STRAIN CHARACTERISATION, ANTIBIOTIC RESISTANCE AND *MECA* GENE ANALYSIS OF STAPHYLOCOCCI

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DECLARATION OF INDEPENDENT WORK

I, Anna Alvera Vorster, do hereby declare that this dissertation is my own, unaided work. It is being submitted for the degree M. Med. Sc. at the University of the Orange Free State, Bloemfontein. It has not been submitted before for any degree or any examination in any other university.

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ABSTRACT

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S. aureus, is undoubtedly the most pathogenic of the *Staphylococcus* species, having the ability to produce invasive and toxigenic infections. Historically, the less virulent coagulase-negative staphylococci (CNS) were regarded as clinically insignificant contaminants but they have become increasingly implicated as opportunistic nosocomial pathogens. The increasing frequency of methicillin and multiple-antibiotic resistance in staphylococci over the last four decades has seriously compromised therapeutic options.

The study was designed to (a) identify and type staphylococcal species, (b) undertake standardised antimicrobial susceptibility testing, and (c) determine the prevalence of methicillin resistance in staphylococcal isolates.

Presumptive staphylococcal strains were isolated from the diagnostic microbiology laboratories of Pelinomi (147 strains) and Universitas (144 strains) hospitals. Subsequently, these strains were identified using conventional biochemical methods. Species-specific PCR identification assays were performed on selected CNS strains. Antimicrobial susceptibilities were determined for 13 clinically available antibiotics on 144 staphylococcal isolates

and on selected strains for 5 developmental agents. RAPD and plasmid profiles were generated to assess possible epidemiological strain relatedness. For the detection of methicillin resistance in staphylococci the following methods were used: (a) oxacillin MICs detecting phenotypic methicillin resistance levels (b) a multiplex-PCR detecting the *mecA* gene, and (c) a slide agglutination test (MASTALEX-MRSA) detecting PBP2' production.

The inclusion of bile-aesculin agar plates and a bacitracin susceptibility test into the diagnostic laboratory protocol for the identification of staphylococci would reduce misidentification of non-staphylococcal isolates by 12.8%. Colony morphology in combination with the coagulase test could be instrumental in the improved differentiation of *S. aureus* from CNS. Although expensive, when a rapid and fairly comprehensive identification of CNS species is required, the STAPH ID 32 API system was found to be satisfactory. Due to the apparent inaccuracy of the PCR identification assay based on API, its use in the clinical microbiology laboratory would be argued against; although if standardised and expanded it could be considered for future incorporation in routine practice.

The presence of unique RAPD profiles for each specific *Staphylococcus* species suggests RAPD profiling could offer a molecular identification technique for the majority of commonly isolated CNS in the clinical microbiology laboratory. Good typeability was observed for Primer I and III in CNS strains, however, for *S. aureus* strains, poor typeability and discrimination was observed. It has been

found by other researchers that longer oligonucleotide primers (>10 bp in length) are more efficient for *S. aureus* strain typing, but to the contrary in the present study primers ERIC 1 and 2 were totally unsatisfactory. Combined susceptibility data and plasmid profile analysis revealed strain relatedness in *S. haemolyticus* isolates but RAPD Primers I and III indicated otherwise.

All staphylococcal strains isolated were vancomycin-susceptible. Of the staphylococci isolated in the Universitas hospital, 34.3% were oxacillin-resistant. Similarly, 30.1% staphylococci isolated in Pelinomi hospital were oxacillin-resistant. Resistance to ciprofloxacin, erythromycin, gentamicin and clindamycin was found in 49% of oxacillin-resistant staphylococci. In comparison to the other quinolones tested, moxifloxacin showed superior activity against oxacillin-resistant CNS. The glycylcyclines, LY333328 and Q/D may well be considered excellent alternatives to vancomycin for the treatment of MRSA. Of the developmental agents investigated, linezolid showed consistent *in vitro* activity against all staphylococci.

The inadequacy of a single diagnostic method for the detection of methicillin resistance in staphylococci was evident when comparing (a) susceptibility data, (b) multiplex-PCR for *mecA* gene detection, and (c) PBP2' detection. None of these methods was seen to correlate with each other at the 100%-level. The detection of PBP2' was rapid although, in comparison to *mecA* gene detection and antimicrobial susceptibility tests, inaccurate for the identification of

methicillin resistance in staphylococci. DNA sequencing of a fragment of the *mecA* gene in selected staphylococcal strains revealed minimal sequence variation. This was an indication that variable levels of methicillin resistance in staphylococci can be attributed to different mechanisms of methicillin resistance or variations in the expression of the *mecA* gene, rather than mutations within the gene itself. The low sequence variation observed in the *mecA* gene is primarily responsible for initial assumptions of a clonal origin for methicillin-resistance in staphylococci. As of yet, pharmaceutical companies have failed to produce an analogous antimicrobial agent to β-lactam agents that would be able to specifically target PBP2'. The development of such an agent would be instrumental in the reduction of glycopeptide selection pressure. It is imperative that correct identification, strain typing, susceptibility testing and methicillin resistance detection is performed to direct therapy and epidemiologically monitor methicillin-resistant strain types.

DEDICATION

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"Ek weet, o Here, dat aan die mens sy weg nie toebehoort nie; nie aan 'n man om te loop en sy voetstappe te rig nie."-JER. 10:23

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LIST OF ABREVIATIONS

CNS: coagulase-negative staphylococci

MRSA: methicillin-resistant *S. aureus*

MSSA: methicillin-susceptible *S. aureus*

MSSE: methicillin-susceptible *S. epidemidis*

MRSE: methicillin-resistant *S. epidermidis*

MRSH: methicillin-resistant *S. haemolyticus*

EMRSA: Epidemic methicillin-resistant *Staphylococcus aureus*

ORSA: oxacillin-resistant *S. aureus*

OSSA: oxacillin-susceptible *S. aureus*

ORSE: oxacillin-resistant *S. epidermidis*

ORSH: oxacillin-resistant *S. haemolyticus*

ORCNS: oxacillin-resistant coagulase-negative staphylococci

ORS: oxacillin-resistant staphylococci

GORSA: gentamicin-oxacillin-resistant *S. aureus*

EORSA: Epidemic oxacillin-resistant *S. aureus*

BORSA: borderline methicillin-resistant *S. aureus*

MODSA: moderately methicillin-resistant *S. aureus*

VISA: vancomycin intermediately resistant *S. aureus*

Hetero-VRSA: heterogeneously vancomycin resistant *S. aureus*

***S.*:** *Staphylococcus*

***E.*:** *Enterococcus*

S. aur: *S. aureus*

S. hae:	*S. haemolyticus*
S. epi:	*S. epidemidis*
S. spp:	*Staphylococcus* species
PEN:	penicillin
OXA:	oxacillin
CIP:	ciprofloxacin
TRO:	trovafloxacin
Q/D:	quinopristin/dalfopristin
VAN:	vancomycin
TEC:	teicoplanin
ERY:	erythromycin
AZI:	azhithromycin
GEN:	gentamicin
CLI:	clindamycin
TET:	tetracycline
RIF:	rifampicin
MOX:	moxifloxacin
NNIS:	National Nosocomial Infections Surveillance System
ID:	identification
ND:	not determined
bp:	base pair
kb:	kilo bases
MWM:	Molecular weight Marker

PBP: Penicillin-binding protein

PCR: Polymerase chain reaction

RAPD: Randomly amplified polymorphic DNA

D-nLDH: D-lactate dehydrogenase

MLS: macrolide-lincosamide-streptogramin

MIC: minimum inhibitory concentration

SCV: small colony variants

NCCLS: National Committee for Clinical Laboratory Standards

API: Analytical profile index

TEP: tissue, exudate and prosthesis samples

CHAPTER 1

INTRODUCTION

1.1. Background

In 1995, the World Health Organisation reported that 17 million people died of infectious diseases, warning that the situation was likely to get worse before improving (Jackson, 1996). A major contributor to this problem has been the emergence of multi-resistant bacteria such as *Staphylococcus aureus.* Infections due to methicillin-resistant *S. aureus* (MRSA) strains are the cause of particular problems in hospital intensive care units, where the use of invasive medical devices provides a high risk factor in the development of hospital-acquired infections (Cormican & Jones, 1996). The ever fading distinction between nosocomial and community infection is exemplified by MRSA, by which patients are discharged from hospital with increasing speed and then transferred between community facilities such as nursing homes and day care centers, home and hospital (Cormican & Jones, 1996; Peters & Becker, 1996). The drug of choice for treatment of MRSA infections, and in many cases the last available option is vancomycin, making reports from Japan of MRSA with reduced susceptibility to vancomycin particularly troublesome (Hiramatsu, 1998).

1.2. The clinical significance of staphylococci

S. aureus, is undoubtedly the most pathogenic of the *Staphylococcus* species, having the ability to produce invasive and toxigenic infections (Wagner, 1990).

This well-documented human pathogen may also be considered part of the normal flora as it is carried in the anterior nares, throat, skin and mucous membrane sites by as many as 15% of the healthy human population (Baron, 1992). As a hospital-acquired pathogen, *S. aureus* has been a major cause of morbidity and mortality. A survey conducted in the UK in 1980, showed *S. aureus* to be the second-most common isolate obtained from clinical specimens (Gordon, 1993). *S. aureus* can cause infections of the skin, eye, nose and throat, vagina, and gastrointestinal tract (Howard & Seo, 1994). Proposed mechanisms of pathogenicity include encapsulation, toxin and enzyme production, colonisation of specific host sites and slime production (Howard & Seo, 1995). The *S. aureus* virulence arsenal includes the production of coagulase, haemolysins, leukocidin, enterotoxins, DNAse, hyaluronidase and staphylokinase (Wagner, 1990).

Historically, the less virulent coagulase-negative staphylococci (CNS) were regarded as clinically insignificant contaminants but they have become increasingly implicated as opportunistic nosocomial pathogens in bacteraemia, primarily due to the increased use of foreign and indwelling devices (Kloos & Bannerman, 1994; Jones, 1996; Gribaldo *et. al.*, 1997). According to the National Nosocomial Infections Surveillance System (NNIS), USA, a trend toward fewer urinary tract infections and more bloodstream infections since the early 1980s, has become apparent (Jones, 1996). Many infections attributed to CNS are considered a direct consequence of hospitalisation. CNS are a

ubiquitous group of microorganisms that colonise the epidermis of the skin, adjacent glands and hair follicles. *S. capitis* is isolated from adult scalps, while on the face, four or more different strains of CNS, including *S. epidermidis* and *S. hominis*, are routinely isolated (Edmiston *et. al.*, 1989). The axilla, inguinal and perineal regions, areas of excessive surface humidity, may carry high staphylococcal burdens that preferentially include *S. epidermidis*, *S. hominis*, *S. haemolyticus*, and *S. saprophyticus* (Edmiston *et. al.*, 1989). Other staphylococcal species indigenous to humans are *S. cohnii*, *S. xylosus*, *S. warneri*, *S. saccharolyticus*, *S. auricularis*, *S. caprae*, *S. lugdunensis*, *S. schleiferi*, *S. capitis* subsp. *ureolyticum* and *S. cohnii* subsp. *ureolyticum* (Kloos & Bannerman, 1994).

Reports on surveillance data taken from the NNIS during the late 1980s to the early 1990s, have indicated that CNS are among the most commonly reported nosocomial pathogens, in fifth place at 9% (Kloos & Bannerman, 1994). The major contributing factors to the success of CNS causing infections, are: (a) high hydrophobicity; enabling cells to adhere strongly to polymer surfaces and (b) extracellular slime production; a water-soluble substance that binds loosely to the staphylococcal cell, functioning as a penetration barrier for antibiotics and interfering with host defense mechanisms (Wagner, 1990). To monitor and gain knowledge as to the clinical significance of CNS it is therefore considered important to identify CNS to species level and in some cases with respect to strain types (Gribaldo *et. al.*, 1997).

1.3. Identification

The genus *Staphylococcus* comprises 32 species, of which from a clinical point of view, coagulase-positive *S. aureus* is the most important species (Gribaldo *et. al.*, 1997). A major problem facing the diagnostic laboratory concerning CNS is to distinguish clinically significant pathogenic strains from contaminant strains (Kloos & Bannerman, 1994). If the skin has been damaged by trauma, CNS that are present as commensal flora, can gain entry to the host, adhere, multiply and may develop a pathogenic lifestyle (Howard & Seo, 1994). With the increasing awareness of the pathogenic potential of CNS, the identification of CNS has become a high priority in the diagnostic microbiology laboratory.

A variety of methods have been proposed for the identification of clinically important CNS, including: (a) conventional identification systems (Kloos & Schleifer, 1975), (b) commercial identification systems (Staph-Zym system and Staph ID 32 API system) (Kloos & Bannerman, 1994), (c) penicillin-binding protein (PBP) profiles (Kanda *et. al.*, 1990), (d) polymerase chain reaction identification assays (Gribaldo *et. al.*, 1997), (e) cellular fatty acid analysis (Kotilainen *et. al.*, 1991), (f) pyrolysis-mass spectrometry (Freeman *et. al.*, 1991), (g) plasmid analysis (Pfaller & Hollis, 1989), (h) multilocus enzyme electrophoresis (Musser *et. al.*, 1990), (i) whole cell polypeptide analysis (Clink & Pennington, 1987), and (j) chromosomal restriction endonuclease fingerprinting (Bialkowska-Hobrzanska *et. al.*, 1990). For the purposes of this study, the following species identification systems will be discussed, although

comparisons will be made with other methods: 1) conventional biochemical tests, 2) polymerase chain reaction (PCR) identification assays, 3) randomly amplified polymorphic DNA (RAPD) identification assays.

1.3.1. Conventional biochemical tests (Kloos & Shleifer, 1975).

Conventional methods for the identification of staphylococci rely solely on the determination of specific phenotypic characteristics that aid in species differentiation. The major drawback of this system is the amount of time that is required for analysis to be completed before a final identification is obtained. The Centers for Disease Control *Micrococcaceae* conventional identification system identified 824 CNS to an accuracy level of >95% (Kloos & Bannerman, 1994). In order to make the identification process more accessible for use in the clinical laboratory, several manufacturers have developed intricate yet rapid species identification test systems (Kloos & Bannerman, 1994).

The API Staph ID 32, API Staph IDENT, Staph-Zym and Staph Trac systems are examples of commercially available biochemical identification systems (Kloos & Bannerman, 1994; Renneberg *et. al.*, 1995). Unlike the simplified scheme proposed by Kloos & Schleifer (1975), the identification accuracy of commercial systems is between 70 and 90% (Kloos & Bannerman, 1994). It is expected that identification accuracy will improve as databases expand but their cost may well exclude their use in many diagnostic laboratories.

1.3.2. PCR species identification assays

PCR identification assays were proposed by Gribaldo *et. al.* (1997) as swift and accurate methods for the molecular identification of clinically significant CNS. In principle, three variable regions in the 16S rRNA gene of CNS were investigated to identify oligonucleotide bases that differed between CNS species (Gribaldo *et. al.*, 1997). From this data, species-specific PCR primer pairs were identified to ensure maximum primer specificity.

1.3.3. RAPD identification assays

RAPD typing is a molecular PCR assay that requires no prior knowledge of the DNA template to be analysed. Short primers are selected arbitrarily to hybridise at random sites on the DNA template to initiate DNA polymerisation (Power, 1996). The amplification reaction is performed at low annealing temperatures and is therefore characterised by low stringency. PCR products are separated electrophoretically and yield DNA fingerprints, which differ according to the degree of relatedness of the strains under investigation (Power, 1996).

As with conventional biochemical tests, molecular identification techniques require substantial standardisation and databases before they can be considered for use in a routine clinical laboratory (Kloos & Bannerman, 1994). By combining various genetic techniques, some of the above mentioned problems encountered individually may be overcome, allowing reliable yet rapid identification (Gribaldo *et. al.*, 1997; Kloos & Bannerman, 1994).

1.4. Antibiotic susceptibility: current status

Over the last few decades, emphasis on hospital-acquired infections has shifted towards any bacterial species exhibiting multiple-antibiotic resistance, of which *S. aureus* and CNS constitute a major group (Jones, 1996). Penicillinase production by *S. aureus* strains has risen to >90% worldwide (Cormican & Jones, 1996). NNIS data has shown that methicillin resistance in *S. aureus* developed over 16 years, to have by 1991 reached 29% (Jones, 1996). In certain Japanese hospitals, methicillin resistance in *S. aureus* strains has escalated to >80% (Kono *et. al.*, 1992). In the USA, as many as 70% of CNS in 1991 were seen to be resistant to macrolides, oxacillin, chloramphenicol, clindamycin, trimethoprim/sulfamethoxazole and tetracycline (Jones, 1996). In a study performed in Spain in 1994, 80% of CNS were found to be resistant to more than ten antimicrobial agents (Cercenado *et. al.*, 1996). CNS may justifiably be considered reservoirs of antibiotic resistance genes and may disseminate these determinants among other pathogens in the hospital environment. Thus, it was proposed as early as 1975 that CNS should not be disregarded until their possible clinical relevance has been determined (Kloos & Shcleifer, 1975).

1.4.1. β-lactam antibiotics

Prior to the antibiotic era, *S. aureus* bacteraemia had a mortality rate of >80% (Smith & Wickers, 1960). The situation changed drastically in the 1940s after the introduction of penicillin (Michel & Gutmann, 1997). Nonetheless, this "ideal"

antibiotic never lived up to expectations, for bacteria such as *S. aureus* could soon counter penicillins' mechanism of action by producing penicillinases capable of inactivating penicillin by hydrolysis (Peters & Becker, 1996). This brought about the synthesis of penicillinase-resistant penicillins, i.e. methicillin and oxacillin, in 1959 (Michel & Gutmann, 1997). A mere two years had passed after the introduction of methicillin when the first methicillin-resistant *S. aureus* (MRSA) was described (Jevons, 1961). Ever since then, the frequency of MRSA has risen annually in most countries around the world. This phenomenon is portrayed in Table 1.1.

The relative inefficiency of β-lactam antibiotics against methicillin-resistant and susceptible *S. aureus* strains are shown in Table 1.2 (Cunha *et. al.*, 1997; Fass, 1997; Woodcock *et.al.*, 1997; Sumita *et. al.*, 1995; Kaatz & Seo, 1998; Singh *et. al.*, 1996). Unlike the β-lactam antibiotics penicillins/cephalosporins, carbapenems have maintained effective activity against clinically relevant meticillin-susceptible *S. aureus* (MSSA) strains, but the situation is drastically different with MRSA strains (Table 1.2).

1.4.2. Quinolones

Quinolones are derivatives of nalidixic acid, which was introduced in the 1960s (Endtz *et. al.*, 1997). Today they represent a major class of antimicrobial agents. The early derivatives ciprofloxacin and ofloxacin are highly active against Gram-negative bacteria, but failures in the treatment of serious

Table 1.1. The prevalence and distribution of MRSA.

Country	Year	MRSA (%)	Reference
Japan	1980s	22 – 69	(Mukae *et. al.*, 1990; Tosaka *et.al.*,1992; Oguri, 1992)
	1990s	29 - >80	(Oguri, 1992; Kono *et. al.*, 1992; Hashimoto *et. al.*, 1994)
USA	1980s	3 – 38	(Sarvolatz *et. al.*, 1982)
	1990s	5 – 50	(Baron, 1992; Panlilio *et. al.*, 1992; Jones, 1996)
United Kingdom	1970s	<0.05 – 2	(Cookson & Phillips, 1990)
	1980s	0.31 – 15.5	(Cookson & Phillips, 1990)
Germany	1960s	10 – 17	(Cookson & Phillips, 1990)
	1970 –1990s	<6	(Peters & Becker, 1996)
Denmark	1971	46	(Cookson & Phillips, 1990)
Scandinavia	1970 –1990s	<1	(Peters & Becker, 1996)
Netherlands	1970 –1990s	<2	(Peters & Becker, 1996)
Spain, France, Italy	1970 –1990s	>30	(Peters & Becker, 1996)
Belgium	1970 –1990s	25	(Peters & Becker, 1996)

Table 1.2. Susceptibility of MSSA and MRSA strains to β-lactam agents (Cunha *et. al.*, 1997; Fass, 1997; Woodcock *et. al.*, 1997; Sumita *et. al.*, 1995; Kaatz & Seo, 1997; Singh *et. al.*, 1996)

Antimicrobial agent	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
MSSA isolates		
Ampicillin	>32	>32
Co-amoxiclav	0.25 – 2	0.5 – 16
Cefuroxime	1 – 2	2 – 4
Ceftazidime	16	>16
Ceftriaxone	4	4
Naficillin	0.39 – 0.5	0.5 – 0.78
Cefpodoxime	2	4
Imipenem	0.013 – 0.025	0.025
Meropenem	0.1	0.2
MRSA isolates		
Ampicillin	>32	>32
Co-amoxiclav	2 – 16	16
Cefuroxime	128	128
Ceftazidime	>128	>128
Ceftriaxone	0.39	3.13
Naficillin	12.5 – 100	5 - >100
Cefpodoxime	50	50
Imipenem	3.13	3.13
Meropenem	3.13	3.13 – 12.5

infections caused by Gram-positive bacteria have been encountered (Endtz *et. al.*, 1997). The tendency of quinolones to select for resistant strains of staphylococci, as well as to induce cross-resistance to different classes of antibiotics, argues against their widespread routine use (Jones, 1996). Table 1.3 shows the respective minimum inhibitory concentration (MIC) values of quinolones against MSSA and MRSA isolates.

With the exception of *S. epidermidis*, information on quinolone resistance of CNS is limited as species are not defined and results are often grouped under the general heading of CNS. This can be attributed to over emphasis being placed on *S. aureus* and the lack of realisation that CNS are important pathogens. In turn, information on resistance determinants that may be present in CNS is often incomplete. The MIC values for the "early" quinolones, ciprofloxacin and ofloxacin, range between 0.06 - >100 μg/ml and 0.12 – 8 μg/ml, respectively for MSSA isolates. From these studies, it is clear that ciprofloxacin and ofloxacin developed in the 1980s, today display only limited activity (relative to clinically achievable serum levels) against staphylococci (Eliopoulos, 1995; Giamarellou, 1995). The MIC values for more recent quinolones, like sparfloxacin, range between 0.013 μg/ml and 25 μg/ml. Although the hydrophobic nature of this agent enables it to display greater activity towards staphylococci possessing the *norA* gene. Isolates with topoisomerase mutations exhibit cross-resistance to all members of the quinolone class (Kaatz & Seo, 1997). This is also reflected in the MIC ranges of

Table 1.3. MICs of quinolones against MSSA and MRSA isolates (Cunha *et. al.*, 1997; Fass, 1997; Takahasi *et. al.*, 1996; Woodcock *et. al.*, 1997; Jones *et. al.*, 1996; Hosaka *et. al.*, 1994; Oh *et. al.*, 1996; Coque *et. al.*, 1996; Weiss *et. al.*, 1995)

Antimicrobial agent	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)
MSSA isolates			
Ciprofloxacin	0.12 – 0.78	0.5 – 8	0.06 - >100
Ofloxacin	0.25 – 0.5	1	0.12 – 8
Lomefloxacin	0.78 – 1	2 – 25	0.13 - >100
Sparfloxacin	0.05 – 0.1	0.13 – 12.5	0.013 – 25
Norfloxacin	0.5	1	0.12 – 1
Levofloxacin	0.2	0.063	≤0.008 – 0.13
Clinafloxacin	0.015 - ≤0.12	0.03 -≤0.12	≤0.008 – 0.12
MRSA isolates			
Ciprofloxacin	0.25 – 128	1 – 128	0.1 – 128
Ofloxacin	0.5 - >32	1 - >32	0.25 - >32
Lomefloxacin	1 – 25	2 - >100	0.13 – 128
Sparfloxacin	0.05 – 3.13	0.25 – 25	0.012 – 50
Norfloxacin	0.5	>16	0.12 - >16
Levofloxacin	6.25	25	0.1 – 50
Clinafloxacin	0.06 - ≤0.12	0.5	≤0.008 - 1

lomefloxacin (0.13 - >100 μg/ml). Newer compounds, such as clinafloxacin, with higher intrinsic activity than older compounds against MSSA, have a MIC range of ≤0.008 – 0.12 μg/ml. These agents may still have clinically relevant activity against staphylococcal strains expressing topoisomerase mutational resistance (Kaatz & Seo, 1997).

It is apparent that the newer quinolones show substantially greater potency against the methicillin-resistant staphylococci, sometimes by a 100-fold or greater difference in MIC_{90} values, values that fall well below anticipated serum concentrations (Eliopoulos, 1995). Although staphylococci were initially highly susceptible to quinolones, resistance to ciprofloxacin among MRSA strains escalated from 0% to 78% within 18 months of its introduction into a community hospital in the USA (Cormican & Jones, 1996). The rate of fluoroquinolone resistance development within the CNS has been shown to be similar (Cormican & Jones, 1996).

1.4.3. Glycopeptides

In the past, the medical community was comforted by the knowledge that vancomycin provided effective therapy for all infections caused by MRSA. However, in 1997 the emergence of clinical isolates of *S. aureus* with intermediate resistance to vancomycin (MIC 8 μg/ml) and teicoplanin (MIC 8-16 μg/ml) introduced the possibility that resistance to glycopeptides in *S. aureus* would soon become an important clinical problem (Hiramatsu, 1998). In

addition, it was demonstrated by using *in vitro* and *in vivo* conjugal transfer, the gene complex encoding high-level vancomycin resistance in enterococci could be transferred to *S. aureus* (Noble *et al.*, 1992).

Recently, *S. aureus* strains with reduced susceptibility to vancomycin (MICs 8 μg/ml) were reported in the USA (Martin and Wilcox, 1997). The incidence of vancomycin-intermediately-resistant *S. aureus* (VISA) isolates (MIC 8 μg/ml) was low in Japan, with fewer than one in 1000 of all MRSA strains isolated (Hiramatsu, 1998). However, heterogeneously-resistant MRSA generating cells with varied degrees of vancomycin susceptibility (hetero-VRSA) are quite prevalent. In Japan 1998, more than 9% of MRSA isolates from seven University hospitals, and 1.3% of MRSA strains isolated from 195 non-university sources, displayed hetero-VRSA activity (Hiramatsu, 1998). In contrast with the sporadic reports of vancomycin resistance in *S. aureus* over the past few decades, a number of reports on vancomycin resistance in CNS have been published (Johnson *et. al.*, 1990; Cercenado *et. al.*, 1996; Daum *et. al.*, 1992). This has also been the case for the related glycopeptide, teicoplanin (Mainardi *et. al.*, 1994; Kaatz *et. al.*, 1990). In a study conducted over a five year period in Spain, the most frequently isolated CNS species with reduced susceptibility to teichoplanin were *S. epidermidis*, *S. haemolyticus* and *S. hominis* (Cercenado *et al.*, 1996). Cercenado *et al.* (1996) also reported six CNS strains with MIC values for teicoplanin in excess of 64 μg/ml.

1.4.4. Other antimicrobials

Rifampin has long been recognised as an anti-staphylococcal agent with continued efficacy against methicillin-resistant strains. The usefulness of rifampin monotherapy though is limited because of the rapid emergence of resistance. Rifampin/β-lactam combinations have been shown to render methicillin-resistant strains more susceptible to the β-lactam agent. The synergistic effect is thought to be a result of rifampin causing a decrease in the production of PBP 2' (Brandt *et al.*, 1995). New macrolides have been investigated for their efficacy against CNS but activity is generally comparable to that of erythromycin (Noble, 1997). Resistance to the newer macrolides emerges rapidly due to the prevalence of macrolide-lincosamide-streptogramin (MLS) resistance especially in the CNS (Noble, 1997). This phenotype has been found in isolates of *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. hominis*, *S. simulans*, *S. cohnii*, *S. warneri*, *S. capitis*, *S. xylosus*, and *S. sciuri*.

1.5. Molecular mechanisms of antibiotic resistance

1.5.1. Glycopeptide resistance

The mechanism of glycopeptide resistance in staphylococci is still unknown. Vancomycin-selected laboratory mutants have shown an increase in MICs for both vancomycin and teicoplanin (Moreira *et al.*, 1997). When compared with the parental strain regarding phenotypical features, mutants were seen to exhibit smaller colony size, lower growth rates, larger cell diameter, thicker cell walls and decreased susceptibility to lysostaphin, decreased zone size of β-

haemolysis, and loss of phage susceptibility and capsular type. In addition, some mutants over-produced a D-lactate dehydrogenase (D-nLDH), similar in structure to VanH dehydrogenase found in enterococci. However, insertional inactivation of the *ddh* gene encoding D-nLDH in *S. aureus* mutants did not alter the resistance phenotype, making it a distinct vancomycin resistance mechanism from that employed by enterococci.

An alternative hypothesis explaining glycopeptide resistance in *S. aureus* is based on transpeptidase activity (Moreira *et al.*, 1997). Since transpeptidases bind to the same D-ala-D-ala termini of peptidoglycan precursors to which glycopeptides bind, resistance could result from (a) alterations that increase transpeptidase affinity for the D-ala-D-ala termini, or (b) overproduction of transpeptidases (especially PBP 2a) giving rise to substrate competition, with subsequent inhibition of glycopeptide activity. Alternatively, expression of genes encoding the PBPs may be co-regulated along with genes encoding further protein(s) which may be responsible for glycopeptide resistance (Moreira *et al.*, 1997).

Although many theories linking PBP affinity alterations/increased production with vancomycin resistance have been proposed, the direct role of PBPs in the development of glycopeptide resistance in general remains unclear. Whether resistance to the glycopeptides in staphylococci will be the result of (a) selection of resistant mutants, or (b) transfer of high-level resistance from enterococci, the

emergence of epidemic VRSA/MRSA strains has to be anticipated (Noble, 1997).

1.5.2. β-lactam antibiotic resistance

The mechanism of action for β-lactam antibiotics is mainly directed towards peptidoglycan synthesis. β-Lactam agents are thought to be structural analogous of the terminal di-peptide of the peptidoglycan precursor (Mathews & van Holde, 1990). This enables β-lactams to bind to transpeptidases in place of the natural substrate, forming a stable but inert complex, rendering the transpeptidase inactive in catalysing the final stages of cell-wall synthesis (Peters & Becker, 1996). In *S. aureus* transpeptidase activity is associated with the high-molecular-weight PBPs (PBP1, 2 and 3) which also have the highest affinity for β-lactam antibiotics. Isolates of *Staphylococcus* spp. that are highly resistant to methicillin produce an additional low-affinity PBP, PBP 2'. PBP 2' takes over the role of "normal" transpeptidases, when they are saturated/inactivated by β-lactam antibiotics (Michel & Gutmann, 1997). PBP 2' is encoded by the *mecA* gene and the expression of this resistance determinant confers intrinsic resistance to not only methicillin and oxacillin but to all currently available β-lactam agents. However, not all isolates of staphylococci containing the *mecA* gene are capable of expressing methicillin resistance (Peters & Becker, 1996).

The phenotypic expression of the *mecA* gene is controlled by two regulatory systems, (a) *mecRI – mecI* genetic element in the upstream region negatively controlling *mecA* transcription, and (b) *blaRI – blaI* system which regulates the staphylococcal β-lactamase gene, *blaZ*. The proteins encoded by these two groups of genes have strong sequence similarities and it is thus not surprising that they co-regulate *mecA* expression in staphylococci (Michel & Gutmann, 1997). In theory, highly methicillin-resistant staphylococcal isolates will be devoid of the *mecRI - mecI* and *blaRI - blaI* genetic elements, enabling constitutive PBP 2' synthesis. In certain strains, however, the amount of PBP 2' produced does not correlate with levels of resistance exhibited. These strains are termed heterogeneously methicillin-resistant, all harbour PBP 2', but only one bacterium out of 10^4 - 10^7 expresses high-level resistance to methicillin, whereas in homogeneously methicillin-resistant staphylococcal strains the whole population is highly resistant to methicillin (Ryffel *et. al.*, 1994). Thus, the phenotypic expression of methicillin resistance does not only vary between different isolates, but also within a single isolate.

In addition to the above-mentioned regulatory systems for *mecA* gene expression, at least five 'auxiliary' genes are essential for the expression of methicillin resistance. Inactivation of these *aux* factors (or factor essential for methicillin resistance, *fem* A through E) decreases resistance to methicillin without affecting PBP 2' production, in effect transforming homogeneous to heterogeneous methicillin resistance (Peters & Becker, 1996). As shown in

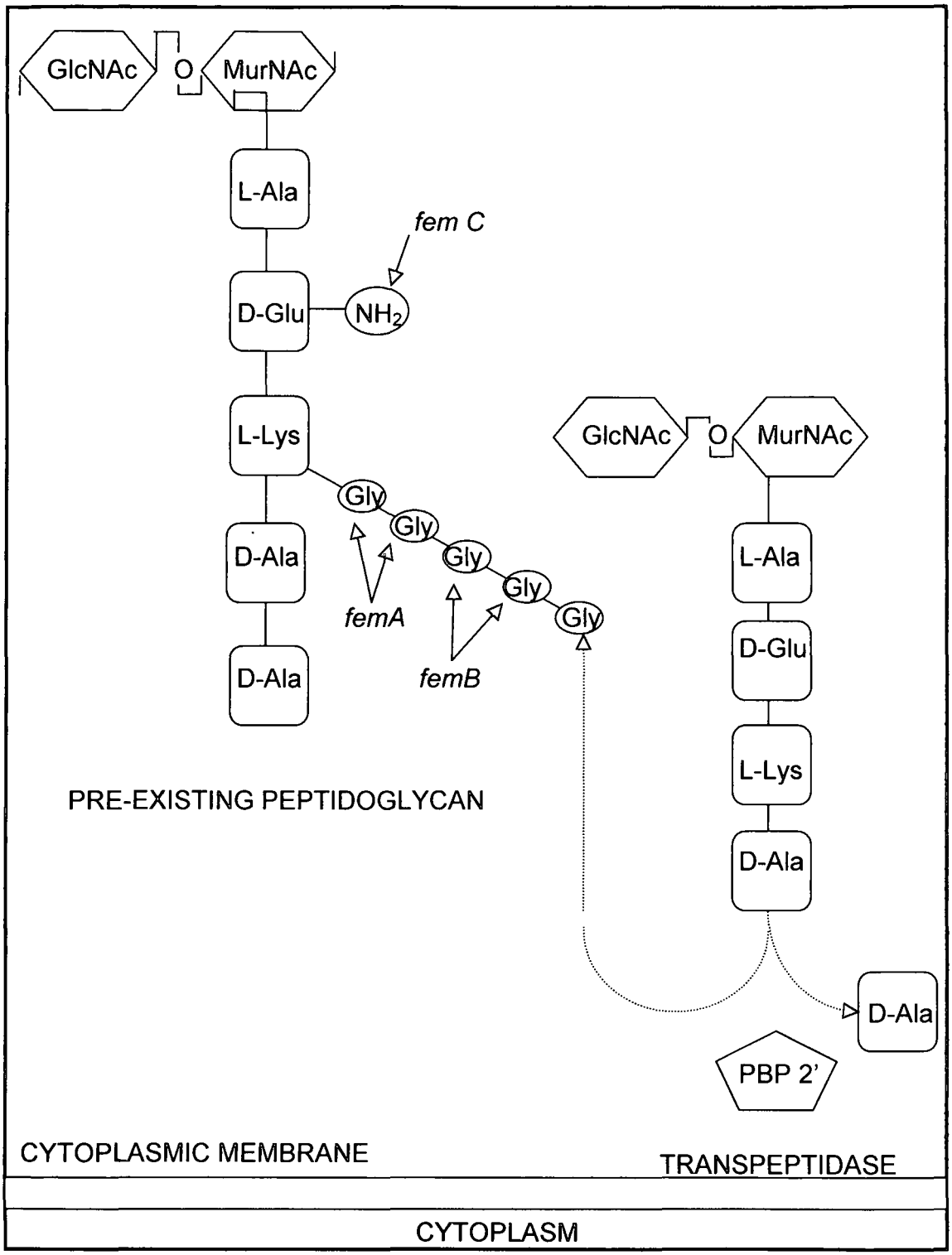


Figure 1.1: Schematic representation of the cell-wall muropeptide sites inactivated by the auxillary genes: *femA*, *femB* and *femC* (Michel & Gutmann, 1997).

Figure 1.1, mutations in *fem* A, B and C genes affect different steps in the peptidoglycan precursor synthesis. The absence of specific *fem* genes results in altered muropeptide structures and in particular alteration of the glycine content of the peptidoglycan side chain. PBP 2' has to recognise an acceptor in the peptidoglycan with a normal pentaglycine side chain to efficiently link the peptidoglycan precursor to the pre-existing cell wall. Modification of the glycine content in the pentapeptide side chain will render the substitute transpeptidase PBP 2' inefficient, resulting in the transition from a homogeneous to a heterogeneous methicillin-resistant population (Michel & Gutmann, 1997). Furthermore, not all isolates of staphylococci expressing phenotypic methicillin resistance, contain the *mecA* gene. At least five other mechanisms explain methicillin resistance found in staphylococcal strains, these include:

1. **The over-expression of β-lactamase, accounting for low-level methicillin resistance (MICs 4 – 8 μg/ml)**

   According to the hypothesis of Montanari *et al.* (1996), a staphylococcal penicillinase, when hyperproduced, could succeed in partially hydrolysing methicillin. *S. aureus* strains exhibiting borderline levels of resistance to methicillin/oxacillin (also known as BORSA) have the following characteristics:

   a. They are reported as neither heterogeneously-resistant to methicillin nor multiply-resistant,

   b. BORSA are hyperproducers of β-lactamase,

c. Strains will become completely susceptible to penicillinase-resistant penicillins in the presence of β-lactamase inhibitors, and

d. BORSA strains do not contain the *mecA* gene.

2. **The over-expression of PBP 4, accounting for low-level methicillin resistance (MICs 4 – 8 μg/ml)**

Staphylococcal PBP 4 is a low-molecular-weight PBP with low β-lactam binding affinity, and was thought to be non-essential in cell growth and viability. However, transpeptidase activity has been demonstrated for PBP 4. The overproduction of PBP 4 by strains of *S. aureus*, has been associated with an increase in the degree to which peptidoglycan is cross-linked, resulting in reduced susceptibilities for methicillin (Henze *et al.*, 1996).

3. **The alteration through acquired mutations of PBPs 1 and 2 that can lower affinity for β-lactams, accounting for low-level methicillin resistance (MICs 4–8 μg/ml)**

Certain strains of staphylococci can produce PBPs with modified capacities to bind β-lactam antibiotics (Michel & Gutmann, 1997). In comparison to the expression of the *mecA* gene, these strains produce only a modest increase in methicillin resistance (also known as MODSA). MODSA strains are characterised by the following:

a. MODSA strains do not contain the *mecA* gene,

b. Strains do not contain any sub-populations of highly resistant cells, and

c. β-lactam binding affinity of PBP 1 and 2 decreases.

4. **The presence of small colony variants (SCV), accounting for high-level methicillin resistance (MICs >8 μg/ml)**

   SCV are a sub-population presenting as slow growing variants that are more resistant to many antimicrobial agents than the original parental population. The slow growth of these variants reduce the effectiveness of cell-wall active antibiotics such as β-lactams. SCV have a slow growth phenotype and show defective respiratory activity. Due to their slow growth, atypical colony morphology and unusual biochemical profiles, SCV are often misidentified in the clinical laboratory (Proctor *et. al.*, 1998).

5. **The production of methicillinases, accounting for low-level methicillin resistance (MICs 4 – 8 μg/ml).**

   The combined effect of methicillinases and other β-lactamases on methicillin might well explain the increase in β-lactamase activity often observed in BORSA. Methicillinase producing staphylococci are susceptible to inhibition by β-lactamase inhibitors such as sulbactam. Methicillinases are inducible and have a lower molecular weight than staphylococcal penicillinases. Unlike staphylococcal penicillinases, methicillinases are active against padac and this property may prove useful in the differentiation between staphylococcal penicillinases and methicillinases (Montanari *et al.*, 1996).

1.5.3. Acquisition and dissemination potential of antibiotic resistance genes

Classically *S. aureus* carries a number of antimicrobial resistance determinants on plamids. Plasmid mediated resistance to penicillin, tetracycline, streptomycin and erythromycin has been described (Noble, 1995).

The chromosomally located *mecA* gene in *S. aureus* has been found to always map in the same location, between the genes encoding protein A and DNA gyrase (Pattee *et. al.*, 1990). In addition to *mecA*, the *mec* locus consists of 30 kb flanking sequences (Beck *et. al.*, 1986). In epidemic MRSA strains, many antibiotic resistance genes are located on the chromosome, especially in the region of the *mec* locus. The insertion sequence *IS257* upstream from the *mec* locus, creates a hotspot for the incorporation of the tetracycline resistance plasmid and erythromycin resistance transposon (Noble, 1997). Thus the plasmid location trend for antibiotic resistance genes in *S. aureus* has shifted towards the chromosome.

There is considerable speculation about the origin and evolution of the *mecA* gene. One hypothesis assumes that a single *mecRI* insertion-deletion event occurred, most likely in a CNS, with subsequent horizontal dissemination of rearranged sequences within the *Staphylococcus* species. This suggests that CNS have played important roles as intermediates and reservoirs for the DNA sequences that eventually appeared in *S. aureus* (Archer *et. al.*, 1994).

1.6. Objectives

The objectives of this study were to:

(i) isolate clinical and environmental strains of *Staphylococcus aureus* and coagulase-negative staphylococci (CNS);

(ii) identify to species level staphylococci isolated from clinical and environmental sources to elucidate (a) the prevalence of staphylococcal species involved in local nosocomial infections and (b) the prevalence of multi-resistant staphylococci in the clinical setting;

(iii) conduct species specific PCR assays and RAPD studies to assist in the identification of CNS;

(iv) determine antimicrobial susceptibilities of staphylococci employing antibiotics in current use and to assess new antibiotics;

(v) analyse RAPD and plasmid profiles of selected *S. aureus* and CNS exhibiting methicillin susceptibility and resistance in order to address staphylococcal strain typing;

(vi) screen for the presence of staphylococcal strains exhibiting decreased levels of susceptibility for vancomycin;

(vii) screen for the presence of the *mecA* gene in methicillin-susceptible and methicillin-resistant staphylococci with the assistance of a multiplex-PCR;

(viii) screen for the presence of the *mecA* gene product (PBP 2') in staphylococcal strains possessing the *mecA* gene;

(ix) sequence PBP2' (*mecA*) genes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains

2.1.1. Isolation of *Staphylococcus* species from raw milk samples

Forty one presumptive staphylococcal strains from raw milk samples were kindly provided by H. de Beer. These strains were primarily identified as presumptive staphylococcal strains after raw milk samples were plated onto Baird-Parker agar plates. Staphylococcal strains were stored at -70°C in 1 ml freezing mixture containing brain-heart infusion broth (Oxoid Ltd., Basingstoke, Hampshire, England) and a final concentration of 10% glycerol.

2.1.2. Isolation of *Staphylococcus* species from Universitas Hospital

A total of 170 presumptive staphylococcal strains were serially collected from clinical specimens obtained from the Universitas Hospital between April to July 1998. Pure cultures were grown on 6% sheep blood agar (Blood Agar Base, Diagnostics Pasteur, Marnes-la-Coquette, France/ Bacto Agar, Becton Dickinson, Le Pont de Claix, France) plates or MacConkey agar (Oxoid) plates. Staphylococcal strains were stored at -70°C in 1 ml freezing mixture containing brain-heart infusion broth (Oxoid) and a final concentration of 10% glycerol.

2.1.3. Isolation of *Staphylococcus* species from Pelinomi Hospital

Staphylococcus species identified by the diagnostic laboratory Pelinomi Hospital

were kindly forwarded for vancomycin resistance screening. The clinical isolates were collected during the period January – April 1998 and totalled 147 strains. For screening, staphylococcal strains were streaked onto brain-heart infusion (Oxoid) agar plates containing 40 μg/ml or 4 μg/ml vancomycin.

2.2. Primary identification of *Staphylococcus* species

2.2.1. Conventional biochemical tests

All staphylococcal strains collected were identified as staphylococci by the various laboratories. Presumptive staphylococcal strains obtained from clinical and raw-milk samples were identified by means of conventional biochemical tests. These tests included: Gram-stain, catalase production, colony morphology on both Baird-Parker (Oxoid) and blood agar plates, resistance to 0.04 units bacitracin (Davies Diagnostics, Merseyside, UK), aesculin hydrolysis and coagulase production. These biochemical tests resulted in a coagulase split for the subsequent identification of coagulase-negative staphylococcal species with the Staph ID 32 API system.

2.2.2. Analytical profile index (API) 32 ID STAPH system (BioMérieux Vitek, Inc., Mo., USA)

CNS obtained from the Universitas microbiology laboratory and isolated from raw milk samples were submitted to testing with the API system for species identification. Test strains were grown overnight on blood agar plates at 37°C. Each test strain was suspended in 2 ml water and visually standardised to a 0.5

McFarland and diluted to a concentration of approximately 5×10^7 CFU/ml. The cell-suspension was then added to the API strip test ampoules, 50 µl per ampoule. The API 32 ID STAPH system include the following biochemical tests:

Tests for the production of: urease, arginine dihydrolase, arginine-arylamidase, pyrrolidonyl arylamidase, alkaline phosphatase, ornithine decarboxylase, acetoin, β-galactosidase and β-glucuronidase.

A test for the hydrolysis of: aesculin

Tests for the fermentation of: glucose, fructose, mannose, maltose, lactose, trehalose, mannitol, raffinose, ribose, cellobiose, sucrose, turanose, arabinose, N-acetyl-glucosamine

Test for resistance to: novobiocin

Test for the reduction of: nitrates

The urease, arginine dihydrolase and ornithine decarboxylase test ampoules were overlaid with two drops of mineral oil. The API test strips were incubated for 24 h at 37°C in aerobic conditions. After incubation, one drop of NIT 1 reagent (Sulfanilic acid 0.8%, acetic acid 5N) and one drop of NIT 2 reagent (N-N- dimethyl-1-naphthylamine 0.6%, acetic acid 5N) were added to the nitrate reduction test ampoule. One drop VP A reagent (20% KOH) and VP B reagent (α naphtol 12%) were added to the acetoin production test ampoule. Fast Blue BB reagent was added to the β galactosidase, arginine arylamidase, alkaline phophatase and pyrrolidonyl arylamidase test ampoules. Aside from the ribose and cellobiose tests, reaction results in the Staph ID 32 API were used to

construct an eight-digit profile. In cases of low discrimination ribose and cellobiose tests were used as additional criteria. Staph ID 32 API database identification was kindly provided by Mr. P. Cahill, Separations Scientific C.C., Honeydew, South Africa.

2.3. Secondary identification of CNS species

2.3.1. Template DNA preparation

2.3.1.1. Cell lysis

The test strains were grown overnight on blood-agar plates (Diagnostics Pasteur) at 37°C. A quarter plate of confluent growth was suspended in 250 µl TE-buffer (50 mM Tris, pH 7.5, 20 mM EDTA) containing 136 µg/ml lysostaphin (Sigma Chemical Co., St. Louis, MO) and 1.25 ml (10 mg/ml) lysozyme (Boehringer Mannheim, Mannheim, Germany). After the cell- suspension was incubated for 1 h at 37°C, 250 µl Triton-X-buffer (20 mM Tris, pH 7.5, 2% Triton-X-100) was added. Ten micro-liters (4 µg/ml) Proteinase K (E. Merck, Darmstadt, Germany) was then added and tubes incubated for 1 h 37°C. Bacterial lysates were stored at 4°C.

2.3.1.2. Nucleic acid isolation

For the isolation of nucleic acids from staphylococci for RAPD analysis, a high pure PCR template preparation kit (Boehringer) was employed according to manufacturer's protocol. This basically involved the suspension of test strains in 20 µl distilled water and incubation on a heat block for 10 min. at 99°C. Ater the

addition of 80 µl lysostaphin (136 µl/ml) and lysozyme (10 µl/ml), strains were incubated for 1 h at 37°C. One hundred microlitres phosphate buffered saline, 200 µl Binding Buffer and 40 µl Proteinase K (20 mg/ml) were added to the suspension and incubated for a further 10 min at 72°C. Nucleic acids were subsequently allowed to bind specifically to the surface of glass fibres in the presence of chaotropic salt in High Pure filter tubes. Cellular impurities were removed with the addition of Washing Buffer, while nucleic acids were eluated in Elution Buffer. Nucleic acids were stored at 4°C for further analysis.

2.3.2. Rapid PCR identification assay for clinically relevant CNS

PCR identification assays were performed basically as described by Gribaldo *et. al.* (1997). Three species specific primer pairs were employed for the identification of the most commonly isolated CNS in this study: *S. epidermidis*, *S. haemolyticus* and *S. hominis*.

PCR primer pairs:

S. epidermidis	*Sep*:	5'-GGT TCA ATA GTG AAA GAC GGT T
		5'-CTC TAT CTC TAG AGG GGT CAG
S. haemolyticus	*Shl*:	5'-TCT GTT ATT AGG GAA GAA CAT AC
		5'-GAA GGC TCT ATC TCT AGA GTT G
S. hominis	*Shn:*	5'-GTT CGA TAG TGA AAG ATG GCT C
		5'-GGA AAC TTC TAT CTC TAG AAG G

Amplification was performed in a final reaction volume of 25 µl comprising: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP

(Boehringer), 25 pmol of each of the primer and 1.5 μl of the bacterial lysate. The samples were subjected to amplification in a thermal cycler (Perkin Elmer 9600, Norwalk, USA) with pre-denaturation at 96°C for 5 min, primer annealing at 63 °C at which time 0.5 units Taq DNA polymerase (Advanced Biotechnologies, Surrey, UK) was added and a preliminary extension period of 72°C for 5 min. Thirty amplification cycles were then performed: 94°C for 1 min, 63°C for 1 min, 72°C for 1 min. This was followed by a final extension of 72°C for 5 min.

The PCR product (25 μl) was subjected to electrophoresis. Electrophoresis was performed in 1.5% low melting temperature agarose gels (NuSieve GTG, FMC Bioproducts, Rocklands, USA), using Tris-acetic acid-EDTA running buffer [40mM Tris (pH 8.0), 20mM EDTA]. DNA was stained with ethidium bromide (0.5 μg/ml) and products were viewed under UV illumination. A 100 bp molecular weight marker (MWM G) (Gibco BRL, Life Technologies, Paisley,UK) was used as reference for DNA fingerprint sizes in each gel run.

2.3.3. RAPD analysis

RAPD assays were performed employing two ERIC primers as described by Van Leeuwen *et. al.* (1996). Primer III was obtained from a kit containing 10-base oligonucleotide primers, for use in genetic mapping and DNA fingerprinting (Advanced Biotechnologies Ltd., Surrey, UK).

RAPD primers:

ERIC I: 5'-ATG TAA GCT CCT GGG GAT TCA C

ERIC II: 5'-AAG TAA GTG ACT GGG GTG ACG C

PRIMER I : 5'-GTG TGC CGG A (Matthee, et. al., 1997)

PRIMER III: 5'-AGC CAG GCT G

Amplification was performed in a final reaction volume of 25 µl comprising: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM of each dNTP (Boehringer Mannheim), 25 pmol of primer and 1.5 µl of the bacterial lysate. For RAPD assays employing ERIC primers a concentration of 2.5 mM $MgCl_2$ was used, while 4 mM $MgCl_2$ was used for primers I and III. Amplification was performed in a thermal cycler (Perkin Elmer 9600) with pre-denaturation at 94°C for 5 min, primer annealing at 36 °C at which time 0.5 units Taq DNA polymerase (Advanced Biotechnologies) was added and a preliminary extension period of 72°C for 5 min, 94°C for 5 min, 36°C for 5 min and 72°C for 5 min. Forty amplification cycles: 94°C for 1 min, 36°C for 1 min, 72°C for 2 min; with a final 5 min extension period at 72°C. PCR products were visualised as previously described in section 2.3.2. MWM G (Gibco), MWM XIV (Boehringer) and MWM AB (Advanced Biotechnologies) were used as reference for DNA fingerprint sizes.

2.3.4. Plasmid analysis

For the determination of *S. haemolyticus* plasmid profiles, plasmid DNA was extracted with the Wizard® *Plus* Minipreps DNA Purification System (Promega Corporation, Madison, WI, USA). Plasmid DNA extraction was performed in

accordance with the manufacturers guidelines. In brief, the procedure involved the following:

Strains were grown overnight on blood-agar plates at 37°C. Four to five colonies were suspended in 3 ml Mueller-Hinton (Difco Laboratories, Detroit, MI, USA) broth. After overnight incubation, the cell-suspension was centrifuged at 10,000 × g in microcentrifuge for 5 min. The supernatant was discarded and the pellet was re-suspended in 200 µl Cell Re-suspension Solution. A final concentration of 150 µg/ml lysostaphin (Sigma) and 4 µl lysozyme (10 mg/ml, Boehringer) were then added to the Cell Re-suspension Solution to enhance bacterial lysis. The cell-suspension was incubated for 1 h at 37°C. Cell-Lysis Solution (200 µl) was then added to the cell-suspension and mixed by inversion until the suspension cleared. Neutralization Solution (200 µl) was then added to the bacterial lysate and tubes were mixed by inversion. The bacterial lysate was centrifuged in a microcentrifuge at 10,000 × g for 5 min. The supernatant was drawn off and mixed with 1 ml resin. The resin/supernatant was passed through a mini-column under vacuum and the column was washed with Column Wash Solution and dried under vacuum. After brief centrifugation to remove excess Column Wash Solution, DNA was eluted from the column by the addition of 50 µl water. The plasmid DNA preparation was stored at 4°C until further analysis. The plasmid DNA preparations (14 – 25 µl) were subjected to electrophoresis. Electrophoresis was performed in 0.7% agarose (Seakem, FMC BioProducts) gels, using Tris-acetic acid-EDTA (40mM Tris (pH 8.0), 20mM EDTA) running

buffer. Plasmids were stained with ethidium bromide (0.5 μg/ml) and viewed under UV illumination. A supercoiled DNA ladder (Promega) was included for the determination of plasmid sizes.

2.4. MIC determinations

MICs were determined by a standardised agar dilution method for staphylococcal strains employing 12 currently available and six developmental antimicrobial agents (Table 2.1.). MICs were determined according to the approved National Committee for Clinical Laboratory Standards protocol (NCCLS, 1998) on Mueller-Hinton agar plates (Difco). Staphylococcal colonies were suspended in 2 ml Mueller-Hinton broth (Difco) to a turbidity equivalent to a 0.5 McFarland containing approximately 5×10^7 CFU/ml. Mueller-Hinton agar (Difco) plates containing antibiotic concentrations ranging 0.06-128 μg/ml were inoculated using the direct colony suspension method. Plates were inoculated using a multi-point inoculator (Mast) and incubated at 35°C for 18 h. For the determination of oxacillin MICs, staphylococcal isolates were tested on Mueller-Hinton agar supplemented with 2% NaCl. MIC tests for the detection of ORSA were incubated for a full 24 h at 35°C.

The MICs were recorded as the lowest concentration of antimicrobial agent that completely inhibited growth, disregarding the growth of five colonies or less. *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were used as NCCLS recommended control strains.

Table 2.1. Antimicrobial agents employed in susceptibility testing:

Currently available antibiotics	**Developmental antibiotics**
penicillin, oxacillin, gentamicin (Sigma Chemical Co., St. Louis, Mo.)	**quinopristin-dalfopristin** (Rhône-Poulenc Rorer, Collegeville, Pa.)
erythromycin, vancomycin (Eli Lilly & Co., Indianapolis, Ind.)	**linezolid** (Pharmacia & Upjohn Inc., Kalamazoo, Mich.)
trovafloxacin [1], **azithromycin** (Pfizer Inc., Groton, Conn.)	**LY 333328** (Eli Lilly & Co., Indianapolis, Ind.)
ciprofloxacin (Bayer AG., Wuppertal, Germany)	**moxifloxacin** (Bayer AG., Wuppertal, Germany)
clindamycin (Pharmacia & Upjhon Inc., Kalamazoo, Mich.)	**CL 329,998, CL 331,002** (Lederle Laboratories, Pearl River, N.Y.)
teicoplanin (Hoechst Marion Roussel, Gerenzano, Italy)	
rifampicin (Restan Laboratories-Mer-National, Craighall)	
tetracycline (Bristol-Myers Squibb, Princeton, N.Y.)	

[1]Currently withdrawn from general clinical use due to adverse renal effects

2.5. *MecA* gene detection and sequencing

2.5.1. Multiplex PCR for *mecA* gene detection

Methicillin-resistant and -susceptible staphylococcal strains isolated from the Universitas Hospital were screened for the presence of the *mecA* gene employing the multiplex PCR identification assay (Geha *et. al.*, 1994). Oligonucleotide primers:

Primer *MecA*1: 5'-GTA GAA ATG ACT GAA CGT CCG ATA A

Primer *MecA*2: 5'-CCA ATT CCA CAT TGT TTC GGT CTA A

Primer X: 5'-GGA ATT CAA A[T/G, 1:1]G AAT TGA CGG GGG C

Primer Y: 5'-CGG GAT CCC AGG CCC GGG AAC GTA TTC AC

Strains were grown overnight on blood-agar plates at 37°C. Two colonies were suspended in 20 µl water. The cell-suspension was heated for 10 min at 99°C on a heating block. To the cell-suspension 80 µl TE-buffer (50 mM Tris, pH 7.5, 20 mM EDTA), 136 µg/ml final concentration of lysostaphin (Sigma), and 10 mg/ml final concentration of lysozyme (Boehringer) were added. Following incubation for 1 h at 37°C, 100 µl Triton-X-buffer (20 mM Tris, pH 7.5, 2% Triton-X-100) and 10 µl Protienase K (Merck) (4 mg/ml) were added to the suspension. The suspension was then incubated for 1 h at 37°C. Bacterial lysates were stored at 4°C.

Amplification was performed in a final reaction volume of 25 µl comprising: 10 mM Tris, 1.5 mM $MgCl_2$, 50 mM KCL, 200 µM of each dNTP (Boehringer), 25

pmol of each *mecA* primer (*mecA*1, *mecA*2), 5 pmol of each universal primer (X, Y) and 1.5 μl of the bacterial lysate. The test samples were subjected to amplification in a thermal cycler (Perkin Elmer 9600) with pre-denaturation at 94°C for 4 min and primer annealing at 50°C at which point 0.5 units of taq DNA polymerase (Advanced Biotechnologies) was added. Thirty amplification cycles were then performed: 94°C for 45 sec, 50°C for 45 sec, 72°C for 1 min. This was followed by a final extension of 72°C for 5 min.

PCR products (25 μl) were subjected to electrophoresis. Electrophoresis was performed in 2.3% low melting temperature agarose gels (NuSieve GTG, FMC Bioproducts) using Tris acetic-acid EDTA running buffer (40mM Tris (pH 8.0), 20mM EDTA). DNA was stained with ethidium bromide (0.5 μg/ml) and viewed under UV illumination. A 100 bp MWM AB (Advanced Biotechnologies) was used as reference for PCR product sizes.

2.5.2. Sequencing of *mecA* gene

Automated sequencing was performed on *mecA* genes of selected strains employing primers designed from known *mecA* gene sequences (Geha *et. al.*, 1994).

Bacterial lysis: Bacterial lysis was performed as previously described in section 2.5.1.

Oligonucleotide primers: *mecA* primers (section 2.5.1.)

Amplification was performed in a final reaction volume of 100 µl comprising: 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM of each dNTP (Boehringer), 100 pmol of each of the *mecA* primers and 6 µl of the bacterial lysate. The test samples were subjected to amplification in a thermal cycler (Perkin Elmer 9600) with pre-denaturation at 94°C for 4 min and primer annealing at 50°C at which time 2 units of Taq DNA polymerase (Advanced Biotechnologies) were added. Thirty amplification cycles were then performed: 94°C for 45 sec, 50°C for 45 sec, 72°C for 1 min. This was followed by a final extension of 72°C for 5 min.

For the isolation of PCR products from a amplification reaction, a PCR Clean-Up Kit (Boehringer) was used according to the manufacturers protocol. This involved:

The extraction of the PCR reaction with an equal volume of chloroform, followed by centrifugation for 5 min at 3000 × g in a microcentrifuge. TE-buffer and 400 µl Nucleic Acid Binding buffer was added to the upper-phase to obtain a final volume of 500 µl. The PCR solution was incubated at room temperature with 10 µl silica suspension. After incubation, the suspension was centrifuged briefly at maximum speed in a microcentrifuge and the supernatant discarded. The pellet was collected and washed in a double centrifugation step involving the addition of 400 µl washing buffer. DNA was eluted from the silica with 50 µl TE-buffer

(10 mM Tris-HCl, 0.1 mM EDTA, pH 8.5) after centrifugation at maximum speed. Samples of the clean PCR product (3 μl) were subjected to electrophoresis. Electrophoresis was performed in 2.3% agarose gels (NuSieve, FMC Bioproducts) using Tris acetic-acid EDTA running buffer (40mM Tris (pH 8.0), 20mM EDTA). DNA was stained with ethidium bromide (0.5 μg/ml) and viewed under UV illumination. After visual DNA estimation, DNA concentrations were standardised by dilution.

Sequencing of the *mecA* gene: Amplification was performed in a final reaction volume of 20 μl comprising: 2 pmol of *mecA1 or mecA2* primer, 8 μl sequencing reagent premix (DYEnamic ET terminator cycle sequencing kit, AEC Amersham), 0.5 units of Taq DNA polymerase, 8 μl water/ PCR product. The PCR products were subjected to 25 amplification cycles in a thermal cycler (Perkin Elmer 9600). Thirty amplification cycles were then performed: 94°C for 45 sec, 50°C for 45 sec, 72°C for 1 min. Sodium acetate/EDTA buffer (2 μl) and 80 μl 95% ethanol were added to the PCR product and the tube placed on ice for 15 min. The mixture was centrifuged in a microcentrifuge for 15 min at 10,000 × g. To the supernatant 300 μl 70% ethanol was added and the tube centrifuged briefly. After the supernatant was discarded, the pellet was air dried for 3 min at 60°C. The pellet was then re-suspended in 4 μl loading dye and loaded onto a lane of a sequencing gel.

2.6. Slide agglutination latex test for the detection of PBP2' (MASTALEX-MRSA™, Mast Laboratories Ltd., Bootle, Merseyside, UK)

MASTALEX-MRSA was performed on clinical oxacillin-resistant and oxacillin-susceptible staphylococcal strains screening positive for the *mecA* gene. Strains were grown overnight on MacConkey-agar (Difco) plates at 37°C. An eighth plate of confluent growth was suspended in 4 drops Extraction Reagent 1 in a microcentrifuge tube. Test samples were placed in a boiling water bath for 3 min, and cooled in an ice-water bath to room temperature. One drop of Extraction Reagent 2 was added to the sample and the mixture was centrifuged at 1500 × g for 5 min in a microcentrifuge. Supernatants (50 µl) were drawn off and mixed with a drop of both test latex and control latex reagents. A positive agglutination reaction with the test latex reagent and not with the control latex reagent indicated the presence of PBP2'- presumptive methicillin-resistant *Staphylococcus* spp.

CHAPTER 3

IDENTIFICATION OF STAPHYLOCOCCI

3.1 Introduction

Currently, there are 32 recognised species in the genus *Staphylococcus*, of which the coagulase-positive species *S. aureus* is the most clinically important (Gribaldo *et. al.,* 1997). During the last several years, CNS that are often associated with immuno-compromised patients have surfaced as important nosocomial pathogens (Gribaldo *et. al.* 1997; Kloos & Bannerman, 1994; Kloos & Schleifer, 1975). With the growing appreciation for their pathogenic potential, the identification of CNS has become a high priority in the clinical microbiology laboratory.

Phenotypic characteristics for the routine differentiation of *Staphylococcus* species from other gram-positive cocci are shown in Table 3.1 (Kloos & Bannerman, 1992). Employing biochemical tests, final identification of specific staphylococcal species requires up to three days of laboratory investigation (Kloos & Bannerman, 1994). The inclusion of lysostaphin testing is advantageous, although lysis of methicillin-resistant and vancomycin hetero-resistant strains is reduced and it carries extra expense that could be beyond the budget of some laboratories. Conventional methods for identification are at present considered adequate and serve as reference for determining the accuracy of other systems (Kloos & Bannerman, 1994).

TABLE 3.1. Differentiation of staphylococci from other gram-positive cocci (Kloos & Bannerman, 1994).

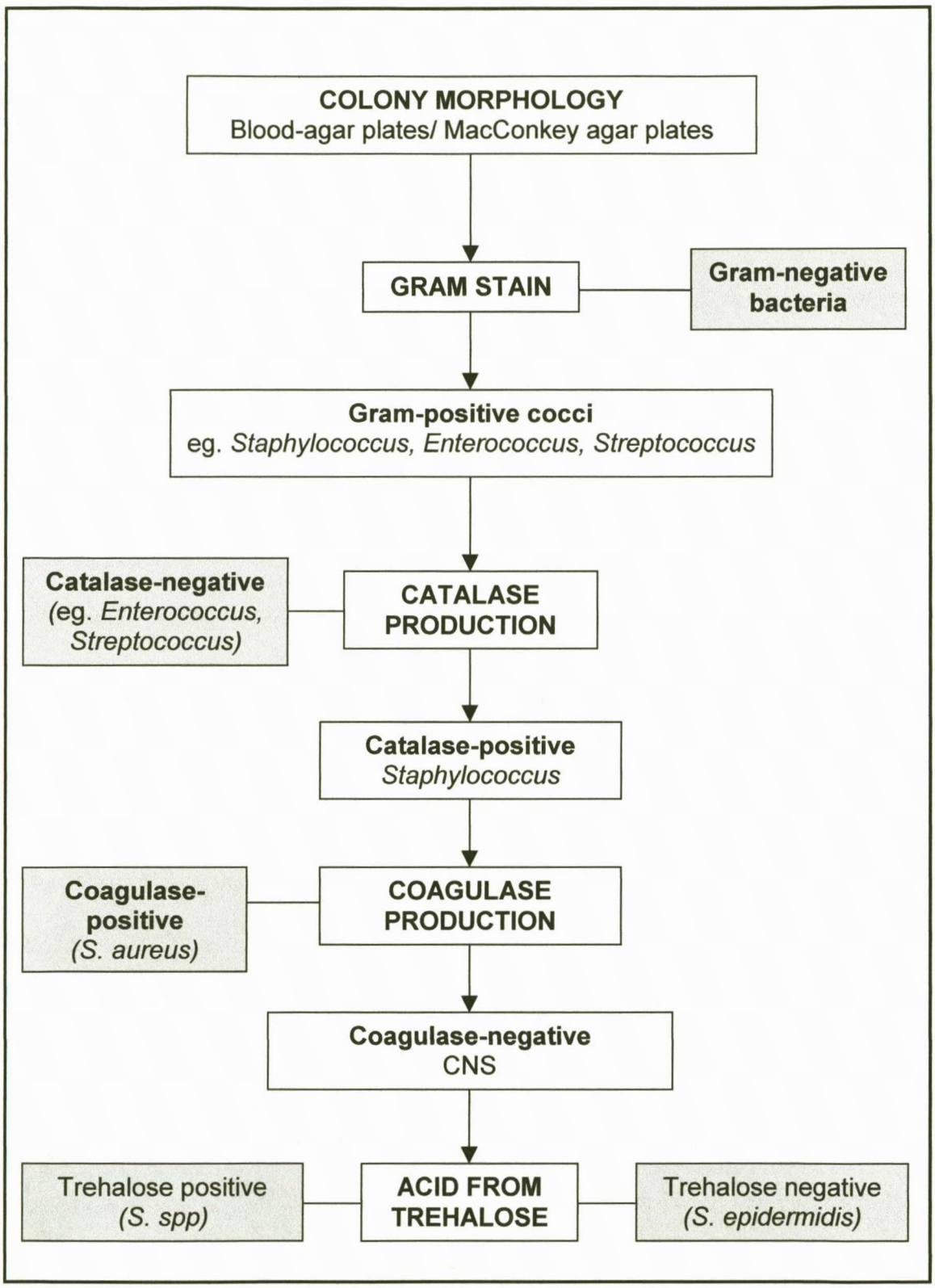
Biochemical test	Genus						
	Staphylococcus	*Enterococcus*	*Streptococcus*	*Aerococcus*	*Planococcus*	*Stomatococcus*	*Micrococcus*
% G+C	33-39	34-42	34-46	35-40	39-52	56-60	66-75
Strict aerobe	-	-	-	-	+	-	+
Facultative anaerobe	d	+	+	+	-	+	-
Strict anaerobe	-	-	d	-	-	-	-
Tetrad cell arrangement	d	-	-	+	d	d	+
Strong adherence on agar	-	-	-	-	-	+	-
Motility	-	d	-	-	+	-	-
Growth on:							
5% NaCl agar	+	+	d	+	+	-	+
6.5% NaCl agar	+	+	d	+	+	-	+
12% NaCl agar	d	(±)	-	+	+	-	D
Schleifer-Krämer agar	+	(±)	-	ND	ND	ND	-
P agar in 18 h	+	±	-	-	-	-	-
Catalase	+	-	-	-	+	±	+
Benzidine test	+	-	-	-	+	+	+
Modified oxidase test	-	-	-	-	ND	-	+
Anaerobic acid from glucose	d	+	+	(+)	-	+	-
Aerobic acid from glycerol	+	d	d	ND	-	d	-
Resistance to:							
Lysostaphin (200 µg/ml)	-	+	+	+	+	+	+
Erythromycin (0.04 µg/ml)	+	+	-	ND	ND	ND	-
Bacitracin (0.04 U)	+	+	d	-	ND	-	-
Furazolidone (100 µg)	-	-	-	-	-	d	+

Key: **+**, >90% positive; **-**, >90% negative; **±**, >90% weakly positive; **d**, 11-89% positive; **ND**, not determined

Some laboratories chose to alter extensive identification schemes by restricting the number of biochemical tests employed. This is done on the basis that *S. aureus, S. epidermidis, S. haemolyticus, S. lugdenensis* and *S. saprophyticus* are the staphylococcal species most commonly associated with human infections (Kloos & Bannerman, 1994). A schematic representation for the routine identification of human *Staphylococcus* species in the Universitas clinical microbiology laboratory is shown in Figure 3.1. This sequence of reactions is quite sufficient for routine purposes, however, emphasis is only placed on *S. aureus* and *S. epidermidis*. The format does not effectively differentiate staphylococci from other gram-positive organisms, as only catalase-production and colony morphology provides a split between these groups of organisms. Some bacteria (eg. enterococci and streptococci) produce peroxidase that catalyses the breakdown of hydrogen peroxide providing a weakly positive catalase reaction which can be mistaken as a truly positive catalase reaction (Baron *et. al.*, 1994). Furthermore, some strains of micrococci produce a yellow pigment, which may be confused with staphylococcal colony morphology (Kloos & Bannerman, 1994).

In contrast, for meaningful routine identification of CNS conventional biochemical tests are less than satisfactory due to variable expression of certain phenotypic characters and limited data bases. There is the requirement for DNA based approaches for rapid and accurate species identification. Gribaldo *et. al.* (1997) designed a PCR identification technique for differentiating the most

Figure 3.1. Schematic representation of the identification of *Staphylococcus* species by the clinical microbiology laboratory at the Universitas Hospital



commonly isolated CNS species. Primers were designed to amplify three variable regions found in the 16S rRNA gene.

3.2. Results and Discussion

3.2.1. Conventional biochemical tests

The biochemical tests used for the differentiation of clinical and environmental staphylococci received from the various laboratories are shown in Figure 3.2. The inclusion of Baird-Parker agar proved useful for the presumptive selection of staphylococcal species, in particular *S. aureus*. On Baird-Parker agar, 99.9% of *S. aureus* strains produced colonies that exhibited typical morphology, while only 70.4% of CNS colonies conformed. Despite these inaccuracies, Baird-Parker in combination with catalase, bile-aesculin hydrolysis and bacitracin susceptibility provides sufficient differentiation for identifying staphylococci.

Table 3.2 shows the strains that were misidentified by the diagnostic microbiology laboratory according to the identification scheme used in the present study. The catalase test revealed 24 catalase-negative strains identified as staphylococci by the clinical microbiology laboratory. Of these 24 catalase-negative gram-positive cocci, 22 strains had the ability to hydrolyse aesculin. Susceptibility to 0.04 U bacitracin is primarily used for the identification of micrococci, susceptibility was observed with seven gram-positive cocci received from the clinical laboratories. From the results shown in Table 3.2, and the data given in Table 3.1 it is clear that micrococci present with a very similar

Figure 3.2. Schematic representation of tests employed for the identification of staphylococci. The numbers of isolates that diverged or conformed to a positive staphylococcal strain are given in brackets. **P**= Pelinomi strains, **U**= Universitas strains and **E**= strains isolated from raw milk samples.

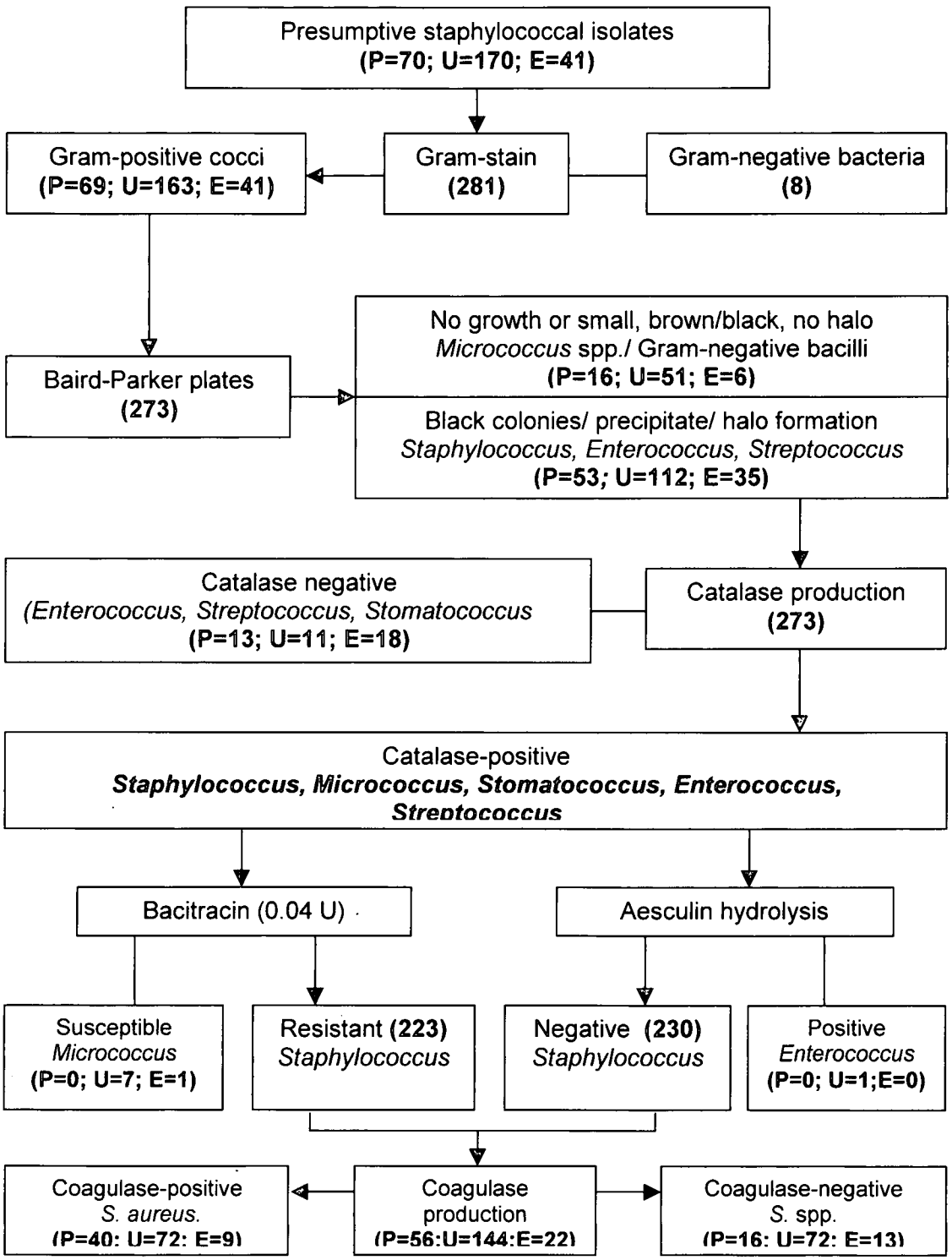


TABLE 3.2. Gram-positive cocci other than staphylococci that were identified by the microbiology laboratory as *Staphylococcus* species.

Strain No.	Laboratory identification	Biochemical tests					
		GS	BP[a]	CAT	BAE[b]	BAC[c]	COA
U55	*S.* species	-	atypical	+	-	resistant	-
U66	*S.* species	-	atypical	+	-	resistant	-
U67	*S. epidermidis*	-	atypical	+	-	resistant	-
U105	*S.* species	-	typical	+	-	resistant	-
U114	*S.* species	-	typical	+	-	resistant	-
U118	*S.* species	-	typical	+	-	resistant	-
P23	*S. epidermidis*	-	atypical	-	-	resistant	-
U73	*S.* species	-	typical	+	+	resistant	-
U75	*S.* species	+	atypical	-	-	resistant	-
U87	*S.* species	+	atypical	-	-	resistant	-
U45	*S.* species	+	atypical	-	+	resistant	-
U58	*S.* species	+	typical	-	+	resistant	-
U59	*S.* species	+	atypical	-	+	resistant	-
U60	*S.* species	+	atypical	-	+	resistant	+
U62	*S.* species	+	typical	-	+	resistant	-
U79	*S.* species	+	typical	-	+	resistant	-
U80	*S.* species	+	typical	-	+	resistant	-
U84	*S.* species	+	typical	-	+	resistant	-
U90	*S.* species	+	atypical	-	+	resistant	-

continued

TABLE 3.2. *continued*

Strain No.	Laboratory identification	Biochemical tests					
		GS	BP[a]	CAT	BAE[b]	BAC[c]	COA
P97	*S.* species	+	typical	+	+	resistant	-
P10	*S. epidermidis*	+	typical	-	+	resistant	-
P11	*S. epidermidis*	+	typical	-	+	resistant	-
P17	*S. aureus*	+	typical	-	+	resistant	-
P24	*S. epidermidis*	+	typical	-	+	resistant	-
P33	*S.* species	+	typical	-	+	resistant	-
P37	*S. aureus*	+	typical	-	+	resistant	-
P47	*S. aureus*	+	typical	-	+	resistant	-
P66	*S. epidermidis*	+	typical	-	+	resistant	-
P68	*S. aureus*	+	typical	-	+	resistant	-
P70	*S. aureus*	+	typical	-	+	resistant	-
P16	*S.* species	+	atypical	-	+	resistant	-
P40	*S.* species	+	typical	-	+	resistant	-
U36	*S. epidermidis*	+	typical	-	+	resistant	-
U17	*S.* species	+	atypical	+	-	susceptible	-
U18	*S. epidermidis*	+	atypical	+	-	susceptible	-
U57	*S.* species	+	atypical	+	-	susceptible	-
U72	*S.* species	+	typical	+	-	susceptible	-
U81	*S.* species	+	atypical	+	-	susceptible	-
U170	*S.* species	+	typical	+	-	susceptible	-
U112	*S. aureus*	+	typical	+	-	susceptible	+

Key: U, Universitas hospital; **P**, Pelinomi hospital; **GS**, Gram-stain; **BP**, Baird-Parker plates; **CAT**, catalase; **BAE**, bile-aesculin plates; **BAC**, bacitracin; **COA**, coagulase.
[a] colony morphology; [b] aesculin hydrolysis; [c] susceptibility to 0.04 U bacitracin

identification profile to that of staphylococci. The simple inclusion of bile-aesculin agar plates and a bacitracin susceptibility test into the diagnostic laboratory protocol would reduce the misidentification of non-staphylococcal isolates by 12.1%.

In the case of the Universitas microbiology laboratory, identification of staphylococci is restricted to *S. aureus, S. epidermidis* and *S.* spp. Poor correlation (84.6%) was found on comparing the coagulase tests performed by the Universitas diagnostic laboratory and the coagulase tests performed for the identification of *S. aureus* in this study. This was also the case for the coagulase test performed by the Pelinomi diagnostic laboratory correlating with a mere 83.9% of the coagulase test performed for the purposes of this study. In addition to *S. aureus; S. schleiferi*, S. *intermedius* and *S. lugdenensis* are also coagulase producers, however, these strains are usually differentiated from *S. aureus* on the basis of penicillin resistance. Another explanation for the poor correlation found between the respective coagulase tests performed, could have been the use of human plasma instead of the proposed rabbit plasma. Human plasma should be avoided unless it is controlled for clotting capability and for the absence of inhibitors. The human plasma used in both coagulase tests was untreated. For the purposes of this study, a single batch of plasma was used to limit any possible influence that inhibitors may have had on the coagulase test. Furthermore, any additional CNS profiling as *S. aureus* with the STAPH ID 32 API were added to the list of *S. aureus* isolates identified with the coagulase

test. However, this is not ideal for the purposes of the diagnostic laboratory. In addition to coagulase, a test screening for DNase activity, used for the recognition of potentially pathogenic staphylococci, should be included for the differentiation of *S. aureus* from CNS. Selective media such as Baird-Parker may also assist in the identification of *S. aureus* isolates.

3.2.2. Evaluation of the Staph ID 32 API system

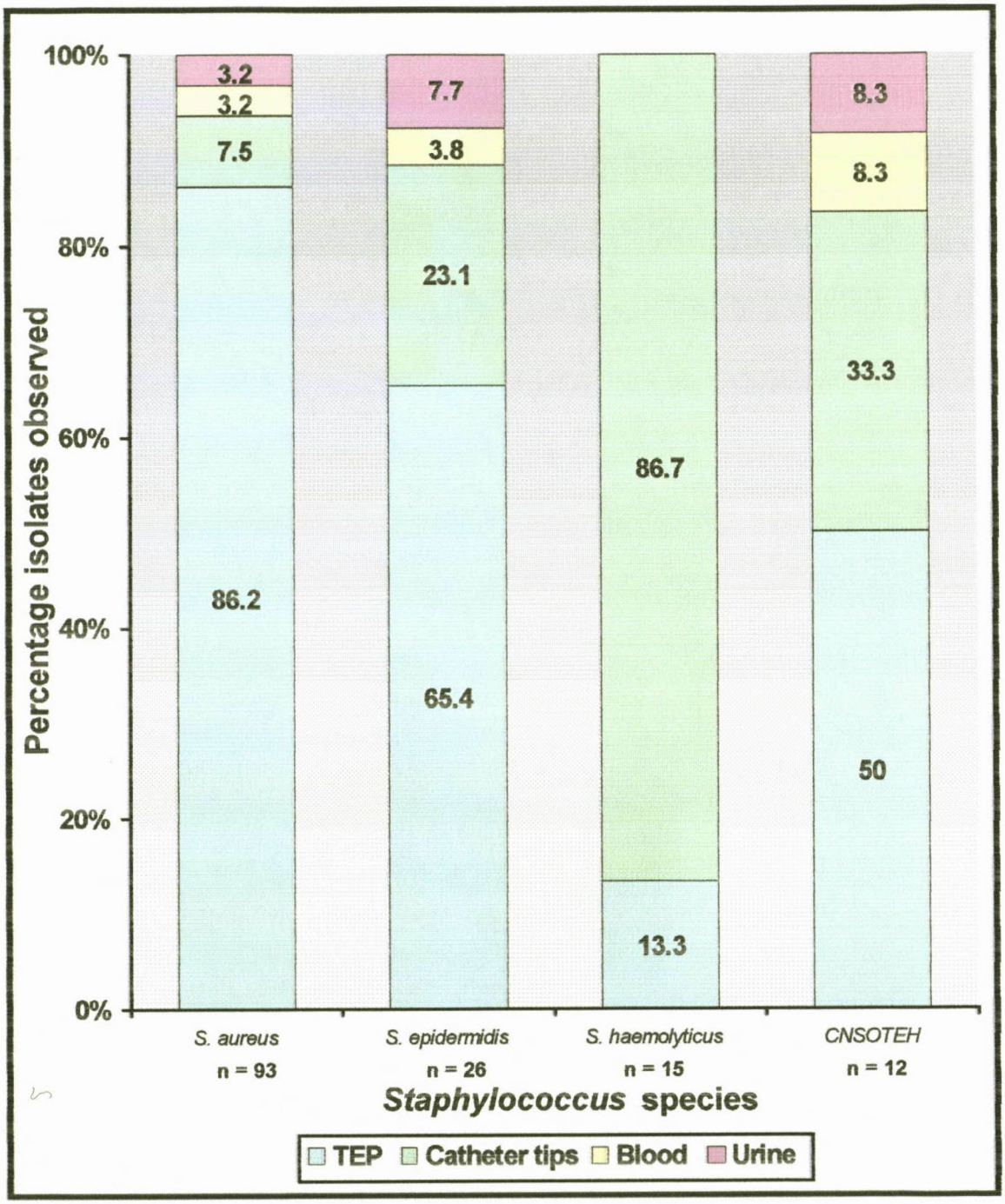
Of the 52 clinical staphylococcal isolates investigated, results obtained employing the STAPH ID 32 API system were as follows: good identification (>90% ID accuracy), 30 strains (57.7%); acceptable identification (>75 - <90% ID accuracy), 10 strains (19.2%); for 12 strains (23.1%) identification was problematic (<75% agreement). The species, *S. epidermidis*, is split by the API system into two sub-species: *S. epidermidis 1* and *S. epidermidis 2*. This resulted in good identification for eight *S. epidermidis* strains at the genus level, but also a ≤75% API species identification accuracy. When percentage identification accuracy was combined for these subspecies, overall identification of the 52 isolates resulted in the following: 38 strains good identification (>90% ID accuracy); 6 strains acceptable (>75 - <90% ID accuracy) with 8 strains giving problem identifications (<75% ID accuracy). An acceptable identification value of 84.6% was therefore achieved. The 12 CNS obtained from raw milk samples showed a similar acceptable identification value of 83.3%.

S. epidermidis was the most commonly isolated CNS obtained from the Universitas microbiology laboratory, justifying in part, the focus placed by the medical microbiology laboratory on the identification of this coagulase-negative *Staphylococcus* species only. However, the differentiation of *S. epidermidis* based on a single biochemical test (trehalose fermentation), did lead to the incorrect identification of 14 *S. epidermidis* strains as *S.* spp. and two *S.* spp. as *S. epidermidis* strains. These results suggest that diagnostic microbiology laboratories should persist with conventional methods for the routine identification of staphylococcal strains. For the identification of clinically rare or phenotypically variable CNS strains, the Staph ID 32 API system is certainly of value.

3.2.3. Distribution of staphylococci: species and isolation sites

Ninety-two of the 144 staphylococcal isolates were *S. aureus* strains. Most CNS infections were caused by *S. epidermidis* (17.4% of all the 144 staphylococcal strains isolated), followed by *S. haemolyticus* (10.4%) and *S. hominis* (2.8%) (Figure 3.3). The percentage distribution of staphylococcal strains isolated from blood; catheter tips; urine; tissue, exudate and prosthesis samples (TEP) is shown in Figure 3.3. *S. aureus* and *S. epidermidis* strains were predominantly isolated from TEP samples. This reflects the opportunistic nature of these bacteria to cause infections in patients where natural host defense barriers (eg. skin) has been damaged by trauma or surgery for prosthesis.

Figure 3.3. The percentage of staphylococcal strains isolated from blood; catheter tips; urine; tissue, exudate and prosthesis specimens.



Key: **TEP,** tissue, exudate, and prosthesis samples; **CNSOTEH,** CNS other than *S. epidermidis* and *S. haemolyticus*.

A low percentage of *S. aureus* and CNS were isolated from blood. Nevertheless, evidence provided by blood cultures is largely indirect and in the case of localised infections, it is necessary to identify the organism at the focus of infection. Noticeably, 86.7% of *S. haemolyticus* strains were found in catheter tips, suggesting an enhanced ability of the organism to adhere to polymer surfaces and multiply. CNS, other than *S. epidermidis* and *S. haemolyticus,* were most commonly isolated from urines. However, in the present study, not a single *S. saprophyticus* strain was isolated from urinary specimens, which might reflect API inaccuracies. Due to the high frequency of *S. haemolyticus* isolates found in indwelling devices, it is recommended that laboratory identification of CNS, should be extended to include not only *S. epidemidis*, but also *S. haemolyticus.*

Most of the currently recognised staphylococcal species have been isolated from the raw milk of cows, sheep and goats. In the present study, 10 *S. aureus*, 4 *S. hominis*, 2 *S. warneri*, 1 *S. chromogenes*, 3 *S. hyicus*, one *S. simulans* and one *S. capitis* were isolated from raw milk samples. Similarly, Harvey & Gilmour (1985) found *S. aureus* and *S. hominis* to be the most frequently isolated staphylococci from raw cows milk, while *S. warneri, S. chromogenes, S. hyicus* and *S. simulans* could be isolated at a lower frequency. *S. capitis* was the only species not isolated in 1983 by Harvey & Gilmour (1990).

3.2.4. Evaluation of a species specific PCR technique for the identification of CNS

For both Sep (*S. epidermidis*) and Shn (*S. hominis*) primer pairs, PCR products of 833-852-bp were amplified and visualised, while for the Shl (*S. haemolyticus*) primer pair, a 593-bp PCR product was observed (Figures 3.4.1-3.4.3). The results of the species-specific PCR identification assays performed on 34 CNS strains are shown in Table 3.3. The primer pair Sep was in agreement for 9 of 18 API identified *S. epidermidis* strains (Figure 3.4.1). The primer pair Shl identified five of twelve API *S. haemolyticus* strains correctly (Figure 3.4.2.). Only one of four API identified *S. hominis* strains was confirmed with the Shn primer pair (Figure 3.4.3.).

The overall specificity of the PCR assay for the identification of the CNS investigated based on the API profile identification system was only 44.1%. Correlation between the API system with ≥ 90% identification accuracy and PCR species-specific primer identification was even lower at 26.9%; with correlation between the API ≤75% identification accuracy and PCR identification being 50%. One of the major problems of the PCR assay was non-specific identification; as shown in Table 3.3, five strains (14.7% of strains tested) were identified as two different species. The inclusion of universal primers, could have assisted in determining false negative PCR results that could have arisen from lysis difficulties.

Figure 3.4.1.

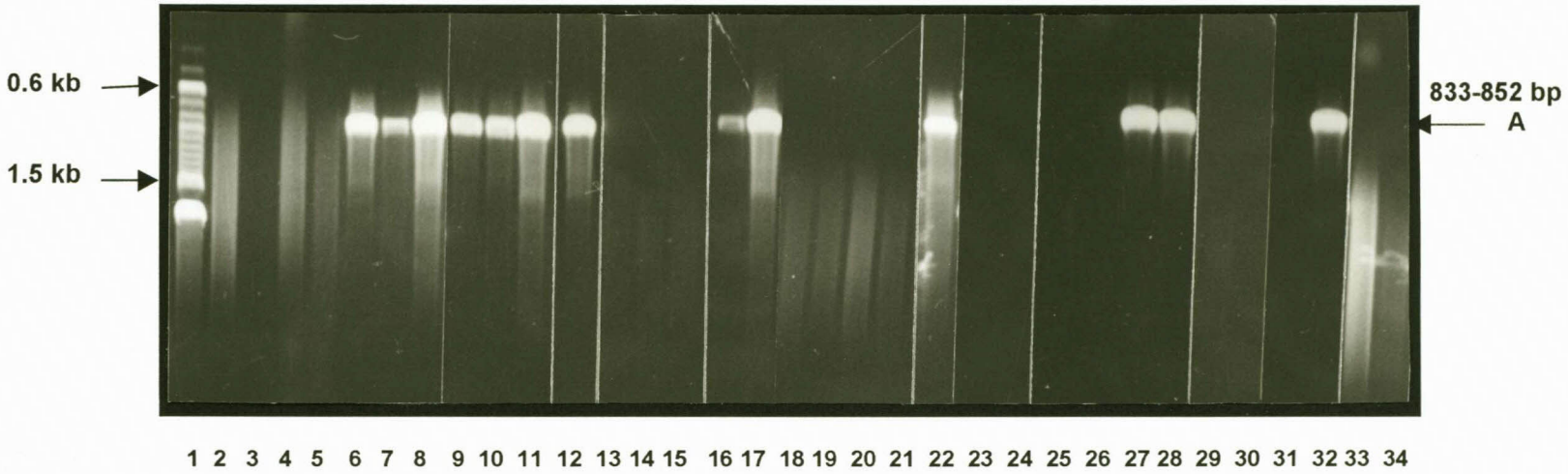


Figure 3.4.1. Species-specific PCR identification, employing the *Sep* primer pair. ***Lane 1:*** MWM G, ***Lanes 2 to 19:*** *S. epidermidis* strains: ***2:*** strain U77, ***3:*** strain U82; ***4:*** strain U122, ***5:*** strain U39, ***6:*** strain U129, ***7:*** strain U142, ***8:*** strain U141, ***9:*** strain U99, ***10:*** strain U155, ***11:*** strain U40, ***12:*** strain U73, ***13:*** strain U47, ***14:*** strain U31, ***15:*** strain U10, ***16:*** strain U97, ***17:*** strain U46, ***18:*** strain U14, ***19:*** strain U48. ***Lanes 20 – 30:*** *S. haemolyticus* strains: ***20:*** strain U123, ***21:*** strain U16, ***22:*** strain U70, ***23:*** strain U131, ***24:*** strain U69, ***25:*** strain U124, ***26:*** strain U88, ***27:*** strain U4, ***28:*** strain U28, ***29:*** strain U11, ***30:*** strain U93. ***Lanes 31 to 34:*** *S. hominis* strains: ***31:*** strain U148, ***32:*** strain U24, ***33:*** strain U26, ***34:*** strain U63. (**A**) *Sep* primer pair, species-specific PCR product.

Figure 3.4.2.

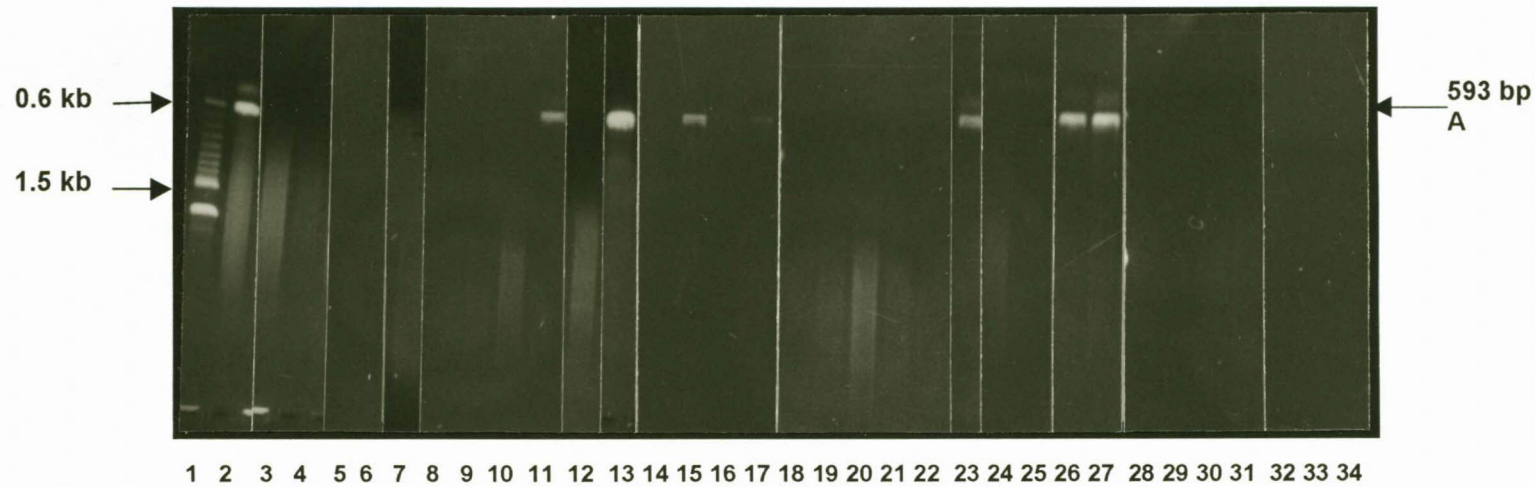


Figure 3.4.2. Species specific PCR identification employing *Shl* primer pair. ***Lane 1:*** MWM G, ***Lanes 2 – 13:*** *S. haemolyticus* strains: ***2:*** strain U70, ***3:*** strain U88, ***4:*** strain U131, ***5:*** strain U16, ***6:*** strain U124, ***7:*** strain U93, ***8:*** strain U123, ***9:*** strain U42, ***10:*** strain U11, ***11:*** strain U28, ***12:*** strain U69, ***13:*** strain U4. ***Lanes 14 – 30:*** *S. epidermidis* strains: ***14:*** strain U77, ***15:*** strain U82, ***16:*** strain U99, ***17:*** strain U14, ***18:*** strain U39, ***19:*** strain U73, ***20:*** strain U46, ***21:*** strain U10, ***22:*** strain U141, ***23:*** strain U40, ***24:*** strain U129, ***25:*** strain U142, ***26:*** strain U97, ***27:*** strain U47, ***28:*** strain U48, ***29:*** strain U155, ***30:*** strain U122. ***Lanes 31 – 34:*** *S. hominis* strains: ***31:*** strain U148, ***32:*** strain U26, ***33:*** strain U24, ***34:*** strain U63. (**A**) *Shl* primer pair, species specific PCR product.

Figure 3.4.3.

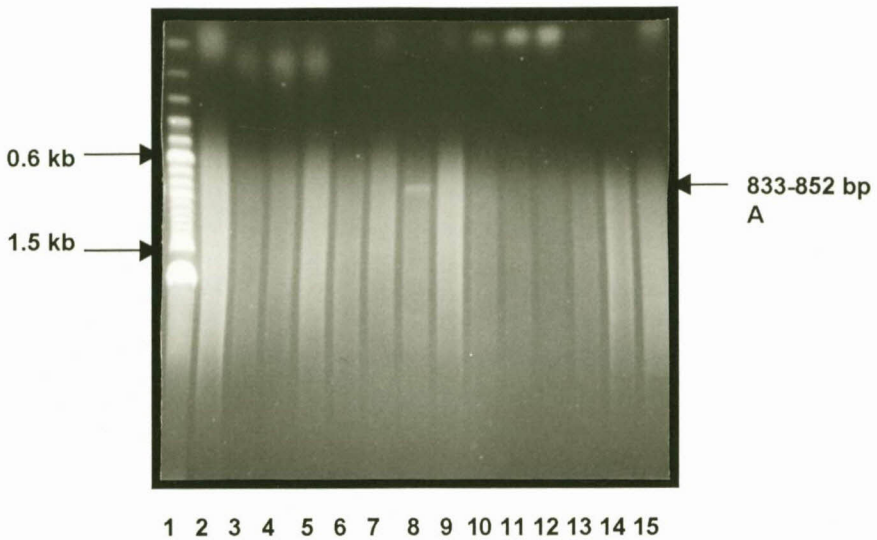


Figure 3.4.3. Species-specific PCR identification employing *Shn* primer pair. ***Lane 1:*** MWM G, ***Lanes 2 – 7:*** *S. epidermidis* strains: ***2:*** strain U141, ***3:*** strain U82, ***4:*** strain U47, ***5:*** strain U10, ***6:*** strain U46, ***7:*** strain U142. ***Lanes 8 – 11:*** *S. hominis* strains: ***8:*** strain U24, ***9:*** strain U26, ***10:*** strain U148, ***11:*** strain U63. ***Lanes 12 – 15:*** *S. haemolyticus* strains: ***12:*** strain U123, ***13:*** strain U88, ***14:*** strain U11, ***15:*** strain U93. (A) Shn primer pair, species-specific PCR product.

Table 3.3. Comparison of *Staphylococcus* species as identified by the API system and PCR assays.

Lab. No.	Staph ID 32 API system		Species specific PCR ID assay			
	Organism	% ID	Sep	Shl	Shn	PCR ID
U123	*S. haemolyticus*	98.5	-	-	-	Unidentified
U16	*S. haemolyticus*	98.5	-	+	ND	*S. haemolyticus*
U70	*S. haemolyticus*	98.5	+	+	ND	Non-specific
U88	*S. haemolyticus*	98.8	-	-	-	Unidentified
U28	*S. haemolyticus*	98.8	+	-	ND	*S. epidermidis*
U11	*S. haemolyticus*	98.8	-	-	-	Unidentified
U93	*S. haemolyticus*	99.7	-	+	ND	*S. haemolyticus*
U131	*S. haemolyticus*	99.9	-	-	-	Unidentified
U42	*S. haemolyticus*	99.9	-	+	ND	*S. haemolyticus*
U69	*S. haemolyticus*	99.9	-	-	-	Unidentified
U4	*S. haemolyticus*	99.9	+	-	ND	*S. epidermidis*
U124	*S. haemolyticus*	99.9	-	+	ND	*S. haemolyticus*
U148	*S. hominis 2*	44.1	-	-	-	Unidentified
	S. chromogenes	30.4				
	S. aureus	23.5				
U26	*S. hominis 2*	79.1	-	-	-	Unidentified
	S. aureus	20.7				
U24	*S. hominis 2*	80.3	+	-	+	Non-specific
	S. hominis 1	18.3				
U63	*S. hominis 2*	91.8	-	-	-	Unidentified
U129	*S. epidermidis 1*	-	+	-	ND	*S. epidermidis*
	A. viridans	-				
U141	*S. epidermidis 2*	32	+	-	ND	*S. epidermidis*
	S. epidermidis 1	27.3				
	S. chromogenes	19.8				

continued

Table 3.3. *continued*

Lab. No.	Staph ID 32 API system		Species specific PCR ID assay			
	Organism	% ID	Sep	Shl	Shn	PCR ID
U77	*S. epidermidis 1*	42.5	-	-	-	Unidentified
	S. hominis 2	42.1				
U82	*S. epidermidis 1*	42.5	-	+	ND	*S. haemolyticus*
	S. hominis 2	42.1				
U142	*S. epidermidis 2*	62.1	+	-	ND	*S. epidermidis*
	S. epidermidis 1	34.6				
U97	*S. epidermidis 2*	65.4	+	+	-	Non-specific
	S. epidermidis 1	35.5				
U155	*S. epidermidis 1*	69.5	+	-	ND	*S. epidermidis*
	S. epidermidis 2	29				
U122	*S. epidermidis 1*	72	-	-	-	Unidentified
	S. epidermidis 2	26.3				
U99	*S. epidermidis 1*	77.8	+	ND	-	*S. epidermidis*
	S. chromogenes	18.4				
U39	*S. epidermidis 1*	86	-	-	-	Unidentified
	S. epidermidis 2	7.3				
U73	*S. epidermidis 1*	91.5	+	-	+	Non-specific
U14	*S. epidermidis 1*	92.1	-	+	ND	*S. haemolyticus*
U46	*S. epidermidis 1*	92.5	+	-	ND	*S. epidermidis*
U40	*S. epidermidis 2*	93.6	+	+	-	Non-specific
U31	*S. epidermidis 1*	97	-	-	-	Unidentified
U48	*S. epidermidis 1*	98	-	-	-	Unidentified
U47	*S. epidermidis 1*	98	-	+	ND	*S. haemolyticus*
U10	*S. epidermidis 1*	98.2	-	-	-	Unidentified

ND, not determined, as results had either concurred or a PCR product of predicted size had been obtained with Sep or Shl primers.

Furthermore, two API *S. haemolyticus* strains (99.9% ID accuracy) were identified as *S. epidermidis* by PCR, while three API *S. epidermidis* strains (16.6%) were identified by PCR as *S. haemolyticus*.

The rapidity of PCR identification assays offers an alternative to the API system for the identification of clinical CNS. However, the inaccuracy experienced, based on PCR/API correlation for identifying the most commonly isolated CNS in this study, argues against its use in the clinical microbiology laboratory. The PCR method requires standardisation, the introduction of universal primers for the detection of false negatives and extensive species comparative investigations before it can be considered for incorporation into routine practice.

CHAPTER 4

STRAIN CHARACTERISATION

4.1. Introduction

The remarkable propensity of epidemic MRSA strains to colonise and disseminate rapidly in the hospital environment, has increased pressure on the diagnostic laboratory for early and specific diagnosis (Aires de Sousa *et. al.*, 1996). Ever since the emergence of these epidemic strains in the 1980s, research has focused on the dissemination of MRSA in the hospital setting (Van Leeuwen *et. al.*, 1996). The ubiquitous nature of CNS (particularly *S. epidermidis*) and recent realisation of pathogenic potential, has necessitated the development of methods for CNS strain deliniation (Van Straten *et. al.*, 1996).

Global epidemiological surveillance programs require reliable techniques with the capacity to differentiate independent strains from clonally related strains. Epidemiological typing techniques should ideally be technically elementary, exhibit high discriminatory power, be non-labour intensive and inter-laboratory standardised (Van Leeuwen *et. al.*, 1996). The data gained from epidemiological typing studies promote improved detection, prevention, and therapy, and an increased understanding of the pathogenisis of staphylococcal infections (Van Straaten *et. al.*, 1996).

RAPD analysis has been proposed as one of the few genotyping systems to conform with standards set for strain delineation in bacteria such as staphylococci, although its inter-laboratory reproducibility requires improvement (Van Leeuwen *et. al.*, 1996). Highly conserved, repetitive DNA sequences are usually considered quite rare in the prokaryotic genome. Exceptions to this rule are multiple gene copies and transposable elements (insertion sequences) (Hulton *et. al.*, 1991). Besides well-defined repetitive sequences, the prokaryotic genome contains repetitive DNA sequences with no apparent function other than self-preservation. These sequences of unknown function are highly species specific and form the basis of RAPD typing (Hulton *et. al.*, 1991). Primers are selected arbitrarily or semi-selectively (repetitive DNA sequences) that hybridise at random sites in the genome to initiate DNA polymerisation (Power, 1996). After amplification, the PCR products are electrophoresed and DNA fingerprints visualised.

Typically primers are chosen that are 10 bases in length (eg. Primer I and III used in this study), and with low annealing temperatures to decrease amplification stringency a multiple PCR banding profile is obtained (Power, 1996). It has been suggested that primers greater than eight nucleotides in length do not generally significantly increase the information content of RAPDs (Power, 1996). However, in practice, RAPD typing of MRSA was found to be more successful using primers longer than 10 nucleotides in length (Van Belkum *et. al.,* 1993). ERIC 1 & 2 are two such primers, that are basically directed

against recently described enterobacterial repetitive intergenic consensus (ERIC) sequences; a family of highly conserved repetitive DNA sequences first described in *Salmonella typhimurium* (Hulton *et. al.*, 1991).

Aires de Sousa *et. al.* (1996) assessed ERIC primer performance against a total of 183 staphylococcal isolates from eight Portuguese and one Spanish hospital. The combination of ERIC 1 & 2 primers produced a total of ten different RAPD types. In a further study conducted in the Netherlands seven different RAPD types were produced with the ERIC 1 & 2 primer combination (Van Belkum *et. al.*, 1997). Although some variation was observed for this primer combination, major cluster formation was observed for the strains investigated. Yet in an earlier study performed in Poland only two different ERIC RAPD pattern types were found (Trzcinski *et. al.*, 1994). Strain typing from this study was seen to be quite homogeneous and discrimination for the majority of isolates was not possible. Furthermore, the above mentioned studies only explored the application of the ERIC primers to the epidemiological typing of *S. aureus* and not in CNS isolates.

The reproducibility of the RAPD assay can be increased by consistency (decrease variables) concerning reaction volumes, reagent concentrations, the thermostable DNA polymerase employed (even to same batch), thermal cycler, procedures for visualisation and by nominating only a few proficient technical staff (Power, 1996). A major drawback in the standardisation of the RAPD

technique is the method used for the preparation of template DNA. For RAPD typing in the diagnostic laboratory in order to provide a result within a realistic time frame, the use of crude template DNA preparations are advocated. However, variable quantities of chromosomal and extrachromosomal DNA are generated by boiling of the test organism, inhibitors of DNA polymerase may be present in crude bacterial lysates and DNA may shear resulting in different DNA templates. Crude preparations of DNA were therefore not recommended by Power (1996). It should, be noted that multiple manipulations necessary for obtaining purified DNA template can increase shear/lower DNA integrity that will also adversely influence PCR product size and band number. Yet another factor that affects RAPD results, is the interpretation of the PCR fragments produced. Power (1996) suggested that a two or three band difference in the RAPD pattern implies a different strain, while four band or greater differences suggests a different species. When employing the rapid cell lysis method for the preparation of template DNA, RAPD profiles can not be analysed using the above mentioned criteria due to the low reproducibility of weak bands. For analysis of RAPD profiles, using crude DNA preparations, emphasis is placed on strong bands. A cluster of strains may, therefore not have exactly identical RAPD profiles, but will share similar prominent/strong RAPD band profiles. The RAPD patterns created are also useful for the development of species specific primers or if a unique amplicon is identified, strain specific DNA probes (Van Leeuwen *et. al.*, 1996). An alternative to the RAPD technique for intra-species strain typing is the use of plasmid profiles. Van Straten *et. al.* (1996) found

plasmid profiles to be the most accurate method for the determination of relatedness amongst staphylococcal isolates. This does not eleviate problems that can be encountered if a plasmid is lost during sub-culturing. Thus, it was also stressed that plasmid profiles in conjunction with antimicrobial susceptibility patterns should be used as discriminatory criteria for strain deliniation.

4.2. Results and Discussion

4.2.1. RAPD assays with ERIC 1 & 2 primers

The results of RAPD assays, employing the primers ERIC 1 & 2 are shown in Figure 4.1. The following strains were included to create preliminary representative RAPD profiles (Figure 4.1.): two *S. aureus*, three *S. epidermidis* and two strains of *S. haemolyticus*. To assess optimal reaction conditions amplification was performed with two separate $MgCl_2$ concentrations. However, no improvement was observed for 2.5 mM $MgCl_2$ in comparison to 4 mM $MgCl_2$. In fact, poor typeability and discrimination was observed for both ERIC 1 (Figure 4.1.a) and ERIC 2 (Figure 4.1.b) primers against the five strains tested. As it had been suggested that primers longer than ten oligonucleotide bases in length can show improved typeability and discrimination for *S. aureus* strains (Van Belkum *et. al.*, 1993), nine *S. aureus* strains were included (Figure 4.2). In a further attempt to improve performance of the ERIC 2 primer, the annealing temperature was decreased to 25 °C (Figure 4.2.). Again, no improvement in the typeability or discrimination of the ERIC RAPD assay was observed at the decreased annealing temperature.

Figure 4.1.A.

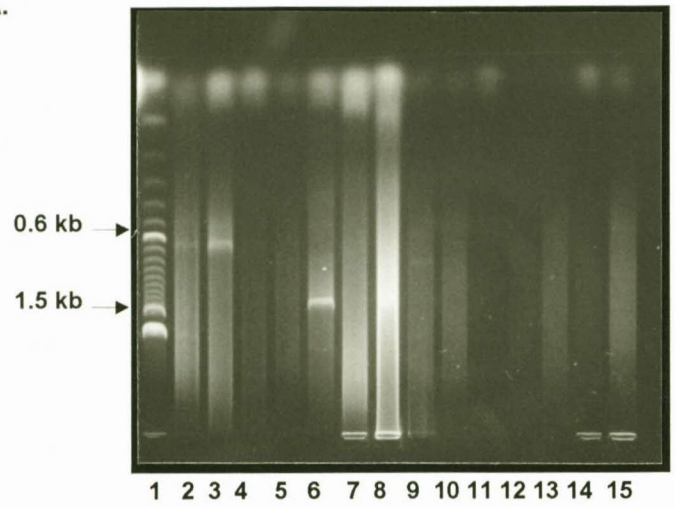


Figure 4.1.B.

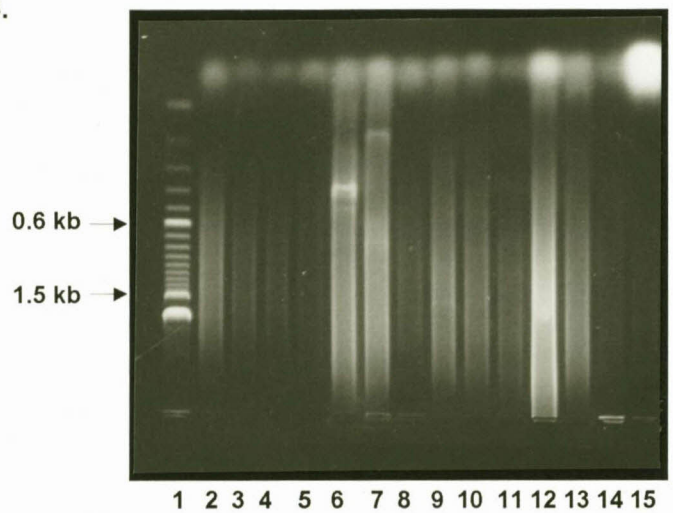


Figure 4.1. RAPD analysis of staphylococci employing ERIC 1 & ERIC 2 primers.

(A) ERIC 1: ***Lane 1:*** MWM G. ***Lanes 2 – 8:*** 2.5 mM $MgCl_2$. ***Lanes 9 – 15:*** 4 mM $MgCl_2$. (***2 & 9:*** strain U25, ***3 & 10:*** strain U107, ***4 & 11:*** strain U142, ***5 & 12:*** strain U99, ***6 & 13:*** strain U82, ***7 & 14:*** strain U70, ***8 & 15:*** strain U11)

(B) ERIC 2: ***Lane 1:*** MWM G. ***Lanes 2 – 8:*** 2.5 mM $MgCl_2$. ***Lanes 9 – 15:*** 4 mM $MgCl_2$. (***2 & 9:*** strain U25, ***3 & 10:*** strain U107, ***4 & 11:*** strain U142, ***5 & 12:*** strain U99, ***6 & 13:*** strain U82, ***7 & 14:*** strain U70, ***8 & 15:*** strain U11)

Figure 4.2.

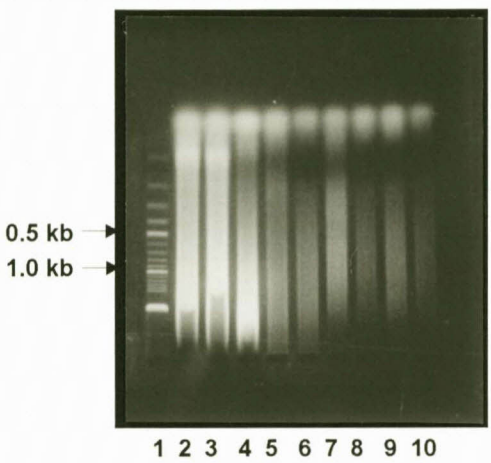


Figure 4.2. RAPD analysis of *S. aureus* strains employing ERIC 1 & ERIC 2 primers. ***Lane 1:*** MWM XIV, ***2:*** strain U65, ***3:*** strain U107, ***4:*** strain U131, ***5:*** strain U27, ***6:*** strain U25, ***7:*** strain U154, ***8:*** strain U29, ***9:*** strain U113, ***10:*** strain U41

4.2.2. RAPD assays with Primers I & III

On analysing the RAPD profiles created with Primer I, major PCR products could be assigned as unique for each species. The unique RAPD I profiles for the different staphylococcal species investigated are shown in Figures 4.3.1 to 4.3.3. Species specific bands are shown schematically in Figure 4.4 at 900 bp for *S. aureus* (additional bands at 300 bp & 600 bp), 1200 bp for *S. epidermidis* (additional bands at 400 bp & 600 bp), 1100 bp for *S. haemolyticus* (additional bands at 600 bp & 800 bp) and 1000 bp for *S. hominis* (additional band at 800 bp). Two *S. aureus* strains presented with atypical RAPD profiles, (Figure 4.5.3 lanes 7 and 9), one *S. epidermidis* (Figure 4.5.1 lane 17) and one *S. hominis* (Fig. 4.5.3 lane 40) strain did not concur. For *S. haemolyticus* API and RAPD I profile results were in total accordance (Figure 4.5.2). Although only four *S. hominis* strains were isolated, one strain possessed a different RAPD profile to that of the other strains. Excellent species typeability was achieved with Primer I.

The entire RAPD profiles could further be used for discriminating between unrelated species. Two major clusters were formed from the 19 *S. epidermidis* strains studied. This could not be attributed to the presence of both *S. epidermidis* subspecies as strain U141 was the only isolate of *S. epidermidis* 2 identified by the Staph ID 32 API system. A single cluster was evident for *S. haemolyticus* strains, while three different RAPD profiles were observed for *S. aureus*.

Figure 4.3.1. Schematic representation of RAPD I profiles for the differentiation of *S. epidermidis* isolates

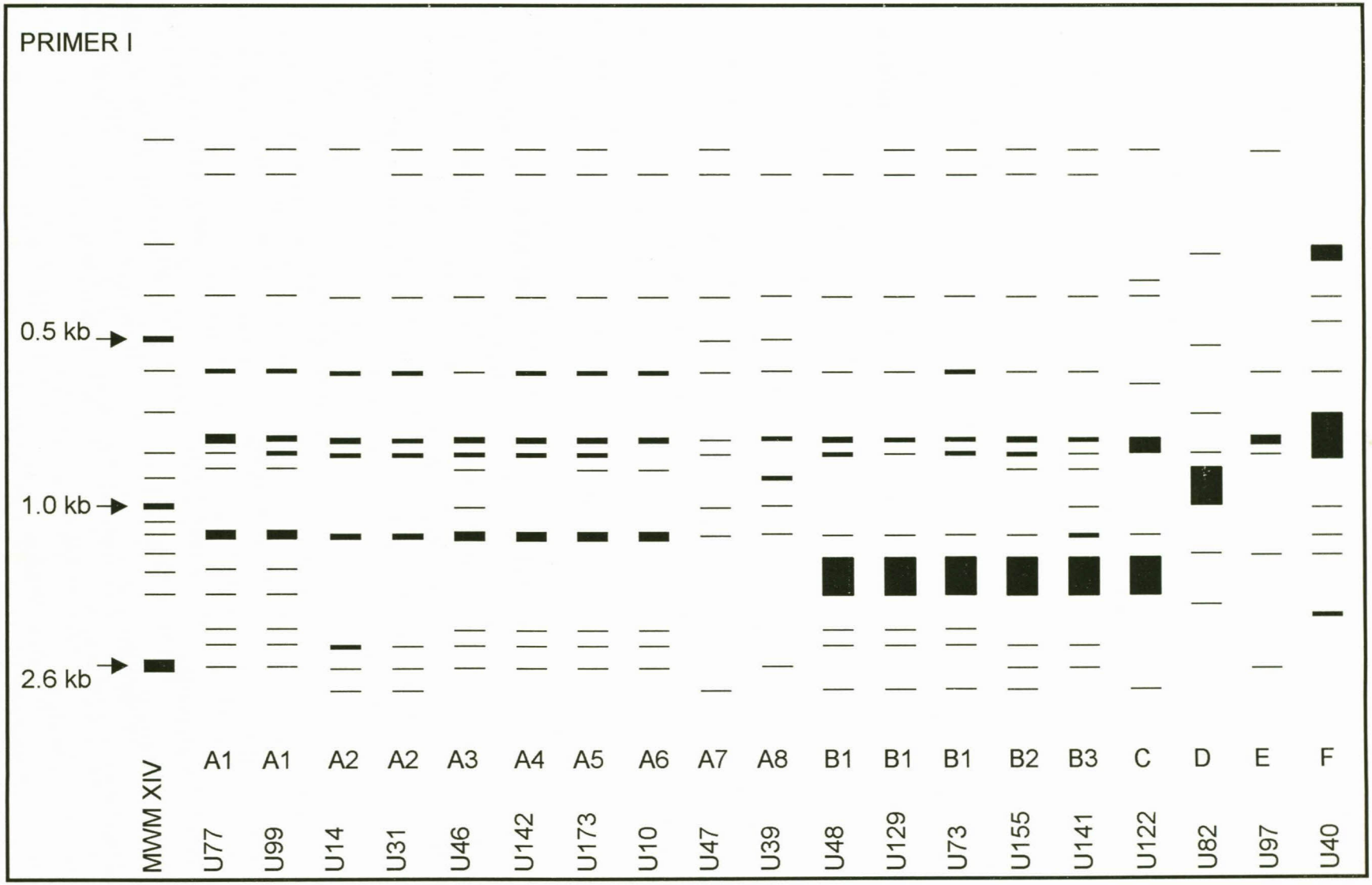


Figure 4.3.2. Schematic representation of RAPD I profiles for the differentiation of *S. haemolyticus* isolates

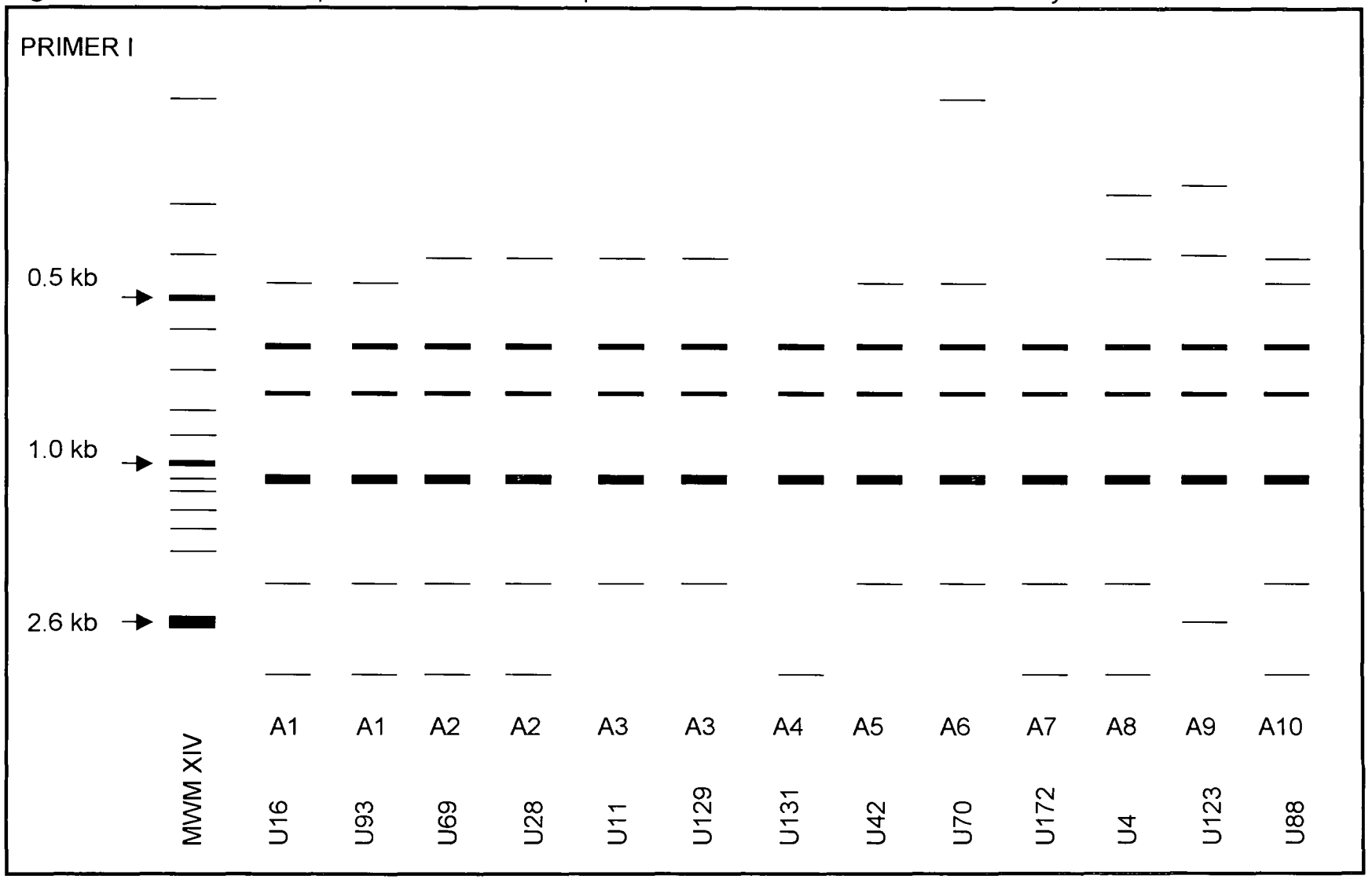


Figure 4.3.3. Schematic representation of RAPD I profiles for the differentiation of (A) *S. aureus* and (B) *S. hominis*

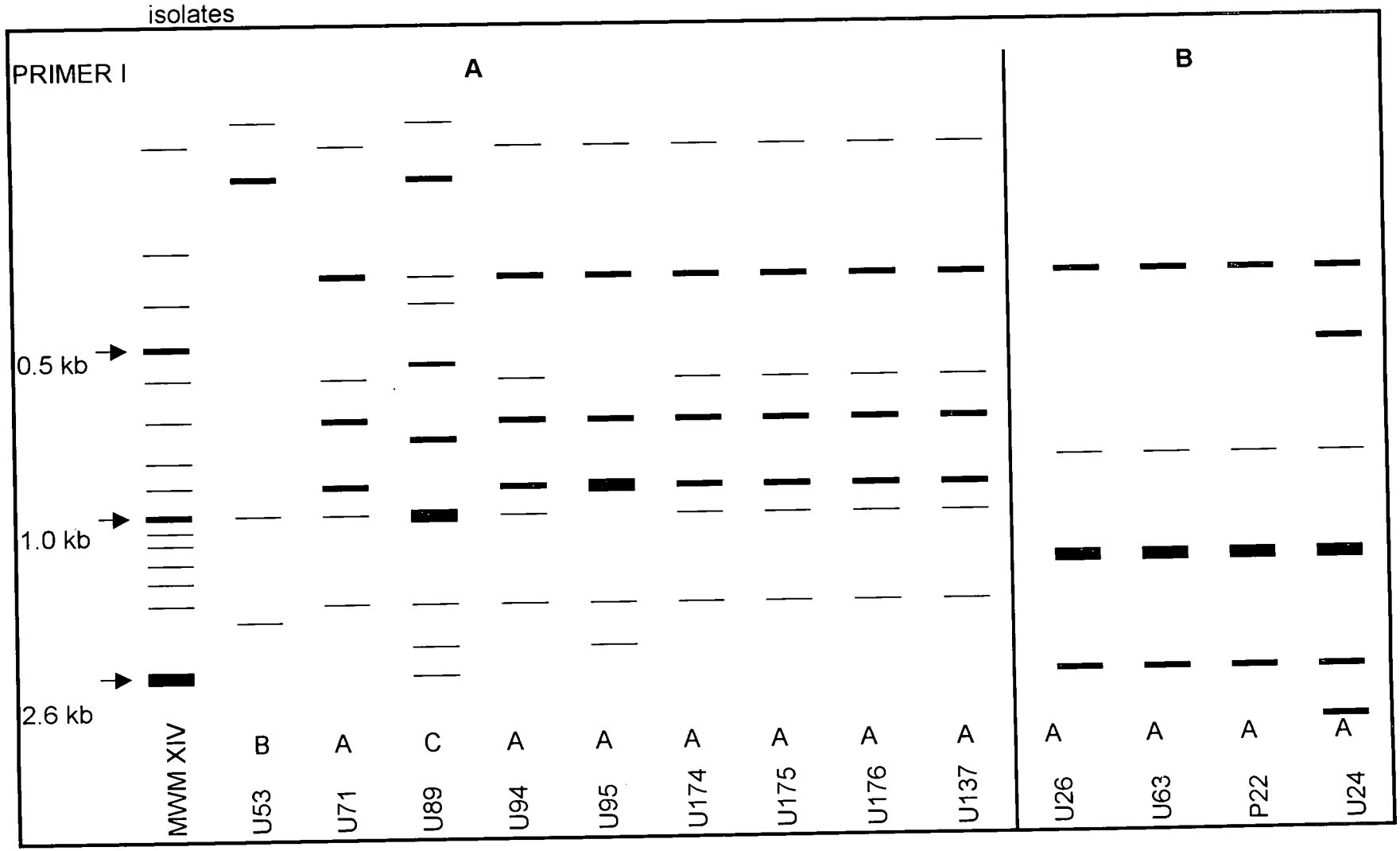


Figure 4.4. Schematic representation of RAPD profiles obtained with Primer I for *S. aureus*, *S. epidermidis*, *S. haemolyticus* and *S. hominis*. (→) Additional bands assisting RAPD profiling

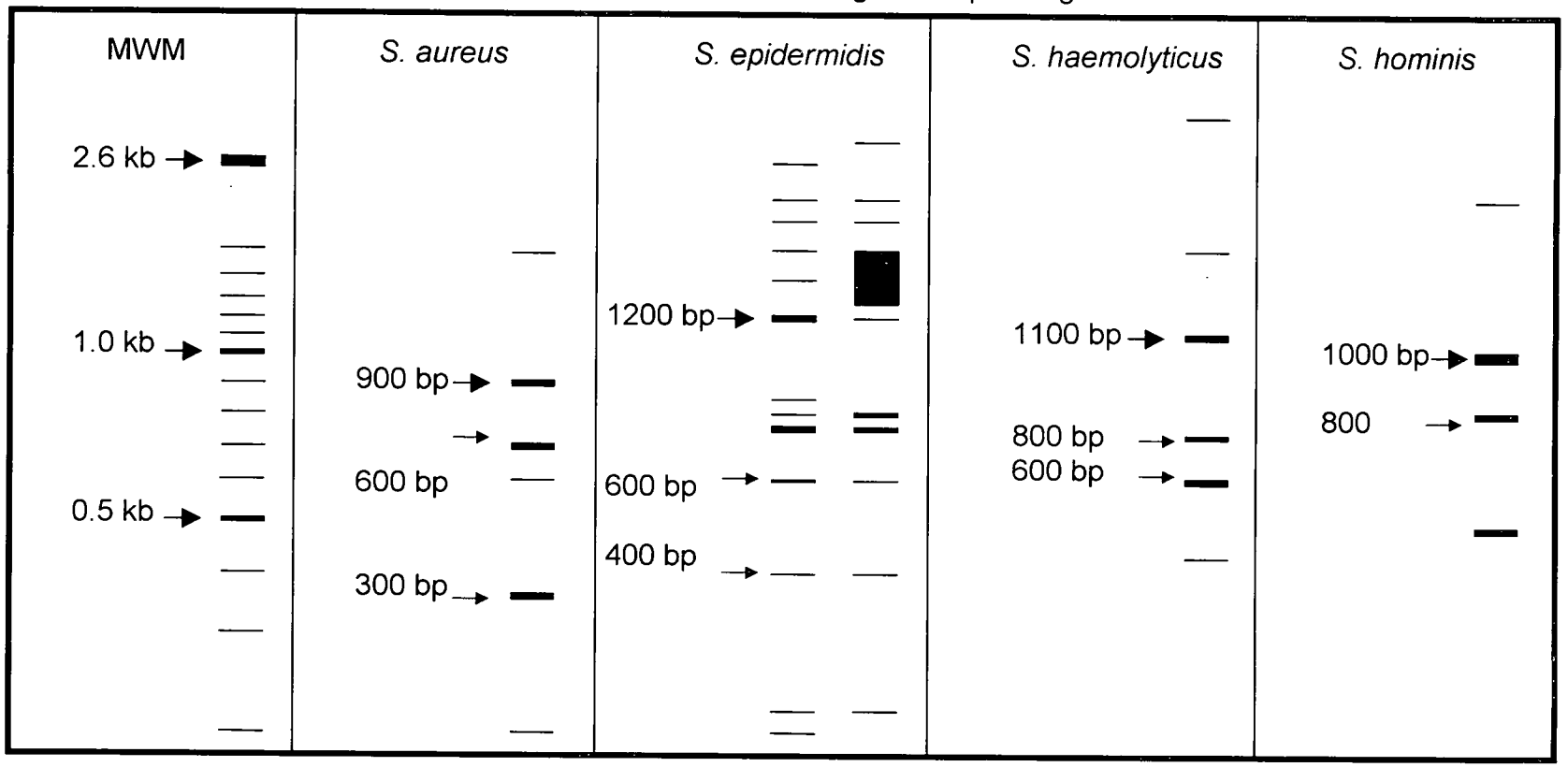


Figure 4.5.1.

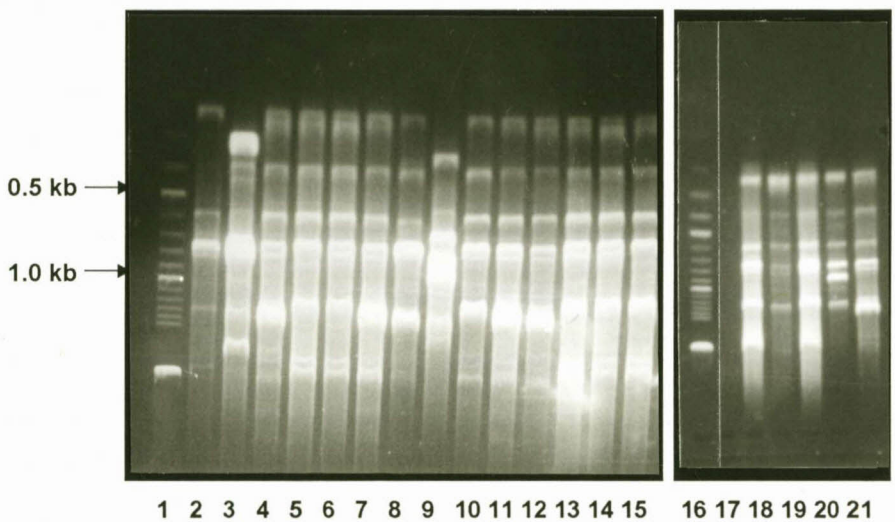


Figure 4.5.1. RAPD I profiles for *S. epidermidis* (RAPD I profile numbers in brackets). ***Lanes 1:*** MWM XIV, ***2:*** strain U97 (E), ***3:*** strain U40 (F), ***4:*** strain U73 (B1), ***5:*** strain U77 (A1), ***6:*** strain U99 (A1), ***7:*** strain U155 (B2), ***8:*** strain U122 (C), ***9:*** strain U82 (D), ***10:*** strain U46 (A3), ***11:*** strain U48 (B1), ***12:*** strain U129 (B1), ***13:*** strain U142 (A4), ***14:*** strain U173 (A5), ***15:*** strain U10 (A6), ***16:*** MWM XIV, ***17:*** strain U14 (A2), ***18:*** strain U47 (A7), ***19:*** strain U31 (A2), ***20:*** strain U39 (A8), ***21:*** strain U141 (B3).

Figure 4.5.2.

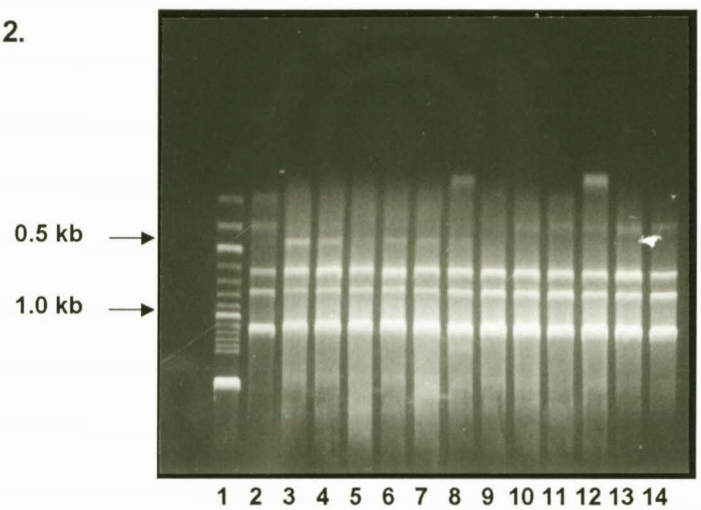


Figure 4.5.2. RAPD I profiles for *S. haemolyticus* (RAPD I profile numbers in brackets). ***Lane 1:*** MWM XIV, ***2:*** strain U123 (A9), ***3:*** strain U88 (A10), ***4:*** strain U16 (A1), ***5:*** strain U131 (A4), ***6:*** strain U93 (A1), ***7:*** strain U42 (A5), ***8:*** strain U70 (A6), ***9:*** strain U172 (A7), ***10:*** strain U69 (A2), ***11:*** strain U4 (A8), ***12:*** strain U28 (A2), ***13:*** strain U11 (A3), ***14:*** strain U129 (A3).

Figure 4.5.3.

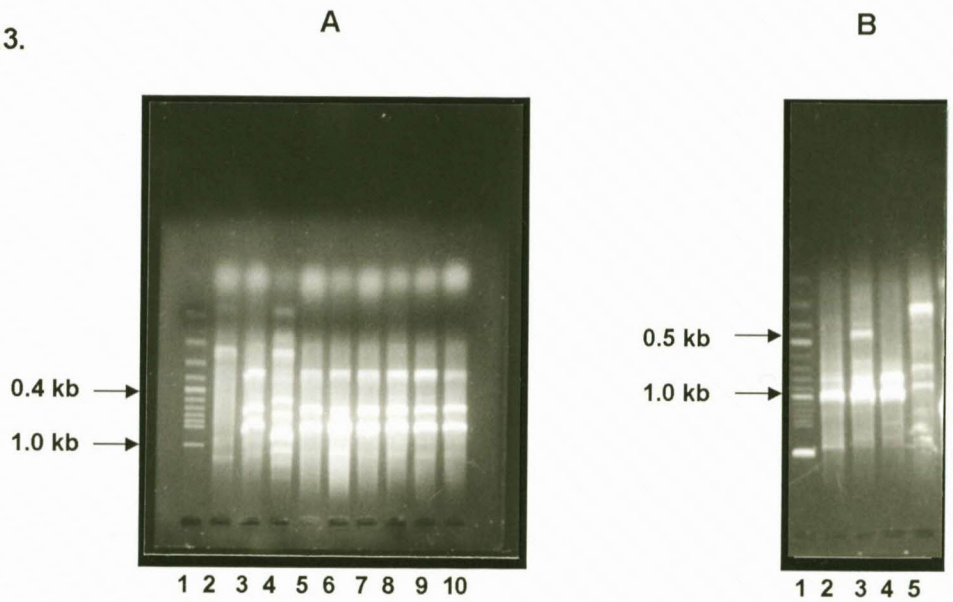


Figure 4.5.3. RAPD I profiles for *S. aureus* and *S. hominis* (RAPD I profile numbers in brackets).

(A) *S. aureus* strains: ***Lane 1:*** MWM AB, ***2:*** strain U53 (B), ***3:*** strain U71 (A), ***4:*** strain U89 (C), ***5:*** strain U94 (A), ***6:*** strain U95 (A), ***7:*** strain U174 (A), ***8:*** strain U175 (A), ***9:*** strain U176 (A), ***10:*** strain U137 (A).

(B) *S. hominis* strains: ***Lanes 1:*** MWM XIV, ***2:*** strain U26 (A), ***3:*** strain U22 (A), ***4:*** strain U63 (A), ***5:*** strain U26 (A).

The reproducibility of the RAPD assay is shown in Figure 4.6. The RAPD assay was performed for nine MRSA strains on two separate occasions with both Primer I and III. For Primer I, from independent experiments, only two strains produced the same RAPD profile. Furthermore, on the first occasion (Figure 4.6.1) four strains produced less than two bands. Primer III produced hardly any bands on the first occasion (Figure 4.6.1). Because all parameters of the method were the same on both occasions, a possible explanation for poor reproducibility could be the method used for preparing the DNA template. As previously mentioned in section 4.1, the preparation of DNA template by bacterial lysis alone can affect the reproducibility of a RAPD assay. A possible solution for this problem is the purification of template DNA before fingerprints are generated. The effect of template DNA purity on RAPD typing of MRSA is shown in Figure 4.7. Crude DNA preparations (Figure 4.7.1) were compared to purified DNA preparations (Figure 4.7.2); the number of bands observed in the crude DNA preparation were lower than those seen using a purified DNA preparation. This could have resulted due to (a) the presence of DNA polymerase inhibitors or (b) incomplete lysis in the crude DNA preparations, but also excessive shear when preparing purified template. The addition of a DNA purification step in the RAPD assay, does increase the time required for completion and cost.

Primer III showed acceptable discrimination and typeability for *S. haemolyticus* strains.

Figure 4.6.1.

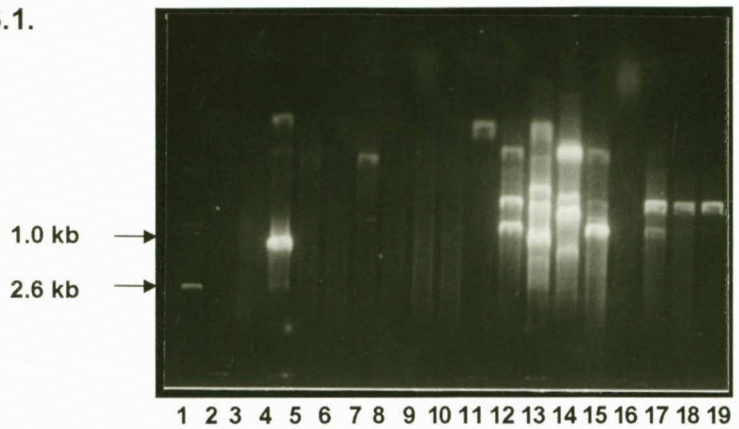


Figure 4.6.2.

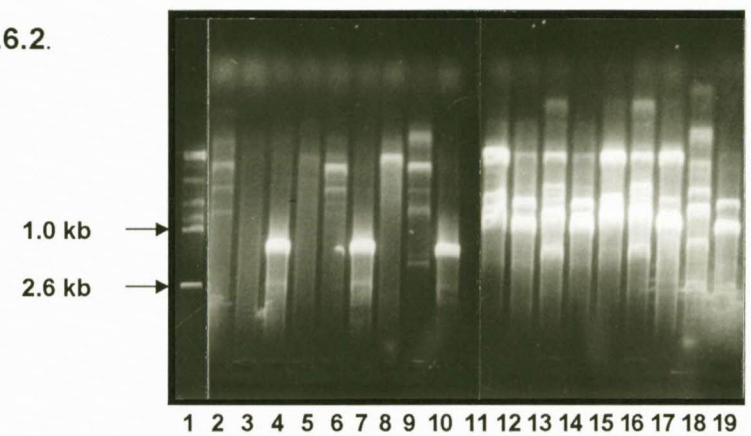


Figure 4.6. The reproducibility of RAPD primer I and III in *S. aureus* strains.

Figure 4.6(1) 1st RAPD assay for nine *S. aureus* strains: ***Lane 1:*** MWM XIV, ***Lanes 2 – 10:*** Primer III, ***Lanes 11 – 19:*** Primer I. (***Lanes 2 & 11:*** strain U110, ***3 & 12:*** strain U103, ***4 &13:*** strain U177, ***5 & 14:*** strain U166, ***6 &15:*** strain U159, ***7 & 16:*** strain U65, ***8 & 17:*** strain U35, ***9 & 18:*** strain U160, ***10 & 19:*** strain U166.)

Figure 4.6(2) 2nd RAPD assay for nine *S. aureus* strains: ***Lane 1:*** MWM XIV, ***Lanes 2 – 10:*** Primer III, ***Lanes 11 – 19:*** Primer I. (***Lanes 2 & 11:*** strain U110, ***3 & 12:*** strain U103, ***4 &13:*** strain U177, ***5 & 14:*** strain U166, ***6 &15:*** strain U159, ***7 & 16:*** strain U65, ***8 & 17:*** strain U35, ***9 & 18:*** strain U160, ***10 & 19:*** strain U166.)

Figure 4.7.1.

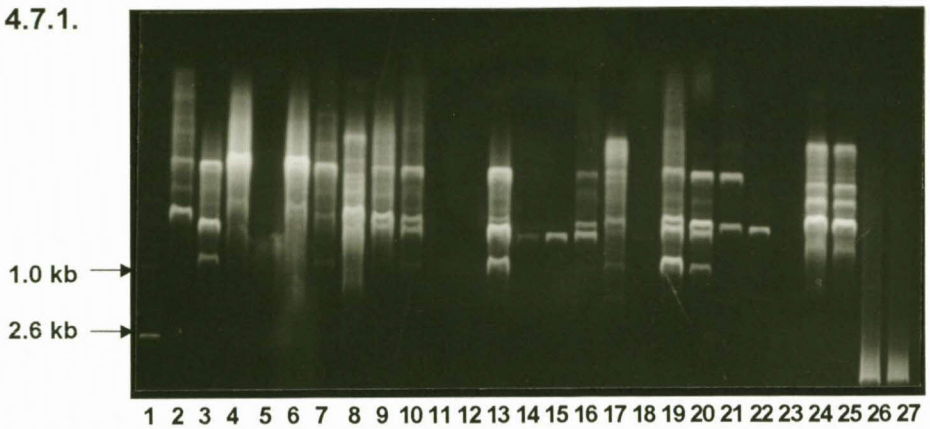


Figure 4.7.2.

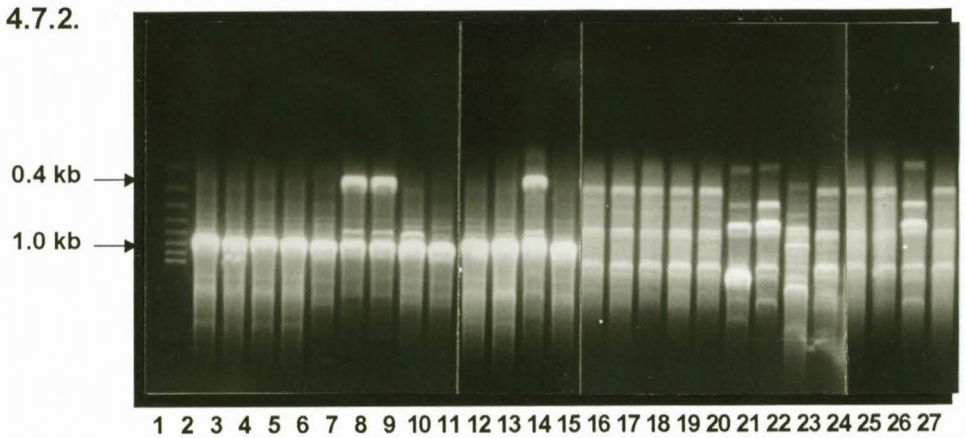


Figure 4.7.1. RAPD profiles with Primer I & III, using crude DNA preparations of *S. aureus* strains. ***Lane 1:*** MWM XIV, ***Lanes 2-14:*** Primer I. ***Lanes 15-27:*** Primer III. (***Lanes 2 & 15:*** strain U151, ***3 & 16:*** strain U110, ***4 & 17:*** strain U2, ***5 & 18:*** strain U103, ***6 & 19:*** strain U3, ***7 & 20:*** strain U65, ***8 & 21:*** strain U117, ***9 & 22:*** strain U25, ***10 & 23:*** strain U36, ***11 & 24:*** strain U177, ***12 & 25:*** strain U161, ***13 & 26:*** strain U159, ***14 & 27:*** strain U160)

Figure 4.7.2. RAPD profiles with Primer I & III, using purified DNA template of *S. aureus* strains. ***Lane 1:*** MWM AB, ***Lanes 2-14:*** Primer I, ***Lanes 15-27:*** Primer III. (***Lanes 2 & 15:*** strain U151, ***3 & 16:*** strain U110, ***4 & 17:*** strain U2, ***5 & 18:*** strain U103, ***6 & 19:*** strain U3, ***7 & 20:*** strain U65, ***8 & 21:*** strain U117, ***9 & 22:*** strain U25, ***10 & 23:*** strain U36, ***11 & 24:*** strain U177, ***12 & 25:*** strain U161, ***13 & 26:*** strain U159, ***14 & 27:*** strain U160)

Two clusters of three strains each were formed by Primer III for *S. haemolyticus* (Figure 4.8.2; Figure 4.10.1). The typeability of Primer III for *S. epidermidis* was good, however, discrimination was less effective for this primer, with five *S. epidermidis* strains producing exactly the same RAPD profile (Figure 4.8.1; Figure 4.10.2). For the typing of *S. aureus* strains the purified DNA template method was used with Primers I & III (Figures 4.9.1 and 4.9.2). Primer III produced three major clusters for *S. aureus*. Although typeability was good with the purified DNA method, discrimination between unrelated strains of *S. aureus* was poor (Figure 4.7.2).

The results obtained from RAPD assays, API identification, antibiograms and plasmid profiles are summarised in Table 4.1. Three major *S. aureus* strain clusters were produced with Primer III. No correlation was found between the *S. aureus* strain clusters produced by Primer III and the antibiograms of the respective strains. The discrimination between unrelated *S. aureus* strains was not improved by combining results obtained from Primer I & III (Table 4.1). A cluster of eight *S. epidermidis* strains gave a single RAPD profile with Primer I, but in combination with Primer III, good discrimination was found for unrelated strains. The Primer I & III combination produced only two *S. epidermidis* strains with the same RAPD profiles. In addition, based on susceptibility data, differentiation between these strains (U14, U31) was not possible. The *S. epidermidis* strains, U14 and U31, were isolated nine days apart from each other, from different wards and patients.

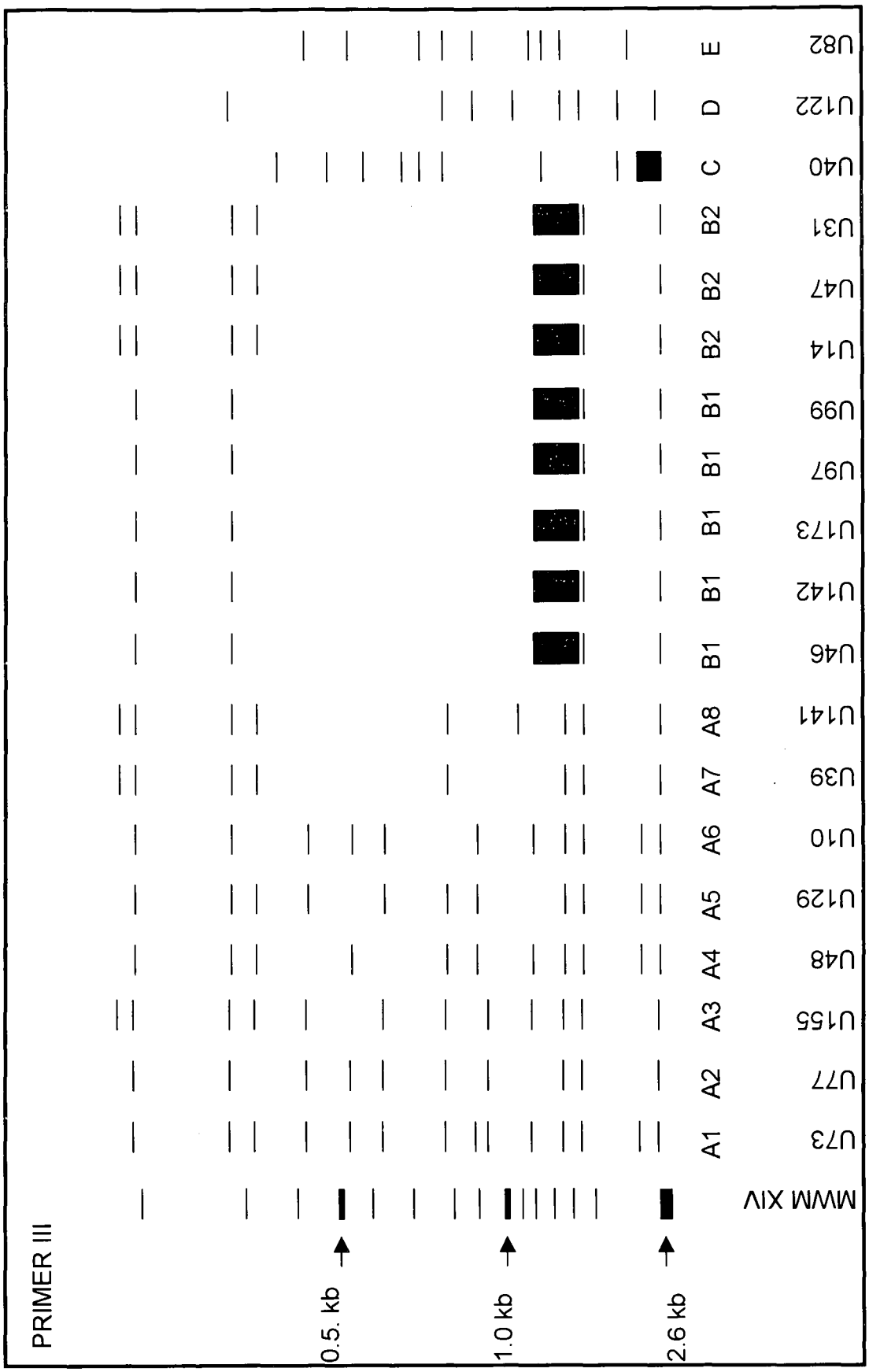


Figure 4.8.1. Schematic representation of RAPD III profiles for the differentiation of *S. epidermidis* strains.

Figure 4.8.2. Schematic representation of RAPD III profiles for the differentiation of *S. haemolyticus* strains.

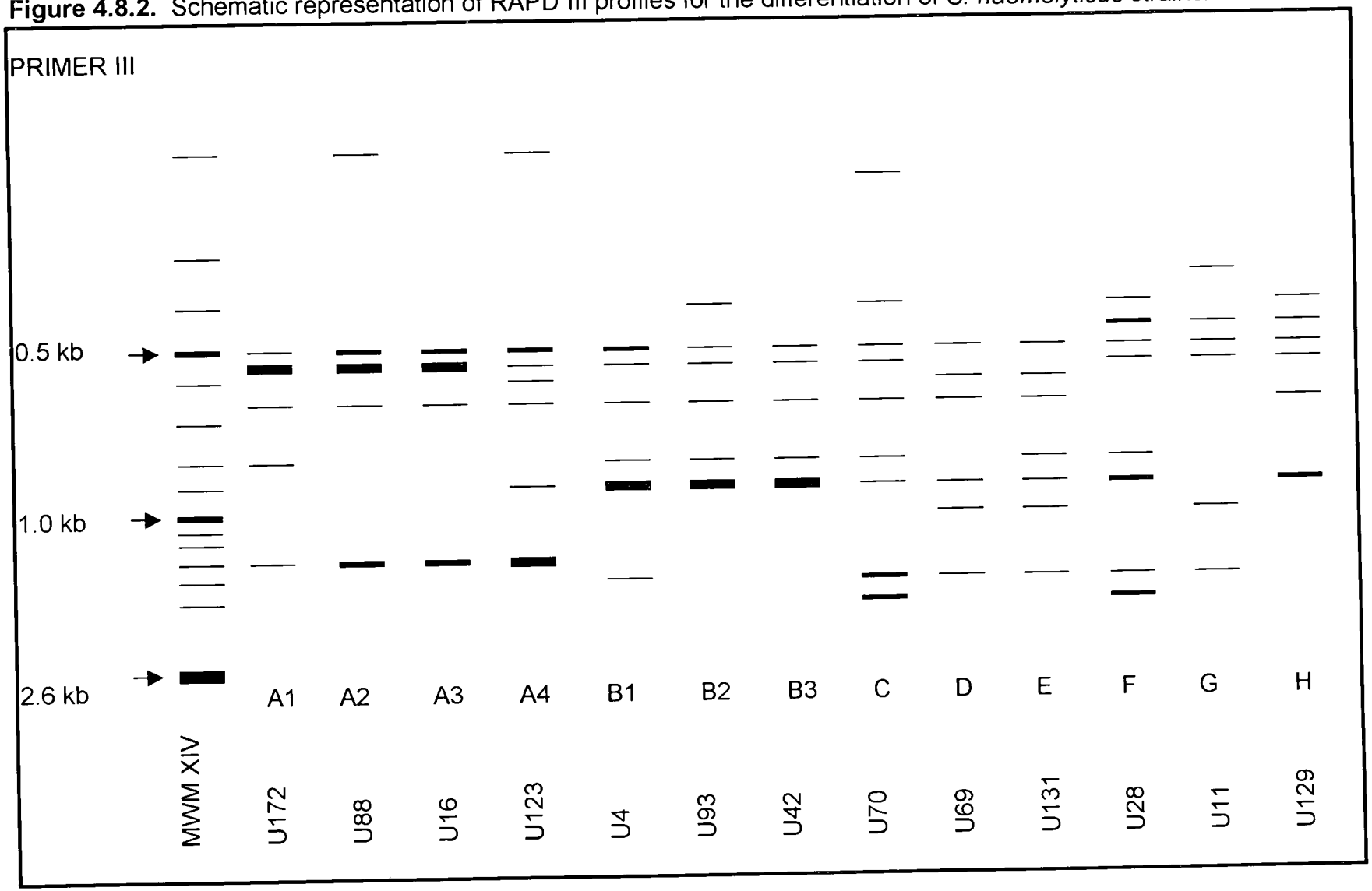
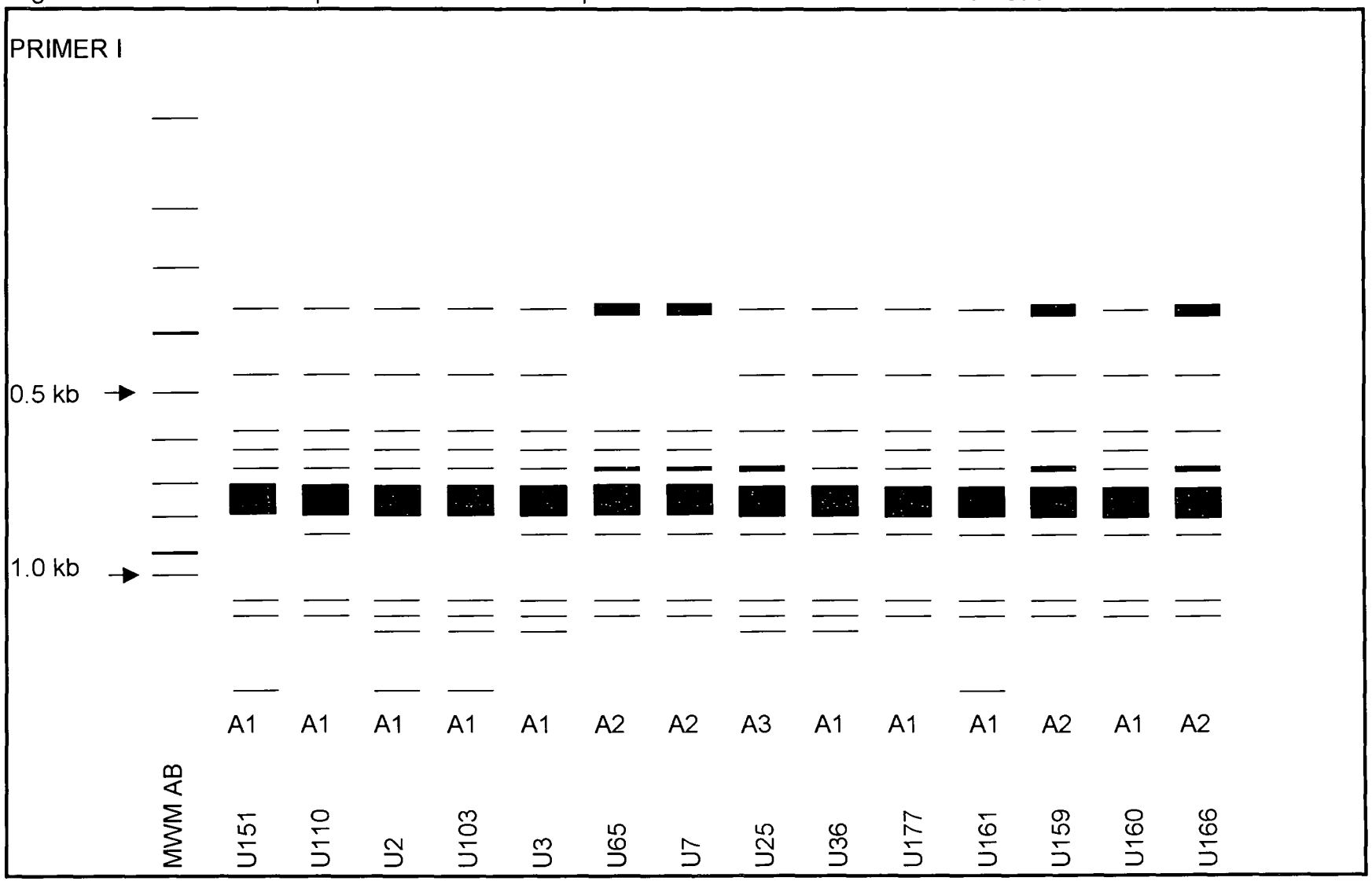


Figure 4.9.1. Schematic representation of RAPD I profiles for the differentiation of *S. aureus* strains.



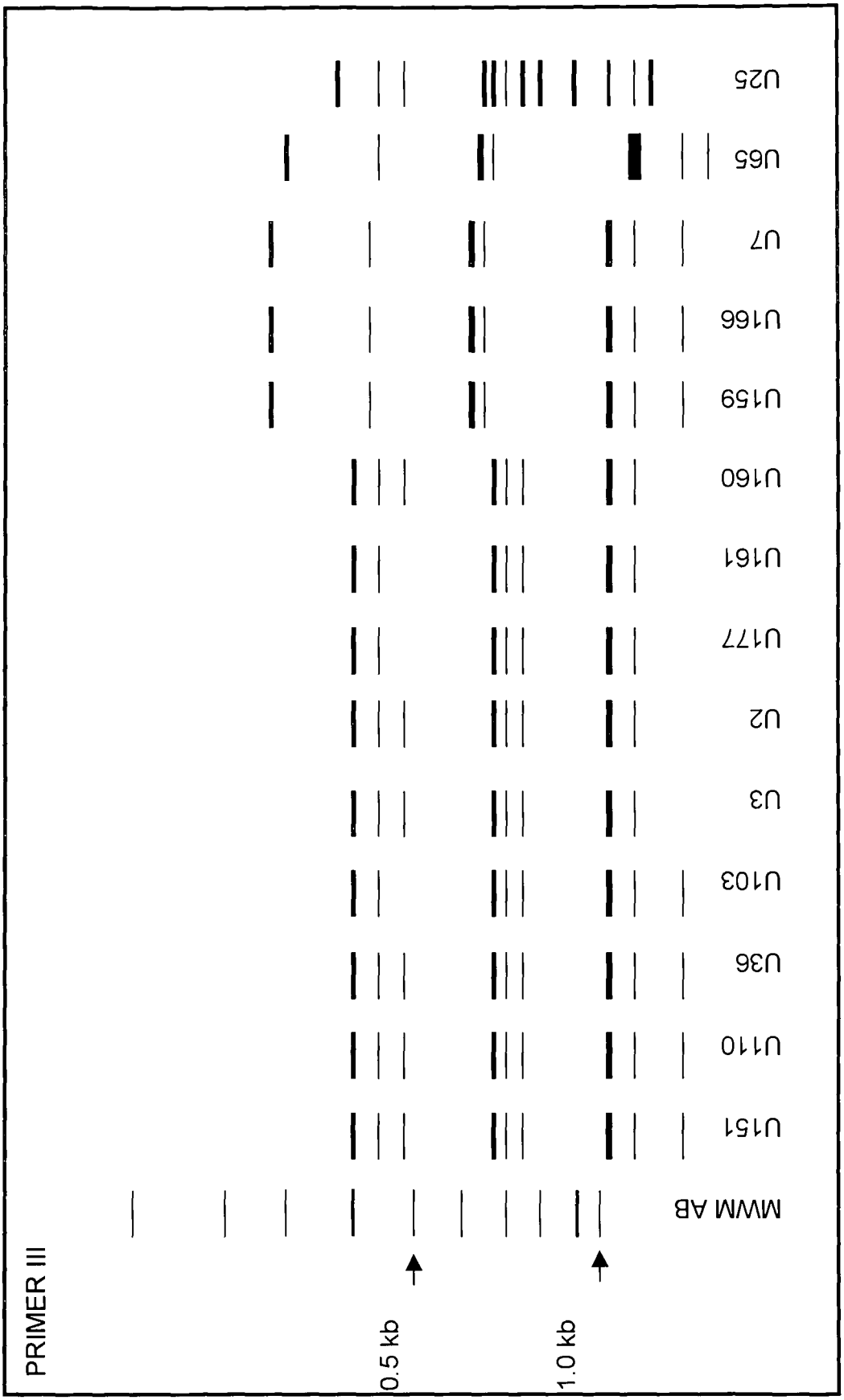


Figure 4.9.2. Schematic representation of RAPD III profiles for the differentiation of *S. aureus* strains

Figure 4.10.1.

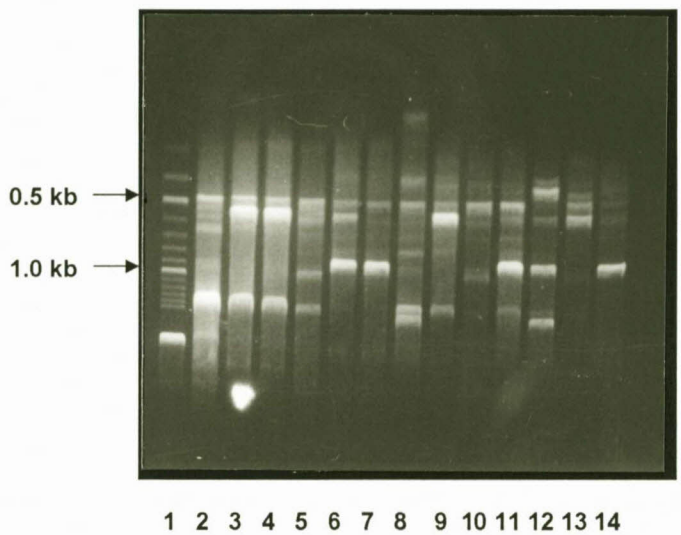


Figure 4.10.1. RAPD III profiles for *S. haemolyticus* strains (RAPD III profile numbers in brackets).

Lane 1: MWM XIV, ***2:*** strain U123 (A4), ***3:*** strain U88 (A2), ***4:*** strain U16 (A3), ***5:*** strain U131 (E), ***6:*** strain U93 (B2), ***7:*** strain U42 (B3), ***8:*** strain U70 (C), ***9:*** strain U172 (A1), ***10:*** strain U69 (D), ***11:*** strain U4 (B1), ***12:*** strain U28 (F), ***13:*** strain U11 (G), ***14:*** strain U129 (H).

Figure 4.10.2.

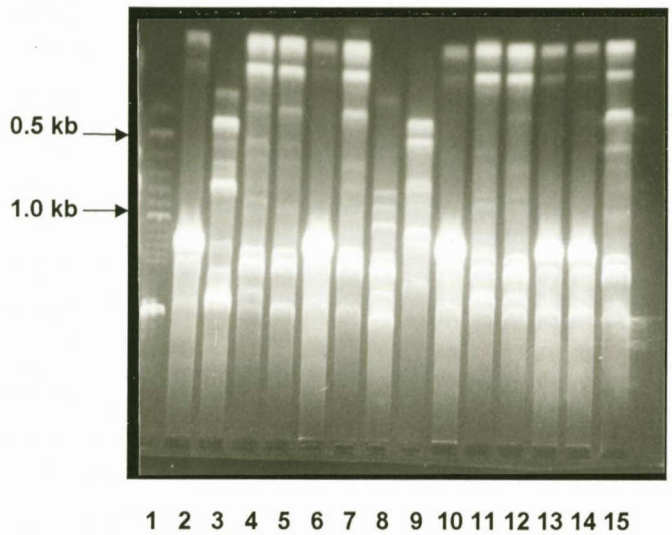


Figure 4.10.2. RAPD III profiles for *S. epidermidis* strains (RAPD profile numbers in brackets).

Lane 1: MWM XIV, ***2:*** strain U97 (B1), ***3:*** strain U40 (C), ***4:*** strain U73 (A1), ***5:*** strain U77 (A2), ***6:*** strain U99 (B1), ***7:*** strain U155 (A3), ***8:*** strain U122 (D), ***9:*** strain U82 (E), ***10:*** strain U46 (B1), ***11:*** strain U48 (A4), ***12:*** strain U129 (A5), ***13:*** strain U142 (B1), ***14:*** strain U173 (B1), ***15:*** strain U10 (A6),

Table 4.1. Comparative strain analysis: data obtained from RAPDs, API identification, susceptibility tests and plasmid analysis.

Strain	API identification	%ID	RAPD		Antibiogram										Plasmid profile
U16	*S. haemolyticus*	98.5	A1	A3	P^R	O^R	C^R	Tr^R		E^R	A^R	G^R	Cl^R			B
U93	*S. haemolyticus*	99.7	A1	B2	P^R	O^R				E^R	A^R	G^R	Cl^R	Te^R	R^R	A2
U88	*S. haemolyticus*	99.8	A10	A2	P^R	O^R	C^R	Tr^R		E^R	A^R			Te^R		D
U69	*S. haemolyticus*	99.9	A2	D	P^R	O^R	C^R			E^R	A^R	G^R	Cl^R			A1
U28	*S. haemolyticus*	98.8	A2	F	P^R	O^R				E^R	A^R	G^R	Cl^R	Te^R		A1
U11	*S. haemolyticus*	98.8	A3	G	P^R	O^R	C^R	Tr^R		E^R	A^R	G^R	Cl^R		R^R	B
U129	*S. haemolyticus*	99.9	A3	H	P^R	O^R	C^R			E^R	A^R		Cl^R	Te^R		C
U131	*S. haemolyticus*	99.9	A4	E	P^R	O^R	C^R			E^R	A^R	G^R	Cl^R	Te^R		A1
U42	*S. haemolyticus*	99.9	A5	B3	P^R	O^R	C^R		Ti^R	E^R	A^R	G^R	Cl^R	Te^R		A3
U70	*S. haemolyticus*	98.5	A6	C	P^R	O^R	C^R			E^R	A^R	G^R	Cl^R		R^R	A1
U172	*S. haemolyticus*	99.8	A7	A1	P^R	O^R	C^R	Tr^R		E^R	A^R	G^R	Cl^R			B
U4	*S. haemolyticus*	99.9	A8	B1	P^R	O^R	C^R			E^R	A^R	G^R	Cl^R			C
U123	*S. haemolyticus*	98.5	A9	A4	P^R	O^R	C^R			E^R	A^R	G^R	Cl^R	Te^R		A1

Table 4.1. *continued*

Strain	API identification	ID %	RAPD		Antibiogram	Plasmid profile
U77	*S. epidermidis*	42.5	A1	A2	P^R Te^R	ND
U99	*S. epidermidis*	77.8	A1	B1	P^R Te^R	ND
U14	*S. epidermidis*	92.1	A2	B2	P^R O^R C^R E^R A^R G^R Cl^R	ND
U31	*S. epidermidis*	97.0	A2	B2	P^R O^R C^R E^R A^R G^R Cl^R	ND
U46	*S. epidermidis*	92.5	A3	B1	P^R O^R Te^R	ND
U142	*S. epidermidis*	62.1	A4	B1	P^R O^R E^R A^R G^R Cl^R R^R	ND
U173	*S. epidermidis*	92.5	A5	B1	P^R O^R	ND
U10	*S. epidermidis*	98.2	A6	A6	P^R	ND
U47	*S. epidermidis*	98.0	A7	B2	P^R O^R Te^R	ND
U39	*S. epidermidis*	86.0	A8	A7	P^R E^R A^R Te^R	ND
U73	*S. epidermidis*	91.5	B1	A1	P^R C^R E^R A^R Cl^R R^R	ND
U48	*S. epidermidis*	98.0	B1	A4	P^R O^R C^R E^R A^R G^R Cl^R R^R	ND
U129	*S. epidermidis*	<42.0	B1	A5	P^R O^R C^R Tr^R E^R A^R G^R Cl^R Te^R R^R	ND
U155	*S. epidermidis*	69.5	B2	A3	P^R O^R E^R A^R G^R Cl^R	ND
U141	*S. epidermidis*	32.00	B3	A8	P^R O^R C^R E^R A^R G^R Cl^R Te^R R^R	ND
U122	*S. epidermidis*	72.0	C	D	E^R A^R	ND
U82	*S. epidermidis*	42.5	D	E	P^R O^R E^R A^R G^R Cl^R	ND
U97	*S. epidermidis*	65.4	E	B1	P^R E^R A^R Cl^R	ND
U40	*S. epidermidis*	93.6	F	C	P^R E^R A^R Te^R	ND

Table 4.1. *continued*

Strain	API identification	ID %	RAPD		Antibiogram										Plasmid profile
U151	*S. aureus*[a]	ND	A1	A1	P^R	O^R	C^R			A^R	G^R		Te^R	R^R	ND
U110	*S. aureus*	ND	A1	A1	P^R	O^R	C^R		E^R	A^R	G^R	Cl^R	Te^R	R^R	ND
U103	*S. aureus*	ND	A1	A1	P^R		C^R		E^R	A^R		Cl^R	Te^R	R^R	ND
U36	*S. aureus*	ND	A1	A1	P^R	O^R	C^R		E^R	A^R	G^R	Cl^R		R^R	ND
U2	*S. aureus*	ND	A1	A2	P^R	O^R			E^R	A^R				R^R	ND
U3	*S. aureus*	ND	A1	A2	P^R	O^R			E^R	A^R	G^R			R^R	ND
U177	*S. aureus*	ND	A1	A2	P^R								Te^R		ND
U161	*S. aureus*	ND	A1	A2	P^R	O^R	C^R		E^R	A^R	G^R	Cl^R	Te^R	R^R	ND
U160	*S. aureus*	ND	A1	A2	P^R	O^R					G^R				ND
U7	*S. aureus*	ND	A2	B	P^R	O^R			E^R	A^R	G^R	Cl^R			ND
U159	*S. aureus*	ND	A2	B	P^R	O^R	C^R		E^R	A^R		Cl^R	Te^R	R^R	ND
U166	*S. aureus*	ND	A2	B	P^R	O^R	C^R				G^R		Te^R	R^R	ND
U65	*S. aureus*	ND	A2	C	P^R										ND
U25	*S. aureus*	ND	A3	D	P^R	O^R				A^R	G^R				ND

Key: ND, not-determined; P^R, penicillin; O^R, oxacillin; C^R, ciprofloxacin; Tr^R, trovafloxacin; Ti^R, teicoplanin; E^R, erythromycin; A^R, azithromycin; G^R, gentamicin; Cl^R, clindamycin; Te^R, tetracycline; R^R, rifampicin.

[a] *S. aureus* identified with conventional method of identification

4.2.3. Plasmid analysis

From susceptibility data, it was expected that strains of *S. haemolyticus* would cluster together. The *S. haemolyticus* isolates all produced β-lactamases and plasmid profiles were analysed to elucidate extra chromosomal similarities. The plasmid profiles of the 13 *S. haemolyticus* strains are shown in Figure 4.11. Plasmid analysis revealed a common plasmid at 1.0 kb. Combined susceptibility and plasmid analysis data revealed epidemiological relatedness between these thirteen strains, although for Primer III, eight RAPD profiles were formed for *S. haemolyticus* (Table 4.1).

Comparisons between species-specific PCR, RAPD and Staph ID 32 API identification systems, can be made from Table 4.2. The overall correlation between API ≥ 90% identification accuracy and RAPD Primer I identification was 100%. This is in contrast to the correlation between API ≥ 90% identification accuracy and PCR species-specific identification, which was only 30.4%. The overall correlation between API ≤75% accuracy and RAPD I identification was 75%.

The results of the present study suggests that RAPD profiling offers a method of molecular identification for the most commonly isolated CNS. The RAPD technique in combination with phenotypic identification and/or susceptibility data can certainly improve the quality of epidemiological investigations.

Figure 4.11.

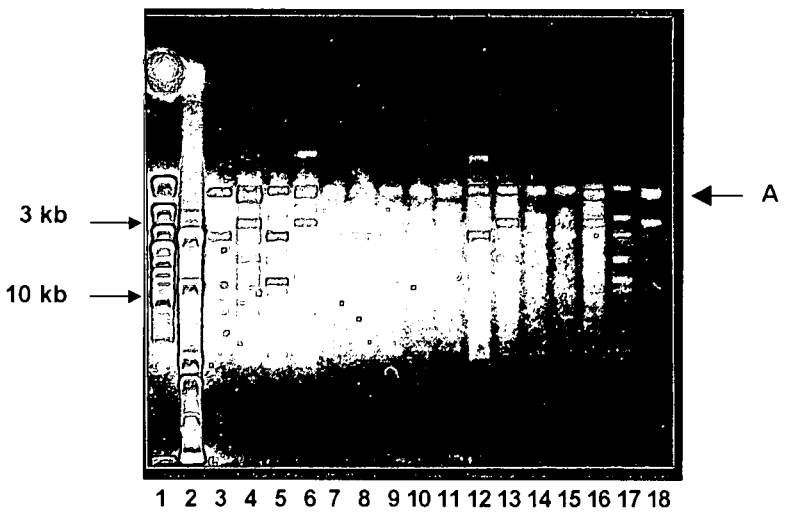


Figure 4.11. Plasmid profiles for *S. haemolyticus* strains (Profile numbers in brackets, Table 4.1). ***Lane 1:*** MWM (Promega), ***2:*** *tetM* conjugative plasmid control *Neisseria gonorrhoeae*; ***3:*** strain U87 (A1), **4:** strain U11 (B), ***5:*** strain U129 (C), ***6:*** strain U88 (D), ***7:*** strain U123 (A1), ***8:*** strain U131 (A1), ***9:*** strain U56 (A1), ***10:*** strain U70 (A1), ***11:*** strain U69 (A1), ***12:*** strain U93 (A2), ***13:*** strain U25, ***14:*** strain U42 (A3), ***15:*** strain U28 (A1), ***16:*** strain U16 (B), ***17:*** strain U4 (C), ***18:*** strain U172 (B). **A:** Common 1.0 kb plasmid found in *S. haemolyticus* isolates.

Table 4.2. Comparisons between API, SSPCR and RAPD identification systems

API			SSPCR*				RAPD			
Species	**No. strains**	**%ID**	**Sep**	**Shl**	**Shn**	**Species ID**	**1a**	**1b**	**2**	**Species ID**
S. epidermidis 1/2 (n = 19)	9	>90%	4	3	1	7 *S. epidermidis*†	5	4	0	18 *S. epidermidis*
	2	75–90%	1	-	-	3 *S. haemolyticus*†	-	2	-	
	8	<75%	5	2	-		2	5	1	
S. haemolyticus n = 13	13	>90%	3	6	-	5 *S. haemolyticus*† 2 *S. epidermidis*	13	-	-	13 *S. haemolyticus*
S. hominis n = 4	1	>90%	1	-	-	1 *S. hominis*	1	-	-	3 *S. hominis*
	3	75-90%	-	-	1	1 *S. epidermidis*	2	-	1	

* SSPCR did not provide a definitive PCR product for 14 strains.

† For 5 strains two different species were assigned to a strain.

CHAPTER 5

ANTIMICROBIAL SUSCEPTIBILITY

5.1. Introduction

Despite attempts at eradication, oxacillin-resistant *S. aureus* (ORSA) has become firmly established as an important pathogen in hospitals around the world. Although no cross-resistance is present, ORSA strains do tend to be resistant to a variety of antimicrobial agents including macrolides, aminoglycosides, tetracycline and quinolones (Peters & Becker, 1996). This is also the case for oxacillin-resistant CNS (Cercenado *et. al.*, 1996). With the recognition in the 1980s that single strain types of ORSA were causing nosocomial outbreaks in the UK, USA and Australia, increased time was spent on the management of ORSA (Cookson & Phillips, 1990).

In 1986, guidelines for the control of epidemic ORSA (EORSA) were published by the combined working party of the Hospital Infection Society and British Society for Antimicrobial Chemotherapy. This was the first time in the UK, that EORSA were accepted as being effectively out of control (Kerr *et. al.*, 1990). When the incidence of oxacillin-resistant staphylococci builds to high levels, the empiric use of vancomycin increases in the hospital. This increased vancomycin selection pressure in turn results in an increase in resistance to this agent among enterococci and CNS (McManus *et. al.*, 1989). A good example of this phenomenon was found in Japan during 1998 (Hiramatsu, 1998). As the

incidence of ORSA in Japanese hospitals rose during the 1990s to >80%, noticeably, a number of ORSA strains with reduced susceptibilities to vancomycin were concurrently reported (Hiramatsu, 1998). Furthermore, in a survey conducted in the USA, ORSA infections resulted in an increase in the length of hospitalisation. Thus, treatment/clearance of ORSA/MRSA is 68% more costly than treating a OSSA/MSSA infection with antimicrobial agents, the cost of vancomycin alone being 43% higher than oxacillin. The requirement for intravenous vancomycin administration which in turn predisposes to prolonged hospitalisation also significantly increases costs (Hershow *et. al.*, 1992).

When ORSA were first described in the 1960s *S. aureus* tended to carry resistance genes on plasmids, but in the 1990s, most of these genes became chromosomally located (Noble, 1997). The main reason for this was the presence of an insertion sequence IS257 immediately upstream from the methicillin resistance gene complex, which acted as a "hot spot" for capturing antibiotic resistance genes. The tetracycline resistance plasmid, pT181, inserts in this region and is flanked by yet another IS element with genes for mercury resistance. Upstream of this complex is the transposon Tn*554* which encodes erythromycin resistance. The transposon incorporating gentamicin resistance (Tn*4001*) first described in enterococci, has been shown to be transmissible to staphylococci (Archer *et. al.*, 1998).

Antimicrobial susceptibilities of both *S. aureus* and CNS strains isolated from clinical sources in the Bloemfontein area were therefore determined and the efficacy of alternate therapeutic agents investigated.

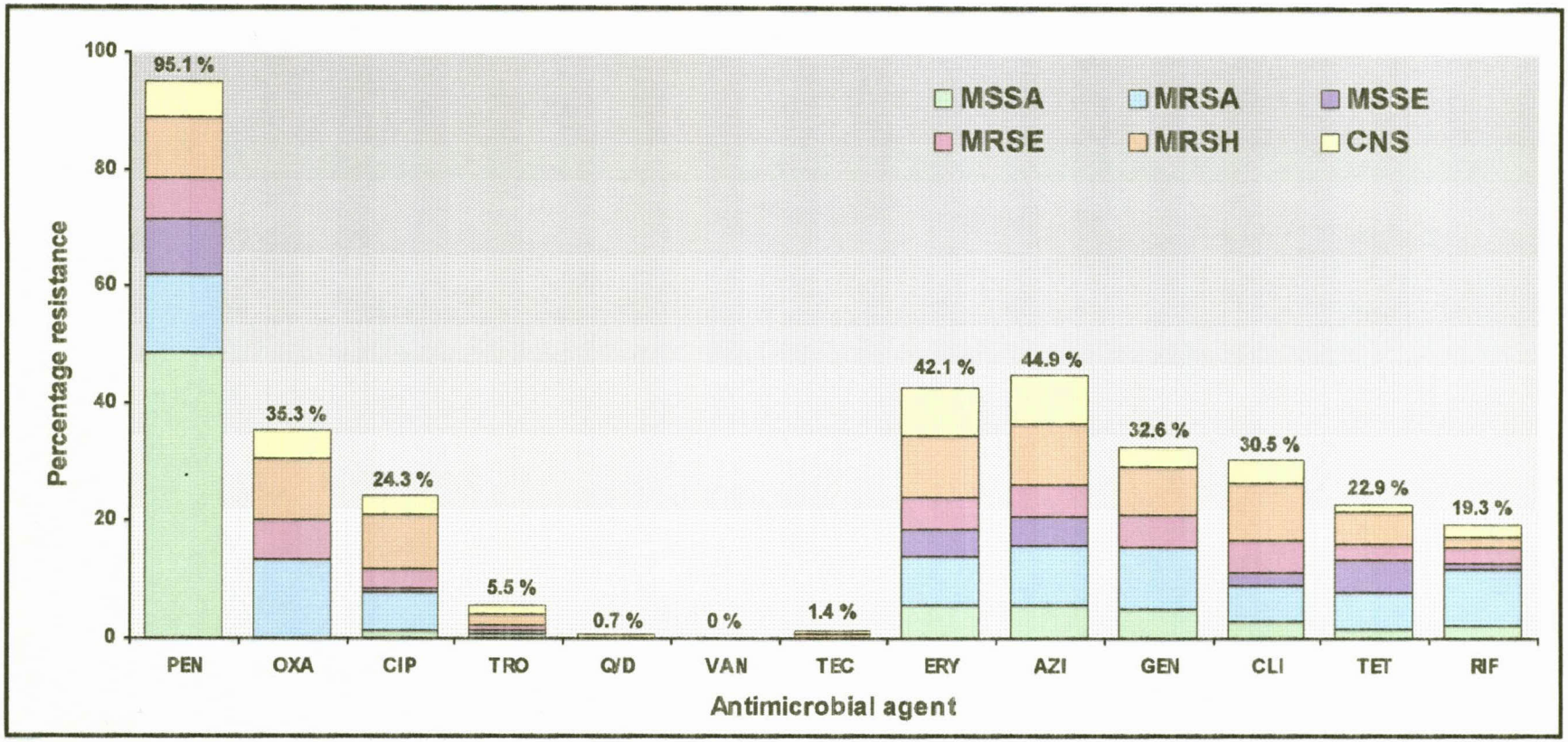
5.2. Results and duscussion

5.2.1. ß-Lactam agents

The inefficacy of penicillin against clinical staphylococcal strains isolated in the Universitas hospital is shown in Figure 5.1. Of a total of 144 clinical staphylococcal isolates investigated, 137 strains were found to be resistant to penicillin. Of these 137 penicillin-resistant staphylococci, 16 strains (11,7%) did not produce penicillinase(s). These findings were similar to those found in Europe and the USA (Gordon , 1993; Jones, 1996). Despite the overwhelming presence of penicillin resistance in clinical staphylococcal strains, worldwide (>95%) and locally (>95%), penicillin is still included in the regimen for treatment of staphylococcal infections (Jones,1996). The main reason for this being the discouragement of the empirical treatment of penicillin-susceptible/ non-ß-lactamase producing staphylococci with penicillinase-resistant penicillins such as methicillin/oxacillin.

Overall, oxacillin resistance demonstrated in the staphylococci isolated between April to July 1998 in the Universitas hospital was 34.3% (Figure 5.1). Oxacillin resistance was more prevalent amongst CNS (79.3%) than in *S. aureus* strains (20.7%).

Figure 5.1. Percentage resistance of clinical staphylococcal strains to 13 clinically available antibiotics



PEN, penicillin; **OXA**, oxacillin; **CIP**, ciprofloxacin; **TRO**, trovafloxacin; **Q/D**, quinopristin-dalfopristin; **VAN**, vancomycin; **TEC**, teicoplanin; **ERY**, erythromycin; **AZI**, azithromycin; **GEN**, gentamicin; **CLI**, clindamycin; **TET**, tetracycline; **RIF**, rifampicin; **MSSA**, methicillin-susceptible *S. aureus*, **MRSA**, methicillin-resistant *S. aureus*; **MSSE**, methicillin-susceptible *S. epidermidis*; **MRSE**, methicillin-resisitant *S. epidermidis*; **MRSH**, methicillin-resistant *S. haemolyticus*; **CNS**, coagulase-negative staphylococci.

Noticeably, not a single strain of *S. haemolyticus* isolated showed susceptibility to either penicillin or oxacillin. Without exception, the 15 *S. haemolyticus* strains isolated, produced ß-lactamase(s). Furthermore, the level of resistance was found to be higher than that exhibited by any of the other CNS isolated. This is reflected in the penicillin and oxacillin MIC_{50} values for *S. haemolyticus* which were 128 µg/ml and >128 µg/ml, respectively (Table 5.1). The high-level of resistance against penicillin might be explained by the overproduction of chromosomal and/or plasmid mediated ß-lactamases. As previously mentioned in section 1.5.2, overproduction of ß-lactamases in *S. aureus* can contribute significantly to the level of methicillin resistance that can be achieved.

5.2.3. Multi-drug resistance

Figure 5.2. shows the extent to which multi-drug resistance was found in oxacillin-resistant staphylococci. On comparing multi-drug resistance in *S. aureus* (26.3%) and CNS (62.5%), it is evident that CNS may justifiably be considered reservoirs of antibiotic resistance genes with the capability of disseminating these factors among other hospital pathogens. Twenty-five oxacillin-resistant clinical staphylococcal strains (49%) were resistant to ciprofloxacin, erythromycin, gentamicin and clindamycin. This incidence is lower than that demonstrated by strains isolated in Spain in 1994, 70% (Cercenado *et. al.*, 1996)

Table 5.1. Comparative activities of thirteen antibiotics against staphylococci

Antibiotic	Susceptibility breakpoint [a]	% susceptible	MIC (µg/ml) 50% [b]	90% [b]	Range
***Staphylococcus aureus*: oxacillin-susceptible (n = 73)**					
Penicillin	≤0.125	4.1	8	32	≤0.06-128
Oxacillin	≤2	100	0.5	2	0.125-2
Ciprofloxacin	≤1	97.3	0.25	1	0.125-64
Trovafloxacin	≤2	98.6	≤0.06	≤0.06	0.125-8
Q/D [c]	≤1	98.6	1	1	0.5-2
Vancomycin	≤4	100	1	2	0.5-2
Teicoplanin	≤8	100	1	2	0.5-4
Erythromycin	≤0.5	89.1	0.25	1	0.125->128
Azithromycin	≤2	90.4	1	2	0.25->128
Gentamicin	≤4	91.8	0.25	2	≤0.06-32
Clindamycin	≤0.5	95.9	0.125	0.25	≤0.06->128
Tetracycline	≤4	97.3	0.25	4	0.125-32
Rifampicin	≤1	97.3	0.25	0.5	<0.06->128
***Staphylococcus aureus*: oxacillin-resistant (n = 19)**					
Penicillin	≤0.125	0	128	>128	2-128
Oxacillin	≤2	0	64	128	4-128
Ciprofloxacin	≤1	47.4	2	32	0.125-64
Trovafloxacin	≤2	94.8	0.125	1	≤0.06-8
Q/D	≤1	94.8	1	1	0.5-2
Vancomycin	≤4	100	1	2	0.5-2
Teicoplanin	≤8	100	2	4	0.5-4
Erythromycin	≤0.5	15.8	>128	>128	0.25->128
Azithromycin	≤2	15.8	>128	>128	0.25->128
Gentamicin	≤4	10.5	16	32	0.25-64
Clindamycin	≤0.5	52.63	0.25	>128	≤0.06->128
Tetracycline	≤4	15.8	8	32	2-64
Rifampicin	≤1	21.1	>128	>128	≤0.06->128

continued

Table 5.1. *continued*

Antibiotic	Susceptibility breakpoint [a]	% susceptible	MIC (µg/ml) 50% [b]	90% [b]	Range
***Staphylococcus epidermidis*: oxacillin-susceptible (n = 15)**					
Penicillin	≤0.125	6.7	2	8	≤0.06-16
Oxacillin	≤2	100	1	2	0.125-2
Ciprofloxacin	≤1	86.7	0.25	2	≤0.06-64
Trovafloxacin	≤2	93.3	≤0.06	0.5	≤0.06-4
Q/D	≤1	93.3	0.5	1	0.25-2
Vancomycin	≤4	100	1	2	0.5-2
Teicoplanin	≤8	100	2	8	0.5-8
Erythromycin	≤0.5	53.3	0.25	>128	0.125-128
Azithromycin	≤2	53.3	0.5	>128	0.5->128
Gentamicin	≤4	100	≤0.06	2	≤0.06-2
Clindamycin	≤0.5	80	0.125	>128	≤0.06->128
Tetracycline	≤4	40	8	64	0.25-64
Rifampicin	≤1	93.3	0.25	0.5	≤0.06->128
***Staphylococcus epidermidis*: oxacillin-resistant (n = 10)**					
Penicillin	≤0.125	0	8	16	4-64
Oxacillin	≤2	0	8	16	4-64
Ciprofloxacin	≤1	50	0.5	64	0.125-128
Trovafloxacin	≤2	60	≤0.06	4	≤0.06-8
Q/D	≤1	90	0.5	1	0.25-2
Vancomycin	≤4	100	2	2	1-2
Teicoplanin	≤8	100	4	8	2-8
Erythromycin	≤0.5	20	>128	>128	0.25->128
Azithromycin	≤2	20	>128	>128	0.5->128
Gentamicin	≤4	10	32	64	4-128
Clindamycin	≤0.5	20	>128	>128	0.125->128
Tetracycline	≤4	40	8	64	0.25-128
Rifampicin	≤1	50	0.25	>128	≤0.06->128

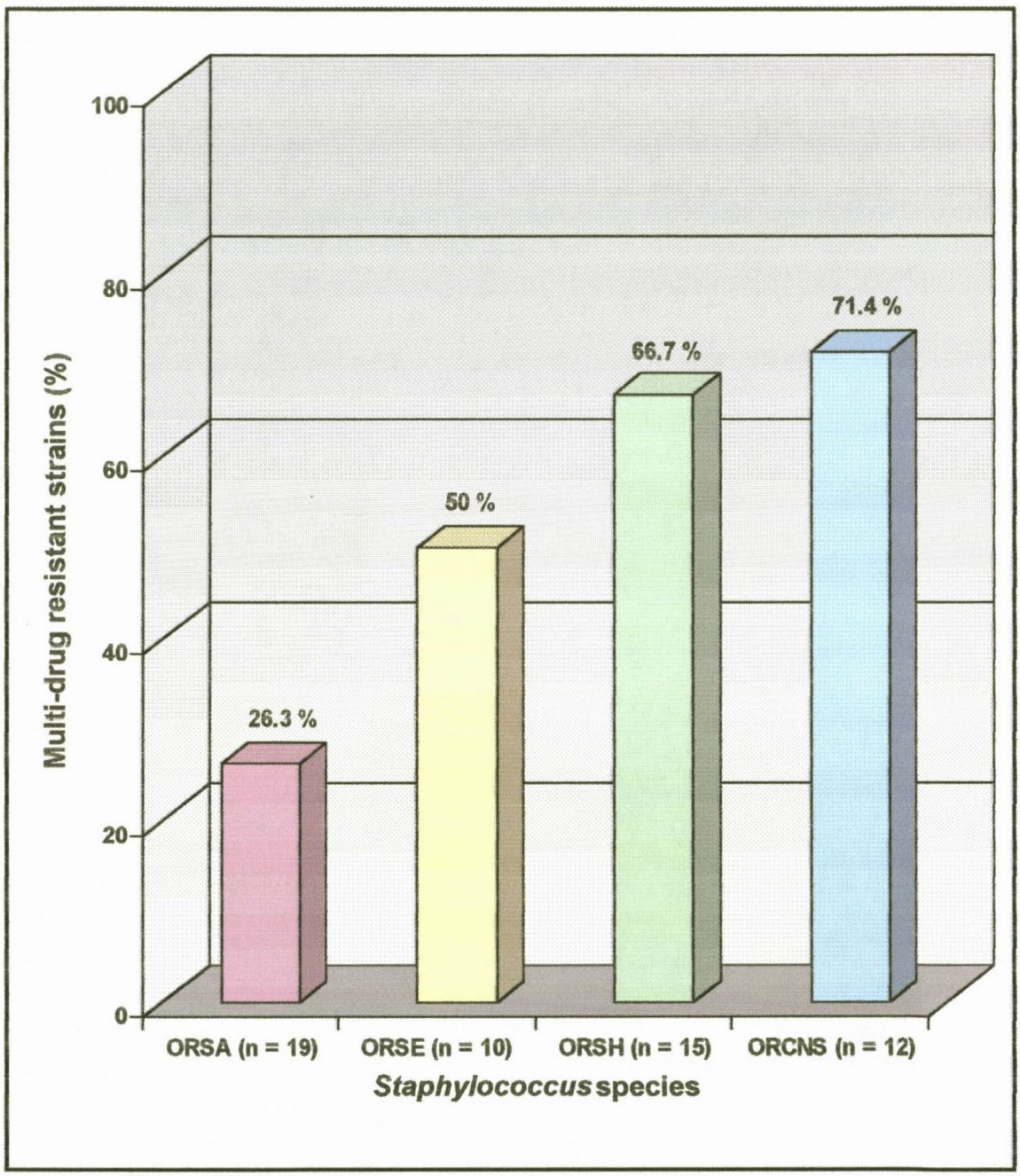
continued

Table 5.1. *continued*

Antibiotic	Susceptibility breakpoint [a]	% susceptible	MIC (µg/ml) 50% [b]	90% [b]	Range
***Staphylococcus haemolyticus*: oxacillin-resistant (n = 15)**					
Penicillin	≤0.125	0	128	128	64->128
Oxacillin	≤2	0	>128	>128	>128
Ciprofloxacin	≤1	6.7	32	128	0.25-128
Trovafloxacin	≤2	73.3	2	16	≤0.06-16
Q/D	≤1	71.4	1	2	0.25-2
Vancomycin	≤4	100	2	2	1-2
Teicoplanin	≤8	71.4	8	16	4-32
Erythromycin	≤0.5	0	64	64	32-64
Azithromycin	≤2	0	128	128	64->128
Gentamicin	≤4	6.7	16	32	4-32
Clindamycin	≤0.5	6.7	>128	>128	0.125->128
Tetracycline	≤4	28.6	128	>128	1->128
Rifampicin	≤1	85.7	0.5	>128	≤0.06->128
***Staphylococcus* spp. coagulase-negative: (n = 12)**					
Penicillin	≤0.125	25	1	128	≤0.06-128
Oxacillin	≤2	41.7	4	>128	≤0.06->128
Ciprofloxacin	≤1	58.3	0.25	128	≤0.06-128
Trovafloxacin	≤2	83.3	≤0.06	8	≤0.06-16
Q/D	≤1	66.7	1	2	0.5-4
Vancomycin	≤4	100	1	2	1-2
Teicoplanin	≤8	83.3	2	16	1-32
Erythromycin	≤0.5	0	>128	>128	16->128
Azithromycin	≤2	0	>128	>128	32->128
Gentamicin	≤4	50	1	32	≤0.06-64
Clindamycin	≤0.5	41.7	1	>128	0.125->128
Tetracycline	≤4	66.7	4	16	0.125->128
Rifampicin	≤1	66.7	0.25	64	≤0.06->128

[a] Susceptibility breakpoints defined by NCCLS (1998); [b] 50% and 90%, MIC_{50} and MIC_{90}, respectively; [c] Q/D, quinopristin-dalfopristin.

Figure 5.2. Multi-drug resistance in oxacillin-resistant staphylococcal (ORS) strains. Additional resistance to: quinolones, macrolides, aminoglycosides and clindamycin.



ORSA, oxacillin-resistant *S. aureus*; **ORSE**, oxacillin-resistant *S. epidermidis*; **ORSH**, oxacillin-resistant *S. haemolyticus*; **ORCNS**, oxacillin-resistant coagulase-negative staphylococci

5.2.4. Quinolones

Trovafloxacin showed good *in vitro* activity against OSSA and oxacillin-susceptible coagulase-negative staphylococci OSCNS with MIC_{90} values two-fold lower than the NCCLS-approved susceptibility breakpoint concentration (Table 5.1). Trovafloxacin susceptibility (MICs ≤ 2 μg/ml) for OSSA (98.6%) and ORSA (94.8%) were comparable. In contrast, to ciprofloxacin OSSA strains were considerably more susceptible (MICs ≤ 1 μg/ml) (97.3%) than ORSA (47.4%). However, a decrease in activity was observed for trovafloxacin in oxacillin-resistant coagulase-negative staphylococci (ORCNS) with MIC_{90} values of 4 μg/ml (*S. epidermidis*) and 16 μg/ml *(S. haemolyticus)*.

Ciprofloxacin (MIC_{90} range 1 - 128 μg/ml) was generally two- to five-fold less active than trovafloxacin (MIC_{90} range ≤0.06 - 16 μg/ml) for all staphylococci strains tested (Table 5.2.). The MIC distribution patterns for trovafloxcacin and ciprofloxacin in *S. aureus* and CNS are shown in Figure 5.3. The bi-modal distribution pattern for ciprofloxacin and trovafloxacin is indicative of a mutational event at the chromosomal level within *S. aureus* and CNS strains affecting susceptibility of both quinolones.

Both trovafloxacin and moxifloxacin showed excellent activity against OSSA, MIC_{50} ≤0.06 μg/ml/ MIC_{90} 0.25 μg/ml and MIC_{50} ≤0.06 μg/ml/ MIC_{90} 1.0 μg/ml respectively.

Table 5.2. Comparative activities of clinically available and developmental antibiotics against staphylococci

Antibiotic	Susceptibility breakpoints[a]	% susceptible	MIC (μg/ml)		
			50% [b]	90% [b]	Range
***Staphylococcus aureus*: oxacillin-susceptible (n = 11)**					
Linezolid	— [c]	—	2	2	1-2
Vancomycin	≤4	100	1	2	1-2
Teicoplanin	≤8	100	1	4	0.5-4
LY 333328	—	—	4	8	4-8
Tetracycline	≤4	90.9	0.5	2	0.5-16
CL 331,002	—	—	0.5	1	0.5-2
CL 329,998	—	—	1	2	0.5-2
Ciprofloxacin	≤1	81.8	0.25	32	0.125-64
Trovafloxacin	≤2	90.9	≤0.06	0.25	≤0.06-8
Moxifloxacin	—	—	≤0.06	1	≤0.06-2
***Staphylococcus aureus*: oxacillin-resistant (n = 19)**					
Linezolid	—	—	2	2	1-2
Vancomycin	≤4	100	1	2	1-2
Teicoplanin	≤8	100	2	4	1-8
LY 333328	—	—	4	8	4-8
Tetracycline	≤4	31.6	16	16	0.5-16
CL 331,002	—	—	0.5	2	0.5-2
CL 329,998	—	—	1	2	0.5-2
Ciprofloxacin	≤1	52.6	0.5	32	0.25-64
Trovafloxacin	≤2	94.7	0.125	1	≤0.06-8
Moxifloxacin	—	—	0.125	2	≤0.06-2

continued

Table 5.2. *continued*

Antibiotic	Susceptibility breakpoints[a]	% susceptible	MIC (µg/ml) 50%[b]	90%[b]	Range
***Staphylococcus epidermidis*: oxacillin-resistant (n = 10)**					
Linezolid	—[c]	—	2	2	2
Vancomycin	≤4	100	2	2	2
Teicoplanin	≤8	90	4	8	2-16
LY 333328	—	—	16	16	8-16
Tetracycline	≤4	40	8	16	0.5-16
CL 331,002	—	—	2	16	0.25-16
CL 329,998	—	—	1	8	0.5-8
Ciprofloxacin	≤1	40	32	64	0.25-128
Trovafloxacin	≤2	50	2	4	≤0.06-16
Moxifloxacin	—	—	1	2	≤0.06-4
***Staphylococcus haemolyticus*: oxacillin-resistant (n = 15)**					
Linezolid	—	—	1	2	1-2
Vancomycin	≤4	100	1	2	1-2
Teicoplanin	≤8	73.3	8	16	8-16
LY 333328	—	—	16	16	8-16
Tetracycline	≤4	26.7	8	16	1-16
CL 331,002	—	—	16	16	1-16
CL 329,998	—	—	4	8	1-8
Ciprofloxacin	≤1	6.7	16	128	0.125-128
Trovafloxacin	≤2	80	2	16	≤0.06-16
Moxifloxacin	—	—	1	4	≤0.06-4

[a] Susceptibility breakpoints defined by NCCLS (1998); [b] 50% and 90%, MIC_{50} and MIC_{90}, respectively; —[c] Susceptibility breakpoints not available.

Figure 5.3. MIC distribution patterns for clinically available antibiotics

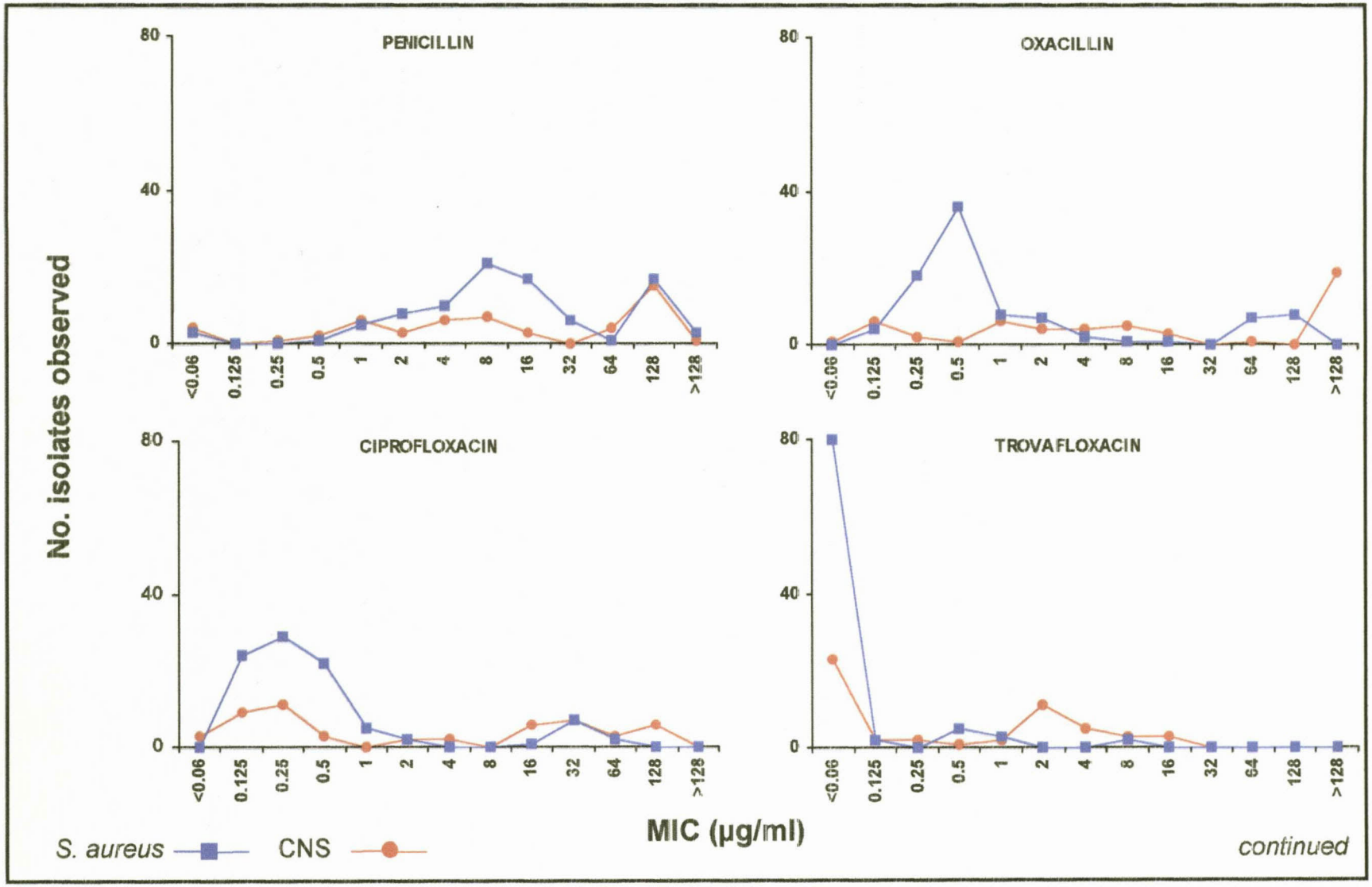


Figure 5.3. *continued*

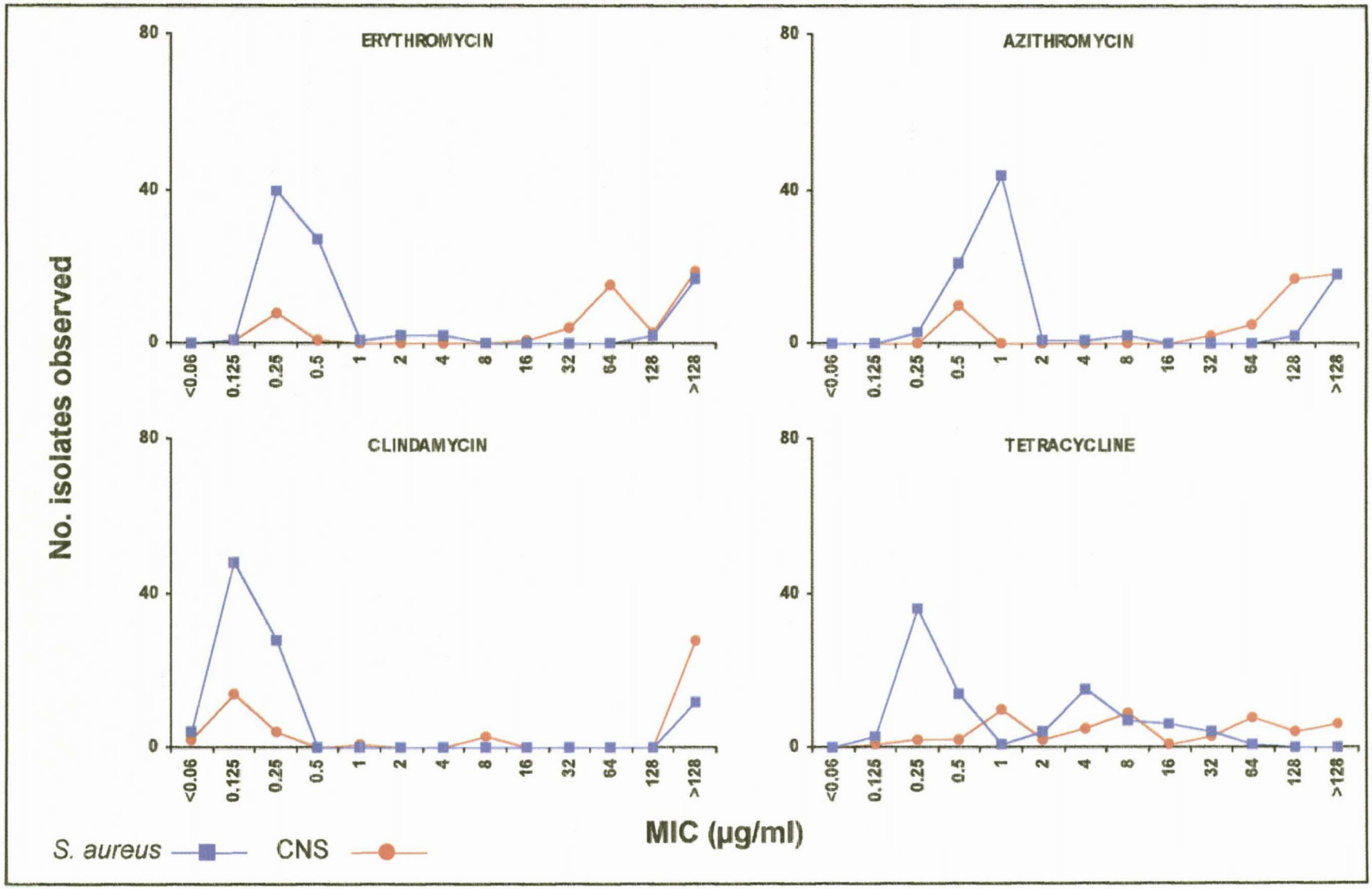
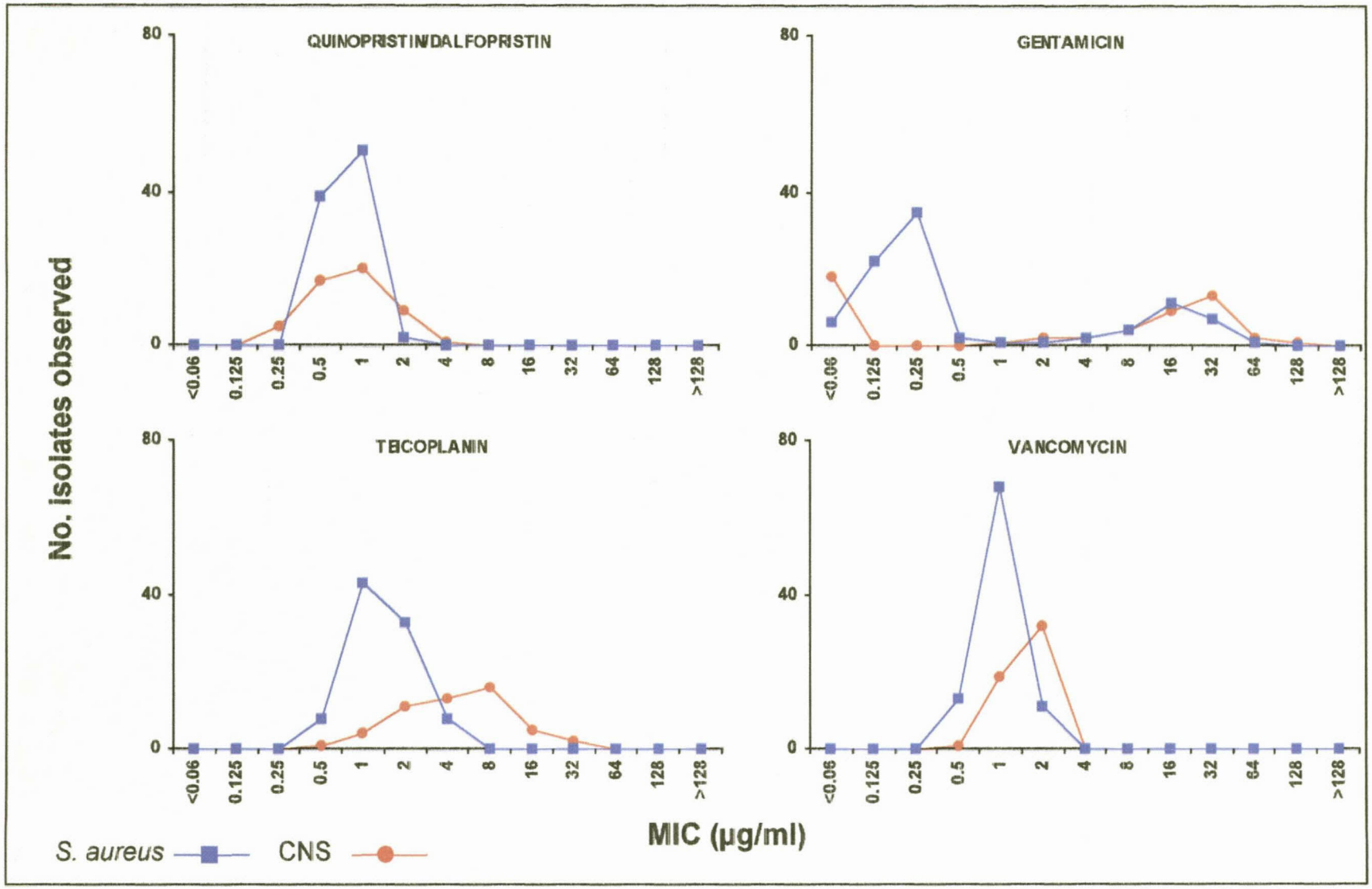


Figure 5.3. *continued*



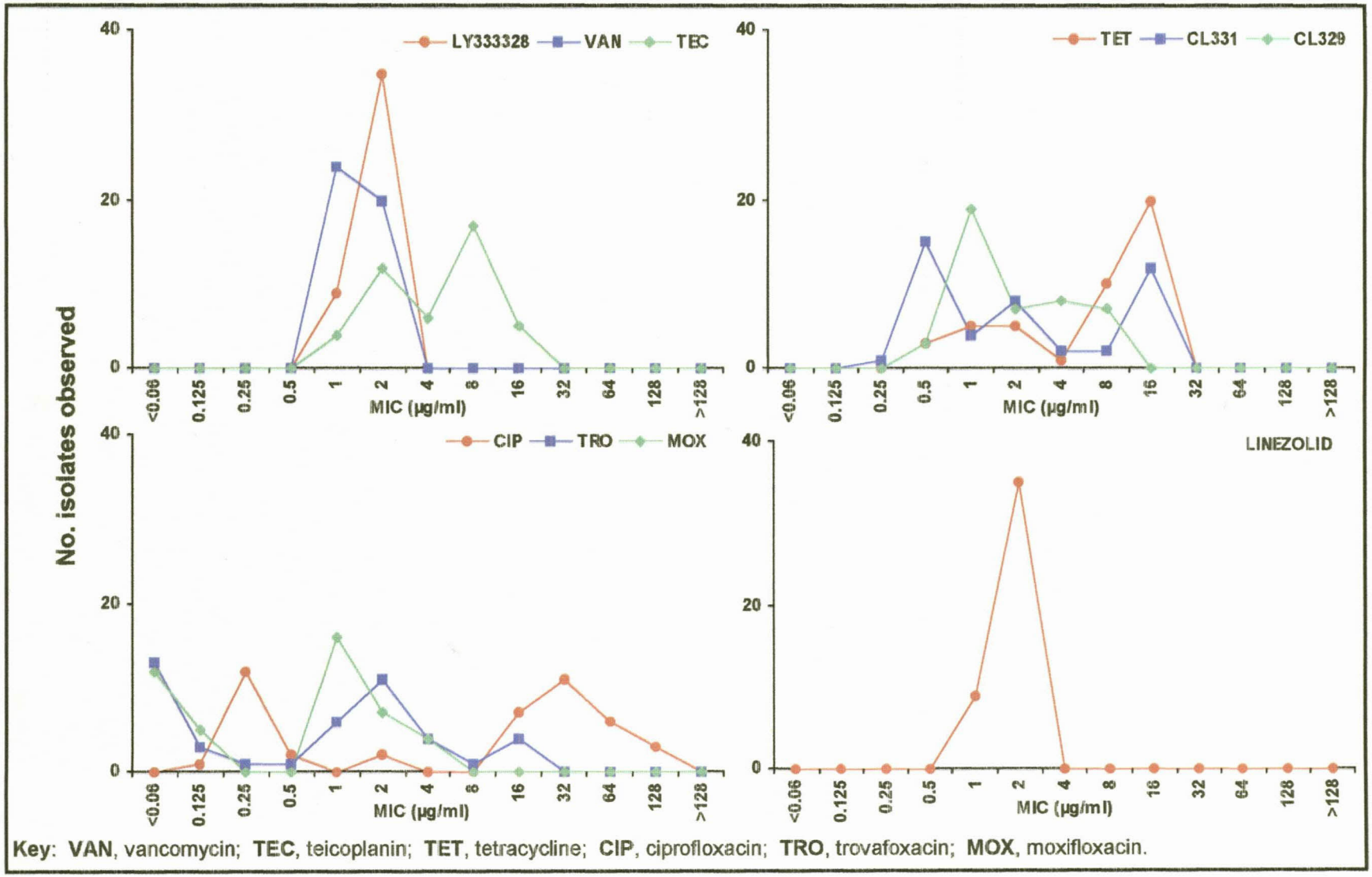
As a single ORSA strain with a MIC_{90} value of 1 μg/ml and one of only 11 OSSA strains (MIC 2μg/ml) had higher MIC_{90} values for moxifloxacin than trovafloxacin a statistical/experimental error may well have been responsible. Trovafloxacin and moxifloxacin exhibited good activity against ORSA with 90% of strains being inhibited by concentrations of 1 μg/ml and 2 μg/ml, respectively (Table 5.2). This is in contrast to ciprofloxacin (MIC_{90} 32 μg/ml) which is at least four-fold less active than the other quinolones tested against ORSA.

Although moxifloxacin showed improved activity against ORCNS in comparison to the other quinolones, concentrations that inhibited 50% of ORSA strains (MIC 0.125 μg/ml) were at least three-fold lower than concentrations necessary to inhibit 50% of ORCNS (MIC 1 μg/ml) (Table 5.2). Comparative bimodal MIC distribution patterns for moxifloxacin, ciprofloxacin and trovafloxacin in oxacillin-resistant staphylococci are shown in Figure 5.4. This indicates again that moxifloxacin, like ciprofloxacin and trovafloxacin, is influenced by a single mutation at the chromosomal level. These findings are comparable to those of Woodcock *et. al.* (1997) and Jones (1996), that also demonstrated bi-modal MIC distribution patterns for moxifloxacin, trovafloxacin and ciprofloxacin.

5.2.5. Glycopeptides

Between 20/01/98 and 16/02/98 sixty-nine presumptive staphylococcal strains from Pelinomi Hospital were sequentially screened for vancomycin resistance.

Figure 5.4. MIC distribution patterns for clinically available and developmental antibiotics



Key: **VAN**, vancomycin; **TEC**, teicoplanin; **TET**, tetracycline; **CIP**, ciprofloxacin; **TRO**, trovafoxacin; **MOX**, moxifloxacin.

Of these strains 16% grew on plates containing 40 μg/ml vancomycin, however, subsequent MICs were ≤ 4 μg/ml. Between 16/02/98 and 06/04/98 an additional seventy-eight presumptive staphylococcal strains were screened for vancomycin resistance. Of these strains 73% grew on plates containing 4 μg/ml vancomycin. The MIC values for strains isolated from Pelinomi hospital are shown in Table 5.3. However, again the MIC ranges for vancomycin (0.25 – 2 μg/ml) and teicoplanin (0.5 –8μg/ml) did not correlate with growth on screening plates containing 4 μg/ml vancomycin. This is most likely due to the method of vancomycin resistance detection used, as inoculum effect complicates screening of cell wall active agents. Since July 1998 a variety of methods have been employed for the detection of ORSA with decreased susceptibilities to vancomycin. E-tests were used for environmental strains allowing for fast and reliable screening. Currently, population analysis profiles are the most reliable method for the detection of VISA or hetero-VRSA.

All the staphylococcal isolates investigated were susceptible to vancomycin (MICs ≤ 4 μg/ml). Teicoplanin showed good activity against ORSA and oxacillin resistant *S. epidermidis* (ORSE) isolates with >90% of strains being susceptible. Decreased activity was observed for teicoplanin against oxacillin-resistant *S. haemolyticus* (ORSH) isolates, with only 71.4% of strains being susceptible (Table 5.2.).

Table 5.3. Comparative activities of thirteen antibiotics against staphylococci selected on 4 µg/ml vancomycin

Antibiotic	Susceptibility breakpoint [a]	% susceptible	MIC (µg/ml) 50% [b]	90% [b]	Range
***Staphylococcus aureus*: oxacillin-susceptible (n = 40)**					
Penicillin	≤0.125	5	8	64	≤0.06->128
Oxacillin	≤2	80	0.5	64	0.125->128
Ciprofloxacin	≤1	95	0.5	0.5	0.125-32
Trovafloxacin	≤2	100	≤0.06	≤0.06	≤0.06-8
Q/D [c]	≤1	100	1	1	0.5-1
Vancomycin	≤4	100	1	1	0.5-2
Teicoplanin	≤8	100	1	2	0.25-4
Erythromycin	≤0.5	75	0.5	>128	0.25->128
Azithromycin	≤2	75	1	>128	0.25->128
Gentamicin	≤4	82.5	0.25	16	≤0.06-32
Clindamycin	≤0.5	95	0.125	0.25	0.125->128
Tetracycline	≤4	75	0.5	16	0.125-32
Rifampicin	≤1	92.5	0.125	0.125	<0.06->128
***Staphylococcus aureus*: oxacillin-resistant (n = 18)**					
Penicillin	≤0.125	5.2	4	32	≤0.06->128
Oxacillin	≤2	52.6	2	32	0.25-128
Ciprofloxacin	≤1	89.5	0.25	1	0.125-64
Trovafloxacin	≤2	94.7	≤0.06	0.25	≤0.06-8
Q/D	≤1	78.9	1	2	0.5-8
Vancomycin	≤4	100	1	2	0.5-2
Teicoplanin	≤8	100	2	8	0.5-8
Erythromycin	≤0.5	26.3	64	>128	0.25->128
Azithromycin	≤2	26.3	128	>128	0.5->128
Gentamicin	≤4	63.2	1	16	≤0.06-16
Clindamycin	≤0.5	63.2	0.125	>128	≤0.06->128
Tetracycline	≤4	36.8	16	32	0.25-32
Rifampicin	≤1	57.9	0.5	>128	≤0.06->128

[a] Susceptibility breakpoints defined by NCCLS (1998); [b] 50% and 90%, MIC_{50} and MIC_{90}, respectively; [c] Q/D, quinopristin-dalfopristin.

The MIC_{90} values of LY 333328 against oxacillin-resistant staphylococcal strains were comparable to those of oxacillin-susceptible staphylococci (MIC_{90} 8/16 μg/ml). Teicoplanin and LY 33328 (MIC_{90} 2 and 16 μg/ml) were generally two- to three-fold less active than vancomycin against oxacillin-resistant staphylococci (MIC_{90} 2 μg/ml) (Table 5.2).

5.2.6. Gentamicin

Gentamicin resistance was found in 32.6% of clinical staphylococcal isolates obtained from the Universitas hospital. The percentage of *S. aureus* strains exhibiting methicillin and gentamicin resistance was 16. 3%. Such strains are known as gentamicin- oxacillin- resistant *S. aureus* (GORSA). The percentage of GORSA strains isolated in a hospital environment is highly significant because aminoglycosides are considered an alternative to vancomycin for the treatment of ORSA infections. Furthermore, the treatment of oxacillin-resistant staphylococcal infections with intravenous vancomycin is more costly than treatment with gentamicin, because of longer hospitalisation periods. Thus, 16.3% of ORSA could not be treated with aminoglycosides, because gentamicin resistance may serve as a marker for resistance to other aminoglycosides. In staphylococci, resistance to gentamicin, tobramycin and kanamycin is usually mediated by a plasmid encoded bifunctional protein that specifies 6' acetyltransferase [AAC (6')] and 2" phosphotransferase [APH (2")] aminoglycoside modifying activities. Strains carrying this aminoglycoside resistance determinant on a transposable element (Tn*4001*) were first described

in Australia 1986, but have since disseminated throughout the world, especially to the USA (Townsend, 1983).

5.2.7. Macrolides

The macrolides, erythromycin and azithromycin, exhibited similar activity against all staphylococcal strains tested, with susceptibility to erythromycin at 42.1% and azithromycin at 44.9%. Both azithromycin and erythromycin show comparable bimodal MIC distribution patterns (Figure 5.2). Again, this is indicative of a single mechanism of resistance, affecting macrolide susceptibility as a group.

5.2.8. Developmental agents

The glycylcyclines (CL 331,002 and CL 329,998) were considerably more active than tetracycline against ORSA strains (MIC_{90} 2 μg/ml versus 16 μg/ml) (Table 5.2.). Although, the activities of the glycylcyclines (MIC_{90} 8 – 16 μg/ml) were comparable to tetracycline (MIC_{90} 16 μg/ml) in ORCNS. In OSSA strains, the activity of tetracycline was only one- to two-fold less than the suggested susceptibility breakpoint (MIC_{90} ≤4 μg/ml) with the glycylcyclines showing similar limited activity. Figure 5.4. compares the MIC distribution patterns for tetracycline, CL 331,002 and CL 329,998. All three agents showed multi-modal distribution patterns, indicative of mutational events influencing MIC values for oxacillin-resistant staphylococci.

Quinopristin/dalfopristin (Q/D) showed superior activity than the other agents tested against ORSA and ORSE strains with susceptibility >90% of strains being susceptible. However, a decrease in activity was observed for Q/D in ORSH strains with susceptibility being reduced to 71.4%. Q/D may well be considered an alternative to vancomycin for the treatment of ORSA, although decreased susceptibility was seen in certain groups of CNS. Against all staphylococcal strains tested, the activity of linezolid (MIC_{50} 2 μg/ml) was consistent throughout (Table 5.2).

The high frequency of multiple-drug-resistant staphylococcal strains warrants the continued search for new antimicrobial agents with increased anti-staphylococcal activity. The decreased activity of new antimicrobial agents (glycylcyclines and moxifloxacin) in ORCNS suggests that the selection of resistant bacteria or development of resistance during therapy may pose possible future problems, which has been the scenario for many of the currently available anti-staphylococcal agents. If *in vitro* results are supported by clinical trials, moxifloxacin, linezolid and LY 333328 could play significant roles in the treatment of staphylococcal infections.

CHAPTER 6

METHICILLIN RESISTANCE

6.1. Introduction

In 1986 it was recognised that epidemic methicillin-resistant *S. aureus* (EMRSA) were effectively out of control and that, at the time, infection control programs already in place were wholly inadequate for their control (Cookson & Phillips, 1990). Recommendations embodied in the new guidelines did little more than reiterate the codified principles of prevention stipulated in the 1959 Ministry of Health's memorandum on Staphylococcal Infection in Hospitals. This memorandum served as guidance for the control of certain highly virulent epidemic types of *S. aureus*, notably phage type 80, the hospital staphylococcus whose prevalence was largely due to excessive and indiscriminate use of antibiotics. Therefore, history was repeating itself and at the time microbiologists could not help but ask "What if...".

Main recommendations of the 1986 guidelines for successful containment (Gordon, 1993)

1. Close liaison between Infection Control Officer (ICO), Infection Control Nurse (ICN) and involved clinician to decide policy.
2. Flag positive patients' case notes with MRSA sticker warning of future risk on transfer or readmission.
3. Rapid bacteriological detection of carriers and cases of infection.

4. Prompt screening of transfers from affected hospitals or on repatriation from abroad, especially if from ICU.
5. Isolation nursing in single cell of known or suspected carriers and cases of infection.
6. Cohort nursing in four or six-bedded wards if single cell not available.
7. Full isolation nursing policy to be implemented with use of gloves, protective clothing and strictly enforced scrupulous antiseptic hand washing before and after patient contact. This applied to all categories of staff and visitors.
8. Treatment of carriage sites and superficial lesions with antiseptics.
9. Treatment of MRSA infections to reduce risk of dissemination.
10. Restrict movement of patients and staff from affected to non-affected ward/area.
11. Amend antibiotic usage in ward or hospital on advice of ICO to reduce antibiotic selection pressure.
12. Terminal disinfection of ward/area with phenolic disinfectant.
13. Keep accurate records of MRSA infections and other relevant epidemiological information.
14. Test for clearance: full body screen sampled weekly, or may restrict to previous positive sites. Three sets of negative screening swabs at weekly intervals or over 2 week period, required for clearance.
15. Patient can normally be discharged home without significant risk of community spread, the earlier the better to limit risk of hospital dissemination.

It was not long before dissenting voices argued that the new policy was redundant or would prove ineffective or even counter productive by disturbing commensal ecosystems with the risk of colonisation by more harmful pathogens than MRSA. Critics and defenders advanced cogent arguments on both sides (Gordon, 1993).

Some of the arguments against the control policy were:

(A) It is difficult, if not impossible, to predict the course of events when MRSA is introduced into a hospital. Sometimes nosocomial transmission is limited, while in other instances, rapid spread of the organism throughout the hospital occurs. Thus, it is difficult to know when to implement control measures. Furthermore, transmission appears to depend in part upon the patient population into which the organism is introduced, and possibly the characteristics of the strain (Boyce, 1991).

(B) No controlled trails had been done to establish the efficacy of control measures commonly employed in MRSA outbreaks. Hospitals implement from two to ten control measures, often simultaneously, understandable but often it becomes difficult to determine the extent to which each measure independently contributes to outbreak control (Boyce, 1991).

(C) MRSA's failure to become the predominant staphylococcus in domiciliary sepsis suggests that they must possess some cellular defect, since their resistance to many antibiotics and antiseptics should have given these organisms a decisive advantage over other strains of *S. aureus*.

Nevertheless, it is ultimately the therapeutic implications of methicillin-resistance on which all arguments lose their validity and flounder. Methicillin resistance is a marker for (a) resistance to all ß-lactams, the penicillins and cephalosporins; (b) probable multiple-resistance to the majority of available non-ß-lactam antibiotics; and (c) expensive therapy, with serious implications for therapeutic options. Selection of chemotherapy for MRSA is limited to those antibiotics against which resistance is readily acquired (e.g. fusidic acid, rifampicin, quinolones) or to those which are complex, toxic (drug monitoring) and expensive (require intravenous administration) e.g. teicoplanin, vancomycin (Gordon, 1993).

No single laboratory method can detect all methicillin-resistant staphylococci. The main reason for this is that methicillin resistance is not encoded for by a single genetic determinant in staphylococci. The direct determination of antimicrobial susceptibilities to oxacillin remains the most reliable and efficient way for testing for the methicillin resistance phenotype in clinical staphylococcal isolates. Oxacillin is used instead of methicillin because it predicts methicillin resistance best under laboratory testing conditions. Susceptibility tests do, however, have some limitations in accuracy due to heterogeneous phenotypic expression of methicillin resistance in certain staphylococcal strains.

It has been proposed that accuracy would be improved with the addition of a multiplex-PCR assay for the detection of the *mecA* gene under controlled PCR conditions (Geha *et. al.*,1997).

Because the *mecA* gene is not always expressed, detection of PBP 2' is considered to provide a better indication of methicillin resistance. This may be accomplished by the analysis of PBP profiles which is time consuming and expensive or with a now commercially available slide agglutination assay. In the rapid slide agglutination test, latex particles are sensitised with monoclonal antibodies directed against PBP 2'.

Screening of staphylococcal isolates for the presence of the *mecA* gene and sequences on selected isolates was therefore performed and results correlated with MICs and the agglutination assay.

6.1. Results and discussion

6.1.1. Multiplex-PCR for identification of oxacillin-resistant staphylococci.

One hundred and fourty four staphylococcal strains obtained from clinical specimens were screened for the presence of the *mecA* gene employing a multiplex-PCR. Of the 144 staphylococci, 93 were oxcacillin-susceptible and 51 oxacillin-resistant. For *mecA*-positive strains two distinct DNA fragments, a 479-bp 16 S rRNA-specific product (universal primers) and the 310-bp *mecA*-specific product, were amplified and visualised (Figure 6.1). For *mecA*-negative strains only the 479-bp 16 S rRNA-specific product was observed (Figure 6.2).

Figure 6.1.

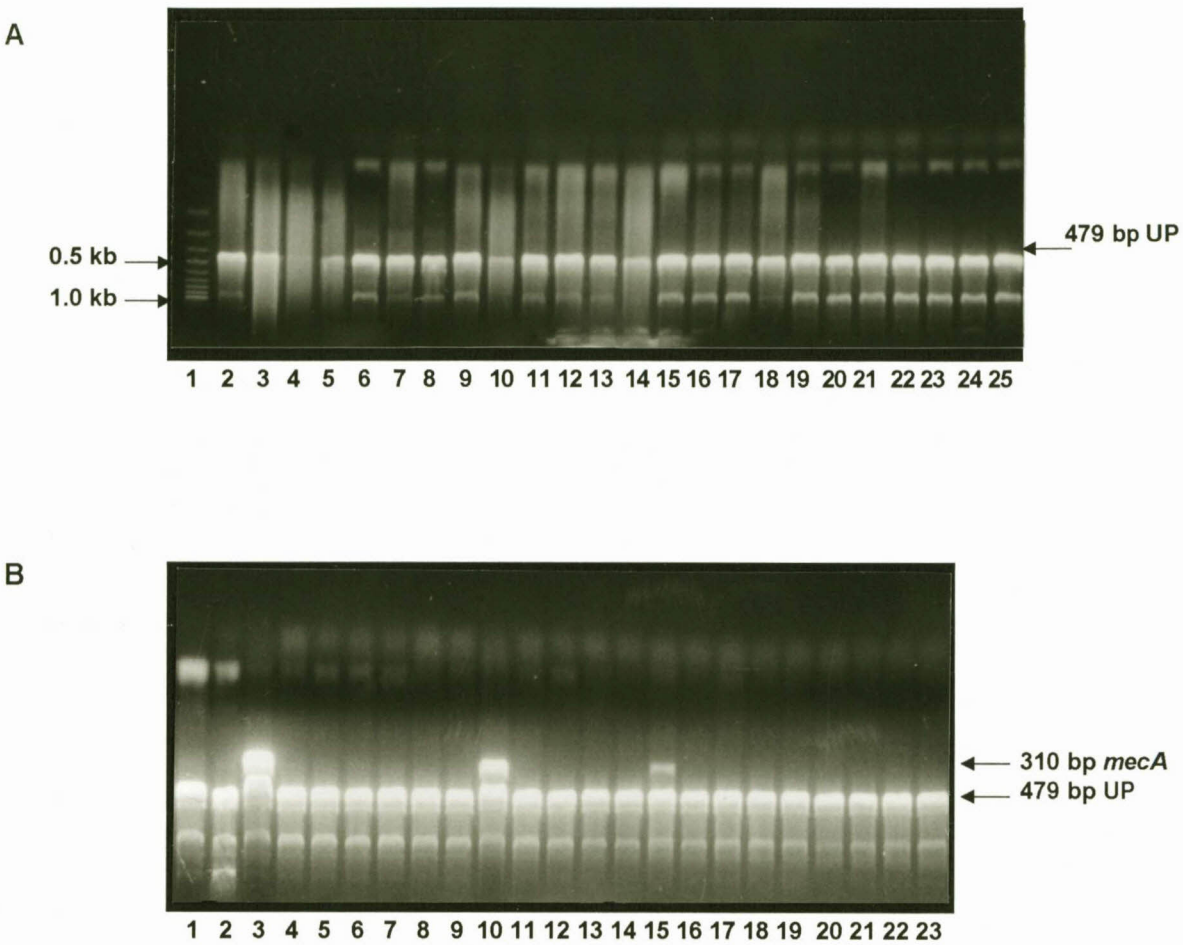


Figure 6.1. *MecA* gene detection in oxacillin-susceptible staphylococci.

Figure 6.1(A): ***Lane 1:*** MWM XIV, ***Lanes 2 – 25:*** *mecA*-negative oxacillin-susceptible staphylococci (**UP:** 479 bp universal primer PCR product).

Figure 6.1(B): ***Lanes 1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22, and 23:*** *mecA*-negative oxacillin-susceptible staphylococci, ***Lanes 3, 10 and 15:*** *mecA* positive oxacillin-susceptible staphylococcal strains (***mecA***: 310 bp *mecA* gene PCR product; **UP:** 479 bp universal primer PCR product).

Figure 6.2.

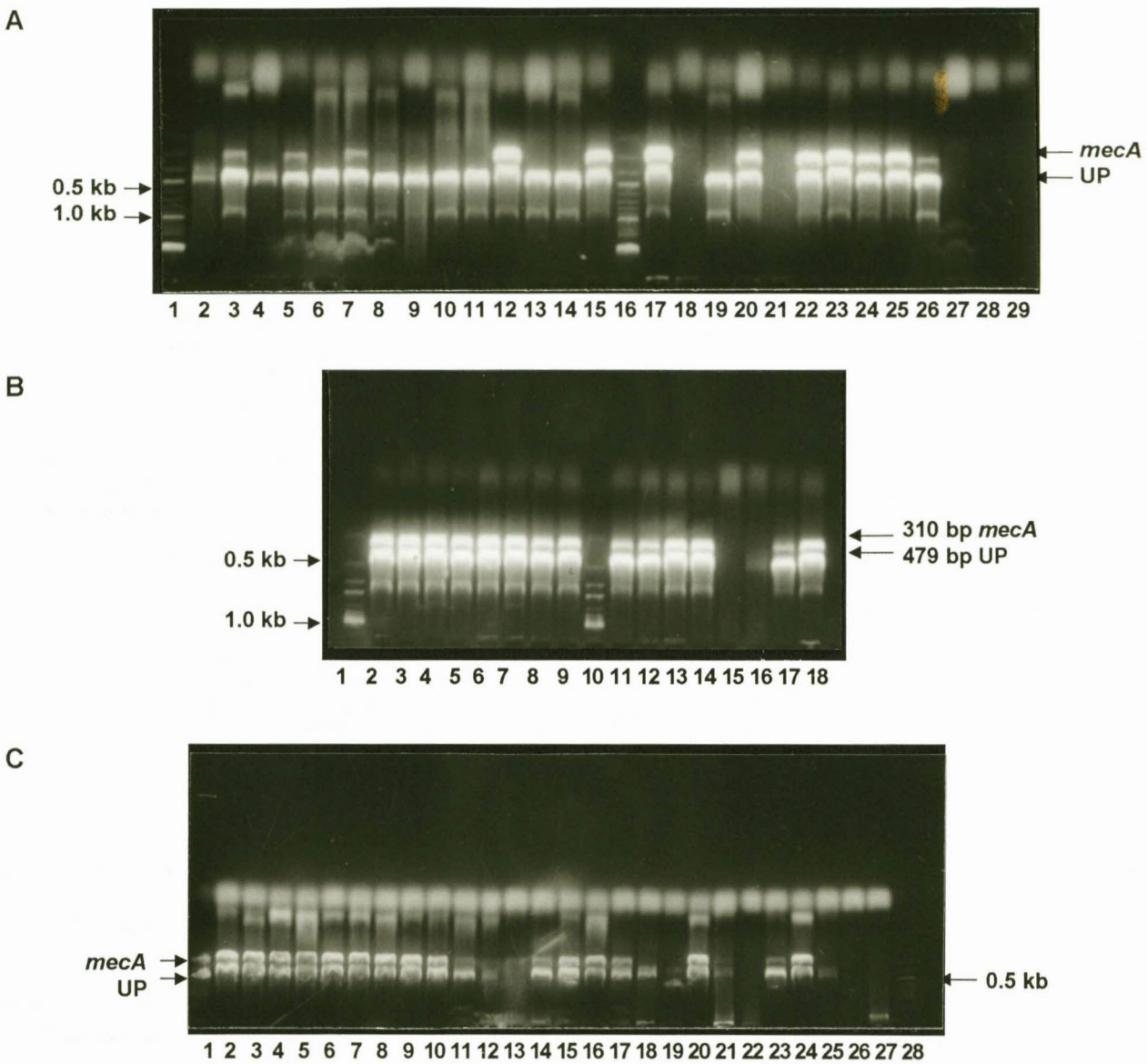


Figure 6.2. *MecA* gene detection in oxacillin-resistant staphylococci.

Figure 6.2(A): ***Lanes 1 & 16:*** MWM XIV, ***Lanes 18, 21, 27, 28 & 29:*** no PCR product, ***Lanes 2, 4, 6, 8, 9, 10, 11, 13, 14, 19:*** *mecA*-negative oxacillin-resistant staphylococci ***Lanes 3, 5, 7, 12, 15, 17, 20, 22 - 26:*** *mecA*-positive oxacillin-resistant staphylococci.

Figure 6.2(B): ***Lanes 1 & 10:*** MWM AB, ***Lanes 2 – 9, 11 – 14, 17, 18:*** *mecA*-positive oxacillin-resistant staphylococci, ***Lane15 & 16:*** no PCR product.

Figure 6.2(C): ***Lane 28:*** MWM XIV, ***Lanes 12, 13, 22, 26, 27:*** no PCR product. ***Lanes 1 – 11, 14, 15, 16, 17, 20, 21, 23, 24:*** *mecA*-positive oxacillin-resistant staphylococci, ***Lanes 18, 19, 25:*** *mecA*-negative oxacillin-resistant staphylococci.

The multiplex-PCR technique applies the 16S rRNA gene detection method as an internal control mechanism for failure of DNA extraction and PCR target-sequence amplification. In strains for which no PCR product was observed, the bacterial lysis procedure or PCR-amplification was repeated rather than the result being considered *mecA*-negative. From 14 non-product reactions, 12 strains revealed a 16S rRNA PCR product upon repeating the test.

Of the 93 oxacillin-susceptible staphylococci tested, eight strains (8.6%) screened *mecA*-positive (Figure 6.1.-A). Of the 51 oxacillin-resistant staphylococci tested, two (3.9%) strains screened *mecA*-negative (Figure 6.2.-B). This finding concurred with results from the USA in which 5.5% oxacillin-resistant strains screened *mecA*-negative (Geha *et. al.*, 1997). However, the percentage of *mecA*-positive oxacillin-susceptible strains in the present study was higher (8.6%) than reported by Geha *et. al.* (1994) [1.3%]. The overall correlation between phenotypic (MIC values) and genotypic (multiplex-PCR) data for the 145 staphylococci tested was 83.8%.

The PCR assay was comparable to susceptibility data that identified intrinsic methicillin resistance in *S. aureus* and CNS, 94.5% and 96.9% respectively. After further evaluation, of the two *mecA*-negative oxacillin-resistant staphylococcal strains, other mechanisms for methicillin/oxacillin resistance were considered as possible explanations for the lack of correlation between genotypic and phenotypic results (Table 6.1.). In strains *S. chromogenes* U147 and *S. aureus* U61, the most likely causes of high-level methicillin resistance

(MICs >128 μg/ml) observed were (a) the presence of small colony variants (SCV) and/or (b) hyperproduction of β-lactamase, since both U147 and U61 produced β-lactamase.

Complications that may arise in the laboratory detection of methicillin-resistant staphylococci are reflected in the eight *mecA*-positive yet oxacillin-susceptible staphylococcal strains identified. MIC values could be incorrect, a possible explanation for this phenomenon might be the variables that influence oxacillin susceptibility testing such as pH, temperatures and salt concentrations. Such strains would be deemed oxacillin-susceptible based on susceptibility data, and the patient subjected to inappropriate β-lactam antibiotic treatment. Due to the inducibility of the expression of the *mecA* gene in the presence of β-lactams, all *mecA*-positive, oxacillin-susceptible staphylococci have the potential to become highly-resistant. Since vancomycin continues to be the only reliable drug for the eradication of intrinsically methicillin-resistant staphylococci, the preservation of vancomycin specifically for this purpose, may help to control the high costs associated with its over use and development of resistance to this glycopeptide. These findings suggest that the presence of the *mecA* gene is important in interpreting susceptibility data. MIC results should not be used as sole criteria for the identification of methicillin-/oxacillin-resistant staphylococci. The implementation of *mecA* gene screening will depend on the volume of isolates tested on a routine basis. The higher the volume of specimens, the more time and money will be saved.

Table 6.1. Characteristics of *mecA*-negative oxacillin-resistant staphylococci.

Strain No.	U147	U61
Species	*S. chromogenes*	*S. aureus*
Oxacillin MIC (μg/ml)	>128	128
16 S rRNA product	+	+
MecA gene product	-	-
β-Lactamase producer	+	+
PBP2' detection	-	-
Interpretation	Small colony variants	

Table 6.2. Comparitive MIC values and PBP2' production of selected staphylococcal strains

	PBP2' production	Oxacillin MIC (μg/ml)		
		MIC_{50}	MIC_{90}	MIC range
MecA+, Ox^R	+(37)	128	>128	4->128
(n = 47)	-(10)	8	16	4-16
MecA+, Ox^S	+(5)	-	-	0.125-2
(n = 8)	-(3)	-	-	0.125-1
Non-PCR-product	+(1)	-	-	>128
(n = 2)	-(1)	-	-	4
MecA-, Ox^R	-(2)	-	-	128->128
(n = 2)	+(0)	-	-	
Key: Ox^R, oxacillin-resistant; Ox^S, oxacillin susceptible				

Genotypic detection of drug resistance will undoubtedly become an important component in the diagnostic laboratory and guidelines for interpretation of *mecA* gene presence will need to be formally addressed.

6.1.2. Evaluation of the MASTALEX-MRSA slide agglutination test for the detection of PBP2'

A total of 59 *mecA*-positive staphylococcal isolates were tested for the production of PBP2'. These strains included 48 *mecA*-positive oxacillin-resistant, eight *mecA*-positive oxacillin-susceptible, two *mecA*-negative oxcacillin-resistant staphylococci and two non-PCR-product strains. Of the 47 *mecA*-positive, oxacillin-resistant staphylococci 37 (77.1%) strains produced PBP2' (MASALEX-MRSA). Of the eight *mecA*-positive oxacillin-susceptible staphylococci screened with the MASTALEX-MRSA test, five (62.5%) strains produced PBP2'. This is an indication of inconsistencies in oxacillin susceptibility data. Variability in pH, temperature and salt concentrations are most likely responsible for these inconsistancies. Neither *mecA*-negative oxacillin-resistant strains produced PBP2', while one of the non-PCR-product oxacillin-resistant strains produced PBP2'.

The comparison between PBP2' production and susceptibility data obtained for 47 *mecA*-positive, oxacillin-resistant staphylococci are shown in Table 6.2. Although, susceptibility data reflect a resistance phenotype, expression of the

mecA gene was not detected with the MASTALEX-MRSA in 10 *mecA*-positive staphylococcal strains. The MIC_{50} value (128 μg/ml) for 37 *mecA*-positive, oxacillin-resistant, PBP2'-producing strains was four-fold higher than that of the 10 non-PBP2' producing strains (MIC_{50} 8 μg/ml). The NCCLS provides a oxacillin-resistant breakpoint MIC in staphylococci MIC ≤4 μg/ml, however, the borderline resistance phenotype has been seen to vary from study to study, with MICs of 1 to 16 μg/ml (Geha *et. al.* 1994). The 10 *mecA*-positive, oxacillin-resistant, non-PBP2' producing staphylococci identified in this study may be classified as being borderline methicillin-resistant. It should be kept in mind that (a) perhaps MASTALEX-MRSA is not capable of detecting PBP2' in low concentrations and (b) other mechanisms for methicillin resistance may be contributing to the elevation of oxacillin MIC observed. Since nine of the ten strains were β-lactamase producers, the hyperproduction of β-lactamase could be considered as the major mechanism of resistance for these strains.

The MASTALEX-MRSA slide latex test for the detection of PBP2' can be performed on 48 strains within the hour. In comparison to multiplex-PCR methods there is the possibility of false negative results that lowers the sensitivity of MASTALEX-MRSA, but the method is very simple to perform and results are readily available.

6.2. *MecA* gene sequencing

A 240-bp region of the *mecA* gene (1.2 kb DNA sequence) was sequenced in 26 staphylococcal strains. These isolates included eight *S. aureus,* five *S. epidermidis* and 13 *S. haemolyticus* strains. Comparisons of the *mecA* gene sequences are shown in Table 6.3. Minimal sequence variation was observed for the 26 staphylococcal strains investigated. For *S. haemolyticus* strains, 76.9% (10 strains) showed no sequence variation, while for *S. aureus* and *S. epidermidis* strains, minor sequence variation was seen with 87.5% (7 strains) and 60% (3 strains), respectively. After further examination of the staphylococcal species sequenced, three clusters of nucleotide base changes at positions 250, 258 and 331 were observed (Table 6.3). As a consequence of these nucleotide changes, amino acid alterations occurred at positions 84, 86 and 111 (Table 6.4). No correlation was found on comparing production of PBP2' and nucleotide/amino acid changes in the sequenced staphylococcal strains (Table 6.5). The finding of an amino acid change at cluster position 84 in different strains was similar to that described in a *S. sciuri* strain by Wu *et. al.* (1996). The results reflect the highly conserved nature of the *mecA* gene within staphylococcal species. The high-level of sequence homology found in staphylococcal *mecA* genes has been the major contributing factor to the theory that *mecA* genes are of clonal origin.

Table 6.3. Nucleotide changes detected in the *mecA* gene of thirteen staphylococcal strains (Wu *et. al.*, 1996)

Strain No.	Nucleotide																																																	
	212	214	229	234	241	250	255	256	257	258	260	263	269	277	293	299	306	313	317	318	322	323	324	326	329	330	331	332	334	336	355	366	370	388	402	404	405	407	413	419	420	421	422	423	425	427	428	429	433	434	435
CTR	A	A	G	A	C	A	A	A	A	A	A	T	A	A	A	A	A	T	G	T	A	T	T	A	G	C	A	A	G	T	G	G	A	A	A	G	A	T	A	A	A	A	G	C	T	C	A	T	G	A	A
U152					G			T			T																				C									G	G						T	A			
U113																																					C	C													
U4				T		**T**	T			**G**																										C	C						T			G					
U155						**C**																					**G**	C	C																G						
U154																																														G				T	
U107	T	C				**T**				**C**			T																				T																		T
U29																											**C**																								
U141			T											C								G																	C												
U31						**G**				**C**			T												C		**T**																						A		
U41																																T																			
U113																																										G		A							
U124		G				**T**								T		G											**T**								T	T															
U25									T	**C**		G		G	T		T	G	C	C	C		C	C		T				G				T																	

Key: **CTR**, control sequence; **N** (shaded), basepair change clusters; **A**, adenine; **T**, thymine; **C**, cytosine; **G**, guanine.

Table 6.4. Amino-acid changes observed for 13 staphylococcal strains

AAP	CTR	U152	U113	U4	U155	U154	U107	U29	U141	U31	U41	U113	U124	U25
68	Lys													Thr
71	Asn						Ile							
72	Ser						Arg						Gly	
77	Asp								Tyr					
78	Ile			Ile										
81	Gln	Glu												
84	Lys			Stop	Gln		Stop			Glu			Stop	
85	Ile			Ile										
86	Lys	Stop		Arg			Asn			Asn				Ile
87	Lys	Ile												
88	Val													Gly
90	Lys						Ile			Ile				
92	Lys													Lys
93	Lys								Gln				Stop	
98	Gln													Leu
100	Lys												Arg	
102	Lys													Asn
105	Tyr													Asp
106	Gly													Ala
108	Ile								Ser					Leu
109	Asp													Ala

continued

Table 6.4. *continued*

AAP	CTR	U152	U113	U4	U155	U154	U107	U29	U141	U31	U41	U113	U124	U25
110	Arg									Pro				Arg
111	Asn				Ala			His		Tyr			Tyr	
112	Val				Leu									Val
119	Glu	Gln												
122	Met										Ile			
124	Lys						Stop							
130	Ser													Cys
134	Pro												Pro	
135	Gly		Gly	Ala									Val	
136	Leu		Thr											
138	Cys								Thr					
140	Gln	Arg												
141	Ser			Ile								Gly		
142	Ile				Arg									
143	His	Leu		Asp		Asp								
145	Glu					Val	Asp			Lys				

Key: **Gly**, Glycine, **Ala**, Alanine; **Val**, Valine; **Leu**, Leucine; **Ile**, Isoleucine; **Ser**, Serine; **Cys**, Cysteine; **Thr**, Threonine; **Met,** Methionine; **Tyr,** Tyrosine; **Pro**, Proline; **His**, Histidine; **Lys**, Lysine; **Arg**, Arginine; **Asp**, Aspartic acid, **Glu**, Glutamic acid; **Asn**, Asparagine; **Gln,** Glutamine. **AAP**: amino acid position; **CTR:** control sequence.

Table 6.5. Comparative MICs, PBP2' detection and amino-acid alterations for 26 staphylococci

Strain	Species	Oxacillin MIC (μg/ml)	PBP2' detection	Amino acid changes
U107	*S. aur*	0.125	+	71, 72, 84, 86, 90, 124,145
U131	*S. aur*	0.25	+	141
U27	*S. aur*	0.25	+	
U25	*S. aur*	0.5	+	68,86, 88, 92, 98, 102, 105, 106, 108, 109, 110, 112, 130
U154	*S. aur*	1	+	143, 145
U29	*S. aur*	4	-	111
U113	*S. aur*	8	+	142
U41	*S. aur*	64	+	122
U47	*S. epi*	8	-	
U14	*S. epi*	8	-	
U155	*S. epi*	16	+	84, 111, 112, 142
U31	*S. epi*	16	-	84, 86, 90, 110, 111, 145
U141	*S. epi*	64	+	77, 93, 108, 138
U152	*S. hae*	>128	+	81, 86, 87, 119, 140, 143
U4	*S. hae*	>128	+	78, 84, 85, 86, 135, 141, 143
U11	*S. hae*	>128	+	
U16	*S. hae*	>128	+	
U28	*S. hae*	>128	+	
U42	*S. hae*	>128	+	
U69	*S. hae*	>128	+	
U88	*S. hae*	>128	+	
U70	*S. hae*	>128	+	
U123	*S. hae*	>128	+	
U124	*S. hae*	>128	+	72, 84, 93, 98,111, 134, 135,
U133	*S. hae*	>128	+	119, 140,143
U153	*S. hae*	>128	+	

CHAPTER 7

GENERAL DISCUSSION

In clinical microbiology laboratories in South Africa, many sacrifices are made for the cause of saving time and money. One such example is the over-simplification of the identification of staphylococci. When Kloos & Shleifer (1975) proposed their simplified scheme for the identification of clinically significant staphylococci in 1975, it was done with regard for both *S. aureus* and CNS. Over the last few decades, this scheme has been down scaled to suite the needs of individual clinical microbiology laboratories. The Universitas microbiology laboratory has reduced the original 23 conventional biochemical tests proposed by Kloos & Schleifer (1975) for the differentiation between staphylococci and other gram-positive bacteria to a single biochemical test, that being catalase production. The price paid for cost-effectiveness is evident in that 15.7% of gram-positive cocci other than staphylococci were incorrectly identified as staphylococci. The inclusion of bile-aesculin agar plates and a bacitracin susceptibility test into the diagnostic laboratory protocol for the identification of staphylococci would reduce misidentification of non-staphylococcal isolates by 11.3%.

Originally, the identification of staphylococcal species was limited to a coagulase test. This provided differentiation between the more virulent *S. aureus* and less virulent coagulase-negative staphylococci. However, alterations in the cell wall

of methicillin-resistant staphylococci and recently described staphylococci with reduced susceptibilities to vancomycin has led to indifferent levels of coagulase production in certain *S. aureus* strains (Moreira *et. al.*, 1997). This warrants the re-assessment of the coagulase test as sole criteria for differentiating between pathogenic and potentially pathogenic strains. The poor correlation (83.4%) between the two independent coagulase tests performed in this study is of great concern. Care should always be taken to perform identification tests in accordance with the proposed methods. This includes the use of untreated human plasma in the coagulase test, which was most likely the reason for the observed inconsistencies in the coagulase test performed by the Universitas microbiology laboratory. Since more than 90% of *S. aureus* strains produce colonies exhibiting typical morphology on Baird-Parker plates, colony morphology in combination with the coagulase test could be instrumental in the improved differentiation of *S. aureus* from CNS.

The recent recognition of the potential pathogenicity of CNS especially in association with immuno-compromised patients, has warranted the inclusion of additional identification methods in diagnostic protocols. The Universitas microbiology laboratory has included the trehalose test for differentiation between *S. epidermidis* and other CNS species. Again, as in the case of the catalase test, the use of a single determinant for the identification of a single species, is far from ideal. Identification accuracy is reliant on the number of biochemical tests performed, the STAPH ID 32 API system has been reported to

incorporate the minimum number of biochemical tests required for the identification of staphylococcal species.

In an attempt to save time, identification accuracy can also suffer. In comparison to the conventional identification scheme proposed by Kloos & Shcleifer (1975), the API system does save up to 48 h. However, due to the limited phenotypic characteristics used and the database that has been built to date, the overall identification accuracy of the API system applied to CNS in this study was only 84.6%. The API system was not specifically designed for the clinical laboratory and does identify clinically insignificant *Staphylococcus* species. Although expensive, when a rapid and fairly comprehensive identification of CNS species is required, the STAPH ID 32 API system is satisfactory.

S. epidermidis (26 strains) and *S. aureus* (93 strains) comprised 81.5% of staphylococci isolated from the Universitas hospital. This justifies in part the emphasis placed on these two species of staphylococci as opposed to other CNS. As these two species are included in the staphylococcal identification protocol of the Universitas laboratory based purely on the most commonly isolated staphylococci from clinical specimens; then *S. haemolyticus* (15 strains) comprising 10.3% of isolates, is a strong contender for inclusion in the scheme. From TEP-cultures, 65% of isolates were *S. epidermidis,* reiterating its importance especially in patients where natures defense barriers have been

compromised. Although a low percentage (13.7%) *S. haemolyticus* strains was isolated from TEP-cultures, a much higher percentage was found in catheter tips. Noticeably, not a single *S. saprophyticus* strain was identified by API perhaps indicating inherent identification inaccuracies in the system. It might, however, be an indication of the dissemination/colonisation potential of this species in the short space of time in which staphylococci were isolated for the purposes of this study. The potential of CNS to develop into epidemic populations should not be dismissed and shifts in all clinically significant CNS species should be constantly monitored by employing accurate identification methods.

In an attempt to find a midway point between cost-effectiveness and time spent in the identification of staphylococci, a species-specific PCR identification assay was assessed. The species-specific PCR primers developed for clinically significant staphylococci by Gribaldo *et. al.* (1997) were used on staphylococci identified by the API system. One of the major problems encountered in using the species-specific PCR format of Gribaldo *et. al.* (1997) was the absence of a universal primer. It is recommended due to lysis problems encountered with staphylococci, that inclusion of universal primers would have eleviated the problem of false-negative results. As previously mentioned, cell wall alterations associated with methicillin resistance, affects not only coagulase production, but also lysostaphin lysis susceptibility. Yet another problem encountered with the species-specific PCR identification assay was non-specific species identification.

In comparison to the API identification system, the species-specific PCR identification assay focuses only on clinically significant CNS species and dramatically reduces costs. However, in comparison with the API identification system, the overall specificity of the species-specific PCR assay for the identification of clinically significant CNS was only 44.1%. Due to the apparent inaccuracy of the PCR identification assay based on API, its use in the clinical microbiology laboratory would be argued against; although if standardised and expanded it could be considered for future incorporation into routine practice.

In the early 1990s in the USA, *S. aureus* was already the second-most common isolate obtained from clinical specimens (Cormican & Jones, 1996). This effectively meant that if epidemic MRSA strains were allowed to spread through a hospital, a high percentage of patients would require isolation, extended hospitalisation and expensive glycopeptide therapy. One of the major recommendations for the control of epidemic MRSA in the hospital environment is epidemiological surveillance, the purpose of this being prevention. If a patient with an epidemic MRSA could be identified, he/she could be isolated, preventing the spread of epidemic strains throughout the hospital. An epidemic MRSA outbreak is characterised by the ability of a subset of staphylococci to disseminate rapidly. These strains may cluster together within a short period if time (<2 months), carrying high-level methicillin/oxacillin resistance (MIC >16 μg/ml) in association with multiple resistance (i.e. resistance to macrolides, quinolones, aminoglycosides and/or clindamycin).

In an effort to find a rapid, molecular epidemiological typing technique for staphylococci, which also has high discriminatory power, RAPD profiles were examined for 45 staphylococci. Crude DNA template preparations were used in the study for several reasons: (a) time required for species and strain delineation is short, (b) to reduce extensive DNA shearing in preparing the DNA template opposed to the purified DNA template, and (c) reduce strain typing costs. Eleven major RAPD patterns were observed employing RAPD primer I. Primer I effectively grouped the staphylococci according to API identification data, suggesting an acceptable level of species discrimination. The presence of a unique RAPD profile for each specific *Staphylococcus* species suggests RAPD profiling could offer a molecular identification technique for the majority of commonly isolated CNS in the clinical microbiology laboratory.

Primer I and also primer III showed good typeability for the staphylococci examined, using the crude DNA template method. The discriminatory power of primer III was acceptable for *S. haemolyticus* strains, differentiating between epidemiological unrelated strains. However, for *S. epidermidis*, discrimination between unrelated staphylococci was optimal when data obtained from both primers were combined. Primer I and III showed good reproducibility and discriminatory capacity when employed together providing a rapid method for strain typing CNS. Due to the pathogenic potential of CNS it is recommended that these strains are constantly monitored for the emergence of any epidemic strain types.

Individually and in combination, primers I and III showed poor discriminatory capacity for typing *S. aureus* strains. It has been found by other researchers that longer oligonucleotide primers (>10 bp in length) are more efficient for *S. aureus* strain typing, but to the contrary, in the present study primers ERIC 1 and 2 were totally unsatisfactory. ERIC 1 and 2 exhibited no discriminatory nor typing capacity for the strains investigated.

Plasmid analysis proved useful for the determination of extra-chromosomal epidemiological relatedness. The 15 *S. haemolyticus* strains isolated from the Universitas hospital exhibited similar susceptibility data, not only in the presenting antibiograms, but also in the resistance levels achieved for β-lactams, quinolones, macrolides, aminoglycosides and clindamycin. Combined susceptibility data and plasmid profile analysis revealed strain relatedness primarily due to the presence of a 1.0 kb plasmid in the majority (14/16) of *S. haemolyticus* isolates, but primer III data suggested limited strain similarity.

Between April and July 1998, 34.3% of staphylococci isolated from the Universitas hospital were oxacillin-resistant. Such a high level of oxacillin-resistance is particularly troublesome for the following reasons: (a) intrinsic β-lactam resistance, (b) additional multiple resistance to quinolones, macrolides, aminoglycosides and clindamycin, and (c) subsequent vancomycin selection pressure created by oxacillin resistance/over use.

Despite the high levels of penicillin resistance found in the staphylococci isolated from the Universitas hospital (95.1%) and world wide (>80%), penicillin is included in the NCCLS proposed regimen for the testing of antimicrobial resistance in staphylococci. Possible explanations for this might be the discouragement of the empirical treatment of all methicillin-susceptible staphylococci with penicillinase-resistant penicillins. Penicillin is also a marker for β-lactam resistance and the presence of β-lactamases in certain staphylococcal strains. Noticeably, oxacillin resistance was more than 50% higher in the CNS as opposed to *S. aureus*. The main reason for this being, the eradication of *S. aureus* (the identified pathogen in most cases of staphylococcal infection) during methicillin-treatment, while the effect of the antimicrobial agent on normal flora of which CNS constitutes a major part, is often neglected. As vancomycin treatment is expensive, toxic and labour intensive, alternatives are often sought for the treatment of methicillin-resistant staphylococci. Alternative antibiotics recommended by NCCLS are erythromycin, ciprofloxacin, gentamicin and clindamycin. When MIC values for these clinically available agents were examined, it was found that 49% of oxacillin-resistant staphylococci were resistant to all four antibiotics. Multi-drug resistance was higher in CNS (62.5%) than in *S. aureus* (29.4%), again reiterating the accumulation of antibiotic resistance genes in CNS.

In certain parts of the world, such as Australia, gentamicin and oxacillin resistance are found in such close liaison in *S. aureus* strains, that they are described as gentamicin-oxacillin-resistant *S. aureus* (GORSA) (Townsend, 1995). These strains are rapidly disseminating throughout the world. In the Universitas hospital, 16.3% of ORSA could not have been treated with aminoglycosides. The dissemination of GORSA strains in a hospital environment further increases the empirical treatment of *Staphylococcus* infections with vancomycin. The continued loss of clinically available antibiotics warrants the search of new antibiotics with increased anti-staphylococcal activity.

The developmental quinolones, trovafloxacin and moxifloxacin, provide useful alternatives to ciprofloxacin in the treatment of methicillin-resistant staphylococcal infections. Both trovafloxacin and moxifloxacin showed excellent activity against all oxacillin-susceptible staphylococci and oxacillin-resistant *S. aureus* strains. In comparison to the other quinolones tested, moxifloxacin showed improved activity against oxacillin-resistant CNS. All three quinolones displayed bimodal MIC distribution patterns, indicating a mutational event at the chromosomal-level that exhibits reduced susceptibility across the quinolone group of antibiotics. Against oxacillin-resistant *S. aureus* strains the glycylcyclines showed improved activity over tetracycline. The glycylcyclines, LY333328 and Q/D may well be considered as alternatives to vancomycin treatment for methicillin-resistant *S. aureus*, however, decreased susceptibility

was observed for CNS, in particular *S. haemolyticus*. Of the developmental agents tested, linezolid showed consistent *in vitro* activity against all staphylococci.

The potential sequential effects oxacillin/penicillin and aminoglycoside resistance has on staphylococci for the selection of strains with reduced susceptibilities to the glycopeptides are shown in Figure 7.1. An example of the selection pressure on glycopeptides in staphylococci was found in the present study with *S. haemolyticus* strains. High-level methicillin and penicillin resistance was found with multiple-drug resistance presented in most of these strains. In addition to this, *S. haemolyticus* showed reduced susceptibility to teicoplanin.

The inadequacy of a single diagnostic method for the detection of methicillin resistance in staphylococci is evident when comparing (a) susceptibility data, (b) multiplex-PCR for *mecA* gene detection, and (c) PBP2' detection. None of these methods were seen to correlate with each other at the 100%-level. The lack of *mecA* gene detection in oxacillin-resistant staphylococci indicated the presence of mechanisms other than PBP2' production. The presence of SCV and overproduction of ß-lactamase were considered the most likely reasons for high-level oxacillin resistance (MICs ≥128 μg/ml) observed for mecA-negative oxacillin-resistant strains. Eight *mecA*-positive oxacillin-susceptible staphylococci were evident leading to a slight overestimate to that of the

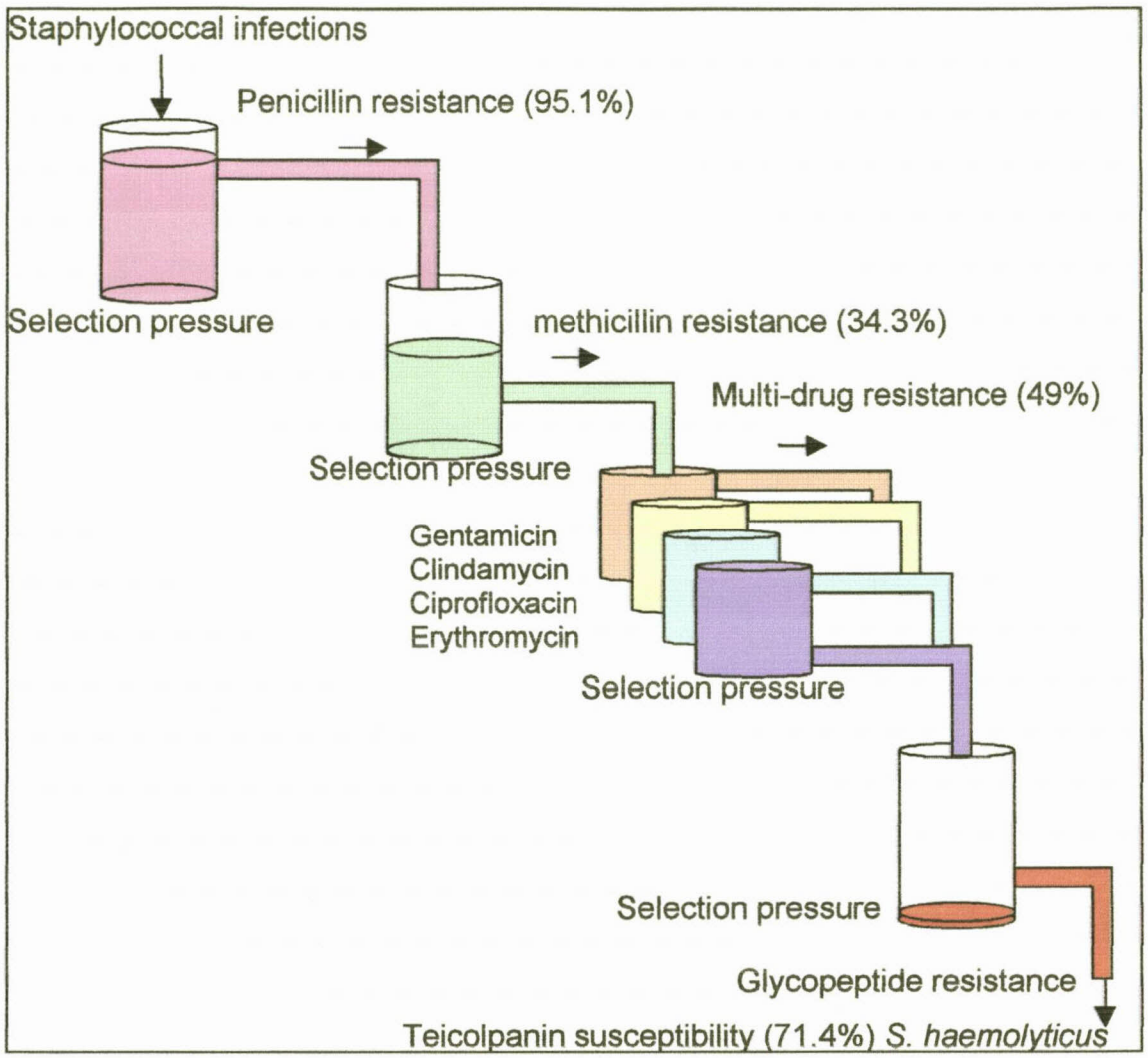


Figure 7.1. The sequential effect that selection pressure on β-lactams, aminoglycosudes, macrolides, quinolones and clindamycin has on the selection of glycopeptide resistance in staphylococci (*S. haemolyticus* in this study). When penicillin resistance places significant pressure on clinicians for the empirical treatment of staphylococci with methicillin, this results in increased selection pressure for methicillin resistance, which in turn will result in the increased use of other clinically available antibiotics. When multi drug resistance has built up to significant levels, the empirical treatment of staphylococcal infections with glycopeptides escalates, which in turn result in increased selection pressure for glycopeptide resistance.

susceptible phenotype, explained by the lack of the consistent phenotypic expression of the *mecA* gene.

The *mecA*-positive staphylococci identified in this study were also evaluated for the expression of the *mecA* gene and therefore the production of PBP2'. Of the *mecA*-positive staphylococci screened for the expression of the *mecA* gene, 87.5% produced PBP2'. The MIC_{50} values of PBP2' producing strains were four-fold higher than for non-PBP2' producers. The 10 oxacillin-resistant *mecA* positive non-PBP2' producers had a MIC range of 4-16 μg/ml. The hyperproduction of β-lactamase(s) was considered for the methicillin resistance phenotype in these strains. However, the MASTALEX-MRSA slide agglutination test does not detect low amounts of PBP2' in certain staphylococcal strains. This has to be taken into consideration for the presentation of a borderline-resistance phenotype/ genotype. The detection of PBP2' was rapid although, in comparison to *mecA* gene amplification detection and antimicrobial susceptibility tests was inaccurate for the identification of methicillin resistance in staphylococci. From these results, it is evident that the clinical microbiology laboratory can not rely on susceptibility data alone for the identification of methicillin resistance in staphylococci. Ideally, susceptibility data should be combined with direct *mecA* gene detection.

DNA sequencing of a fragment of the *mecA* gene in selected staphylococcal strains revealed minimal sequence variation. Three altered amino acid clusters were observed for four staphylococcal strains. However, these alterations did not correlate with susceptibility data, nor did it with PBP2' production. This is an indication that variable levels of methicillin resistance in staphylococci can be attributed to different mechanisms of methicillin resistance or variations in the expression of the *mecA* gene, rather than mutations within the gene itself. The low sequence variation that was observed in the *mecA* gene is primarily responsible for initial assumptions of the clonal origin for methicillin-resistance in staphylococci. As of yet, pharmaceutical companies have failed to produce an analogous antimicrobial agent to β-lactam agents that would be able to specifically target PBP2'. The development of such an agent would be extremely effective and instrumental in the reduction of glycopeptide selection pressure. An antibiotic targeting a penicillin-binding protein such as PBP2' would then be subject to resistance development due to subsequent PBP alterations. The possible significance of the observed amino acid alterations in the gene encoding PBP2' in certain staphylococci in the present study, would only then be realised.

The development of methicillin resistance in a hospital setting results in extended hospitalisation costs and expensive antimicrobial therapy. Effective assessment and control of methicillin-resistant staphylococci is dependent on the ability of the clinical microbiology laboratory to (a) accurately identify

staphylococcal species, (b) perform epidemiological surveillance rapidly and efficiently, (c) undertake standardised susceptibility testing for the identification of potentially epidemic strains, and (d) determine the prevalence of the *mecA* gene in all staphylococcal populations. If one of these links in the chain is weak or even non-existent, a true assessment of the effect of epidemic methicillin-resistant staphylococcal strains can not be made, nor can any control policies be effectively implemented.

Staphylococci have the remarkable ability to add virulence, dissemination, colonisation and antibiotic resistance factors to its arsenal. Today, MRSA are amongst the most threatening of nosocomial pathogens. Regarding the management of methicillin-resistant staphylococci, it is essential to apply common sense and maintain proficient surveillance programmes.

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