the gel.

Figure 3.9

Digitised representation of all EMRSA-16 *Hha*I REFP's



Lanes 1-9: *Hha*I REFP type E16-H1; lanes 10 - 11: REFP type E16-H3; lanes 12 - 14: REFP type E16-H2; lane 15: REFP type E16-H5; lanes 16 - 17: REFP type E16-H4.

When S_D analysis was performed on the genomic variants within this group, a range of 91 - 98% similarity was found, with a mean S_D of 94% as illustrated in Figure 3.10.

S_D analysis of inter-group matching showed that this strain was genetically distinct from EMRSA-1 and EMRSA-15. When matched with EMRSA-1, S_D values ranged from 62 - 76% with a mean value of 69% and when matched with EMRSA-15, S_D values ranged from 70 - 73 with a mean of 72%.

The comparison of EMRSA-1, EMRSA-15 and EMRSA-16 using S_D analysis was valuable in the assessment of the discriminatory power of *Hha*I REFP's. In each instance minor variations were demonstrated within the group but between each group substantial diversity was evident. This indicated that *Hha*I REFP's not only reflected similarity when strains were closely related but also that they did not do so through a lack of discriminatory power.

Figure 3.10

The range of S_D values found among EMRSA-16 variants

(iv) Other MRSA

(a) "Stobhill type"

From the total of 18 MRSA that did not conform to any recognised epidemic group, ten isolates were shown to be genetically related by *Hha*I REFP analysis. Six of these isolates originated from wards within Stobhill Hospital whereas a further four isolates were from different hospitals (see Figure 3.14, isolates AB146, 147, 149 and 151). Eight variants were found, with 3 isolates from Stobhill belonging to a single REFP type (AB152, AB160, and AB166). Chromosomal fragments amenable to computer analysis ranged in size from approximately 4.6kb - 17kb. A digitised representation of these variants is shown in Figure 3.11.

S_D analysis of these variants indicated a range of 76 - 100%, with a mean S_D of 89%. Although two isolates differed in two fragments (AB146 and AB163), they matched at 100%. Again this was due to computer software limitations. When the matching pair analysis was repeated allowing for no fragment size variation, the S_D values ranged from 69 - 96% with a mean S_D of 84%. This showed a margin of error of 5.5% between the two variations in analysis. The range in S_D values for this group is shown in Figure 3.12.

S_D analysis of inter-group matching showed that this group was genetically distinct from EMRSA-1, EMRSA-15 and EMRSA-16. When matched with EMRSA-1, S_D values ranged from 55 - 71% with a mean value of 60%, when matched with EMRSA-15, S_D values ranged from 47 - 62 with a mean of 53% and when matched with EMRSA-16, S_D values ranged from 47 - 67 with a mean of 57%.

Figure 3.11

Digitised representation of *Hha*I REFP's of the MRSA " type" found to be prevalent in Stobhill Hospital

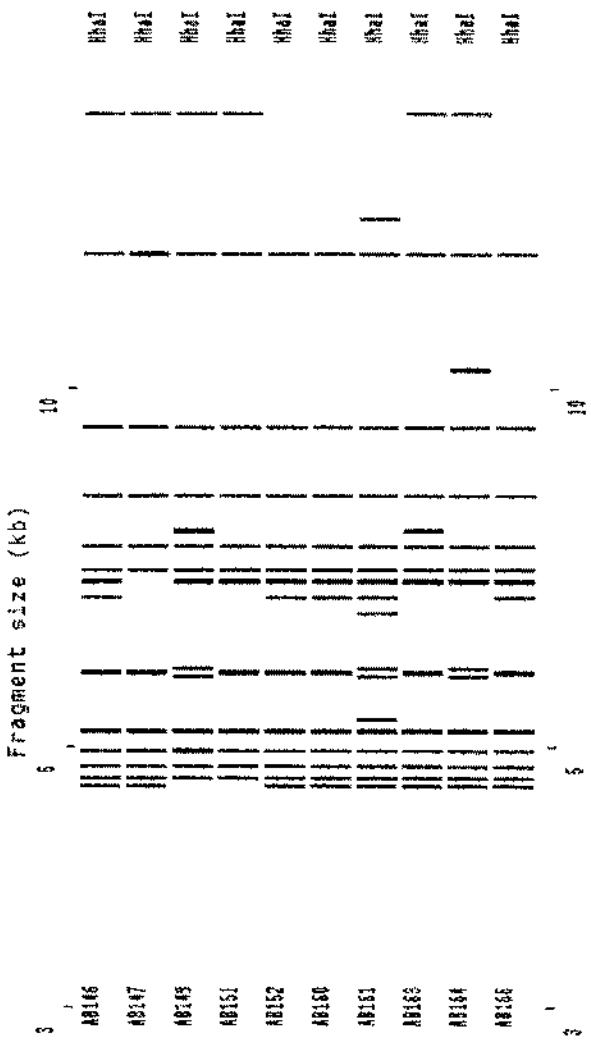
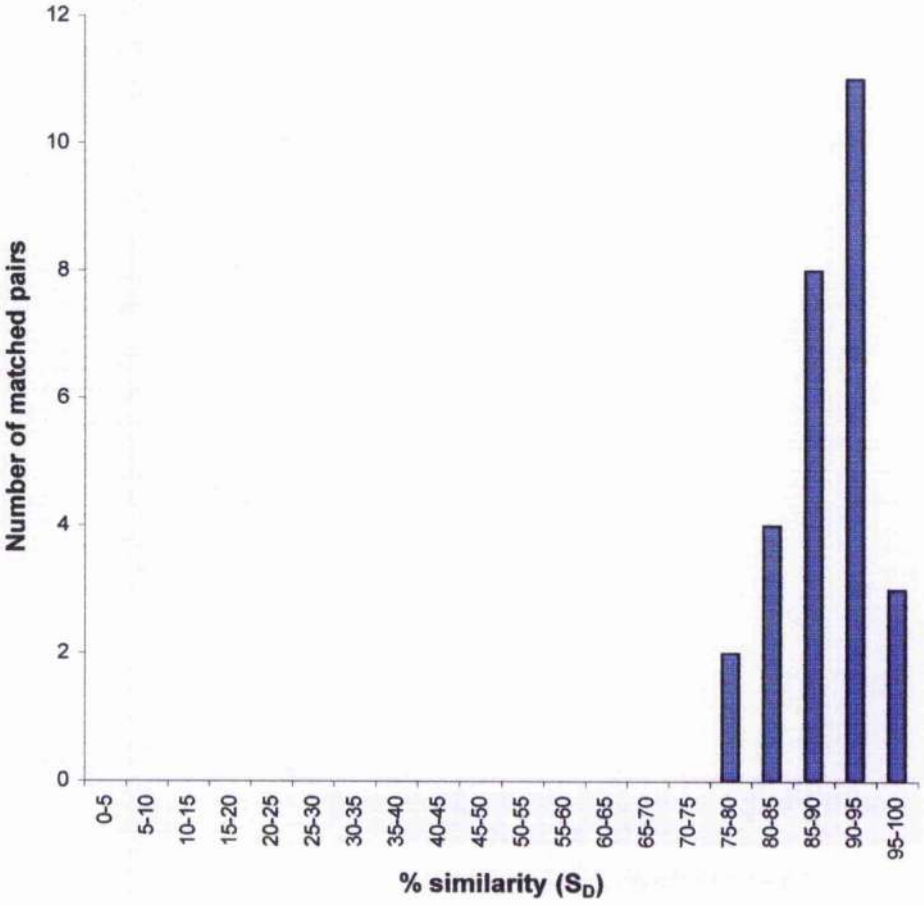


Figure 3.12

The range of S_D values found among MRSA isolates belonging to the tentatively named "Stobhill clone"



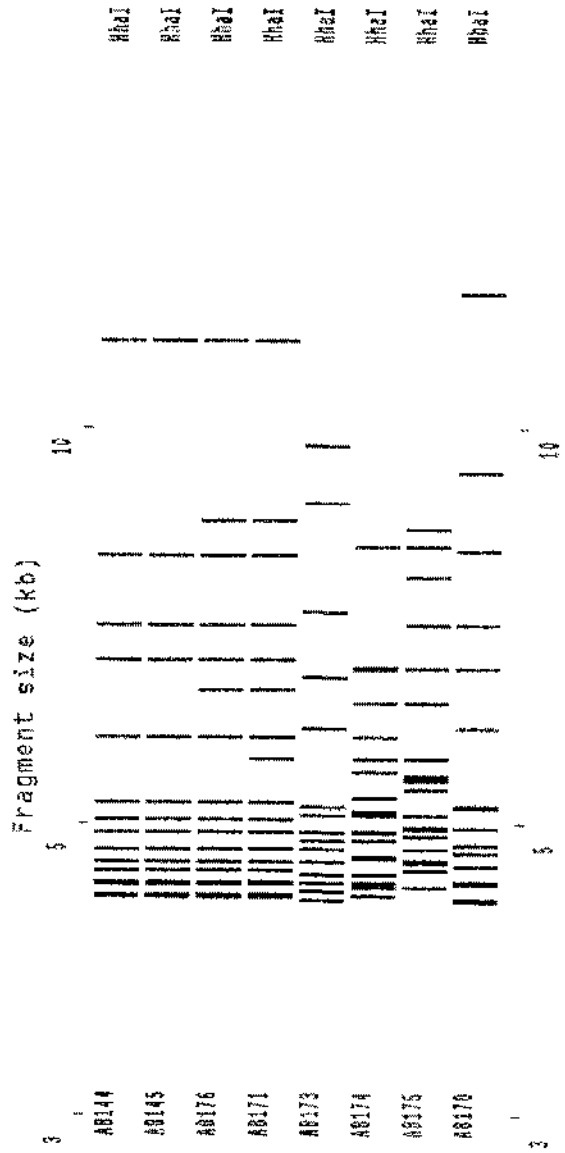
(b) Sporadic MRSA

Eight isolates were defined as "sporadic" on the basis that they were not phenotypically recognisable as epidemic strains, and were revealed by their *Hha*I REFP's to be the most diverse MRSA group. When S_D analysis was performed on these isolates, a range of 69 - 100% similarity was found, with a mean S_D of 82%. Two isolates from different wards at the same hospital matched at 100% (AB144 & AB145). Another two isolates, AB171 & AB176 were closely related to each other, having only a one fragment difference (S_D 97%) and to the two identical isolates (S_D 90% & 93% respectively). The other four isolates of this group appeared to be more diverse both by visual inspection of gel photographs and by computer analysis. A digitised representation of *Hha*I REFP's of these isolates is shown in Figure 3.13.

The analysis of this group of isolates by *Hha*I REFP's indicates the ability of the technique to discriminate among different strains of MRSA and also to sub-type within a single strain. The data also suggests that as in the case of MSSA isolates, genetic relationships between epidemiologically unrelated MRSA strains can also be found. MRSA having had less time to diverge than MSSA are predicted to be somewhat less diverse therefore it was not unexpected that the S_D values indicated this, although this observation must be balanced with the fact that the MSSA group contained more than twice the number of isolates.

Figure 3.13

Digitised representation of *Hha*I REFP's from isolates initially classed as sporadic MRSA



Examples of *Hha*I REFP's of MRSA that belonged to the Stobhill group and other mixed MRSA are shown in Figure 3.14.

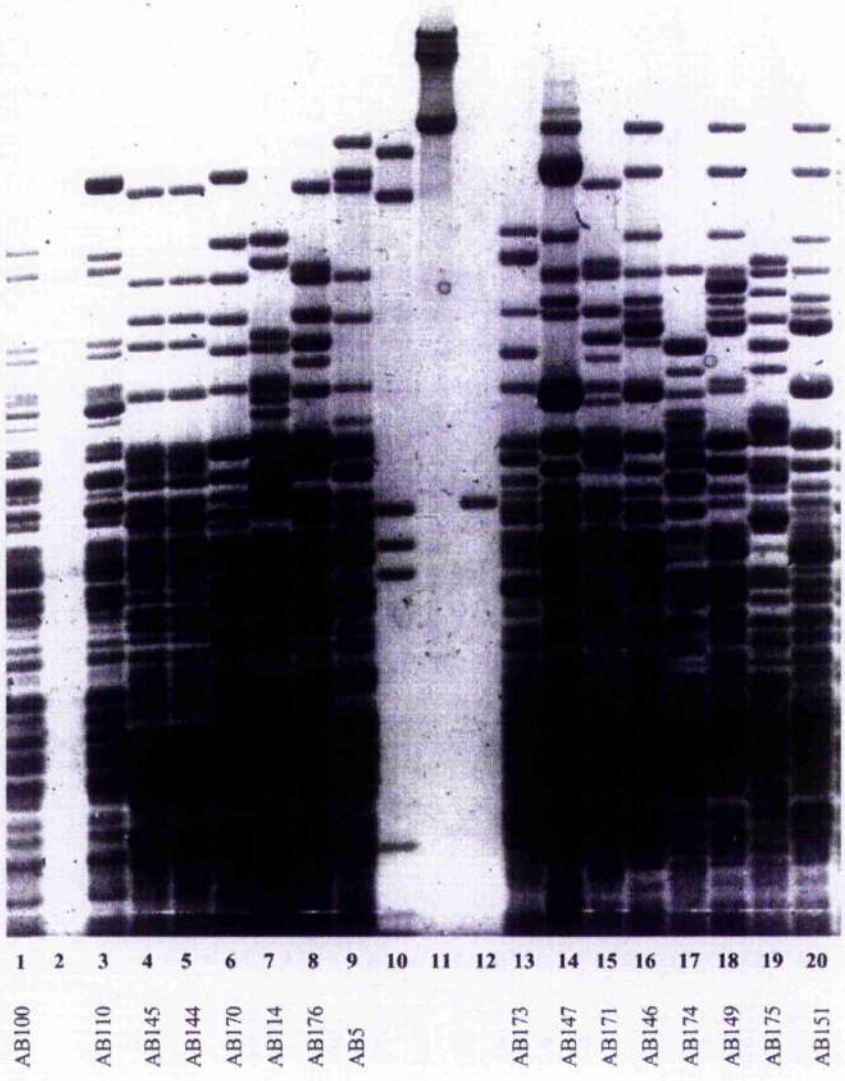
Lanes 10, 11 and 12 contained, respectively, *Pst*I, *Kpn*I and *Hha*I digests of phage lambda DNA. Lanes 1, 3 and 7 contained REFP's of EMRSA-15 isolates (AB100, Dumfries Royal Infirmary; AB118, Yorkhill Hospital; AB114, Vale of Leven Hospital) which prior to this study had not been recognised as such. Lane 9 contained a Lisbon type H1 REFP (AB5). No DNA was present in lane 2, while lanes 4 and 5 contained REFP's from identical sporadic isolates (S_D = 100%) obtained from different wards at the same hospital (AB145 and AB144, RAH). The REFP's in lanes 8 and 15 (AB176, Vale of Leven; AB171, Crosshouse) were very similar (S_D = 91%). Phenotypically, these isolates were almost identical, but they differed in phage type.

The REFP's in lanes 14, 16, 18 and 20 (AB147, AB146, AB149 and AB151 respectively) were from isolates originally included in the "Other" MRSA group, however visual and S_D analysis revealed them to be closely related to each other and to the Stobhill clone. The true degree of genomic relatedness between these four isolates on this gel was somewhat complicated by the presence of a number of intensely staining fragments which may have been of plasmid origin.

Finally, the isolates in lanes 13, 17 and 19 (AB173, AB174, and AB175) were sporadic MRSA, unrelated both phenotypically and genotypically.

Figure 3.14

Gel photograph showing *Hha*I genomic REFP's of a mixed selection of MRSA isolates



3.2 Genotyping of *Staph. aureus* control strains with the restriction endonuclease *Sau3A*I

Digestion with *Sau3A*I yielded fewer discernible fragments under identical electrophoresis conditions and did not appear to be as discriminatory as *Hha*I. Isolates that showed identical *Hha*I fingerprints also showed identical *Sau3A*I fingerprints although the converse of this was not always true. This indicated a greater degree of discrimination with *Hha*I.

Because of the preliminary results obtained with *Sau3A*I and the expense of this enzyme, it was decided only to perform fingerprinting on a selection of the total number of isolates. REFP's of a selection of MRSA that were shown to be genetically diverse with *Hha*I were digested with *Sau3A*I and are shown in Figure 3.15.

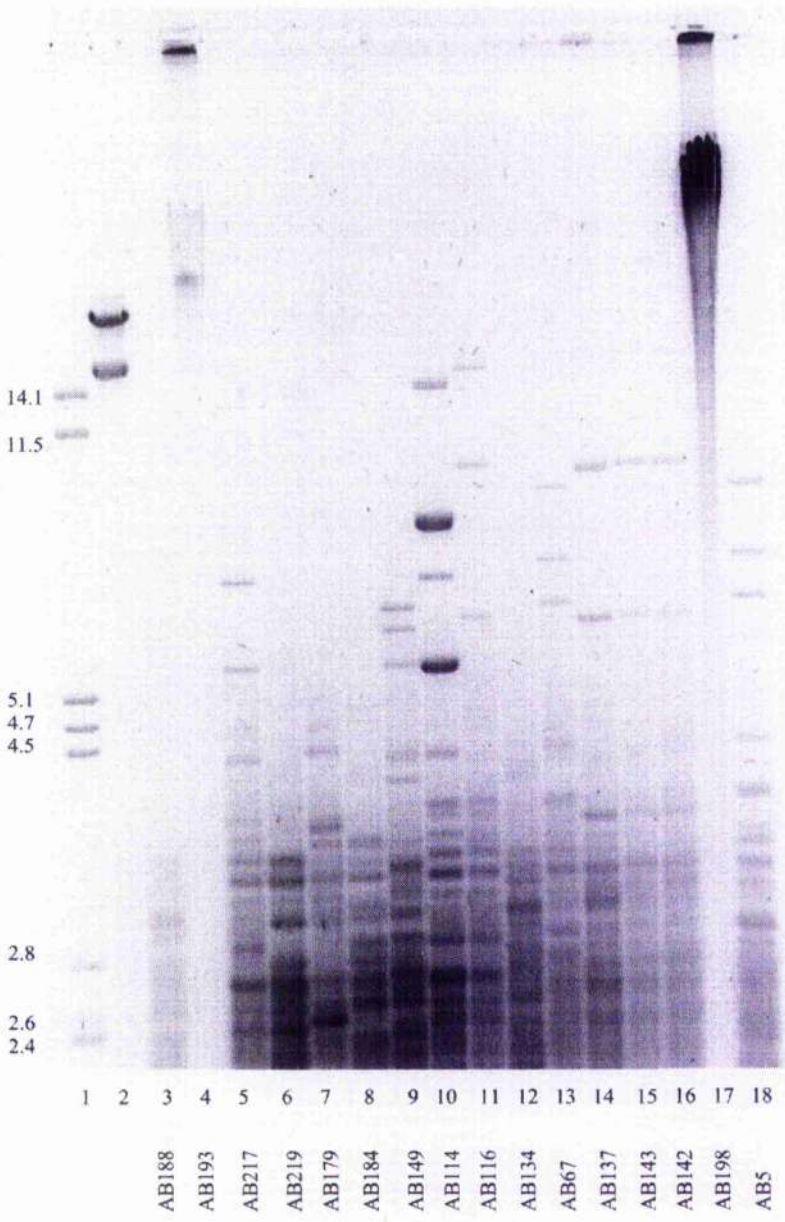
Lanes 1 and 2 showed, respectively, *Pst*I and *Kpn*I REFP's of phage lambda DNA. Lanes 3 - 8 showed MSSA REFP's from nasal (3&4), nosocomial infections (5&6) and bovine sources (7&8). Lane 9 contained a sporadic MRSA. Lanes 10 and 11 contained EMRSA-15 REFP's. The EMRSA-15 in lane 11 was both genetically (type 1) and phenotypically (phage type 75w) typical of the strain. However, the EMRSA-15 in lane 10 was a *Hha*I variant (type 2) and also a *Sau*3AI genomic variant and was also the only EMRSA-15 to differ markedly phenotypically (phage type 6w 42E 47w 75w and erythromycin sensitive). The two intensely stained fragments are probably plasmid DNA. The figure shows these isolates to have distinct *Sau3A*I fingerprints. The isolate in lane 10 was also shown to have a different plasmid

fingerprint to that of other EMRSA-15s. Clearly, further work is necessary to establish the relationship of this isolate to other EMRSA-15's.

Lane 12 showed an EMRSA-16 REFP. As is also shown in Figure 3.8, the *Sau3A*I cleaved the isolates of this strain into numerous small fragments that were not amenable to computer analysis. Lanes 13 and 18 contained typical Lisbon isolate REFP's (*Hha*I type H1). Lanes 14, 15 and 16 contained EMRSA-1 REFP's which had minor differences in their *Hha*I fingerprints but have identical *Sau3A*I fingerprints. The DNA in lanes 4 and 17 was from MSSA which for reasons as yet unknown, failed to digest on two separate occasions. One possible explanation may be that these isolates are producers of *Sau3A*I. Organisms introduce methylations into their DNA to protect themselves from the action of the restriction enzymes they produce.

Figure 3.15

Gel photograph showing *Sau3A*I genomic REFP's of a selection of diverse *Staph. aureus* isolates.



Lanes 1 and 2 contain respectively, REFP's of *Pst*I and *Kpn*I digests of phage lambda DNA as size markers. Numbers on the left of the figure indicate the sizes of lambda fragments in kb.

3.3 Genotyping of Lisbon strain MRSA with *Hha*I

Having established that *Hha*I was highly discriminating in differentiating different strains of *Staph. aureus*, ninety-three isolates of Lisbon strain MRSA were genotyped using this enzyme.

Digestion and electrophoresis with *Hha*I produced 18 - 24 discernible fragments that were amenable to computer analysis. These ranged in size from approximately 3.7kb to 15kb as shown in Table 3.1.

Sixty-eight isolates (73%) gave a genomic fingerprint identical to the index case and were designated *Hha*I type H1.

A typical H1 type was selected at an early stage of the study and digests of this were used on subsequent gels to determine the strain's genomic stability and also to control the DNA extraction and digestion technique. This was the study isolate designated AB5. The REFP of this control isolate remained stable following daily subculture on horse blood agar both at 37°C and 42°C over a six month period. The origins and dates of isolation of the Lisbon variants where known, are given in Table 3.2.

Seven isolates lacked the 5.8kb fragment of type H1 and were designated type H2. REFP's of six isolates showed an additional fragment of 6.4kb and were designated type H3. Two isolates lacked the 8kb fragment of type H1 and were designated type H10. Ten isolates gave unique fingerprints and were designated types H4 - 9 and H11 -14. Fragments lost or gained in these isolates are shown in Table 3.1.

All apparent fragment variation occurred among fragments greater than or equal to 5.3kb.

Table 3.1

Genotypic variation seen among Lisbon strain variants (H2 – H14) as compared to the predominant strain type H1

H1 Approximate Fragment sizes (Kb)	*(No.)*	*H2*	*H3*	*H4*	*H5*	*H6*	*H7*	*H8*	*H9*	*H10*	*H11*	*H12*	*H13*	*H14*
15.0	(1)													
12.6	(2)					Abs								
11.7	(3)													
8.0	(4)									Abs			Abs	
6.8	(5)													
5.8	(6)	Abs			Abs									
5.3	(7)			Abs	Abs				Abs					
5.1	(8)													
4.9	(9)													
4.7	(10)													
4.6	(11)													
4.5	(12)													
4.3	(13)													
4.2	(14)													
4.1	(15)													
4.0	(16)													
3.95	(17)													
3.9	(18)													
3.8	(19)													
3.7	(20)													
Additional Fragments			6.4			6.4	6.4 10.1	6.4 6.6 7.7	5.5		6.2 6.4 7.4 8.3	6.2 6.4	6.2 8.15	6.4 8.3
DNA loss / gain		-5.8	+6.4	-5.3	-11.1	-6.2	+16.5	+20.7	+0.2	-8.0	+28.3	+12.6	+6.35	+14.7

Abs: Absent

Table 3.2

Origins of Lisbon strain variants

LISBON VARIANT	*LOCATION*	*DATE*
H2	Glasgow Royal Infirmary	12.06.91
	Glasgow Royal Infirmary	10.06.93
	Glasgow Royal Infirmary	10.06.93
	Glasgow Royal Infirmary	30.06.93
	Glasgow Royal Infirmary	10.11.93
	Canniesburn Hospital	20.07.93
	Stobhill Hospital	02.05.96
H3	Western Infirmary Glasgow	01.12.90
	Royal Alexandra Hospital	07.06.92
	Glasgow Royal Infirmary	30.10.92
	Queen Elizabeth Hospital	NK
	Crosshouse Hospital	NK
	Crosshouse Hospital	NK
H4	Dumfries Royal Infirmary	13.08.93
H5	Vale of Leven Hospital	08.12.93
H6	Canniesburn Hospital	20.10.92
H7	Western Infirmary Glasgow	NK
H8	NK	NK
H9	Law Hospital	30.07.93
H10	Glasgow Royal Infirmary	21.02.95
	Glasgow Royal Infirmary	NK
H11	Crosshouse Hospital	16.01.91
H12	Glasgow Royal Infirmary	22.09.91
H13	Lightburn Hospital	16.11.94
H14	Ballochmyle Hospital	16.09.91

NK: Not Known

Figure 3.16 shows a gel photograph of a selection of Lisbon strain isolates following *Hha*I digestion and electrophoresis.

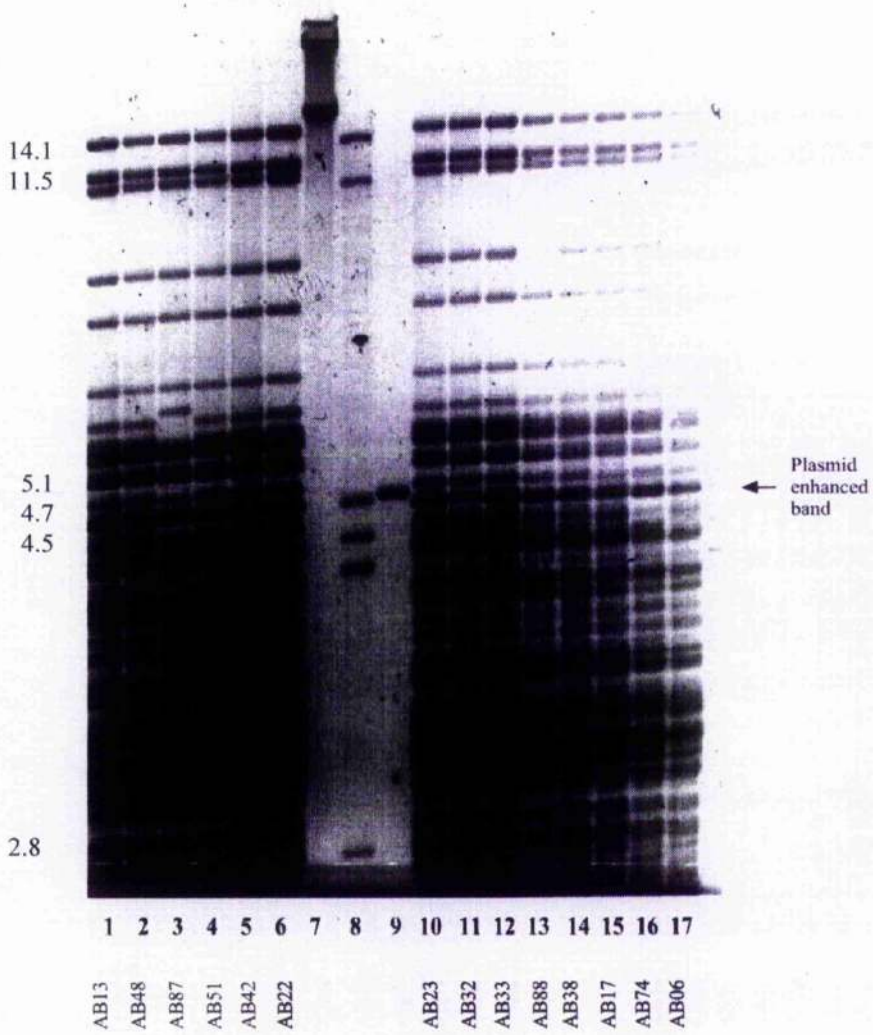
Lanes 3, 13 and 16 contained DNA from Lisbon variants H9, H10 and H2 respectively. Lanes 7, 8 and 9 contained *Kpn*I, *Pst*I and *Hha*I digests respectively of phage lambda DNA. All other lanes contained DNA from Lisbon type H1. The isolate in lane 11 was later found to be plasmid - free. The gel photograph shows that it lacked the enhanced fragment shown by all the other isolates, as indicated by the arrow.

A digitised representation of the fourteen molecular variants of this strain is shown in Figure 3.17. Note that fragment 12 of variant H3 is less intense than the equivalent sized fragment of the other variants. This was due to this variant being plasmid free whereas all others possessed an identical plasmid which when digested with *Hha*I yielded a fragment of approximately 5kb that enhanced the density of this band (see also Figure 3.16)

S_D analysis of the Lisbon variants gave a range of 86 - 100%, with a mean S_D of 95%. Two matches occurred at 100%; H1 and H9, and H3 and H13. Although the isolates within each pair had identical numbers of fragments (20 and 21 respectively) there were small differences in the size of a single fragment which was within the set 5% fragment size variation. These isolates had been run together on at least two occasions and fragment size differences were deemed to be genuine, although beyond the analytical resolution limits of the computer system. Thus the computer based analysis potentially overestimated similarity compared to visual inspection which recognised subtle differences as significant.

Figure 3.16

Gel photograph showing *Hha*I genomic REFP's of a selection of Lisbon strain isolates

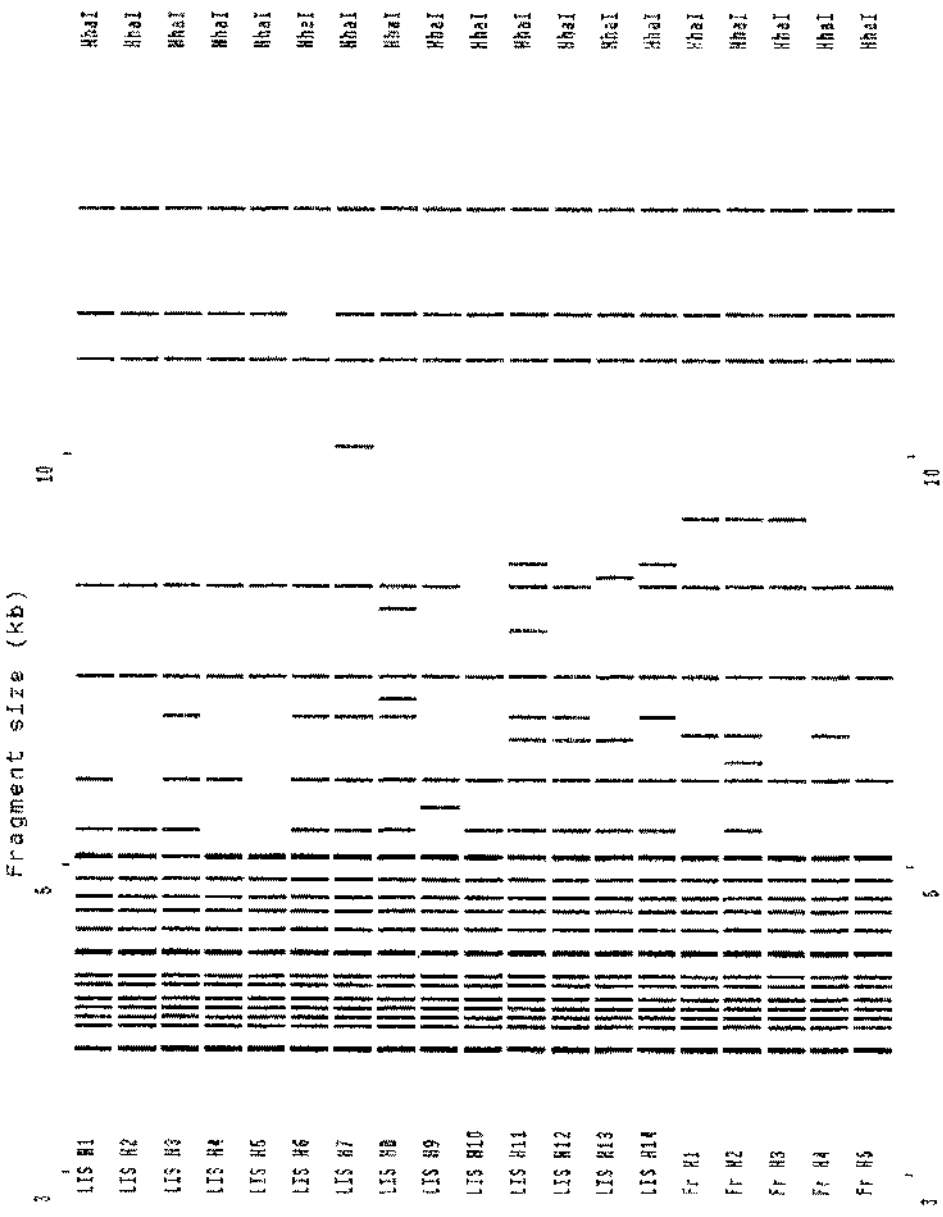


Lane 3, variant H9; Lane 13, variant H10; Lane 16, variant H2; Lane 11, plasmid free isolate of type H1; Lanes 7, 8 and 9, *Kpn*I, *Pst*I and *Hha*I digests respectively of phage lambda DNA; all other lanes, variant H1.

Numbers on left of figure indicate *Pst*I size marker fragments in kb.

Figure 3.17

Digitised representation of *Hha*I REFP's illustrating the range of genotypic variation found among Lisbon and French strain MRSA



Lisbon variant H4 and French variant H5 have identical REFP's.

S_D analysis of inter-group matching showed that this strain was genetically distinct from EMRSA-1, EMRSA-15, EMRSA-16 and the Stobhill clonal group. When matched with EMRSA-1, S_D values ranged from 62 - 78% with a mean value of 71%. When matched with EMRSA-15, S_D values ranged from 65 - 82% with a mean of 74%. When matched with EMRSA-16, S_D values ranged from 44 - 64% with a mean of 55% and when matched with the Stobhill clone, S_D values ranged from 43 - 67%, with a mean of 52%.

From the results of *Hha*I genomic REFP typing, a possible evolutionary sequence of events was proposed to account for the variation in the REFP's of the Lisbon strain in Scotland following its introduction. This is shown in Figure 3.18.

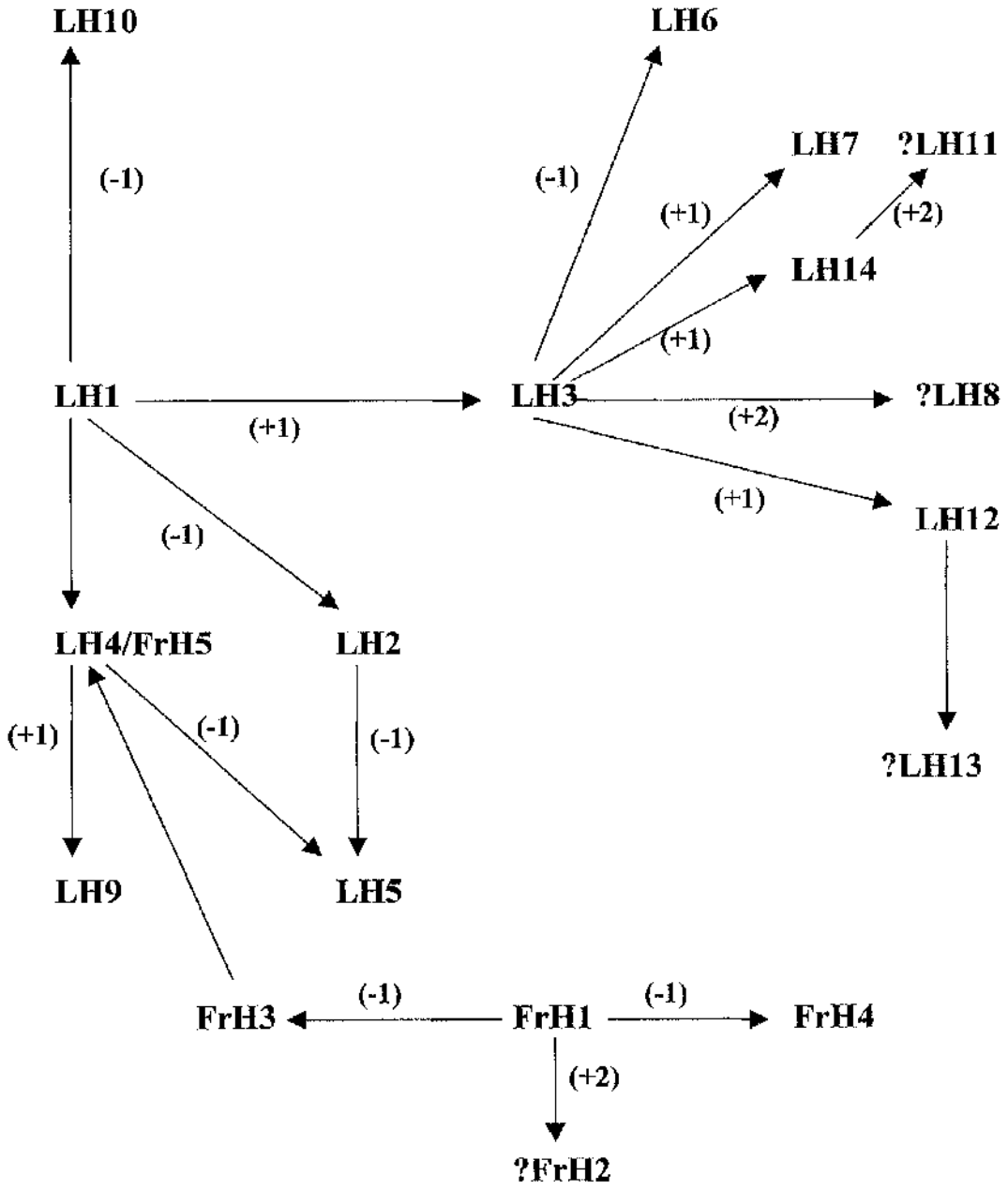
3.4 "French strain" MRSA

Nine isolates from Glasgow Royal Infirmary, that included one from a member of staff, were typed with *Hha*I. These isolates although related by comparison of their REFP's showed a number of polymorphisms and could be subdivided into five subtypes designated FH1 - 5, consisting of 2, 3, 2, 1 and 1 isolates respectively. Digitised representations of the *Hha*I REFP's of these isolates are shown in Figure 3.17. From this data it was found that an isolate of the French strain (AB 228) was identical in genomic REFP to Lisbon variant H4 (AB 82).

When S_D analysis was performed on these isolates, a range of 91 - 98% similarity was found, with a mean S_D of 95%. When Lisbon and French variants were matched with one another, S_D values ranged from 86 - 100% with a mean S_D of 94%. Given the high S_D values found when isolates from the other MRSA clonal groups were matched internally, (Table 3.3) and the considerably lower S_D values found when matching clonal groups with each other, (Table 3.4) this is strong evidence to suggest that the French and Lisbon isolates belong to or are derived from a common clonal ancestor as proposed in Figure 3.18.

Figure 3.18

Lisbon strain (LH) and French strain (FrH) *Hha*I variants: possible evolutionary sequence



Figures in brackets represent DNA fragments lost or gained.

Variants prefixed by "?" denote an evolutionary sequence involving more than one genetic event.

Table 3.3

Dice coefficients of intra-group matching

GROUP	*S_D Range*	*Mean S_D*
MSSA	33 - 93	66
EMRSA-1	84 - 100	92
EMRSA-15	98 - 100	99
EMRSA-16	91 - 100	94
STOBHILL MRSA	76 - 100	89
LISBON MRSA	86 - 100	95
FRENCH MRSA	91 - 100	95
SPORADIC MRSA	69 - 100	82

Table 3.4

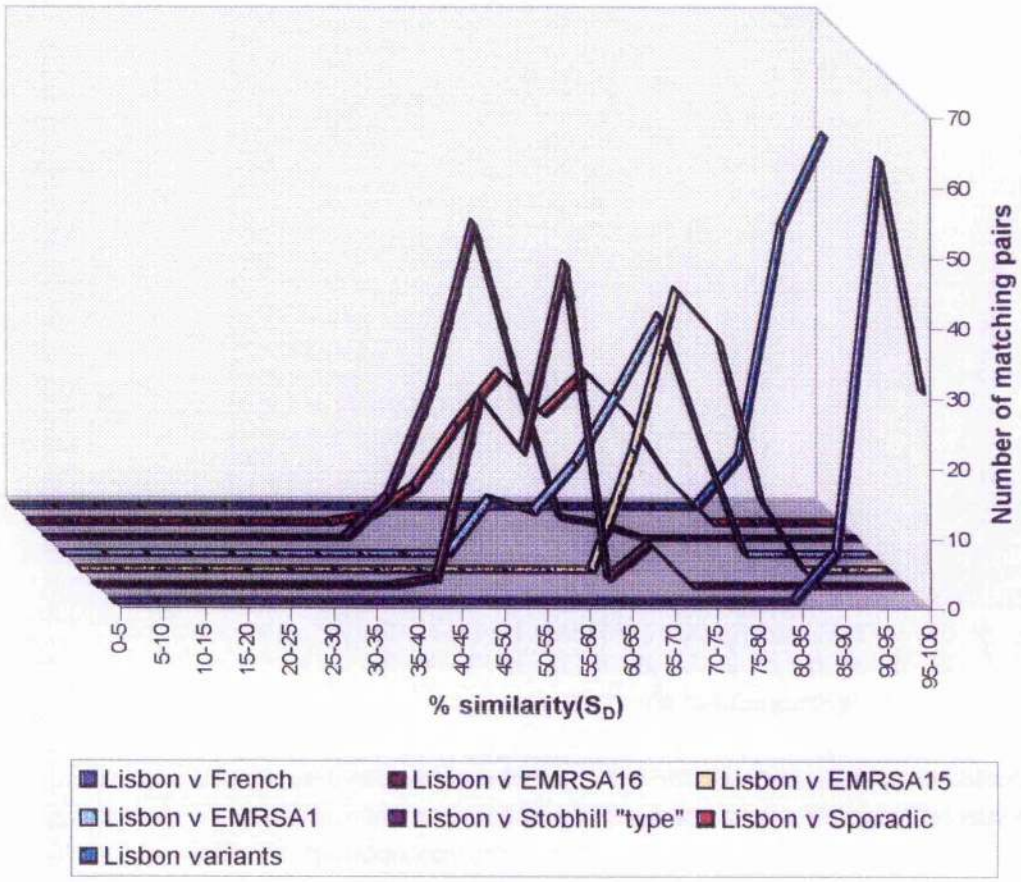
Dice coefficients of inter-group matching

MATCHED GROUPS	*S_D Range*	*Mean S_D*
EMRSA-1 v EMRSA-15	62 - 79	70
EMRSA-1 v EMRSA-16	62 - 76	69
EMRSA-1 v STOBHILL	55 - 71	60
EMRSA-15 v EMRSA-16	70 - 73	72
EMRSA-15 v STOBHILL	47 - 62	53
EMRSA-16 v STOBHILL	47 - 67	57
LISBON v EMRSA-1	62 - 78	71
LISBON v EMRSA-15	65 - 82	74
LISBON v EMRSA-16	44 - 64	55
LISBON v STOBHILL	43 - 67	52
LISBON v FRENCH	86 - 100	94

Figure 3.19 illustrates graphically, the matching of Lisbon strain variants with the variants from the other MRSA groups. The results indicate that the Lisbon and French MRSA were closely related and should be considered as variants that were introduced into Glasgow on two separate and epidemiologically unrelated occasions.

Figure 3.19

S_D values of Lisbon strain variants matched with each other and with variants from the other MRSA groups



3.5 Genotyping of Lisbon strain MRSA with *Sau3A*I

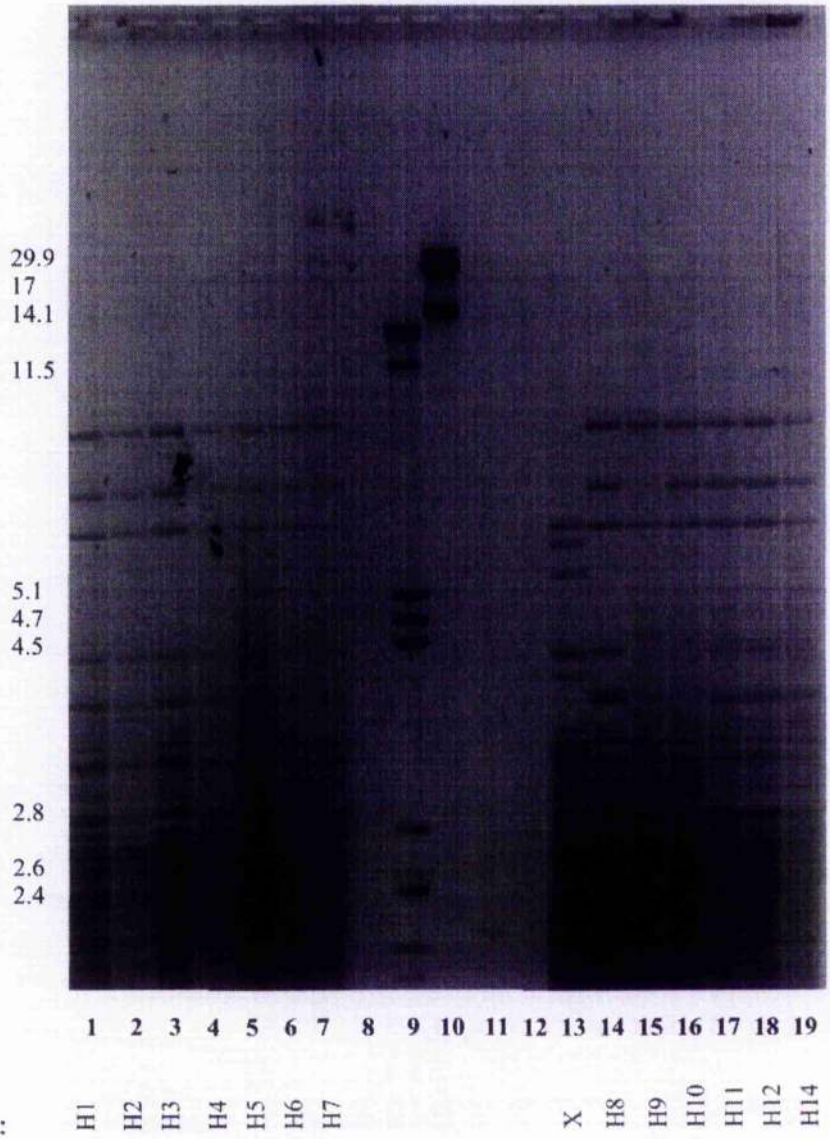
Digestion of the fourteen Lisbon *Hha*I variants with *Sau3A*I yielded 7 variants with 7 isolates belonging to a single type, as shown in Figure 3.20. *Hha*I types H1, H3, H4, H6, H11, H12, and H14 had identical REFP's and were designated *Sau3A*I Type 1. *Hha*I types H2 and H7 were designated *Sau3A*I Type 2 and *Hha*I types H5, H8, H9 and H10 were designated *Sau3A*I Type 3, 4, 5 and 6 respectively.

Twenty-three isolates of the major *Hha*I type, H1 were chosen at random and digested with *Sau3A*I. One variant was found (AB07) with a two-fragment difference as shown in Figure 3.21.

As the restriction enzyme *Hha*I gave clearer fingerprints with a greater number of discernible fragments, it was decided to use this data for statistical analysis.

Figure 3.20

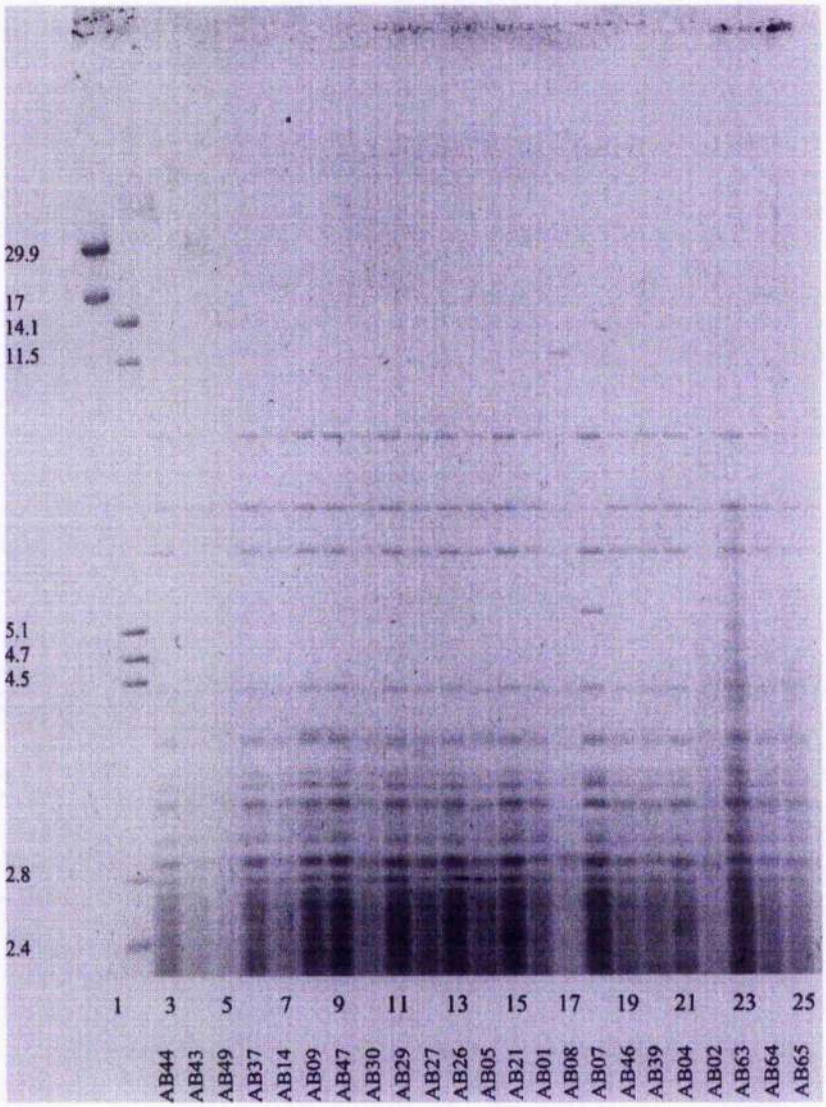
Gel photograph showing *Sau3A*I REFP's of Lisbon strain *Hha*I variants



Lanes 9 and 10 contained respectively, *Pst*I and *Kpn*I digests of phage lambda DNA. Numbers on left of figure denote lambda fragment sizes in kb. Lane 13 (x) contained DNA from a non-Lisbon strain MRSA.

Figure 3.21

Gel photograph showing *Sau*3AI REFP's of Lisbon strain type H1 isolates



Lanes 1 and 2 contained respectively, *Kpn*I and *Pst*I digests of phage lambda DNA. Numbers on left of figure denote lambda fragment sizes in kb. Isolate AB07 in lane 18 was a *Sau*3AI variant.

3.6 Construction of phylogenetic trees

Figures 3.22 - 3.24 show the three dendrograms obtained. RMS values ranged from 8.2 – 9.0. Figure 3.23, the backward run file, was chosen for the analysis as it gave the lowest RMS value. In each case MRSA variants from the same clonal group clustered together and no close relationships were apparent between the different clonal groups. The MSSA formed a number of "loose" unrelated clusters with large genetic distances between them. Unusually some MSSA appeared to be more distantly related to each other than to the single isolate of *Staph. hyicus* and is probably a result of data saturation. Each of the 3 trees indicated that the Lisbon and French strains were very closely related.

Essentially, the dendrograms reflected what was shown by the S_D analysis of the REFP's, that each MRSA clonal group has diverged little within the group but diverged considerably from other MRSA clonal groups, with the possible exception of EMRSA-1 and the clonal group endemic to Stobhill Hospital during 1993/4.

Figure 3.22

Unrooted dendrogram of MRSA and MSSA generated by the neighbor-joining algorithm from data input 1 (forward)

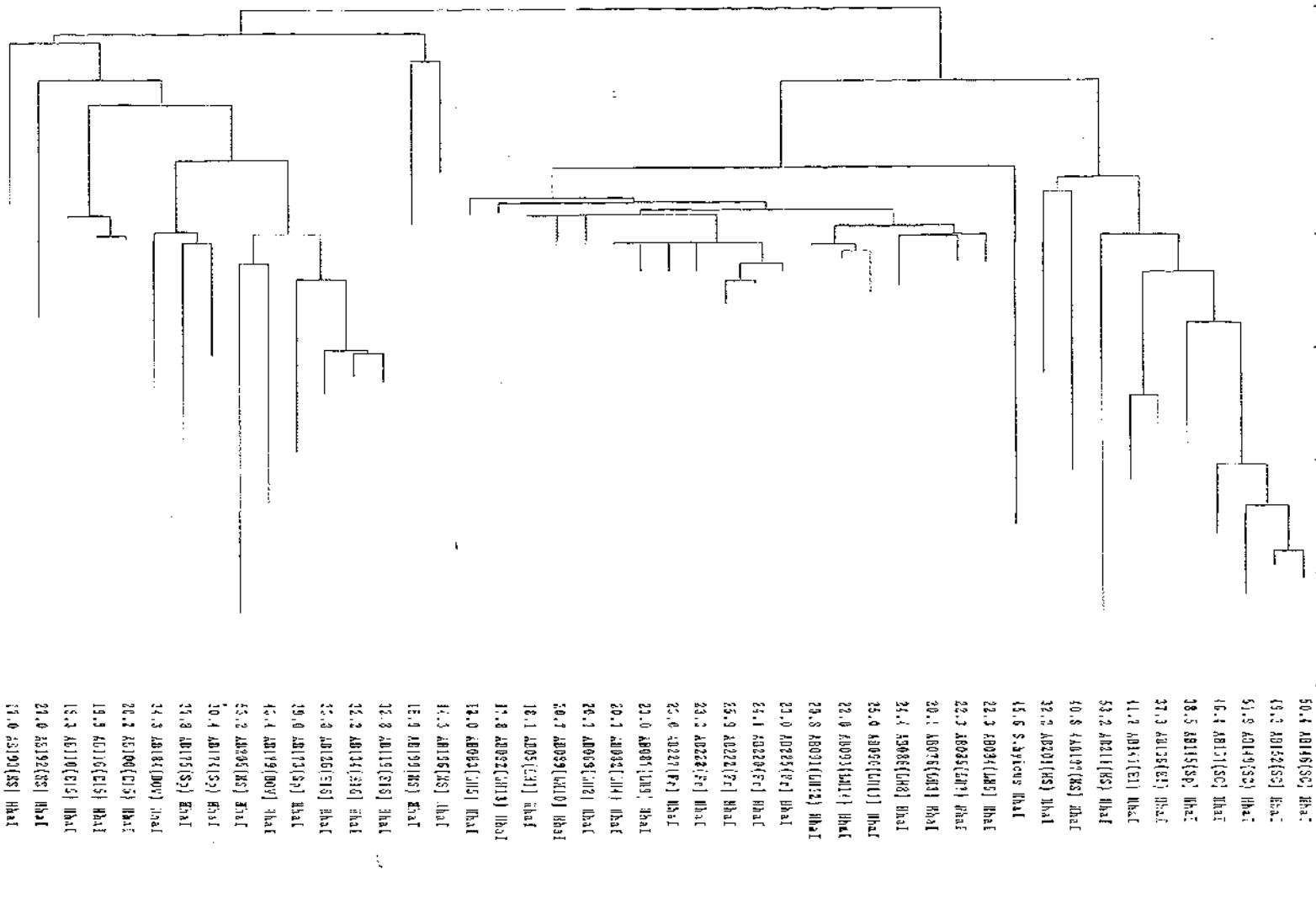
R.M.S. difference between distances from file and tree : 8.2

Figure 3.23

Unrooted dendrogram of MRSA and MSSA generated by the neighbor-joining algorithm from data input 2 (backward)

Figure 3.24

Unrooted dendrogram of MRSA and MSSA generated by the neighbor-joining algorithm from data input 3 (random)



3.7 Phenotyping of *Staph. aureus* strains

(a) Antimicrobial susceptibility testing

(i) Methicillin-sensitive Staph. aureus

This group of isolates was a heterogeneous collection of strains from bovine mastitis, community nasal isolates, GP isolates and isolates from nosocomial infections. Most were sensitive to all anti-microbial agents tested with the exception of penicillin, to which most isolates were resistant. Several strains also showed decreased susceptibility or were fully resistant to ciprofloxacin. One bovine MSSA isolate (AB179) was penicillin and ciprofloxacin resistant. Of the isolates from nasal carriers, one was ciprofloxacin resistant (AB192) and one tetracycline resistant (AB194).

A group of three MSSA from clinical sites (AB198, 199 and 200) were resistant to penicillin, erythromycin and clindamycin, however, *Hha*I fingerprinting indicated that these were genetically unrelated (S_D = < 85%). These results suggested that antibiograms were of little value in differentiation among this heterogeneous group of MSSA.

(ii) EMRSA-1

Eight isolates representative of EMRSA-1 that caused outbreaks of infection in London and in Brisbane, Australia were studied. These were uniformly resistant to penicillin, methicillin, erythromycin, clindamycin, tetracycline, trimethoprim, streptomycin and sensitive to fusidic acid, ciprofloxacin, mupirocin, chloramphenicol, rifampicin and netilmicin. One of

the Australian isolates, AB139 was gentamicin resistant and sulphonamide sensitive and AB137 differed solely in sulphonamide sensitivity. Isolate AB139 lacked a fragment common to all the other EMRSA-1 but it is unlikely that this is related to the isolates' resistance to gentamicin.

Interestingly all the isolates within this small group were trimethoprim resistant, which is somewhat unusual among MRSA strains.

(iii) EMRSA-15

The nineteen EMRSA-15 isolates varied little in antibiogram typing. Fourteen isolates were resistant to penicillin, methicillin erythromycin and ciprofloxacin, and sensitive to clindamycin, fusidic acid, mupirocin, tetracycline, chloramphenicol, rifampicin, sulphonamide, trimethoprim and all aminoglycosides. Three isolates were erythromycin sensitive (AB103, 104 and 111) and all belonged to *Hha*I variant type 15-H1 therefore erythromycin resistance could not be linked to any variation in genotype. Two were sensitive to both erythromycin and ciprofloxacin (AB100 and AB114) and belonged to the *Hha*I variant type 15-H2, therefore as ciprofloxacin and erythromycin resistance is the normal state for most MRSA, it is possible that lack of resistance to both of these agents is connected to this genotypic variation. Four of the five erythromycin-sensitive isolates were from different hospitals.

(iv) EMRSA-16

All of the seventeen EMRSA-16 isolates were resistant to penicillin, methicillin, erythromycin, clindamycin, ciprofloxacin, neomycin and kanamycin and sensitive to fusidic acid, tetracycline, chloramphenicol, rifampicin, streptomycin, netilmicin and amikacin. Mupirocin resistance was detected in nine isolates. High level resistance to mupirocin was not tested for. Seven isolates showed combined trimethoprim and gentamicin resistance and four of these were also sulphonamide resistant. Sulphonamide resistance was also seen in one of the mupirocin resistant isolates.

Kanamycin resistance in EMRSA-16 was interesting. By searching results in the database of the Scottish MRSA Reference Lab. (SMRL) it was noted that all Scottish EMRSA-16 isolates were kanamycin and tobramycin resistant. Although kanamycin resistance was seen in some other isolates in combination with gentamicin resistance, very few other isolates were resistant to kanamycin and tobramycin together. This suggested that kanamycin resistance is a good marker for identification of EMRSA-16, however this alone will not differentiate an EMRSA-16 from a Lisbon isolate.

The EMRSA-16 isolates showed a greater degree of variation than EMRSA-15 in antibiogram and also at genetic level. None of the observed antibiotic variation could be attributed conclusively to observable changes in genotype.

Details of the antibiotic variation seen among EMRSA-16 are shown in Table 3.5.

Table 3.5

Antibiogram variation detected among isolates of EMRSA-16

Designated No.	*CD*	*MUP*	*SU*	*TM*	*GM*
AB 119	R	S	S	R	R
AB 120	S	S	R	R	R
AB 121	R	S	R	R	R
AB 122	R	S	R	R	R
AB 123	S	S	R	R	R
AB 124	R	S	S	S	S
AB 125	R	R	S	S	S
AB 126	R	R	S	S	S
AB 127	R	S	S	S	S
AB 128	R	R	S	S	S
AB 129	R	R	S	S	S
AB 130	R	R	R	S	S
AB 131	R	R	S	S	S
AB 132	R	S	S	S	S
AB 133	R	R	S	R	R
AB 134	R	R	S	R	R
AB 135	R	R	S	S	S

CD, clindamycin; MUP, mupirocin; SU, sulphamethoxazole; TM, trimethoprim; GM, gentamicin.

(v) "Other" MRSA

All of thirty-three ("Stobhill type" plus "Sporadic" group) isolates were resistant to penicillin and methicillin and sensitive to chloramphenicol and rifampicin. All but one were sensitive to trimethoprim. Two isolates (AB144 & AB145) were resistant to all aminoglycosides tested. Both of these isolates had identical antibiograms, identical *Hha*I REFP's and were isolated at the same hospital (RAH). Tetracycline and ciprofloxacin resistance varied considerably in this group, nineteen and thirteen isolates were resistant respectively. Seven isolates were resistant to fusidic acid. Two isolates sensitive to erythromycin were epidemiologically unrelated. One isolate was mupirocin resistant (AB151). This was isolated from Monklands Hospital and was shown by *Hha*I REFP to be related to the Stobhill MRSA clonal type. Antibiogram details of this "non-epidemic" group of MRSA are shown in Table 3.6

These results suggest a heterogeneous group of isolates, which confirms the results of *Hha*I genotyping. Overall, antibiogram typing showed each epidemic MRSA type had specific traits that could be used to make a presumptive identification of the clonal type, e.g. EMRSA-16 were all resistant to kanamycin.

Table 3.6

Antibiogram variation detected among isolates of "non-epidemic" MRSA

Designated No.	ERY	FUS	CIP	MUP	TET	SU	TM	NM	KM	SM	GM	NET	AMI
AB 144	R	S	S	S	R	S	S	R	R	R	R	R	R
AB 145	R	S	S	S	R	S	S	R	R	R	R	R	R
AB 146	R	S	S	S	R	S	S	S	S	S	S	S	S
AB 147	R	S	S	S	R	S	S	S	S	S	S	S	S
AB 148	R	S	R	S	S	S	S	S	S	S	S	S	S
AB 149	R	R	S	S	R	S	S	S	S	S	S	S	S
AB 150	R	S	S	S	S	S	S	S	S	S	S	S	S
AB 151	R	S	S	R	R	S	S	S	S	S	S	S	S
AB 152	R	S	R	S	S	S	S	S	S	S	S	S	S
AB 153	R	R	R	S	R	S	S	S	S	S	S	S	S
AB 154	R	S	R	S	S	S	S	S	S	S	S	S	S
AB 155	R	S	R	S	S	S	S	S	S	S	S	S	S
AB 156	R	S	R	S	R	S	S	S	S	S	S	S	S
AB 157	R	S	R	S	S	S	S	S	S	S	S	S	S
AB 158	R	S	R	S	R	S	S	S	S	S	S	S	S
AB 159	R	S	S	S	R	S	S	S	S	S	S	S	S
AB 160	R	S	R	S	S	S	S	S	S	S	S	S	S
AB 161	R	R	R	S	R	S	S	S	S	S	S	S	S
AB 162	R	S	S	S	R	S	S	S	S	S	S	S	S
AB 163	R	S	S	S	R	S	S	S	S	S	S	S	S
AB 164	R	S	S	S	R	S	S	S	S	S	S	S	S
AB 165	R	S	S	S	R	S	S	S	S	S	S	S	S
AB 166	R	S	R	S	S	S	S	S	S	S	S	S	S
AB 167	R	S	S	S	R	R	S	S	S	S	S	S	S
AB 168	R	S	R	S	R	S	S	S	S	S	S	S	S
AB 169	R	R	S	S	R	S	S	S	S	S	S	S	S
AB 170	R	S	S	S	S	S	S	S	S	S	S	S	S
AB 171	R	R	S	S	S	S	S	S	S	S	S	S	S
AB 173	R	S	S	S	R	S	S	S	S	S	S	S	S
AB 174	R	S	R	S	S	S	R	S	S	S	S	S	S
AB 175	S	S	S	S	S	S	S	S	S	S	S	S	S
AB 176	R	R	S	S	S	S	S	S	S	S	S	S	S
AB 177	S	R	S	S	S	S	S	S	S	S	S	S	S

ERY, erythromycin; FUS, fusidic acid; CIP, ciprofloxacin; MUP, mupirocin; TET, tetracycline; SU, sulphamethoxazole; TM, trimethoprim; NM, neomycin; KM, kanamycin; SM, streptomycin; GM, gentamicin; NET, netilmicin; AK, amikacin.

(vi) The "Lisbon strain"

Of ninety-three Lisbon strain isolates, nine antibiogram variants were identified. Eighty-four had identical antibiograms. These were uniformly resistant to penicillin, methicillin, erythromycin, clindamycin, ciprofloxacin, tetracycline, rifampicin, sulphonamide, neomycin, kanamycin, streptomycin, gentamicin, netilmicin, amikacin and sensitive to fusidic acid, mupirocin, chloramphenicol and trimethoprim.

Of the variants, one had acquired trimethoprim resistance and one fusidic acid resistance. Neither isolate was phenotypically distinct by any other typing method and both belonged to the dominant *Hha*I genotype H1.

Six isolates were sensitive to erythromycin and clindamycin and of these, four belonged to genotype H1 and two to genotype H10. Five of the six were isolated from patients at Glasgow Royal Infirmary and one from a patient at Inverclyde Hospital. A single isolate had lost resistance to the aminoglycosides gentamicin and netilmicin and this belonged to the unique genotype H13.

Details of the variation in antibiotic susceptibility among the Lisbon strain isolates are shown in Table 3.7. As with the other EMRSA groups, antibiogram typing alone was shown to be useful for assigning MRSA isolates to this clonal group. This was an entirely expected observation since it was by this method that the strain was first recognised in Glasgow.

Table 3.7

Antibiogram variation detected among Lisbon strain MRSA

GENOTYPE	*Designated No.*	*ERY*	*CD*	*FUS*	*TM*	*GM*	*NET*	*No. of isolates*
LISBON H 1	common pattern	R	R	S	S	R	R	84
LISBON H 1	AB14	R	R	R	S	R	R	1
LISBON H 1	AB35	S	S	S	S	R	R	1
LISBON H 1	AB43	R	R	S	R	R	R	1
LISBON H 1	AB55	S	S	S	S	R	R	1
LISBON H 1	AB56	S	S	S	S	R	R	1
LISBON H 1	AB57	S	S	S	S	R	R	1
LISBON H 10	AB88	S	S	S	S	R	R	1
LISBON H 10	AB89	S	S	S	S	R	R	1
LISBON H 13	AB92	R	R	S	S	S	S	1

ERY, erythromycin; CD, clindamycin; FUS, fusidic acid; TM, trimethoprim; GM, gentamicin; NET, netilmicin.

(b) Biotyping

(i) Urease Production

All EMRSA-1 and EMRSA-16 isolates tested were urease positive. All EMRSA-15 isolates tested were urease negative. Of the "other MRSA" isolates, eight of thirty-three isolates were urease positive.

Among the MSSA isolates, thirty-nine of forty-two isolates were urease positive.

Of ninety-three Lisbon isolates all but two were urease positive. The two negative isolates were otherwise phenotypically and genotypically unremarkable.

(ii) Hydrolysis of Tween 80

Five of eight EMRSA-1 isolates hydrolysed Tween 80, as did eighteen of nineteen EMRSA-15 isolates and all seventeen EMRSA-16 isolates tested. Of the "Other MRSA" group, eight of thirty-three hydrolysed Tween 80 as did thirty-seven of forty-two MSSA. Among Lisbon isolates only one of ninety-three was found to hydrolyse Tween 80. This isolate was otherwise phenotypically and genotypically unremarkable.

As with the urease test these results suggested that within individual clones, the Tween 80 reaction remained relatively stable and may be a useful strain marker. When results of urease and Tween 80 were combined this gave a very useful aid to detection of the most prevalent epidemic strains as illustrated in Table 3.8.

Table 3.8

Use of simple biotype to distinguish between the Lisbon strain and the current most prevalent epidemic MRSA strains

	TWEEN 80	*UREASE*
LISBON STRAIN	NEGATIVE	POSITIVE
EMRSA 15	POSITIVE	NEGATIVE
EMRSA 16	POSITIVE	POSITIVE

(c) Phage typing

The phage typing results were collated together from three separate sources and may therefore have been subject to variation in interpretation.

Phage typing of EMRSA-1 isolates proved problematic. Some isolates failed to grow or grew only weakly on phage typing media and phage patterns when readable proved confusing with many inhibition reactions. It was therefore decided not to include these results in the final analysis. With the exception of one isolate, all EMRSA-15 were either non-typeable or typed as "75 weak" (75w). One isolate typed as 6w/42E/47w/75w. All EMRSA-16 isolates typed as 29/52/75/77/83A/83C. The group of thirty-three "Other MRSA" isolates showed considerable variation in phage type, although some of these types were not distinguishable by definition. The range is illustrated in Table 3.9.

Phage typing was not performed on the MSSA isolates.

Among the seventy-eight Lisbon isolates for which phage typing was performed, eleven different phage types were identified, although as with the "other" MRSA group, some of these were not distinguishable by definition. Approximately half of the isolates belonged to phage type 85 or 29/77/84/85. The range of phage types is illustrated in Table 3.10.

Although EMRSA-15 isolates fall mainly into two categories when phage typed (75w or Non-typeable), non-typability cannot be considered an indicator of a possible EMRSA-15 isolate since it is not a positive phenotypic trait. However, it may be useful as an aid to identifying isolates of EMRSA-15

when taken in the context of other phenotyping results e.g. Tween 80 positive, urease negative, resistance to; methicillin, erythromycin and ciprofloxacin.

Phage typing was less useful for the Lisbon strain as a number of different phage types were recorded.

Table 3.9

Range of phage types found among isolates of "non-epidemic" MRSA

PHAGE TYPE	*No. OF ISOLATES*
85	6
54/85	2
75/85	2
54/85/90	2
6/42E/47/53/54/75/77	2
85/90	1
42E/47/53/54/75/77	1
6/47/54/75/81	1
6/47/54/75/81/85	1
54/77/47/81	1
53	1
53/85	1
53/85/88A	1
53/83A/75/88A	1
54	1
54/84	1
NT	6
ND	2

NT: Not typeable, ND: Not Done

Table 3.10

Range of phage types found among Lisbon strain isolates.

PHAGE TYPE	*No. OF ISOLATES*
29/77/84/85	22
85	21
77/84	9
77/84/85	8
54/77/84/85	5
54/75/84/85	3
77	2
75	1
54	1
84/85	1
54/77/84/85/75	1
NT	4
ND	15

NT: Not typeable, ND: Not done

Summary of phenotyping results

Table 3.11 summarises the phenotypic typing of the study isolates when broken down into their individual groups. It is noteworthy that all MRSA clonal groups fell into distinct groups on the basis of Tween 80 and urease reactions and although EMRSA-1 and 16 isolates were positive for both tests, inclusion of the antibiogram type enabled further discrimination of these two strains.

Table 3.11

Summary of phenotyping results

STRAIN	*UREASE POSITIVE*	*TWEEN POSITIVE*	*PHAGE TYPE* [1]	*RESISTANT*[2]
LISBON	91/93	1/93	29/77/84/85	MET,ERY,CD,CIP
			54/77/84/85	TET,RIF,SU,
				AGL
EMRSA 1	8/8	6/8	ND	MET,ERY,CD,TET
				TM,SU,SM
EMRSA 15	0/19	18/19	75w	MET,ERY,CIP
EMRSA 16	17/17	17/17	29/52/83A/	MET,ERY, CIP NM,
			75/77/83A	KM, CD (15/17)
				MUP (9/17)
STOBHILL	1/25	2/25	6/42E/47/54/75/85	MET, ERY
MRSA				TET (16/25)
OTHER	8/8	7/8	6/42E/47/53/54/75/77	MET, ERY (4/6)
MRSA			42E/47/53/54/75/77.	
			54/77/47/81	
			53/83A/75/88A	
			NT (2)	
			ND (2)	
MSSA	17/17	12/17	ND	PEN (16/17)
				ERY (3/17)
				CIP (2/17)
				TET (1/17)

[1] Not all patterns listed
[2] Abbreviations defined in methods, except AGL: all aminoglycosides
NT: Not typeable, ND: Not done

4. DISCUSSION

Electrophoresis following whole cell DNA digestion with frequent cutting restriction enzymes is a technique that has not been widely used. In instances where it has been used, the enzymes have been six base cutters and the choice of enzyme and electrophoresis conditions has been optimal for the resolution of fragments less than 6kb (six base cutters). Jordens and Hall (1988) used *Bgl*II (recognition sequence, AGATCT) to type epidemic MRSA isolates from the Thames region and although they were able to say that the epidemic isolates had similar REFP's and unrelated MRSA and MSSA isolates had different REFP's, the interpretation of these REFP's appeared to be very difficult from the figures shown and it is doubtful if the technique would have allowed any form of computerised comparison of REFP's. In addition, fragments due to plasmid DNA also caused problems with interpretation. In a later study of Chinese MRSA by the same authors (Hall and Jordens 1989), a similar technique highlighted the presence of an endemic MRSA strain in one hospital while in a second hospital the isolates were of a more heterogeneous nature. Dice coefficients were used in the Chinese study to determine relationships between isolates. As is also applicable with this study, when using S_D analyses to compare isolates it is important to emphasise that it is the similarity of the banding pattern that is being compared, reflecting conservation of restriction sites in the genome. It must be borne in mind that matching fragments do not necessarily mean identical fragments, as two similar sized DNA fragments may have quite different nucleotide sequences.

*Hha*I and *Sau3A*I, used in this study are four base cutters (recognition sequences GCGC and GATC respectively) which had the advantage of

resolving larger DNA fragments. The resolution of larger fragments obtained after *Hha*I digestion and electrophoresis additionally provided a degree of visual quality control, in that partial digestion products were recognisable if present. In addition, the technique overcomes many of the limitations described by Owen (1989).

Resolution of large fragments provides an open analytical window amenable to computerisation and thereby allowing gel to gel comparisons. It can be adapted to a wide range of disparate organisms using a primary screening panel of usually eight enzymes. The main disadvantage of the technique is that a relatively small portion of the genome is compared and thus different enzymes may not be concordant. The presence of plasmid DNA can on occasion complicate REFP interpretation but any such problems can usually be resolved by running purified plasmid DNA digested by the same enzyme in the well adjacent to the genomic digest (Platt *et al.*, 1996). In addition, within this study, experience has indicated that plasmid fragments in a genomic REFP are generally present in higher copy numbers, tend to stain with greater intensity than genomic fragments and are readily recognised visually.

A selection of epidemiologically unrelated MSSA & MRSA were chosen as control groups in order to evaluate the discriminatory power of the restriction enzymes *Hha*I and *Sau3A*I to distinguish both between and within different MRSA strains involved in outbreaks of infection, as a prelude to the detailed investigation of the Lisbon MRSA strain. All strains within a single bacterial species must have a significant amount of DNA sequence in common to be identified to this taxonomic level. It is possible that any given enzyme

will generate analysable fragments from the conserved (species specific) DNA. Such enzymes would have little resolving power and could not be used to infer that identical REFP's of outbreak strains was meaningful. Conversely, where epidemiologically unrelated strains are shown to be distinct, the subsequent demonstration of identity among potential outbreak strains could be taken as evidence to infer close genetic relatedness.

MSSA strains have been undergoing divergent evolution for countless numbers of years therefore a large number of polymorphisms should be expected. It follows therefore, that epidemiologically unrelated isolates of MSSA will have considerable variation in the arrangement of their genomic DNA and that this variation can be detected by evidence of RFLP's in their genomic fingerprints. Conversely, MRSA strains have evolved over a much shorter time scale (less than 40 years) and therefore it was expected that their genomic fingerprints would be less diverse, especially if the theory put forward by Kreiswirth *et al.*(1993), that the acquisition of *mec* by *Staph. aureus* was a unique event and that all modern MRSA strains are derivatives of this single clone, is correct.

Using probes derived from *mec*A and Tn*554* to hybridise to *Cla*I genomic digests of 472 MRSA isolates dating from the earliest isolations in 1961 to early 1990's, Kreiswirth and co-workers found six *mec*A polymorphic types, which could be arranged chronologically, and 29 different Tn*554* types. They also found that with only one exception (a type showing no homology with Tn*554*) each Tn*554* type occurred in combination with one and only one *mec*A pattern suggesting that primary differentiation of *mec*A patterns is

followed by independent evolution of the Tn*554* patterns within each *mec*A family. Their overall results tended to suggest that horizontal transfer of *mec*A after its initial establishment in *Staph. aureus* is extremely rare and that *mec*A may have been acquired by *Staph. aureus* on a single occasion. However, one major criticism of this study must be the use of two markers not found normally in strains of MSSA. To give the study more validity it would have been useful to include the evolution of a suitable marker found in both groups of *Staph. aureus*. Alternatively, Musser and Kapur (1992) used MLEE on a collection of 254 MRSA isolated over a similar time span but not matched to that of Kreiswirth. They found fifteen distinctive electrophoretic types, marking clones and that the *mec* gene was harboured by many divergent phylogenetic lineages representing a large portion of the breadth of chromosomal diversity within *Staph. aureus.* This result was interpreted as evidence that the horizontal transmission of *mec*A had occurred and therefore a number of unrelated MRSA clones exist. In addition, they also found that MRSA isolated soon after introduction of methicillin into clinical use in the 1960's from the UK, Denmark, Switzerland, Uganda and Egypt belonged to a single electrophoretic type (clone) and concurred with the hypothesis of Lacey and Grinstead (1973) that European MRSA recovered in the 1960's and early 1970's are the progeny of a single ancestral cell which acquired the *mec* determinant.

Mussers study although convincing must be criticised for the small number of enzymes tested, they presented no analysis to demonstrate linkage disequilibrium and genetic drift was not excluded.

The findings of studies by Dominguez *et.al* (1994) and Couto *et. al* (1995) using *mec*A hybridisation and PFGE also suggest the possibility of horizontal transmission of *mec* (see below). Given the diversity indicated by *Hha*I genomic REFP's between the Lisbon strain and the EMRSA-1, 15 and 16 strains, these results would perhaps seem to favour Musser's hypothesis as they are so divergent that their independent evolution from a single clone seems unlikely. However, the fact that the technique only examines a small proportion of the genome must be taken into consideration and such conclusions can be dangerous when only a single molecular typing method is used. In other words, the *Hha*I technique does not yield enough evidence to back up or disprove either authors hypothesis. Specifically the *Hha*I technique demonstrates minor variations and allows similarity to be detected over short time spans. A given *Hha*I REFP does not contain sufficient information to maintain the demonstration of similarity over longer time periods due to the occurrence of data saturation.

Since this original work of Kreiswirth, several workers have carried out epidemiological investigations on the spread of MRSA using *Sma*I PFGE in combination with *mec*A and Tn*554* probe hybridisation of *Cla*I chromosomal digests. Tn*554* was originally chosen to provide a higher degree of resolution when used in combination with the *mec*A hybridisation data. It occurs with a frequency of >90% among MRSA isolates, has never been found on a plasmid, is highly specific in its attachment sites and is often present in two or more copies. It contains a single internal restriction site for *Cla*I, therefore a single insertion of the transposon is represented by two hybridising bands.

A genotyping system based on defined chromosomally located variable genetic elements has significant advantages over phenotypic systems such as phage typing, antibiogram, and biotyping because the genetic basis of the phenotypic variability is usually unknown and the observed phenotypic variation can often be due to more than one type of genetic event.

Phage typing of staphylococci for example, lacks a systematic biological basis, is plagued by non-typeable isolates and by unpredictable variability among typeable ones (Bannerman *et. al*, 1995). It is for such reasons that some reference laboratories including CDC in Atlanta have now abandoned phage typing in favour of PFGE as their main typing system for epidemiological investigations of *Staph. aureus*.

The Kreiswirth approach has been used in a number of MRSA investigations and in particular in tracing the spread of the Iberian clone to which the Lisbon strain is closely related. Couto and co-workers (1995) used in addition to standard phenotyping methods, a combination of PFGE, Tn*554* and *mec*A typing to characterise MRSA and MSSA strains collected over a 3 month period in 1993 from a single Portuguese hospital. Their findings suggested that an unusually large number of MRSA clones were present in the hospital at this time (24 different PFGE types among 54 clinical isolates). This led to the suggestion that the hospital had been acting as a reservoir for strains (including the Iberian clone) responsible for outbreaks in other parts of Portugal and Spain.

Their results also indicated a limited clonality of *mec*A in that only 3 *Cla*I-mecA types were found in contrast to the six described previously by

Kreiswirth *et al.* (1993); *mec*A polymorphs I-VI, and the five described by Dominguez *et al.* (1994); *mec*A polymorphs VII-XI. In common with Dominguez, they found that Tn*554* types could be associated with more than one *mec*A polymorph, contrary to the findings of Kreiswirth. The findings that the same *mec*A polymorph can be associated with numerous chromosomal backgrounds as represented by PFGE patterns and that more than one, of previously established *mec*A polymorphs, can be associated with the same PFGE type was also suggestive of the possible horizontal transfer of *mec*A.

In the same study, Dominguez *et al.* applied Dice coefficients to PFGE patterns and found S_D values of 68% between major PFGE types and S_D values of >88% among sub-types, a result which closely parallels the findings of this study using *Hha*I. Another interesting finding of Dominguez was the isolation of MSSA and MRSA isolates with closely related PFGE patterns from the same patient. The PFGE patterns differed only in the fragment carrying *mec* DNA. The patterns suggested a deletion of the *mec* region resulting in an MSSA homolog.

As far as this author is aware, this is the only epidemiological study of MRSA using restriction enzymes that recognise 4 base sequences (frequent cutters). The results of this study suggested the technique may have considerable potential in future investigations of this type since it unequivocally placed all the control MRSA and Lisbon isolates into distinct clonal groups in agreement with phenotyping results while showing a greater diversity among the MSSA isolates. In addition the technique was further able to discriminate sub-types within each clonal group thus allowing a possible

evolutionary sequence to be proposed since the arrival of the Lisbon strain in Scotland (Figure 3.18). Whereas the combined genetic techniques as described above undoubtedly provide a greater depth of epidemiological and evolutionary data, the *Hha*I technique has the advantage of being simple, cost effective and probably within the scope of most routine clinical laboratories with basic equipment. It also has more than enough resolving power to type outbreaks at a local level. Ideally, within the context of an outbreak the chosen technique should identify all outbreak isolates as identical or closely related while eliminating all unrelated isolates. The *Hha*I technique has been shown to fulfil these criteria within the confines of the study. Whereas it was thought initially that the French MRSA was a different strain on the basis of phenotypic tests, genotyping with *Hha*I combined with Dice coefficient analysis showed these isolates to belong to the same clonal lineage as the Lisbon strain.

Although the MSSA isolates were epidemiologically unrelated, the fact that they came from within a relatively small geographical area may have contributed to slightly higher S_D values than expected. The phenotypic tests were of little value in typing the MSSA isolates, as they were for the most part susceptible to most antibiotics with the exception of penicillin and ciprofloxacin to which most isolates were resistant. The high usage of 4-fluoroquinolones since their introduction has led to this valuable drug being mostly ineffective for nosocomially acquired *Staph. aureus* infection. As most isolates were positive for both Tween 80 hydrolysis and urease production, simple biotyping alone or in conjunction with antibiogram could not be used to produce a useful typing scheme for these isolates. However, as most isolates

conformed to a single biotype (penicillin and ciprofloxacin resistant, Tween 80 hydrolysis and urease positive) unusual isolates not conforming to this biotype could be easily recognised.

Within each epidemic MRSA control group high levels of similarity were found, with mean S_D values >90%. From these results an estimate was made of around >85% for the predicted S_D value which would assign isolates to a defined clonal lineage. These isolates had been designated a particular epidemic type previously on a phenotypic basis (EMRSA-1 etc.) and it was unknown how genetically similar they would be to each other within their groups and also between groups.

The epidemic MRSA control groups were selected as belonging to a specific epidemic type based on phenotypic criteria (phage type), and at the time of their selection the genetic relationships between isolates within the same epidemic type was unknown.

EMRSA-1

In 1984 this became the first recognised epidemic strain to be described in the UK (Cookson and Phillips, 1988). The phage-type of the strain at RTD100 was 85 and varied to some extent with 83A and 84 and with experimental phages 88A and 932. Although phage typing of the EMRSA-1 isolates in this study was attempted, the results repeatedly gave a confusing array of mixed reactions and therefore the data was not used.

Just as *Hha*I had demonstrated its ability to discriminate among unrelated strains of MSSA, its ability to recognise genetically related strains was evident with this group of MRSA. Minimal difference was seen between isolates' overall REFP's although on the basis of individual fragment differences *Hha*I was able to subdivide the group into 5 sub-types.

REFP analysis of the EMRSA-1 isolates revealed the three London isolates to be very closely related (a single fragment difference) and slightly more variation was seen between these and Australian isolates. The divergence seen between these two sub-groups probably resulted from different environmental selective pressures. The *Hha*I REFP's of these isolates indicated that they belonged to the same clonal lineage.

When the REFP from the commonest subtype was matched with the MSSA group, an S_D value of 85% resulted between this and AB198, a community nasal isolate. However when the fragment size variation of 5% was reduced to 1% the S_D value dropped to 46% which agreed with the result when the fragments were matched visually and Dice coefficients calculated manually. This indicated that the computer is less sensitive than the eye and highlighted the importance of taking visual analysis of the gel into consideration when interpreting REFP's as the epidemiological data suggested that both these isolates were unrelated.

The fact that the EMRSA-1 isolates were all sensitive to ciprofloxacin distinguished this group from the other EMRSA groups in the study which were resistant with very few exceptions. That all the EMRSA-1 isolates were sensitive to ciprofloxacin may be a throwback to the age of this strain; i.e. it

may have evolved to epidemic status and later lost its environmental niche to other more persistent MRSA lineages before ciprofloxacin became such a widely used agent for staphylococcal infections. Ciprofloxacin was not introduced into clinical practice until the late 1980's, therefore this observation is not surprising given that resistance to this agent has arisen in staphylococci as a result of widespread use, particularly in treatment of MRSA infections. A uniform resistance to tetracycline was seen among the isolates of this group. This was also the case among Lisbon strain isolates and a high proportion of the non-epidemic MRSA. Prior to the appearance of the Lisbon strain in Glasgow, tetracycline resistance among MRSA was also high and probably reflected a high usage of this antibiotic for staphylococcal infection (G. Edwards, personal communication). The fact that the latest epidemic MRSA strains (EMRSA-15 & 16) are generally sensitive to this agent may reflect a decline in its usage. EMRSA-1 is now only occasionally seen in the UK and data from the Scottish MRSA Reference Laboratory (SMRL) suggest that EMRSA-1 is not seen in Scotland. Statistics provided by the SMRL from April 1997 to July 1998 suggest trimethoprim resistance among Scottish MRSA is relatively rare. Among 4267 isolates of EMRSA-15 only 0.33% were resistant to trimethoprim (14 isolates) and of 934 isolates of EMRSA-16 only 2.67% were resistant. Of a total of 5719 MRSA isolates received in this time only 3.5% exhibited resistance to trimethoprim.

The emergence of multi-resistant strains of staphylococci in the 1970's is thought to have resulted by the combination of resistance genes with transposable elements (Lyon and Skurray, 1987). The mobility of transposable

elements enabled firstly the accumulation of resistance determinants on plasmids (which can then disseminate resistance to large numbers of antimicrobial agents through the bacterial population) and secondly, these elements can then transpose to the chromosome.

Conjugative plasmids clearly demonstrate the role played by transposable elements in the evolution of multi-resistance plasmids. The pSK4 plasmids (35kb) can carry up to 3 different transposons – Tn*4001*, which carries resistance determinants to gentamicin, tobramycin and kanamycin, Tn*4003* (trimethoprim resistance) and Tn*4002* (penicillin resistance). Most of this plasmid family also carries additional resistance determinants to antiseptics and disinfectants.

The rapid rise in isolations of EMRSA-15 and 16 since 1993 (CDR weekly Report, January, 1993) has seen a concurrent decline in isolations of all other epidemic MRSA strains including Lisbon. Reasons for the continued rise of these two epidemic types are unclear but may be due to an increased ability to colonise skin and mucous membranes.

EMRSA-15

Having shown that *Hha*I could successfully differentiate among unrelated strains of *Staph. aureus* and also detect genetically similar or identical isolates, the enzyme showed that phenotypically related isolates of EMRSA-15 were also genetically very similar. Of nineteen EMRSA-15 isolates, three genomic variants were found (when the large plasmid fragment was discounted), of 16 (type E15H1), 2 (type E15H2) and one isolate (type

E15H3). The two E15H2 variants (AB100, AB114) were both sensitive to erythromycin and ciprofloxacin. These isolates were from Dumfries and the Vale of Leven respectively. Data from SMRL indicated that of 4267 EMRSA-15 received from April 1997 to July 31st 1998, only 12 were susceptible to both these antibiotics. Most of these 12 were also atypical in other respects such as phage type and PFGE type.

Erythromycin resistance in MRSA is generally associated with possession of the transposable elephant Tn*554* (Phillips and Novick, 1974). Tn*554*, a site-specific transposon, carries the gene *erm*A that encodes inducible resistance to the macrolide lincosamide and streptogramin B (MLS) groups of antibiotics (Weisblum and Demohn, 1969). It also contains the spectinomycin resistance gene *spc*. Tn*554* is unusual in having a high specificity for a primary chromosomal attachment site (*att*554). Tillotson *et al.* (1989) showed that in contrast to earlier results showing extreme site specificity for the transposon (Murphy et al., 1981; Phillips and Novick, 1979), many isolates of *Staph. aureus* contained second inserts at secondary sites on the chromosome. They found that an attachment site for secondary Tn*554* insertion (*att*155) is within or very close to the region of *mec* DNA on the staphylococcal chromosome. A number of different classes of Tn*554* insertions were found with class 1 being the classical primary insertion site and the novel insert in MRSA being designated class 6. This class 6 insert was found in all of 29 MRSA examined but not in any MSSA. Their results suggested an association of the *att*155 region with the *mec* associated DNA but they were unable to provide any direct evidence for this. Current figures of erythromycin resistance in MRSA

certainly suggest a strong correlation of erythromycin resistance with methicillin resistance while among MSSA, erythromycin resistance remains at a much lower frequency.

Erythromycin sensitivity is therefore most likely due to lack of an active copy of Tn*554* or possession of an inactive form. This is easily tested for using a DNA hybridisation protocol and such a test forms the basis of a now commonly used typing scheme for MRSA (Kreiswirth *et al.*, 1993; De Lancastre *et al.*, 1994; De Sousa *et al.*, 1996; Mato *et al.*, 1998). PCR protocols may also be used to detect Tn*554* (Platt and Parsons, unpublished data). Although five erythromycin-sensitive EMRSA-15 were found in the study, no obvious epidemiological link could be established.

Isolate AB114 also had an unusual phage type (6w/42E/47w/75w) which had not been seen among other EMRSA-15 sent to SMRL. Since most EMRSA-15 are either non-typeable or type as 75w this was regarded as an unusual phenotype. It is intended to investigate these observations further with the help of the SMRL. Isolate AB114 also harboured a unique plasmid of 12kb. AB100 was found to be plasmid free and with the exception of AB114, all other EMRSA-15 contained either a 3.8kb or 38kb plasmid or both (plasmid data not shown). An investigation of the role of these plasmids and their relationships if any, to other MRSA plasmids may provide scope for further investigations.

Prior to *Hha*I typing, these two isolates had not been recognised as EMRSA-15's and were originally included in the sporadic MRSA group. This further illustrates the unreliability of phenotyping. Had *Hha*I genotyping been

available at the time of their initial characterisation, these isolates would have been clearly recognised as EMRSA-15. The establishment of detailed accurate epidemiological information is important as it distinguishes the potentially more troublesome epidemic strains from sporadic MRSA isolates. This information may be important to infection control teams as the appropriate action taken when an MRSA is identified may vary depending on whether or not it is an epidemic strain.

The *Hha*I REFP's of these two isolates were identical to the predominant type except that a fragment of 9.0kb (Figure 3.6) replaced the 8.9kb fragment common to the other EMRSA-15's. This may have been due to the loss of a restriction site in the 8.9kb fragment. If the 8.9kb fragment contained a Tn*554* copy, an insertion into this gene could have rendered the gene inactive to result in the loss of erythromycin resistance. The unique phage type of AB114 may be related to its possession of a 12kb plasmid. Curing the isolate of the plasmid and re-testing the phage type would help to confirm this.

Given the number of both phenotypic and genotypic differences found between these two isolates and the other EMRSA-15, it is also possible that these isolates are more distantly related to EMRSA-15 than the *Hha*I REFP's suggest. Typing anomalies such as this can be resolved in some cases by use of a second enzyme. As the *Sau*3A digests in Figure 3.15 showed, isolate AB114 in track 10 gave a considerably different REFP to the other EMRSA-15 in track 11. This result is in conflict with the *Hha*I result. When use of a second enzyme fails to resolve an anomaly then the application of a different molecular typing technique is the next step. In this instance PFGE may provide the

necessary discrimination to confirm or refute that these isolates belong to the EMRSA-15 clonal type.

The other genomic variant, AB110 (E15H3) differed from the predominant type in possessing an additional fragment of approximately 11.6kb. Since the technique examines only a small proportion of the entire genome we can only speculate how this variant arose. Such a large fragment is unlikely to have arisen from the loss of a single restriction site without a noticeable change elsewhere in the REFP pattern. A more likely explanation is that the isolate has gained this extra DNA from an event such as the integration of an insertion sequence, transposon or phage into the genome. No unusual phenotypic traits (such as additional antibiotic resistance) were observed with this isolate which may have indicated the integration of an insertion sequence or transposon. Although *Hha*I genomic REFP's identified all these isolates as belonging to the same clonal lineage, further work will be necessary to establish the detailed genomic relationships between isolates AB100 and 114 and typical EMRSA-15's.

The EMRSA-15 *Hha*I genomic REFP was very different from that of the EMRSA-1 both visually and by computer analysis of S_D values. This could perhaps be interpreted as an indication that they have evolved independently from unrelated MRSA ancestors. Alternatively, if the clonal theory of MRSA evolution is correct and all modern day MRSA have evolved from a single clone, then it may indicate that the two strains have diverged and evolved from the archetypal MRSA at a very early stage in their evolution. If the strains had begun to diverge sufficiently long ago, because of the large number of

mutations or rearrangements that could have occurred within restriction sites data saturation can occur which obscures the genetic relationships between the strains. However on the basis of *Hha*I REFP's alone there is insufficient evidence for such speculation.

As with EMRSA-1, *Hha*I identified minor genetic variations among the group. Other genetic typing schemes can also subtype these epidemic strains and at this stage it is not yet known how the *Hha*I type / subtype correlates with these, e.g. PFGE. A comparative study of *Hha*I typing versus PFGE may be the focus for future work as the two systems may compliment one another by helping to resolve anomalies seen when only one system is used.

For reasons as yet unknown, antibiogram results proved EMRSA-15 to be the least resistant MRSA type. EMRSA-15 may have evolved fairly recently (first recognised in 1991) from the horizontal transfer of *mec* into a fully sensitive MSSA. In which case it may not have had enough time to acquire new resistance determinants; or it may lack some genetic mechanism required for the establishment of new resistance traits whether they be plasmid encoded or on mobile genetic elements.

Outbreaks of EMRSA-15 infection were confirmed at Stobhill Hospital and Glasgow Royal Infirmary by application of this technique. Disruption to the routine work of the laboratory was minimal, illustrating that the technique can be adapted to use in a clinical setting in addition to being a valuable research tool.

In the UK, successful infection control measures rely on ensuring stringent hand washing between handling patients and isolation of patients,

rather than antibiotic usage. This move away from antibiotic usage may in part be responsible for the evolution of MRSA which are less multi-resistant. If there is no selective pressure on the organisms, unnecessary resistance traits will be lost to allow the organism to adapt to its new environment and become genetically more fit. In Southern Europe infection control policies tend to be less aggressive and antibiotic usage is high. In addition, antibiotics are freely available over the counter. These factors may contribute to the predominance of multi-resistant MRSA clones such as Lisbon (Iberian clone). Data from the SMRL is in agreement with this observation that EMRSA-15 is not a multi-resistant MRSA.

The lack of urease activity amongst EMRSA-15 isolates appeared to be an important phenotypic marker because, with the exception of the "Stobhill clone" of MRSA the vast majority of *Staph. aureus* isolates were urease positive. Although this trait when considered alone is of little value in assigning an organism to a particular type, when used in conjunction with other tests such as Tween 80 hydrolysis and antibiogram, most isolates of EMRSA-15, EMRSA-16 and "Lisbon" can be distinguished presumptively.

EMRSA-15 although the predominant strain isolated in the UK at present, is generally regarded as less pathogenic than EMRSA-16, being more commonly associated with colonisation than true infection, however it is difficult to obtain evidence to support such claims.

EMRSA-16

*Hha*I divided the EMRSA-16's into 5 subtypes. Some REFP's contained fragments suggestive of plasmid DNA. All isolates which were resistant to gentamicin and trimethoprim showed these fragments but plasmid profiling and plasmid REFP's (twice) failed to demonstrate any evidence for these being plasmid in origin. It is possible that these fragments may have been part of a transposon that harboured both resistant determinants.

The mean S_D values between EMRSA-16's, EMRSA-15's and EMRSA-1 showed that *Hha*I was very successful in assigning these epidemic strains to a specific lineage and at distinguishing between different epidemic clones (Tables 3.3 and 3.4).

As with EMRSA-1 and 15, isolates in this group were also genetically closely related by virtue of their *Hha*I REFP's. Genetic variation within the strain appeared to be related to isolate location with type E16H2 found exclusively at the Western General Hospital, Edinburgh; type E16H3 found at Monklands Hospital; types E16H4 and E16H5 found at HCI Hospital and one E16H4 variant was also found at Stobhill Hospital. Differing geographic locations may be an important factor in the molecular divergence of MRSA as the organisms are subjected to different selective pressures. This geographic factor was also evident when the variation of the Australian EMRSA-1 was compared to that of the English isolates and illustrates that the technique may be useful in the recognition of endemic strains that have diverged.

Additionally, resistance to clindamycin and kanamycin was expressed by all the EMRSA-16 isolates and these appear to be key markers in the early recognition of this clonal group.

This was the only group in which mupirocin resistance was seen. Mupirocin resistance in staphylococci is either high-level (>256mg/L) or low-level (0.5 – 256mg/L) which is more common (Rahman *et al.*, 1987; Baird and Coia 1987). Mupirocin is an inhibitor of isoleucine tRNA synthetase and low-level resistance was shown to result from a chromosomal mutation and produced an altered enzyme that had a reduced affinity for mupirocin. In contrast, high-level resistance is plasmid mediated by the *mup*A gene (Gilbart *et al.*, 1993). This gene is sometimes flanked by copies of IS*257*, which suggests that the gene can be mobilised. The mechanism of resistance in these isolates was not elucidated at the time of the study therefore it was not known if the mupirocin resistance was due to high or low level resistance, however this will be addressed in future work with the assistance of SMRL.

Although use of mupirocin for the eradication of MRSA colonisation is widespread and common, the incidence of resistance among Scottish MRSA isolates remains low. Among the isolates which have been found to be mupirocin resistant, high level resistance is rare. Of the epidemic strains (EMRSA-15, 16 and Lisbon) resistance is greatest in EMRSA-16 but the frequency of resistance is greatest in the non-epidemic MRSA (data supplied by SMRL).

The isolates from Dumfries showed combined resistance to gentamicin, kanamycin and trimethoprim. Although we were unable to demonstrate

presence of any plasmid in these isolates, a likely explanation for this resistance is the presence of a large conjugative plasmid. Almost all conjugative plasmids encode resistance to gentamicin, kanamycin and tobramycin by virtue of production of a bi-functional 6' acetyltransferase/2'' phosphotransferase. These plasmids can also mediate resistance to ethidium bromide and quaternary ammonium compounds (Lyon and Skurray, 1987). Some of these plasmids (pSK1) also carry the trimethoprim resistance transposon Tn*4003* on which is located the gene for a type S1 DHFR (dihydrofolate reductase). It is possible that transposition of Tn*4003* to the chromosome has occurred in the above isolates.

Kanamycin resistance appears to be a very good strain marker for EMRSA-16. Among the Scottish MRSA where kanamycin resistance occurs in absence of gentamicin and netilmicin resistance, the isolate is almost always an EMRSA-16. This resistance marker in conjunction with a simple biotype (tween/urease) is a very accurate phenotypic indicator of this strain type (although many English EMRSA-16 are gentamicin resistant). All isolates tested in the study were positive for both urease production and Tween 80 hydrolysis. Figures from SMRL indicate that this is also the case on a larger scale. Of 925 isolates of EMRSA-16 sent to SMRL between April 1997 and July 1998, only 6 were urease negative. The preliminary identification of epidemic strains in this manner provides useful information allowing prompt infection control measures to be implemented while detailed typing at a molecular level is carried out.

Other MRSA

Hhal fingerprinting among the mixed group of MRSA highlighted the possible existence of another clonal group, which may be epidemic in nature as it was isolated from a number of unrelated sources. Since the majority of these isolates came from Stobhill it was tentatively named the "Stobhill clone". There did appear to be more variation between REFP's of this group as shown by the wider range of S_D values (76-100%, mean 89%). Again, some of these REFP's were complicated by the presence of plasmid DNA fragments that contributed to the variation seen. As previously mentioned, for optimum interpretation of the genomic REFP's it is probably advisable to run genomic digests in parallel with their corresponding plasmid digests on the same gel. This approach although more time consuming can yield a large amount of useful genetic information and provides understanding beyond minimalist epidemiology. If necessary an attempt can be made to cure isolates of plasmid DNA using growth in novobiocin at 42°C, however the success rate of this approach is varied. Alternatively, the use of a second enzyme may have confirmed these isolates as a distinct clonal group.

This would be another interesting group to type by other molecular methods as a combination of methods may help confirm the clonal status of the group.

By performing PFGE on the isolates they can be matched to PFGE patterns held on computer at SMRL, which should give some indication if the strain was detected within the last 18 months. It may be that the highly successful EMRSA-15, which had not yet reached its epidemic height in

Scotland around 1994/5, has now displaced this strain from the ecological niche it previously occupied. A comparison of S_D values of this group with the other epidemic strains also indicated that it was not related to the EMRSA-1, 15, 16 or Lisbon clones. These results confirm that the technique is both sensitive and specific at detecting individual MRSA clones.

As expected, the *Hha*I REFP's showed MSSA to be more diverse than their MRSA counterparts, since in evolutionary terms MSSA have been evolving for countless numbers of years as opposed to the near 40 years for MRSA.

***Sau3A*I typing**

Initial results with *Sau3A*I were limited. It was found to be less discriminating than *Hha*I only recognising 7 Lisbon variants. This fact is not necessarily a disadvantage as it provides a hierarchical structure, which enhances the overall information obtained.

*Sau3A*I digested the staphylococcal DNA into much smaller fragments (suggesting a far greater number of restriction sites) resulting in fewer discernible bands amenable to computer analysis. Because of the poor preliminary results coupled with the high cost of this enzyme only selected isolates were typed. Typing systems based on results of single enzyme digests can be less reliable than when two enzymes are used.

Use of the technique in a clinical setting

The technique was found to be useful in the investigation of a small cluster of five MSSA bacteraemia's which were thought to be related, within the Coronary Care Unit at Stobhill. Although the isolates were phenotypically indistinguishable, genomic fingerprinting with *HhaI* and *Sau3AI* proved them all to be unrelated. This served as a useful reminder that the technique can be used effectively in epidemiological investigations not only for MRSA but also for outbreaks involving MSSA isolates that may be difficult to type by phenotypic methods. In a veterinary setting the technique has been used in the investigations of bovine *S. aureus*, *S. hyicus* and *S. intermedius* (Platt *et al.*, 1994).

Technical aspects of *Hha*I genomic fingerprinting

Using the protocol as described in the Materials and Methods section it took approximately 3 - 4 days to type an isolate starting with an isolated colony on a blood agar plate. If using the technique to type isolates in an ongoing outbreak this could be seen as a major disadvantage, however in a setting where the technique is used to type a large collection of isolates in a retrospective analysis the time factor is less important. In addition, during an ongoing outbreak, more rapid PCR protocols may provide sufficient resolving power for preliminary results and *Hha*I fingerprinting can provide a degree of fine-tuning to the investigation.

The DNA extraction method used was a disadvantage. It was labour intensive and involved the toxic chemicals phenol and chloroform. One

potential solution is adaptation of commercially available rapid genomic DNA extraction kits; optimally the protocol would provide purified DNA in one day. However, preliminary personal experience of this approach produced disappointing results with DNA yields being consistently low (data not shown). In contrast to this, a benefit of the study extraction protocol was that it gave high yields of DNA sufficient for up to 3 enzyme digests.

The MSSA group chosen as controls ranged in similarity coefficients from 33-93% with a mean of 66% (Table 3.3). Given that this was a heterogeneous group of isolates known to be unrelated, then mean S_D values for related isolates would be expected to be at least in the high 80's or 90's. When the range of MSSA S_D values was looked at in detail, there appeared to be a greater number of matched pairs at the higher end of the curve (70-90%) than might be expected for an apparently heterogeneous group. This may be due to a combination of random matching, the way in which the gels were digitised and the computer software. Although all electrophoresis parameters were kept constant, the concentration of DNA prepared from each isolate tended to vary somewhat. Where the DNA concentration of an isolate was high, this had the effect of making the smaller fragments appear closer together causing a smear effect which made digitising more difficult. Conversely, where DNA concentration was optimal, separation of the smaller fragments was clearer aiding the computer analysis. If two unrelated isolates were run on different gels and one had a high DNA concentration it was possible for them to appear to be more similar than they really were if they had some larger fragments in

common but more variation among smaller fragments which was not picked up on digitising.

The digitising of fragments in the 2 - 4 kb range was probably the main source of coincidental matching as human error in digitising becomes more significant as the fragments become less well resolved. Because of this difficulty in digitising smaller fragments and because very little fragment variation among MRSA was seen below 4kb, it is recommended in future analyses to set this value as the lower limit of the digitising window. The digitising system used was originally developed for use in building databases of plasmid REFP's, for which it has been highly successful as these fragments are generally well resolved following agarose gel electrophoresis (Rankin *et al.*, 1995, Browning *et al.*, 1995). However, the system does appear to have limitations when applied to genomic REFP's due to the difficulty encountered in resolving fragments less than approximately 4kb. Most workers now use fully automated gel documentation systems e.g. Gelcompar™ and Phoretix™, the latter of which has since superseded the digitising system in our department. Although simple to use, these systems are not yet optimal and lack suitable controls. Many users treat these systems as "black box" technology i.e. they have little or no understanding of the software algorithms that produce the final results (dendrograms). Additionally, the manufacturers and marketers of such software may not have a sufficient understanding of molecular phylogenetic tree construction and the algorithms used to run the software may not be optimal. The human eye/brain system is very accurate at picking up any differences when examining REFP's but a camera is more objective in such an

examination. Computers are designed to equal the work of the brain/eye and effective QC is needed for manufacturers to set parameters to achieve this. Although the technology has advanced to such a level that a computer can equal the eye/brain system the algorithms required to run such systems often have not.

The Lisbon Strain

Phenotypically, the Lisbon strain was very stable. Only one and two isolates varied in their Tween 80 and urease reactions respectively. These results indicated that within specific clones of *Staph. aureus,* the urease reaction remained a relatively stable phenotypic trait that may be a useful strain marker when used in combination with other phenotypic characteristics. In addition, most isolates were urease positive which suggested that perhaps this enzyme is present in natural populations of *Staph. aureus* and that those isolates that were urease negative had either lost the gene for urease production, had a defective copy of the gene or expression of the gene was being suppressed by some mechanism.

Minor differences were seen in the antibiograms of nine isolates. The majority of these isolates (six) lacked the erythromycin / clindamycin resistance phenotype (MLS resistance). Loss of resistance to these agents is not uncommon in MRSA and may result from a loss or a lack of expression of Tn*554*.

Tn*554* is ~6.7kb long and contains no restriction sites for *Hha*I (Genebank accession number U36912) therefore any copies of the transposon

in these isolates would be present on fragments >6.7kb. Conversely if a Lisbon variant had deleted a copy of *Tn*554, a deletion in a fragment >6.7kb would be visible on the *Hha*I REFP. The standard Lisbon *Hha*I REFP, which was found in 68 isolates was designated type LH1 and all variants of this pattern were designated types LH2 – LH14. Two of the erythromycin sensitive isolates belonged to the *Hha*I type LH10, characterised by the absence of an 8.0kb fragment present in the LH1 genotype (Table 3.1). A fragment of this size was present in all other Lisbon and French isolates with the exception of LH13. It is possible therefore that these two isolates had lost a copy of Tn*554* from this fragment. Using a PCR protocol based on detection of Tn*554* to characterise MRSA will be the focus of future work in order to add a further level of discrimination to the technique (Platt and Parsons, unpublished data).

The other four isolates of this group belonged to the LH1 genotype, and since no observable DNA loss had occurred a possible explanation for the loss of MLS resistance in these isolates may be due to the loss of expression of the transposon or a mutation in the *erm*A gene.

One isolate (AB92) was susceptible to gentamicin and netilmicin while remaining resistant to streptomycin and kanamycin. Because resistance to aminoglycosides is the normal phenotype of this clone, it is reasonable to assume that this isolate had lost part of this trait. This isolate had a unique *Hha*I genomic REFP (LH13), which corresponded to the loss of the 8.0kb fragment and acquisition of an 8.1 and 6.2kb fragment. It is possible that the loss of this resistance trait correlated with changes in the genomic fingerprint.

Gentamicin resistance in staphylococci is mediated by the transposon Tn*4001* by way of the aminoglycoside modifying enzymes AAC(6′) and APH (2″), however these enzymes also confer coincident resistance to tobramycin and kanamycin (Lyon *et al.*, 1987). Transposon mutagenesis experiments have shown that the protein encoded by the gene *aac*A-*aph*D possesses two domains, one of which produces the kanamycin and tobramycin phenotype, km^r tm^r via the AAC(6′) activity and a second which mediates gentamicin resistance (gm^r) via the APH(2″) activity. It is also known that sequences within the km^r / tm^r domain are essential for correct folding of the putative APH (2″) active site. It may therefore be possible that a single mutation in one of these sequences may have allowed km^r / tm^r to be expressed but resulted in abnormal folding of the APH (2″) active site preventing expression of gm^r.

Two isolates had acquired additional resistance determinants with no change in the standard (LH1) REFP type. One was resistant to fusidic acid the mechanism for which involves a chromosomal mutation. Resistance to this agent tends to be sporadic and is generally as a result of recent exposure and selection. As with rifampicin, resistance is known to arise rapidly due to a spontaneous mutation when the agent is used alone. For this reason fusidic acid is generally used in combination with another antimicrobial agent.

The final antibiogram variant was due to the acquisition of trimethoprim resistance in an isolate belonging to the standard REFP type LH1 (AB43). As has been previously stated, trimethoprim resistance (tp^r) is uncommon in MRSA isolates with the exception of certain defined clonal types e.g. EMRSA-1 and the Brazilian MRSA (De Sousa et al., 1998). It has been

associated with a putative transposon Tn*4003*, a 4.7kb element that contains 3 copies of the insertion sequence IS*257*. This element contains no restriction sites for *Hha*I therefore its presence would have been detected within the window set for *Hha*I genomic typing. The fact that no variation was seen in the genomic REFP therefore suggested that trimethoprim resistance in this isolate was not due to a Tn*4003* insertion.

Although plasmid analysis was not performed on this isolate, a selection of Lisbon isolates were selected for plasmid profiling and REFP analysis. Preliminary results of this typing (data not shown) suggested that most isolates contained a single plasmid that had identical *Hha*I and *Hae*III REFP's. Some isolates were plasmid free (this appeared to have no observable phenotypic effect). Future plasmid analysis of this isolate may help determine the nature of trimethoprim resistance in this isolate. As with Gram negative organisms, trimethoprim resistance in *Staph. aureus* can be either low level (10-500 mg/L) due to overproduction of chromosomal DHFR, or it may be high level (>1000mg/L) which is typically plasmid mediated. A simpler method of determining the nature of this resistance may therefore be to perform a trimethoprim MIC on the isolate.

The window given by *Hha*I enabled ~ 120 – 130kb of DNA to be visualised as discrete fragments that ranged from 3.7 – 15kb. Clearly as the staphylococcal genome measures ~2.7Mb (Pattee *et al.*, 1990), we are looking at a small percentage (~5%) of the total cellular DNA. This type of approach in a typing technique has two potential problems. Firstly, if the genome contains a large number of hyper-variable regions then the technique will be too

sensitive, showing variation perhaps in every isolate examined and therefore data saturation occurs very quickly. Conversely, if the technique targets areas of the genome which are highly conserved then little or no variation will be seen therefore the technique lacks the necessary discrimination needed to be a useful typing tool.

The selected control groups were able to show that the technique does not suffer from either of these pitfalls. By continuous subculture of a representative Lisbon isolate at different temperatures, we were also able to show that the genotype of the strain remained stable over a six month period and that no observable change in phenotype occurred.

Genomic variation seen in the Lisbon strain

When the Lisbon strain first appeared in Scotland it was easily recognised as a new strain of MRSA when isolated from clinical specimens due to its unusual resistance phenotype (in addition to the fact that it was isolated from a patient that had just arrived in the country from Portugal). Prior to this, multi-resistant strains were encountered only infrequently and this strain was even more unusual in being resistant to all aminoglycosides and to rifampicin which proved a good strain marker. Shortly following the recognition of this new MRSA a second multi-resistant strain was recognised. This strain although very similar to the Lisbon strain was epidemiologically unrelated and differed in its degree of resistance to rifampicin. Because of this it was uncertain whether or not this was a different strain or simply a phenotypic variant of the Lisbon strain and since the strain was thought to have been

imported from France it became known locally as the "French strain" MRSA. MRSA with this phenotype were isolated only very occasionally and were soon replaced by the more persistent Lisbon strain.

One of the aims of the thesis was to address this question of the relationship between the two MRSA phenotypes. As Figures 3.17 and 3.18 clearly showed, *Hha*I REFP's confirmed that both "types" belonged to the same clonal lineage and the mean Dice coefficient for matching the two groups was 94% (Table 3.4). It was also apparent from the analysis that the French variant FrH5 was identical in REFP to Lisbon variant LH4 i.e. they were both lacking a 5.3kb fragment present in Lisbon variant H1 (Figure 3.17).

It should be noted that in order to construct the flow diagram outlining the possible evolutionary sequence of the Lisbon strain we have made the important assumption that all variants have descended from the type LH1. This assumption was based on:

1. the index case typing as this REFP
2. 73% of the isolates gave this REFP
3. of the two oldest isolates in the collection, one had the LH1 REFP.

It is recognised that this assumption may be flawed but given the data obtained it is probably a reasonable proposal of events. It may be equally correct to assume that variant LH3 was the original genotype since the first isolation of this type was at the same time (01/12/90). It is possible that this sub-type diverged into a further five sub-types with the loss or gain of a single fragment (assumed to be a single genetic event) whereas LH1 gives rise to only

four. However, as LH1 was the most frequently isolated genotype (from many sites in WOS), this suggested that it was genetically the most stable or "fittest" sub-type. One other possibility for the appearance of variants is the secondary importation of another variant of the Iberian clone MRSA. Given that the French variant of the Lisbon strain was a secondary import, this explanation is quite plausible particularly for those variants that cannot be arrived at by a single step.

In late 1989, a Spanish Reference Laboratory noted an increase in numbers of MRSA belonging to phage type 29/77/84/932. These isolates were found in several hospitals in different cities suggesting spread of an epidemic strain. Aparicio and co-workers (1992) characterised these isolates by phenotypic and genotypic methods. Included in these study isolates were two isolates representative of outbreaks that had occurred in the UK due to the return of an infected patient from Spain in 1990. They used conventional electrophoresis of whole cell DNA with several enzymes but failed to discriminate conclusively between isolates. However, phenotyping of these isolates suggested two variants based on phage typing. Most importantly, the phenotyping suggested that this strain was part of the Iberian Clone and therefore related to the Lisbon strain, which appeared in Scotland also around the same time. This has implications for the evolution of the Lisbon strain in Scotland. It may help explain the existence of variants that cannot be arrived at by a single genetic event from another subtype (Figure 3.18) by implying that such isolates may have arrived in Scotland from other sources not connected to the index case.

As the strain spread to new locations or new patients, it would be subjected to a number of different selection pressures and in so doing may have undergone mutation in the form of deletions, insertions and rearrangements of its genomic DNA. Where these changes occurred within a *Hha*I restriction site they would be detected on REFP typing if the fragments were large enough. Also, if a DNA insertion is large enough it can be detected on the REFP without necessarily being within a restriction site. These types of genetic events can lead to the development of numerous genetic variants of the type strain. The majority of these variants would be unstable, some mutations may eventually be lethal and the variants would therefore be unable to establish themselves. This seems to be the most likely explanation for the existence of the Lisbon sub-types since, with the exception of LH2, LH3 and to a lesser extent LH10, all other variants were encountered once only. In the case of LH2 these isolates span a period of 5 years from 1991 – 96. It is possible that LH1 diverged to give a stable variant that had survived at a low level being detected only infrequently over the years.

The results obtained in this study have shown that the Lisbon strain is very closely related genetically to the Iberian clone first described in Spain by Dominguez and co-workers (1994). Since its discovery, molecular surveillance studies have identified the clone in Spain, Portugal, Belgium, Germany, Italy, Scotland and USA (Mato *et al.*, 1998)

As part of the above collaborative study (Mato) to investigate the geographical spread of the "Iberian" clone of MRSA, a selection of MRSA comprising 7 Lisbon variants, 2 French variants and 4 sporadic MRSA were

sent to Portugal for further epidemiological typing. This included *Sma*I PFGE, *Cla*I-*mec*A typing and *Cla*I-Tn*554* typing. Using these methods previously, Sanches *et al.* (1995) designated the typical Iberian clone the type I::E::A; where I is the *mec* A type, E is the Tn*554* type and A is the pulse field type. Of the Lisbon isolates, 3 conformed to this typical type (LH1, LH5 and LH6). All had unique PFGE types which were sub-types of A (as with the *Hha*I type) and all had the *mec*A polymorph I. Four Lisbon isolates had unique Tn*554* types. One French variant differed from Lisbon in both *mec*A polymorph (VI) and Tn*554* type although it was closely related by PFGE. Most importantly, the results of PFGE / *mec*A / Tn*554* typing confirmed the results of *Hha*I genomic RFFP's in demonstrating that the Lisbon and French strain MRSA are part of the Iberian MRSA clone. In addition, the Tn*554* types appear to have become more diverse among Scottish isolates of the Iberian clone when compared to those from Italy, Spain and Portugal. As yet reasons for this are unclear but this apparent increased Tn*554* diversity may be the focus of future work. One possible mechanism may be different selection pressures exerted by different approaches to antimicrobial usage in the respective countries.

Conclusions

This study has shown that *Hha*I and to a lesser extent *Sau3A*I are valuable tools for epidemiological typing and the successful application of the technique resulted in all of the Study aims being achieved. Epidemiologically unrelated MRSA were shown to be genotypically diverse but to a lesser extent than MSSA; all isolates phenotypically designated as Lisbon strain MRSA

were closely genotypically related consistent with the expansion of a single clone; *Hha*I was sufficiently discriminating to allow the recognition of several molecular variants of this clonal group; and by application of Dice coefficients, no control group was shown to be closely related either to each other or to the Lisbon group.

Future work

Investigation of anomalies

The study has highlighted several interesting features of MRSA genetics which necessitate further investigation. Firstly, the anomalies in some of the study results will be addressed for completeness e.g. the conflicting *Hha*I / *Sau3A*I / phenotype results of the EMRSA-15 variants AB100 and AB114; the additional aminoglycoside resistance in some of the EMRSA-16 isolates; mechanisms involved in loss of aminoglycoside resistance and gain of trimethoprim resistance (AB92 and AB43, respectively) and further investigation of the "French strain" MRSA which yielded an unrelated PFGE type from the collaborative work of Mato *et al.*(1995). A closer investigation of Tn*554* in the Lisbon strain may prove interesting as the preliminary investigations of Mato *et al.* suggested that the Tn*554* types were more diverse among Scottish isolates. Further investigation of the "Stobhill clone" MRSA is also warranted to determine if it is still present in the hospital since the appearance of EMRSA-15 and EMRSA-16.

PFGE comparative study

A comparative study of *Hha*I / *Sau3A*I genotyping and PFGE typing must be a priority as this will yield more valuable information not only on the relative discriminatory powers of the two techniques but also their respective capacity to detect more distant relationships between strains. The time factor involved in obtaining *Hha*I / *Sau3A*I typing results is a considerable drawback to the technique and should also be addressed with the aim of achieving an extraction protocol which provides a result within 24 hours from an overnight broth culture as this is now possible with PFGE (SMRL, unpublished data).

MRSA plasmids

Although outwith the main scope of this thesis, the plasmid data should be investigated in more detail and characterisation of the Lisbon strain plasmid(s) and relationships to other MRSA would also yield valuable data. The preliminary results of plasmid typing suggested that most isolates of Lisbon MRSA possessed a single plasmid which showed no variation on REFP when digested with *Hha*I or *Hae*III and this plasmid appeared to be unrelated to plasmids found in EMRSA-15 and EMRSA-16.

5. APPENDIX I

Media and Reagents

Preparation of Media and Reagents

1. **Brain Heart Infusion Broth** (Oxoid CM225)

   36g of BHI powder were dissolved in one litre of distilled water, distributed in 10ml amounts in glass universals and sterilised by autoclaving at 121°C for 15 minutes.

2. **Todd Hewitt Broth** (Oxoid CM225)

   36.4g of THB powder were dissolved in one litre of distilled water, distributed in 10ml amounts in glass universals and sterilised by autoclaving at 115°C for 15 minutes.

3. **TES Buffer**

Tris base (Sigma)	50mM	3.03g
Sodium chloride (Analar)	50mM	1.46g
Di-sodium EDTA (Sigma)	5mM	0.93g

   Made up as required by dissolving in 400ml of distilled water and adjusting pH to 8.0 with conc. HCl. The remainder of distilled water was added to give a final volume of 500ml. Stored at 4°C.

4. **TESS Buffer**

   Prepared as above with the inclusion of 50mM sucrose (Sigma).

5. **Lysostaphin** (Sigma)

Used at a conc. of 1000units/ml. Reconstituted to this concentration with distilled water and distributed in 1ml amounts in sterile Eppendorf tubes. Stored at -70°C.

6. **Lysozyme** (Sigma)

Prepared freshly each time. Used at a concentration of 40µg/ml. Reconstituted to this concentration with distilled water by adding 0.12g to 3ml of distilled water. This gave enough to lyse 8 isolates.

7. **20 % Sodium dodecyl sulphate** (Sigma)

Prepared by dissolving 2g of SDS in 10ml of sterile distilled water. Stored at room temperature.

8. **Proteinase K** (Sigma)

Reconstituted to 10mg/ml in sterile distilled water. Distributed in 1.3ml amounts in sterile Eppendorf tubes. Each tube contained enough proteinase K for 8 isolates. Stored at -20°C.

9. **Isopropanol** (Sigma)

A working volume only (<50ml) was kept on the bench at any time. The stock bottle was stored in the "flammable" cupboard.

10. Phenol / Chloroform (Analar)

250g of detached phenol crystals was added to 250ml of chloroform in the fume cupboard in a 2 litre flask. This was allowed to stand until completely dissolved. 50ml of TGE was added and mixed well. The mixture was transferred to a dark bottle and the aqueous layers allowed to separate before use. Stored at 4°C.

11. TGE Buffer

Tris (Sigma)	25mM	1.5g
Di-sodium EDTA (Sigma)	10mM	1.85g
Glucose (Analar)	50mM	4.5g

Dissolved in 100ml distilled water, pH adjusted to 8.0 with HCl and made up to 500ml with distilled water. Sterilised by autoclaving at 110°C for 10 minutes. Stored at room temperature.

12. TE Buffer

Tris base (Sigma)	10mM	0.605g
Disodium EDTA (Sigma)	1mM	0.186g

Dissolved in 400ml of distilled water and pH adjusted to 8.0 with HCl. Final volume made up to 500ml with distilled water and autoclaved at 121°C for 15 minutes.

13. TE_{10} Buffer

Tris base (Sigma)	10mM	0.605g
Disodium EDTA (Sigma)	10mM	1.86g

Dissolved in 400ml of distilled water and pH adjusted to 7.8 with HCl. Final volume made up to 500ml with distilled water and autoclaved at 121°C for 15 minutes.

14. 7.5 M Ammonium acetate (Sigma)

57.3g was dissolved in 50ml of distilled water and pH adjusted to 8.0 with glacial acetic acid. Made up to 100ml with distilled water. Stored at room temperature. This was replaced regularly (monthly) as it was unstable.

15. Absolute Ethanol (BDH)

As with isopropanol, only a working volume was kept on bench and the stock bottle stored in "flammable" cupboard.

16. RNase (Sigma)

Prepared at a working concentration of 10mg/ml by dissolving 100mg in 10ml of distilled water. This was then split into three aliquots in glass universals, placed in a beaker of water and boiled for 10 minutes. Stored at 4°C.

17. Tris - borate Buffer (TBE)

Tris base (Sigma)	89mM	53.9g
Boric acid (Sigma)	89mM	27.5g
Di-sodium EDTA (Sigma)	1.25mM	2.3g

Dry chemicals were weighed out for 5L batches of buffer and stored in plastic jars at room temperature until required, then the jar contents were added of to 5L of distilled water. The pH was adjusted to within the range 8.0 - 8.4.

18. Agarose (Sigma)

A 0.6% gel was prepared by dissolving 3g in 500ml of TBE buffer. This was boiled until completely dissolved and cooled to just below 50°C before pouring.

19. Ethidium Bromide (Sigma)

A stock solution of 10mg/ml was made. Gels were stained in used electrophoresis buffer (TBE) containing a final conc. of 0.5 -1.0 μg/ml of ethidium bromide.

N.B. this chemical is a powerful mutagen and gloves were worn at all times when handling.

20. Tracking dye

Sucrose (Sigma)	25g
Sodium acetate (Sigma)	60mg
SDS (Sigma)	100mg
Bromophenol blue (Sigma)	50mg

Dissolved in distilled water and made up to 100ml. Stored at room temperature.

21. 20 % Sodium sulphite (Analar)

Dissolved 200g in 1 litre of distilled water. Stored at room temperature in a dark bottle.

5. APPENDIX II

Example of computer generated matching pair data

Appendix II (a) Computer generated print-out of DNA fragment sizes of Lisbon and French MRSA molecular variants

Track 0		LIS H1		HhaI		20 Sizes													
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	5.77	2	5.32	2	5.09					
4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02					
3.95	2	3.88	2	3.83	2	3.69	3												
Track 1		LIS H2		Hhal		19 Sizes													
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	5.32	2	5.09	3	4.88					
4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02	2	3.95					
3.88	2	3.83	2	3.69	3														
Track 2		LIS H3		Hhal		21 Sizes													
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	6.40	2	5.77	2	5.32					
5.09	3	4.88	2	4.73	2	4.63	2	4.48	2	4.34	3	4.18	2	4.11					
4.02	2	3.95	2	3.88	2	3.83	2	3.69	3										
Track 3		LIS H4		Hhal		19 Sizes													
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	5.77	2	5.09	3	4.88					
4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02	2	3.95					
3.88	2	3.83	2	3.69	3														
Track 4		LIS H5		Hhal		18 Sizes													
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	5.09	2	4.88	2	4.73					
4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02	2	3.95	2	3.88					
3.83	2	3.69	3																
Track 5		LIS H6		Hhal		20 Sizes													
15.03	2	11.70	2	7.99	2	6.85	2	6.40	2	5.77	2	5.32	2	5.09					
4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02					
3.95	2	3.88	2	3.83	2	3.69	3												
Track 6		LIS H7		HhaI		22 Sizes													
15.03	2	12.57	2	11.70	2	10.08	2	7.99	2	6.85	2	6.40	2	5.77					
5.32	2	5.09	3	4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18					
4.11	2	4.02	2	3.95	2	3.88	2	3.83	2	3.69	3								
Track 7		LIS H8		Hhal		23 Sizes													
15.03	2	12.57	2	11.70	2	7.99	2	7.70	2	6.85	2	6.61	2	6.40					
5.77	2	5.32	2	5.09	3	4.88	2	4.73	2	4.63	2	4.48	2	4.34					
4.18	2	4.11	2	4.02	2	3.95	2	3.88	2	3.83	2	3.69	3						
Track 8		LIS H9		Hhal		20 Sizes													
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	5.77	2	5.52	2	5.09					
4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02					
3.95	2	3.88	2	3.83	3	3.69	3												
Track 9		LIS H10		Hhal		19 Sizes													
15.03	2	12.57	2	11.70	2	6.85	2	5.77	2	5.32	2	5.09	3	4.88					
4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02	2	3.95					
3.88	2	3.83	2	3.69	3														

LIS = Lisbon variants
Fr. = French variants

Appendix II (a) Contd.

Track 10		LIS H11		Hhal		24 Sizes								
15.03	2	12.57	2	11.70	2	8.30	2	7.99	2	7.39	2	6.85	2	6.40
6.20	2	5.77	2	5.32	2	5.09	3	4.88	2	4.73	2	4.63	3	4.48
4.34	3	4.18	2	4.11	2	4.02	2	3.95	2	3.88	2	3.83	2	3.69
Track 11		LIS J112		HhaI		22 Sizes								
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	6.40	2	6.20	2	5.77
5.32	2	5.09	3	4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18
4.11	2	4.02	2	3.95	2	3.88	2	3.83	2	3.69	3			
Track 12		LIS H13		HhaI		21 Sizes								
15.03	2	12.57	2	11.70	2	8.15	2	6.85	2	6.20	2	5.77	2	5.32
5.09	3	4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11
4.02	2	3.95	2	3.88	2	3.83	2	3.69	3					
Track 13		LIS H14		HhaI		22 Sizes								
15.03	2	12.57	2	11.70	2	8.30	2	7.99	2	6.85	2	6.40	2	5.77
5.32	2	5.09	3	4.88	2	4.73	2	4.63	3	4.48	2	4.34	2	4.18
4.11	2	4.02	2	3.95	2	3.88	2	3.83	2	3.69	3			
Track 14		Fr H1		HhaI		21 Sizes								
15.03	2	12.57	2	11.70	2	8.97	2	7.99	2	6.85	2	6.23	2	5.77
5.09	3	4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11
4.02	2	3.95	2	3.88	2	3.83	2	3.69	3					
Track 15		Fr H2		HhaI		23 Sizes								
15.03	2	12.57	2	11.70	2	8.97	2	7.99	2	6.85	2	6.23	2	5.97
5.77	2	5.33	2	5.09	3	4.88	2	4.73	2	4.63	3	4.48	2	4.34
4.18	2	4.11	2	4.02	2	3.95	2	3.88	2	3.83	2	3.69	3	
Track 16		Fr H3		HhaI		20 Sizes								
15.03	2	12.57	2	11.70	2	8.97	2	7.99	2	6.85	2	5.77	2	5.09
4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02
3.95	2	3.88	2	3.83	2	3.69	3							
Track 17		Fr H4		HhaI		20 Sizes								
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	6.23	2	5.77	2	5.09
4.88	2	4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02
3.95	2	3.88	2	3.83	2	3.69	3							
Track 18		Fr H5		HhaI		19 Sizes								
15.03	2	12.57	2	11.70	2	7.99	2	6.85	2	5.77	2	5.09	3	4.88
4.73	2	4.63	3	4.48	2	4.34	3	4.18	2	4.11	2	4.02	2	3.95
3.88	2	3.83	2	3.69	3									

NB. Number 2 or 3 beside fragment size indicates whether fragment was estimated as a doublet or triplet.

Appendix II (b) Computer generated print-out of Dice coefficients from matches between Lisbon and French MRSA molecular variants

Fragment sizes between 3.69kb and 15.03kb
Fragment size variation <=5.0%
Similarity coeff. >1.0%

Sim. Coeff (M)	Tk.	Pts	Organism	Enzyme	Tk.	Pts	Organism	Enzyme
97.4 (19)	0	20/20	LIS H1	Hhal	1	19/19	LIS H2	Hhal
97.6 (20)	0	20/20	LIS H1	Hhal	2	21/21	LIS H3	Hhal
97.4 (19)	0	20/20	LIS H1	Hhal	3	19/19	LIS H4	Hhal
94.7 (18)	0	20/20	LIS H1	Hhal	4	18/18	LIS H5	Hhal
95.0 (19)	0	20/20	LIS H1	Hhal	5	20/20	LIS H6	Hhal
95.2 (20)	0	20/20	LIS H1	Hhal	6	22/22	LIS H7	Hhal
93.0 (20)	0	20/20	LIS H1	Hhal	7	23/23	LIS H8	Hhal
100.0 (20)	0	20/20	LIS H1	Hhal	8	20/20	LIS H9	Hhal
97.4 (19)	0	20/20	LIS H1	Hhal	9	19/19	LIS H10	Hhal
90.9 (20)	0	20/20	LIS H1	Hhal	10	24/24	LIS H11	Hhal
95.2 (20)	0	20/20	LIS H1	Hhal	11	22/22	LIS H12	Hhal
97.6 (20)	0	20/20	LIS H1	Hhal	12	21/21	LIS H13	Hhal
95.2 (20)	0	20/20	LIS H1	Hhal	13	22/22	LIS H14	Hhal
92.7 (19)	0	20/20	LIS H1	Hhal	14	21/21	Fr H1	Hhal
93.0 (20)	0	20/20	LIS H1	Hhal	15	23/23	Fr H2	Hhal
95. 0 (19)	0	20/20	LIS H1	Hhal	16	20/20	Fr H3	Hhal
95.0 (19)	0	20/20	LIS H1	Hhal	17	20/20	Fr H4	Hhal
97.4 (19)	0	20/20	LIS H1	Hhal	18	19/19	Fr H5	Hhal
95.0 (19)	1	19/19	LIS H2	Hhal	2	21/21	LIS H3	Hhal
94.7 (18)	1	19/19	LIS H2	Hhal	3	19/19	LIS H4	Hhal
97.3 (18)	1	19/19	LIS H2	Hhal	4	18/18	LIS H5	Hhal
92.3 (18)	1	19/19	LIS H2	Hhal	5	20/20	LIS H6	Hhal
92.7 (19)	1	19/19	LIS H2	Hhal	6	22/22	LIS H7	Hhal
90.5 (19)	1	19/19	LIS H2	Hhal	7	23/23	LIS H8	Hhal
97.4 (19)	1	19/19	LIS H2	Hhal	8	20/20	LIS H9	Hhal
94.7 (18)	1	19/19	LIS H2	Hhal	9	19/19	LIS H10	Hhal
88.4 (19)	1	19/19	LIS H2	Hhal	10	24/24	LIS H11	Hhal
92.7 (19)	1	19/19	LIS H2	Hhal	11	22/22	LIS H12	Hhal
95.0 (19)	1	19/19	LIS H2	Hhal	12	21/21	LIS H13	Hhal
92.7 (19)	1	19/19	LIS H2	Hhal	13	22/22	LIS H14	Hhal
90.0 (18)	1	19/19	LIS H2	Hhal	14	21/21	Fr H1	Hhal
90.5 (19)	1	19/19	LIS H2	Hhal	15	23/23	Fr H2	Hhal
92.3 (18)	1	19/19	LIS H2	Hhal	16	20/20	Fr H3	Hhal
92.3 (18)	1	19/19	LIS H2	Hhal	17	20/20	Fr H4	Hhal
94.7 (18)	1	19/19	LIS H2	Hhal	18	19/19	Fr H5	Hhal

(M) = Matching fragments number
Tk. = Gel track number
Pts. = Number of fragments compared

Sim. Coeff (M)	Tk.	Pts	Organism	Enzyme	Tk.	Pts	Organism	Enzyme
95.0 (19)	1	21/21	LIS H3	HhaI	3	19/19	LIS H4	HhaI
92.3 (18)	1	21/21	LIS H3	HhaI	4	18/18	LIS H5	HhaI
97.6 (20)	1	21/21	LIS H3	HhaI	5	20/20	LIS H6	HhaI
97.7 (21)	1	21/21	LIS H3	HhaI	6	22/22	LIS H7	HhaI
95.5 (21)	1	21/21	LIS H3	HhaI	7	23/23	LIS H8	HhaI
97.6 (20)	2	21/21	LIS H3	HhaI	8	20/20	LIS H9	HhaI
95.0 (19)	2	21/21	LIS H3	HhaI	9	19/19	LIS H10	HhaI
93.3 (21)	2	21/21	LIS H3	HhaI	10	24/24	LIS H11	HhaI
97.7 (21)	2	21/21	LIS H3	HhaI	11	22/22	LIS H12	HhaI
100.0 (21)	2	21/21	LIS H3	HhaI	12	21/21	LIS H13	HhaI
97.7 (21)	2	21/21	LIS H3	HhaI	13	22/22	LIS H14	HhaI
95.2 (20)	2	21/21	LIS H3	HhaI	14	21/21	Fr H1	HhaI
95.5 (21)	2	21/21	LIS H3	HhaI	15	23/23	Fr H2	HhaI
92.7 (19)	2	21/21	LIS H3	HhaI	16	20/20	Fr H3	HhaI
97.6 (20)	2	21/21	LIS H3	HhaI	17	20/20	Fr H4	HhaI
95.0 (19)	2	21/21	LIS H3	HhaI	18	19/19	Fr H5	HhaI
97.3 (18)	3	19/19	LIS H4	HhaI	4	18/18	LIS H5	HhaI
92.3 (18)	3	19/19	LIS H4	HhaI	5	20/20	LIS H6	HhaI
92.7 (19)	3	19/19	LIS H4	HhaI	6	22/22	LIS H7	HhaI
90.5 (19)	3	19/19	LIS H4	HhaI	7	23/23	LIS H8	HhaI
97.4 (19)	3	19/19	LIS H4	HhaI	8	20/20	LIS H9	HhaI
94.7 (18)	3	19/19	LIS H4	HhaI	9	19/19	LIS H10	HhaI
88.4 (19)	3	19/19	LIS H4	HhaI	10	24/24	LIS H11	HhaI
92.7 (19)	3	19/19	LIS H4	HhaI	11	22/22	LIS H12	HhaI
95.0 (19)	3	19/19	LIS H4	HhaI	12	21/21	LIS H13	HhaI
92.7 (19)	3	19/19	LIS H4	HhaI	13	22/22	LIS H14	HhaI
95.0 (19)	3	19/19	LIS H4	HhaI	14	21/21	Fr H1	HhaI
90.5 (19)	3	19/19	LIS H4	HhaI	15	23/23	Fr H2	HhaI
97.4 (19)	3	19/19	LIS H4	HhaI	16	20/20	Fr H3	HhaI
97.4 (19)	3	19/19	LIS H4	HhaI	17	20/20	Fr H4	HhaI
100.0 (19)	3	19/19	LIS H4	HhaI	18	19/19	Fr H5	HhaI
89.5 (17)	4	18/18	LIS H5	HhaI	5	20/20	LIS H6	HhaI
90.0 (18)	4	18/18	LIS H5	HhaI	6	22/22	LIS H7	HhaI
87.8 (18)	4	18/18	LIS H5	HhaI	7	23/23	LIS H8	HhaI
94.7 (18)	4	18/18	LIS H5	HhaI	8	20/20	LIS H9	HhaI
91.9 (17)	4	18/18	LIS H5	HhaI	9	19/19	LIS H10	HhaI
85.7 (18)	4	18/18	LIS H5	HhaI	10	24/24	LIS H11	HhaI
90.0 (18)	4	18/18	LIS H5	HhaI	11	22/22	LIS H12	HhaI
92.3 (18)	4	18/18	LIS H5	HhaI	12	21/21	LIS H13	HhaI
90.0 (18)	4	18/18	LIS H5	HhaI	13	22/22	LIS H14	HhaI
92.3 (18)	4	18/18	LIS H5	HhaI	14	21/21	Fr H1	HhaI
87.8 (18)	4	18/18	LIS H5	HhaI	15	23/23	Fr H2	HhaI
94.7 (18)	4	18/18	LIS H5	HhaI	16	20/20	Fr H3	HhaI
94.7 (18)	4	18/18	LIS H5	HhaI	17	20/20	Fr H4	HhaI
97.3 (18)	4	18/18	LIS H5	HhaI	18	19/19	Fr H5	HhaI

Sim. Coeff (M)	Tk.	Pts	Organism	Enzyme	Tk.	Pts	Organism	Enzyme
95.2 (20)	5	20/20	LIS H6	Hhal	6	22/22	LIS H7	Hhal
93.0 (20)	5	20/20	LIS H6	Hhal	7	23/23	LIS H8	Hhal
95.0 (19)	5	20/20	LIS H6	Hhal	8	20/20	LIS H9	Hhal
92.3 (18)	5	20/20	LIS H6	Hhal	9	19/29	LIS H10	Hhal
90.9 (20)	5	20/20	LIS H6	Hhal	10	24/24	LIS H11	Hhal
95.2 (20)	5	20/20	LIS H6	Hhal	11	22/22	LIS H12	Hhal
97.6 (20)	5	20/20	LIS H6	Hhal	12	21/21	LIS H13	Hhal
95.2 (20)	5	20/20	LIS H6	Hhal	13	22/22	LIS H14	Hhal
92.7 (20)	5	20/20	LIS H6	Hhal	14	21/21	Fr H1	Hhal
93.0 (20)	5	20/20	LIS H6	Hhal	15	23/23	Fr H2	Hhal
90.0 (18)	5	20/20	LIS H6	Hhal	16	20/20	Fr H3	Hhal
95.0 (19)	5	20/20	LIS H6	Hhal	17	20/20	Fr H4	Hhal
92.3 (18)	5	20/20	LIS H6	Hhal	18	19/19	Fr H5	Hhal
93.3 (21)	6	22/22	LIS H7	Hhal	7	23/23	LIS H8	Hhal
95.2 (20)	6	22/22	LIS H7	Hhal	8	20/20	LIS H9	Hhal
92.7 (19)	6	22/22	LIS H7	Hhal	9	19/19	LIS H10	Hhal
91.3 (21)	6	22/22	LIS H7	Hhal	10	24/24	LIS H11	Hhal
95.5 (21)	6	22/22	LIS H7	Hhal	11	22/22	LIS H12	Hhal
97.7 (21)	6	22/22	LIS H7	Hhal	12	21/21	LIS H13	Hhal
95.5 (21)	6	22/22	LIS H7	Hhal	13	22/22	LIS H14	Hhal
93.0 (20)	6	22/22	LIS H7	Hhal	14	21/21	Fr H1	Hhal
93.3 (21)	6	22/22	LIS H7	Hhal	15	23/23	Fr H2	Hhal
90.5 (19)	6	22/22	LIS H7	Hhal	16	20/20	Fr H3	Hhal
95.2 (20)	6	22/22	LIS H7	Hhal	17	20/20	Fr H4	Hhal
92.7 (19)	6	22/22	LIS H7	Hhal	18	19/19	Fr H5	Hhal
93.0 (20)	7	23/23	LIS H8	Hhal	8	20/20	LIS H9	Hhal
90.5 (19)	7	23/23	LIS H8	Hhal	9	19/19	LIS H10	Hhal
97.9 (23)	7	23/23	LIS H8	Hhal	10	24/24	LIS H11	Hhal
97.8 (22)	7	23/23	LIS H8	Hhal	11	22/22	LIS H12	Hhal
95.5 (21)	7	23/23	LIS H8	Hhal	12	21/21	LIS H13	Hhal
97.8 (22)	7	23/23	LIS H8	Hhal	13	22/22	LIS H14	Hhal
90.9 (20)	7	23/23	LIS H8	Hhal	14	21/21	Fr H1	Hhal
91.3 (21)	7	23/23	LIS H8	Hhal	15	23/23	Fr H2	Hhal
88.4 (19)	7	23/23	LIS H8	Hhal	16	20/20	Fr H3	Hhal
93.0 (20)	7	23/23	LIS H8	Hhal	17	20/20	Fr H4	Hhal
90.5 (19)	7	23/23	LIS H8	Hhal	18	19/19	Fr H5	Hhal
97.4 (19)	8	20/20	LIS H9	Hhal	9	19/19	LIS H10	Hhal
90.9 (20)	8	20/20	LIS H9	Hhal	10	24/24	LIS H11	Hhal
95.2 (20)	8	20/20	LIS H9	Hhal	11	22/22	LIS H12	Hhal
97.6 (20)	8	20/20	LIS H9	Hhal	12	21/21	LIS H13	Hhal

Sim. Coeff (M)	Tk.	Pts	Organism	Enzyme	Tk.	Pts	Organism	Enzyme
95.2 (20)	8	20/20	LIS H9	HhaI	13	22/22	LIS H14	HhaI
92.7 (19)	8	20/20	LIS H9	HhaI	14	21/21	Fr H1	HhaI
93.0 (20)	8	20/20	LIS H9	HhaI	15	23/23	Fr H2	HhaI
95.0 (19)	8	20/20	LIS H9	HhaI	16	20/20	Fr H3	HhaI
95.0 (19)	8	20/20	LIS H9	HhaI	17	20/20	Fr H4	HhaI
97.4 (19)	8	20/20	LIS H9	HhaI	18	19/19	Fr H5	HhaI
88.4 (19)	9	19/19	LIS H10	HhaI	10	24/24	LIS H11	HhaI
92.7 (19)	9	19/19	LIS H10	HhaI	11	22/22	LIS H12	HhaI
95.0 (19)	9	19/19	LIS H10	HhaI	12	21/21	LIS H13	HhaI
92.7 (19)	9	19/19	LIS H10	HhaI	13	22/22	LIS H14	HhaI
90.0 (18)	9	19/19	LIS H10	HhaI	14	21/21	Fr H1	HhaI
90.5 (19)	9	19/19	LIS H10	HhaI	15	23/23	Fr H2	HhaI
92.3 (18)	9	19/19	LIS H10	HhaI	16	20/20	Fr H3	HhaI
92.3 (18)	9	19/19	LIS H10	HhaI	17	20/20	Fr H4	HhaI
94.7 (18)	9	19/19	LIS H10	HhaI	18	19/19	Fr H5	HhaI
95.7 (22)	10	24/24	LIS H11	HhaI	11	22/22	LIS H12	HhaI
93.3 (21)	10	24/24	LIS H11	HhaI	12	21/21	LIS H13	HhaI
95.7 (22)	10	24/24	LIS H11	HhaI	13	22/22	LIS H14	HhaI
88.9 (20)	10	24/24	LIS H11	HhaI	14	21/21	Fr H1	HhaI
93.6 (22)	10	24/24	LIS H11	HhaI	15	23/23	Fr H2	HhaI
86.4 (19)	10	24/24	LIS H11	HhaI	16	20/20	FR H3	HhaI
90.9 (20)	10	24/24	LIS H11	HhaI	17	20/20	Fr H4	HhaI
88.4 (19)	10	24/24	LIS H11	HhaI	18	19/19	Fr H5	HhaI
97.7 (21)	11	22/22	LIS H12	HhaI	12	21/21	LIS H13	HhaI
95.5 (21)	11	22/22	LIS H12	HhaI	13	22/22	LIS H14	HhaI
93.0 (20)	11	22/22	LIS H12	HhaI	14	21/21	Fr H1	HhaI
97.8 (22)	11	22/22	LIS H12	HhaI	15	23/23	Fr H2	HhaI
90.5 (19)	11	22/22	LIS H12	HhaI	16	20/20	Fr H3	HhaI
95.2 (20)	11	22/22	LIS H12	HhaI	17	20/20	Fr H4	HhaI
92.7 (19)	11	22/22	LIS H12	HhaI	18	19/19	Fr H5	HhaI
97.7 (21)	12	21/21	LIS H13	HhaI	13	22/22	LIS H14	HhaI
95.2 (20)	12	21/21	LIS H13	HhaI	14	21/21	Fr H1	HhaI
95.5 (21)	12	21/21	LIS H13	HhaI	15	23/23	Fr H2	HhaI
92.7 (19)	12	21/21	LIS H13	HhaI	16	20/20	Fr H3	HhaI
97.6 (20)	12	21/21	LIS H13	HhaI	17	20/20	Fr H4	HhaI
95.0 (19)	12	21/21	LIS H13	HhaI	18	19/19	Fr H5	HhaI
93.0 (20)	13	22/22	LIS H14	HhaI	14	21/21	Fr H1	HhaI
93.3 (21)	13	22/22	LIS H14	HhaI	15	23/23	Fr H2	HhaI
90.5 (19)	13	22/22	LIS H14	HhaI	16	20/20	Fr H3	HhaI
95.2 (20)	13	22/22	LIS H14	HhaI	17	20/20	Fr H4	HhaI

Sim. Coeff (M)	Tk.	Pts	Organism	Enzyme	Tk.	Pts	Organism	Enzyme
92.7 (19)	13	22/22	LIS H14	HhaI	18	19/19	Fr H5	HhaI
95.5 (21)	14	21/21	Fr H1	HhaI	15	23/23	Fr H2	HhaI
97.6 (20)	14	21/21	Fr H1	HhaI	16	20/20	Fr H3	HhaI
97.6 (20)	14	21/21	Fr H1	HhaI	17	20/20	Fr H4	HhaI
95.0 (19)	14	21/21	Fr H1	HhaI	18	19/19	Fr H5	HhaI
93.0 (20)	15	23/23	Fr H2	HhaI	16	20/20	Fr H3	HhaI
93.0 (20)	15	23/23	Fr H2	HhaI	17	20/20	Fr H4	HhaI
90.5 (19)	15	23/23	Fr H2	HhaI	18	19/19	Fr H5	HhaI
95.0 (19)	16	20/20	Fr H3	HhaI	17	20/20	Fr H4	HhaI
97.4 (19)	16	20/20	Fr H3	HhaI	18	19/19	Fr H5	HhaI
97.4 (19)	17	20/20	Fr H4	HhaI	18	19/19	Fr H5	HhaI

171 pairs found

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