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Investigation of Reduced Susceptibility to Glycopeptides among Meticillin-Resistant *Staphylococcus aureus* Recovered in Ireland (1998 to 2004)

Investigation of Reduced Susceptibility to Glycopeptides among Meticillin-Resistant *Staphylococcus aureus* Recovered in Ireland (1998 to 2004)

by

Margaret Fitzgibbon

A thesis submitted for the award of Doctor in Philosophy,
in the Department of Clinical Microbiology,
Faculty of Health Sciences,
School of Medicine,
University of Dublin, Trinity College.

July 2009

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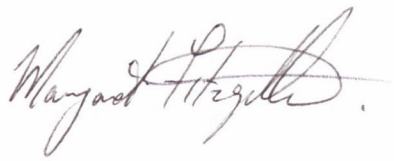
Signed: 

Summary

Meticillin-resistant *Staphylococcus aureus* (MRSA) isolates (n=3,189) from 2990 patients were studied by agar screening and the E-test™ macromethod to investigate reduced susceptibility to glycopeptides among six collections of MRSA recovered between 1998 and 2004. No vancomycin-resistant *S. aureus* or glycopeptide-intermediate *S. aureus* (GISA) were detected but 178 isolates were confirmed by vancomycin population analysis profile (PAP)-area under the curve (vPAP-AUC) ratio determination and/or teicoplanin PAP (tPAP) methods as hetero-GISA (hGISA). Of 139 isolates detected using recommended E-test™ macromethod cut-off values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone, 73 were confirmed as hGISA by vPAP-AUC, 95 by tPAP and 108 by both methods. Lowering the teicoplanin E-test™ macromethod cut-off value to 8 mg/L detected a further 70 hGISA (17 were confirmed by vPAP-AUC and 70 by tPAP). Evaluation of agar screening methods using brain heart infusion (BHI) agar containing 6 mg/L vancomycin (BHIV6) and Mueller-Hinton (MH) agar containing 8 mg/L teicoplanin (MHT8) showed that BHIV6 and MHT8 failed to detect hGISA isolates. MH agar containing 5 mg/L teicoplanin (MHT5) and BHI agar containing 5 mg/L teicoplanin (BHIT5) were evaluated using 10-µl volumes of three inoculum concentrations {bacterial suspensions in saline with densities equivalent to 0.5 and 2.0 McFarland turbidity standards and stationary phase BHI broth subcultures ($MHT5_{0.5}$; $MHT5_{2.0}$; $MHT5_S$; $BHIT5_{0.5}$; $BHIT5_{2.0}$; $BHIT5_S$)}. $BHIT5_{0.5}$ yielded the best performance with a sensitivity of 100% and specificity of 84% for detecting isolates with teicoplanin E-test™ macromethod values of ≥8 mg/L. Screening on $BHIT5_{0.5}$ is useful where screen-positive isolates are investigated with the E-test™ macromethod and confirmed by vPAP-AUC and tPAP methods. Prevalence of hGISA among patients with blood culture MRSA isolates recovered in Irish hospitals between 1999 and 2003 was 2.6% whereas prevalence among patients with isolates from all specimen sites collected during a two-week survey in 1999 was 12%. Prevalence in one hospital fell from 5.3% in 2003 to 1.5% in 2004.

hGISA isolates were typed by antibiogram (A) typing, biotyping and DNA macrorestriction digestion using *Sma*I followed by pulsed-field gel electrophoresis (PFGE). Results of antibiogram typing and PFGE typing were combined to give an A-PFG type. The most frequently occurring A-PFG types were A-PFG 13-00 (ST8-MRSA-II) and A-PFG 14-00 (ST8-MRSA-II) representing 52% and 20% of hGISA isolates,

respectively. In contrast, the prevalence of A-PFG types, A-PFG 13-00 and A-PFG 14-00 among Irish MRSA blood culture isolates during the study period were 14% and 12%, respectively, with the majority of isolates (57%) exhibiting A-PFG 06-01 (ST22-MRSA-IV).

Previous reports have suggested that hGISA isolates carry accessory gene regulator (*agr*) group I or II, are defective in *agr* function (as measured by delta-haemolysin production) and have an increased ability to bind to artificial surfaces such as polystyrene. In the present study, hGISA isolates (n=178) were matched with meticillin-resistant glycopeptide-susceptible *S. aureus* (MR-GSSA) isolates (n=176) exhibiting similar A-PFG types. Both groups were investigated for *agr* type, delta-haemolysin production and ability to bind to polystyrene. Of 178 hGISA isolates, 85% (151/178) belonged to *agr* group I whilst 1% (2/178) and 14% (25/178) belonged to *agr* groups II and III, respectively. However, among 176 MR-GSSA isolates, the majority (84%; 148/176) also belonged to *agr* group I whilst 3.5% (6/176) and 12.5% (22/176) belonged to *agr* groups II and III, respectively. No isolate in this study belonged to *agr* group IV. Forty percent of hGISA isolates and 33% of MR-GSSA isolates lacked delta-haemolysin whilst 70% and 45% of hGISA and MR-GSSA isolates, respectively, were classified as strongly adherent to polystyrene. Thus, none of these traits can be used to distinguish potential hGISA isolates from MR-GSSA isolates.

As expected, no vancomycin resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) were detected among 178 hGISA isolates investigated. An evaluation of phenotypic methods to enhance detection of the resistant phenotype investigating (i) disk diffusion susceptibility testing using low concentration vancomycin and teicoplanin antimicrobial disks, (ii) vancomycin agar screening media containing 4% NaCl and (iii) beta-lactam/vancomycin interaction on vancomycin agar screening media with and without the addition of 4% NaCl, failed to detect all hGISA isolates. Vancomycin selection studies were performed whereby the ability of vancomycin to induce expression of the resistant phenotype among different MRSA strains and a GSSA control strain (*S. aureus* ATCC 29213) was investigated. Results showed that hetero-resistant phenotypes were selected for each isolate investigated and when passaged on drug-free medium for nine days the hetero-resistant phenotype remained stable. Despite the volume of information yielded by the present study, to date there is still no simple phenotypic or genotypic technique available to confirm hetero-glycopeptide resistance in MRSA.

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Abbreviations

A*	antibiogram
ACP	achromopeptidase
agr	accessory gene regulator
AIP	autoinducing peptide
A-PFG*	antibiogram-pulsed-field-group
ARMRL	Antibiotic Resistance Monitoring and Reference Laboratory
AR*	antibiogram-resistogram
AR-PFG*	antibiogram-resistogram pulsed-field-group
ATCC	American Type Culture Collection
AUC	area under the curve
aux	auxillary gene
BHI	brain heart infusion
BHIT4	brain heart infusion agar containing 4 mg/L teicoplanin
BHIT5	brain heart infusion agar containing 5 mg/L teicoplanin
BHIT6	brain heart infusion agar containing 6 mg/L teicoplanin
BHIV2	brain heart infusion agar containing 2 mg/L vancomycin
BHIV4	brain heart infusion agar containing 4 mg/L vancomycin
BHIV5	brain heart infusion agar containing 5 mg/L vancomycin
BHIV6	brain heart infusion agar containing 6 mg/L vancomycin
BHIV8	brain heart infusion agar containing 8 mg/L vancomycin
bp	base pair
BSAC	British Society for Antimicrobial Chemotherapy
BURST	Based Upon Related Sequence Types

*The term antibiogram-resistogram (AR) are used synonymously with antibiogram (A) and for the purpose of work presented in this thesis, antibiogram will be used.

CA	community-acquired
CAMHB	cation-adjusted Mueller-Hinton Broth
CA-MRSA	community-acquired meticillin-resistant *Staphylococcus aureus*
CAPD	continuous ambulatory peritoneal dialysis
CASFM	Comité de l'Antibiogramme de la Société Française Microbiologie
CBA	Columbia agar containing 7% horse blood
CC	clonal complex
ccr	cassette chromosome recombinase
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
CFU/ml	colony forming units per millilitre
CHEF	contour-clamped homogenous electric field
CLSI	Clinical and Laboratory Standards Institute
CoNS	coagulase-negative staphylococci
DNA	deoxyribonucleic acid
EARSS	European Antimicrobial Resistance Surveillance System
EB	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
EMRSA	epidemic meticillin-resistant *Staphylococcus aureus*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
fem	factors essential for meticillin resistance
G	growth
GISA	glycopeptide-intermediate *Staphylococcus aureus*

GMRSA	gentamicin-resistant meticillin-resistant *Staphylococcus aureus*
GRD	glycopeptide resistance detection
GRSA	glycopeptide-resistant *Staphylococcus aureus*
GS	glycopeptide-susceptible
GSSA	glycopeptide-susceptible *Staphylococcus aureus*
H	hospital
h	hour
HA	hospital-acquired
HA-MRSA	hospital-acquired meticillin-resistant *Staphylococcus aureus*
HCA	health-care associated
hGISA	heterogeneous glycopeptide-intermediate *Staphylococcus aureus*
hTISA	heterogeneous teicoplanin-intermediate *Staphylococcus aureus*
hVISA	heterogeneous vancomycin-intermediate *Staphylococcus aureus*
IgG	immunoglobulin G
J-region	junkyard-region
kb	kilobase
L	litre
mg/L	milligram per litre
$MgCl_2$	magnesium chloride
MH	Mueller-Hinton
MHS	Mueller-Hinton agar containing 2% NaCl
MHT5	Mueller-Hinton agar containing 5 mg/L teicoplanin
MHT8	Mueller-Hinton agar containing 8 mg/L teicoplanin
MHV4	Mueller-Hinton agar containing 4 mg/L vancomycin
MHV5	Mueller-Hinton agar containing 5 mg/L vancomycin
MIC	minimum inhibitory concentration

Min	minute
ml	millilitre
MLST	multilocus sequence typing
mm	millimetre
MR-	meticillin-resistant
MR-GSSA	meticillin-resistant glycopeptide-susceptible *Staphylococcus aureus*
MRSA	meticillin-resistant *Staphylococcus aureus*
MRSE	meticillin-resistant *Staphylococcus epidermidis*
MSSA	meticillin-susceptible *Staphylococcus aureus*
NaCl	sodium chloride (salt)
NAG	N-acetyl glucosamine
NAMA	N-acetyl muramic acid
NCCLS	National Committee for Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
NG	no growth
nm	nanometre
NMRSARL	National MRSA Reference Laboratory
NPV	negative predictive value
NT	"no type"
NUT	nutrient agar Tween
ORF	open reading frame
PAP	population analysis profile
PAP-AUC	population analysis profile-area under the curve ratio
PBP	penicillin binding protein
PCR	polymerase chain reaction
PFG	pulsed-field-group
PFGE	pulsed-field gel electrophoresis
PFT	pulsed-field type
pmol	picomole

PPV	positive predictive value
PVL	Panton-Valentine leucocidin
PNSG	poly-*N*-succinyl β-1-6 glucosamine
Q	quarter
QC	quality control
RBC	red blood cell
RE	restriction endonuclease
RFLP	restriction fragment length polymorphism
RO	reverse osmosis
rpm	revolutions per minute
RT	room temperature
s	second
sar	staphylococcal accessory gene regulator
SARI	Strategy for the Control of Antimicrobial Resistance in Ireland
SCC*mec*	staphylococcal cassette chromosome *mec*
spa	*S. aureus* Protein A gene
SSTI	skin and soft tissue infection
ST	sequence type
Stat	stationary phase broth subculture
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TISA	teicoplanin-intermediate *Staphylococcus aureus*
TN	Tris-NaCl
tPAP	teicoplanin population analysis profile
TSB	tryptic soy broth
TSSA	teicoplanin-susceptible *S. aureus*
TSST–1	toxic shock syndrome toxin 1
U	units

µg	microgram
µl	microlitre
UK	United Kingdom
Unf	unfamiliar
UP	ultra pure
UPGMA	unweighted pair group method using arithmetic averages
USA	United States of America
UV	ultra-violet
V	volts
VISA	vancomycin-intermediate *Staphylococcus aureus*
vPAP	vancomycin population analysis profile
vPAP-AUC	vancomycin population analysis profile-area under the curve ratio
VRE	vancomycin-resistant enterococci
VRSA	vancomycin-resistant *Staphylococcus aureus*
VSSA	vancomycin-susceptible *Staphylococcus aureus*
w/v	weight per volume

CHAPTER I

General Introduction

Chapter I General Introduction

1.1 *Staphylococcus aureus*

Staphylococci were first described in 1881 by Ogston, who observed grape-like clusters of bacteria in pus from human abscesses. In 1884, Rosenbach successfully isolated staphylococci in pure culture and due to the golden-yellow pigment of these colonies named the species *Staphylococcus aureus*. Rosenbach showed that this "golden coccus" was responsible for severe wound infections and furunculosis. In the pre-antibiotic era, *S. aureus* was a serious life-threatening pathogen and is a major cause of both hospital and community-acquired infection (Sheagren, 1984a). Approximately 40% of healthy adults are colonized with *S. aureus* and 10% to 20% are persistently colonized (Lowy, 1998).

S. aureus is responsible for a variety of suppurative infections and toxin-mediated diseases in humans. It causes superficial skin lesions such as boils, styes and furunculosis; more serious infections such as mastitis and urinary tract infections; deep tissue infection and invasive disease such as osteomyelitis, endocarditis, pneumonia and meningitis. *S. aureus* is a major cause of hospital-acquired (HA) infection of surgical wounds and infection associated with indwelling medical devices. *S. aureus* causes food poisoning by releasing enterotoxins into food and toxic shock syndrome by the release of superantigens into the bloodstream (Parker, 1983).

Characteristically, staphylococci are gram-positive cocci arranged in grape-like clusters. They are non-motile, non-sporing, non-flagellated, catalase-positive, oxidase-negative facultative anaerobes but in the presence of oxygen, their growth is improved. Colonies of *S. aureus* may appear spherical or ovoid, are golden-yellow in colour and the defining characteristic of this organism is its ability to produce coagulase (Parker, 1983).

1.1.1 Gram-Positive Cell Wall

The staphylococcal cell wall is a rigid matrix consisting of peptidoglycan and ribitol teichoic acid and, in many strains, a polysaccharide capsule covers this complex structure (Sheagren, 1984a). Approximately 90% of the cell wall is constructed of numerous inter-connecting layers of peptidoglycan that consist of a series of murein monomers, each of which has a D-alanyl-D-alanine residue. At least 20 layers of peptidoglycan are stacked together to form the call wall in *S. aureus* (Hiramatsu, 2001).

The monomeric component of peptidoglycan is the murein monomer, which consists of two amino sugars {N-acetyl muramic acid (NAMA) and N-acetyl glucosamine (NAG)} and 10 amino acids. The murein monomer precursor (NAMA and five stem peptides) is synthesised in the cytoplasm, transferred to the outer surface of the cytoplasmic membrane by a lipid carrier during which NAG and five glycine residues are added to form a mature murein monomer. In the cytoplasmic membrane, the mature murein monomer is attached to pre-existing peptidoglycan by the action of two enzymes, glycosyltransferase and transpeptidase. Glycosyltransferase polymerises the murein monomer to produce a nascent peptidoglycan chain. This newly formed peptidoglycan chain is then linked to the pre-existing peptidoglycan layers of the cell wall by the action of transpeptidase {also called penicillin-binding protein (PBP)} during which PBP cleaves in between the D-alanyl-D-alanine residues of the stem peptide and ligates the penultimate D-alanine to the pentaglycine of the neighbouring peptidoglycan chain forming an inter-peptide bridge (Hiramatsu, 2001).

1.1.2 Antibiotic-Resistant S. aureus

The introduction of penicillin for therapeutic use in 1941 marked a historic breakthrough in the treatment of severe *S. aureus* infections. However, many strains developed resistance due to the production of a penicillinase enzyme that degraded the beta-lactam ring of penicillin (LePage *et al.*, 1946; Barber, 1961). By the late 1940s penicillin-resistant *S. aureus* strains were prevalent in hospitals and the rise in prevalence rates throughout the forties was attributed to the spread of penicillinase-producing strains of *S. aureus* by cross-infection (Parker, 1984).

During the 1950s, antibiotic resistance among *S. aureus* strains developed rapidly in hospital environments thus this decade became synonymous with the rise of the "Hospital *Staphylococcus*" (Shanson, 1981). Prevalence rates of both virulent penicillinase-producing staphylococcal strains and multiple-antibiotic resistant strains of *S. aureus* increased and although several new antibiotics such as tetracycline, erythromycin, chloramphenicol and novobiocin were introduced during this decade, isolates with resistance to most of these agents were detected (Shanson, 1981).

The emergence of penicillin-resistant *S. aureus* and its ability to develop resistance to each new antibiotic introduced in the 1950s prompted the development of a new class of penicillins that were specifically targeted against penicillinase-producing strains. In 1959, the semisynthetic penicillinase-stable antibiotic meticillin was developed and cloxacillin and flucloxacillin became the drugs of choice for anti-staphylococcal therapy during the 1960s (Shanson, 1981; Livermore, 2000).

1.2 Meticillin-Resistant *Staphylococcus aureus* (MRSA)

1.2.1 Emergence of MRSA

The introduction of penicillinase-stable penicillins and their subsequent analogues in the 1960s in addition to improved infection control practices in hospitals, resulted in a gradual decline in the prevalence of multiple-antibiotic resistant *S. aureus* strains (Shanson, 1981). However, shortly after meticillin was introduced, the first meticillin-resistant *S. aureus* (MRSA) strain was identified in Britain (Jevons, 1961). Nevertheless, by the mid-1960s, beta-lactam antibiotics were clinically effective in the treatment of staphylococcal infections in hospitals (Shanson, 1981). However, towards the end of the 1960s, reports of MRSA causing severe blood-stream infections began to emerge from European countries (Keane, 1992). At this time MRSA was causing severe clinical problems in Europe but in the United States of America (USA) major clinical problems associated with MRSA did not appear until the 1970s (Shanson, 1992).

1.2.2 Hospital-Acquired MRSA

Throughout the 1960s, MRSA gradually disseminated in hospitals and waves of epidemic strains spread widely causing serious hospital infections worldwide (Lowy, 2003). The 1970s was marked by the emergence of the first gentamicin-resistant strains of *S. aureus* (Speller *et al.*, 1976). *S. aureus* strains resistant to both gentamicin and meticillin were first reported in 1976 and caused outbreaks of hospital infection during the late 1970s and 1980s in the United Kingdom (UK), Ireland and the USA (Shanson, 1981). Several epidemic strains were responsible for hospital outbreaks in the 1980s when a worldwide increase in the prevalence of MRSA was documented (Townsend *et al.*, 1987). Vancomycin became the drug of choice for treating serious life-threatening infections caused by multi-drug resistant MRSA strains

(Sheagren, 1984b; Livermore, 2000). Vancomycin combination therapy (vancomycin and another antimicrobial) was also shown to be successful in treating serious MRSA infection whilst antimicrobial agents such as rifampicin, ciprofloxacin and fusidic acid when used as single agent therapy were associated with resistance (Cafferkey, 1992). The most frequently reported combinations of therapy were vancomycin/aminoglycoside, vancomycin/rifampicin, vancomycin/rifampicin/fusidic acid (Cafferkey, 1992). Newer therapies include rifampicin in combination with daptomycin, minocycline, tigecycline and linezolid (Raad *et al.*, 2007).

In the UK, MRSA strains that spread to two or more patients at two or more hospitals were termed epidemic MRSA (EMRSA) (Livermore, 2000). The first EMRSA strains, EMRSA-1 and EMRSA-2 were recognised in the late 1980s and were soon followed by 12 other EMRSA strains (Marples & Cooke, 1985; Richardson & Marples, 1988; Kerr *et al.*, 1990). EMRSA-15 and EMRSA-16 emerged in the 1990s and rapidly displaced many of the older EMRSA strains. These strains have now spread to and are endemic in many hospitals worldwide (Witte *et al.*, 2001; O'Neill *et al.*, 2001). Another new multi-antibiotic resistant epidemic strain of MRSA (EMRSA-17) was reported in the UK in 2002 (Aucken *et al.*, 2002). EMRSA-17 is resistant to more antibiotics than any previously described EMRSA strains. In addition, borderline resistance to teicoplanin has also been noted in this strain (Aucken *et al.*, 2002).

Although a steady increase in the prevalence of MRSA was noted throughout the 1980s, a report in 1999 suggested that the overall prevalence of *S. aureus* infections had not increased but the percentage of MRSA amongst *S. aureus* isolates had risen dramatically in many countries (Hryniewicz, 1999). A study by Voss *et al.* in 1994 reported that among *S. aureus* isolates, MRSA prevalence rates were less than 1% in northern Europe and more than 30% in

southern/western parts of Europe, respectively (Voss *et al.*, 1994). In the past decade, the prevalence of MRSA has further increased and represents a major cause of nosocomial infections worldwide (Appelbaum, 2006). In 2007, the prevalence of MRSA among blood culture isolates across Europe varied from less than 1% in northern Europe to greater than 40% in southern and western parts of Europe (shown in Figure 1.1). Reports from the USA, Asia and Australia also documented an increase in the incidence of MRSA (Appelbaum, 2006).

What was feared for decades finally became reality in 1997 when the first clinical isolate of *S. aureus* with reduced susceptibility to vancomycin was reported in Japan (Hiramatsu *et al.*, 1997b). Isolates of MRSA with reduced susceptibility to vancomycin have since been reported from around the world (Walsh & Howe, 2002). In 2002, the first vancomycin-resistant *S. aureus* (VRSA) was recovered in the USA (Sievert *et al.*, 2002). Reduced susceptibility to glycopeptides among *S. aureus* isolates is discussed in detail in Section 1.7.2.

Recently, a decrease in the incidence and prevalence of MRSA has been reported in Europe (Monnet & Kristinsson, 2008). Quinupristin/dalfopristin, linezolid, daptomycin and tigecycline are new antimicrobial agents shown to have *in vitro* activity for MRSA and to be superior to vancomycin for treating VRSA (Lentino *et al.*, 2008). New glycopeptides under study with superior pharmacodynamic properties compared to vancomycin include dalbavancin, telavancin and oritavancin (Lentino *et al.*, 2008). Whilst the number of effective antibiotics against MRSA has increased, reports of resistance and clinical failures associated with newer antimicrobials have been documented (Menichetti, 2005). Resistance to linezolid (the first in a new class of antimicrobials, the oxazolidinones) among MRSA isolates has been reported from both the USA and the UK (Peeters & Sarria, 2005; Potoski *et al.*, 2002;

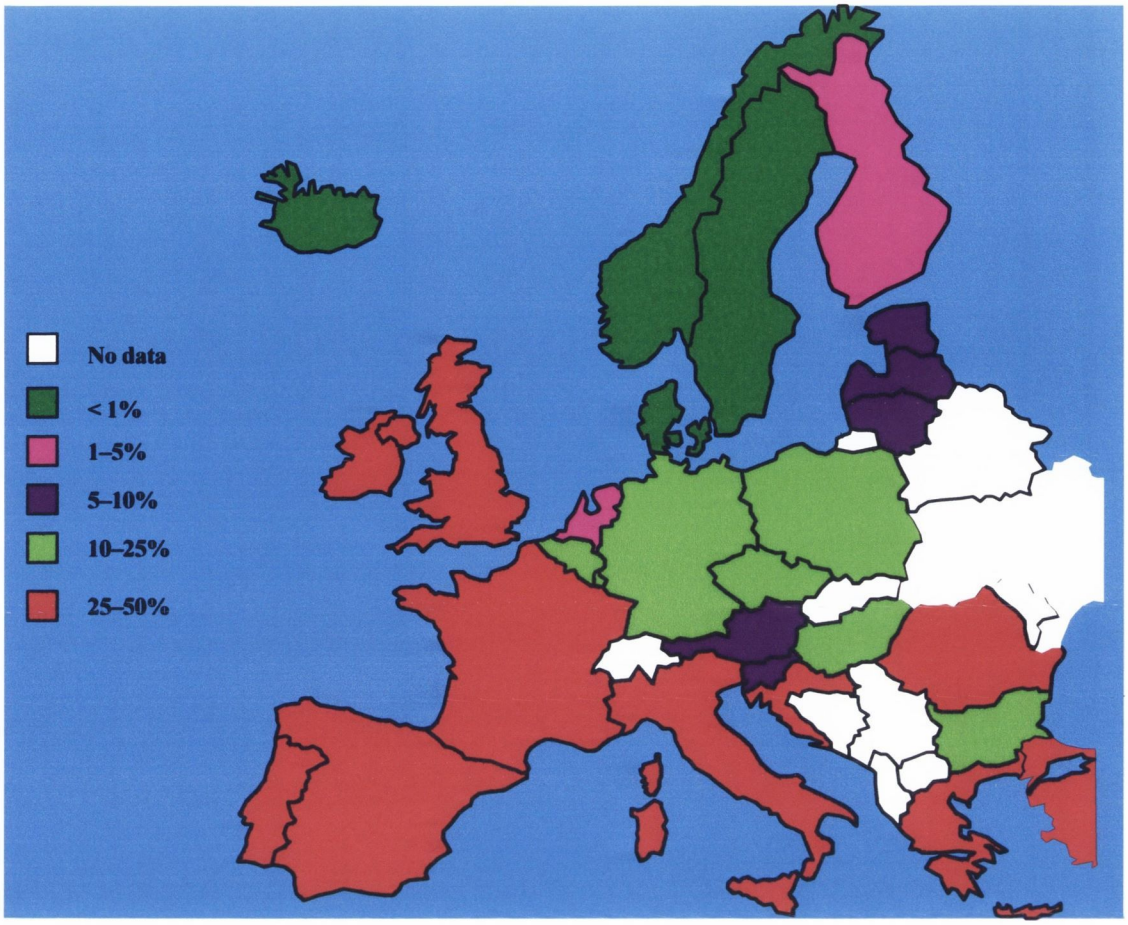


Figure 1.1. Proportion of MRSA among invasive *S. aureus* isolates across Europe in 2007.

Tsiodras *et al.*, 2001; Wilson *et al.*, 2003). Shortly after the introduction of quinupristin-dalfopristin (a streptogramin antibiotic) into clinical medicine, resistance among staphylococcal isolates was reported from France (Haroche *et al.*, 2003). Quinupristin-dalfopristin resistance has also been identified among *S. aureus* isolates exhibiting reduced susceptibility to glycopeptides (Werner *et al.*, 2001). Daptomycin, a cyclic lipopeptide antibiotic with rapid bactericidal activity against Gram-positive pathogens including vancomycin-resistant enterococci (VRE) and MRSA is reported to be a promising therapeutic option for treating serious staphylococcal infections (Steenbergen *et al.*, 2005). Daptomycin exerts its bactericidal effect by causing a calcium ion-dependent disruption of bacterial cell membrane potential resulting in an efflux of potassium, which inhibits RNA, DNA and protein synthesis (Lentino *et al.*, 2008). Since daptomycin is a large molecule (molecular weight, 1,620.67) comparable to vancomycin (molecular weight, 1,485.7), an association between reduced daptomycin susceptibility and *S. aureus* isolates exhibiting reduced susceptibility to glycopeptides has been reported (Cui *et al.*, 2006). Daptomycin treatment failure due to the development of daptomycin resistance in MRSA has also been documented (Mangili *et al.*, 2005). Thus, there are limited therapeutic options for treating serious *S. aureus* infections and in the face of increasing resistance, there is an urgent need for newer antibiotics.

1.2.3 Community-Acquired MRSA

Historically, MRSA has been considered to be a nosocomial problem but in recent years there has been an increase in the number of reports of community-acquired MRSA (CA-MRSA) infection among healthy individuals who have no recognisable health-care associated (HCA) risk factors. HCA risk factors include recent hospitalisation or surgery, indwelling medical devices and catheters, dialysis and residence in a long-term-care facility (Vandenesch *et al.*,

2003). CA-MRSA first gained widespread attention in 1999 following the deaths of four previously healthy children from necrotising pneumonia caused by CA-MRSA in Minnesota and North Dakota (Anon., 1999). Infections caused by CA-MRSA strains have now been reported worldwide and are a growing public health concern (Vandenesch *et al.*, 2003).

Reported prevalence rates of CA-MRSA infections vary widely due to the different definitions used to distinguish between CA-MRSA and hospital-acquired MRSA (HA-MRSA) (Kluytmans-VandenBergh & Kluytmans, 2006). Earlier studies of CA-MRSA strains used time-based definitions to distinguish between CA and HA-MRSA infections. An isolate was classified as CA if it was identified within the first 48–72 hours of hospital admission (Garner *et al.*, 1988; Salgado *et al.*, 2003). Other workers classified strains as CA if no HCA risk factors were identified (Herold *et al.*, 1998; Fridkin *et al.*, 2005; Folden *et al.*, 2005).

Whilst there may be advantages in distinguishing between CA and HA-MRSA infections there are some limitations in defining an isolate as CA or HA-MRSA. Implementing strategies to control MRSA may be markedly affected by the criteria used to determine CA-MRSA and HA-MRSA (Folden *et al.*, 2005). Few reports examining preventative strategies to control CA-MRSA transmission have been documented (Navarro *et al.*, 2008). As HA-MRSA rates are decreasing across Europe, CA-MRSA is now spreading to many parts of the world with varying prevalence rates and the spread to healthcare settings is an emerging problem (Navarro *et al.*, 2008). In two previous studies, discrepancies in determining the prevalence of CA-MRSA were identified due to the different definitions used to describe CA- and HA-MRSA isolates (Folden *et al.*, 2005; Salgado *et al.*, 2003).

Several studies have shown that CA-MRSA strains possess specific features which distinguish them from HA-MRSA strains (Vandenesch *et al.*, 2003; Diep *et al.*, 2004; Carleton *et al.*, 2004). Most CA-MRSA strains harbor the *lukF-PV* and *lukS-PV* determinants, which encode the Panton-Valentine leucocidin (PVL), a pore-forming toxin that damages peripheral mononuclear cells by necrosis or apoptosis. CA-MRSA strains tend to be susceptible to most antimicrobial agents other than beta-lactams and tend not to be related to genotypes that are endemic in hospitals (Vandenesch *et al.*, 2003; Diep *et al.*, 2004; Carleton *et al.*, 2004; Etienne, 2005; Boyle-Vavra & Daum, 2007).

CA-MRSA mainly cause skin and soft tissue infections (SSTI), but are also recovered from more severe infections such as necrotising pneumonia and necrotising fasciitis (Lina *et al.*, 1999; Miller *et al.*, 2005; Kluytmans-VandenBergh & Kluytmans, 2006). PVL has been postulated as one of the key virulence determinants in these CA-MRSA infections, although recent studies have reported that not all CA-MRSA strains carry *pvl* genes, thus PVL may not be critical for the pathogenesis of CA-MRSA infections (Said-Salim *et al.*, 2005; Diep *et al.*, 2006; Etienne, 2005; Vandenesch *et al.*, 2003; Rossney *et al.*, 2007).

1.2.4 MRSA in Animals

With the ever-increasing rise in prevalence rates of MRSA in healthcare settings and in the community, a recent worrying development has been the isolation of MRSA from animals, in particular companion animals. MRSA was first reported in animals in 1972 (Devriese *et al.*, 1972). In 1975, 68 MRSA isolates recovered from milk samples from cows with mastitis in 20 Belgian dairy herds were reported, and it was suggested that the isolates originated from a single human source (Devriese & Hommez, 1975). Over the past three decades occasional

reports of MRSA from animals were documented, but in recent years, the numbers of reports have increased (Devriese & Hommez, 1975; Hartmann *et al.*, 1997; Tomlin *et al.*, 1999; Lee, 2003; Goni *et al.*, 2004; Seguin *et al.*, 1999; O'Mahony *et al.*, 2005; Strommenger *et al.*, 2006; Rich, 2005; Rich & Roberts, 2004; Weese, 2004).

The risk of cross-transmission of MRSA between pets and humans is curious. A recent report documented the transmission of a PVL-positive MRSA strain between humans and a dog (van Duijkeren *et al.*, 2005). A study in Canada documented the transmission of MRSA between horses and horse personnel (Weese *et al.*, 2005). In the Netherlands where the prevalence of MRSA among clinical isolates is <1%, a high prevalence rate of MRSA among pigs and pig farmers has been reported (Voss *et al.*, 2005; Armand-Lefevre *et al.*, 2005; Huijsdens *et al.*, 2006). In one study, the prevalence of MRSA among a group of 26 regional pig farmers was 760 times greater than among the general Dutch population (Voss *et al.*, 2005). In the past, aggressive infection control measures have been successful in eradicating MRSA infections in hospital environments and therefore, continued vigilance and systematic studies in veterinary settings are needed to prevent MRSA in animals becoming an endemic problem.

1.3 Mechanisms of Meticillin Resistance

1.3.1 Penicillin Binding Proteins

Beta-lactam antibiotics exert their antimicrobial activity by binding to the enzymatic site of PBPs, disrupting cell wall synthesis and thus inhibiting growth (Waxman & Strominger, 1983). Transpeptidases or PBPs are essential cell-membrane-bound proteins that catalyse the crosslinking of the peptidoglycan network during the final stages of peptidoglycan biosynthesis (Georgopapadakou *et al.*, 1982). PBPs were first demonstrated in 1973 by

labelling with radioactive penicillin G and thus were called PBPs (Blumberg & Strominger, 1974; Nguyen-Disteche *et al.*, 1998). *S. aureus* strains that are susceptible to beta-lactam antibiotics have four PBPs. MRSA strains produce an extra PBP, PBP2a or PBP2' which has decreased binding affinity for beta-lactam antibiotics (Hartman & Tomasz, 1984; Reynolds & Brown, 1985). In MRSA strains, when the essential PBPs have been inactivated by beta-lactams, PBP2a continues to function and can perform all the PBP reactions necessary for cell wall assembly (Reynolds & Brown, 1985).

1.3.2 Staphylococcal Cassette Chromosome mec

Meticillin resistance develops when meticillin-susceptible *S. aureus* (MSSA) acquires an additional fragment of DNA called the staphylococcal cassette chromosome *mec* (SCC*mec*), which carries the gene encoding meticillin resistance, *mecA* (Hiramatsu, 2001). SCC*mec* is a genomic island of approximately 30 to 50 kilobase pairs (kb) that integrates in the *S. aureus* chromosome at a unique attachement site (*attBscc*) close to the origin of replication. The *attBscc* site is located in an open reading frame (ORF) of unknown function designated *orfX*. Integration occurs within the 3' end of *orfX* while preserving the integrity of the ORF (Ito *et al.*, 1999).

The SCC*mec* element contains the *mec* gene complex and the cassette chromosome recombinase (*ccr*) gene complex. The *mec* gene complex consists of the *mecA* gene encoding PBP2a and the *mecA* regulatory genes, *mecI* and *mecR1*. Five classes of the *mec* gene complex (A-E) have been described in staphylococcal species (Katayama *et al.*, 2001; Lim *et al.*, 2003). The *mec* classes A, B, C and E have been previously identified in MRSA isolates (Ito *et al.*, 2004; Katayama *et al.*, 2001; Lim *et al.*, 2003).

The *ccr* gene complex contains three site-specific recombinase genes, *ccrA* and *ccrB* in combination (*ccrAB*) or *ccrC* alone, which are responsible for the mobility of SCC*mec* (Katayama *et al.*, 2000; Ito *et al.*, 2001; Ito *et al.*, 2004). Five allotypes of the *ccr* gene complex have been described and named *ccrAB1* (type 1), *ccrAB2* (type 2), *ccrAB3* (type 3), *ccrAB4* (type 4) and *ccrC* (type 5) (Ito *et al.*, 2001; Ito *et al.*, 2004). The region bordering the *mec* and *ccr* complexes are designated the junkyard-region (J-region) because they contain genes that are non-essential components of SCC*mec* (Ito *et al.*, 2003). The J-region of SCC*mec* is divided into three regions, J1 to J3. The J1 region ranges from the chromosome right junction to the *ccr* genes while the J2 region spans the region from the *ccr* genes to the *mec* gene complex. The J3 region is located between the *mec* gene complex and the left extremity of SCC*mec* (Deurenberg *et al.*, 2007; Chongtrakool *et al.*, 2006).

At least six structurally different SCC*mec* types (SCC*mec* I-VI) have been identified in *S. aureus* (Deurenberg *et al.*, 2007; Oliviera *et al.*, 2006; Shore *et al.*, 2008). Three additional new types and numerous variants have also been reported (Shore *et al.*, 2008). A schematic diagram of SCC*mec* types identified in *S. aureus* is shown in Figure 1.2. All share the same chromosomal integration site, characteristic nucleotide sequences at the chromosome-SCC*mec* junction, the presence of *ccr* genes and a conserved genetic organisation around the *mec* gene complex. Each SCC*mec* type consists of a unique combination of the *ccr* and *mec* gene complexes and variants of each SCC*mec* type are defined by the J-regions (Deurenberg *et al.*, 2007; Oliviera *et al.*, 2006).

Although significant advances have been made in understanding the SCC*mec* element, assigning a universal nomenclature to SCC*mec* types has remained problematic. Whilst one

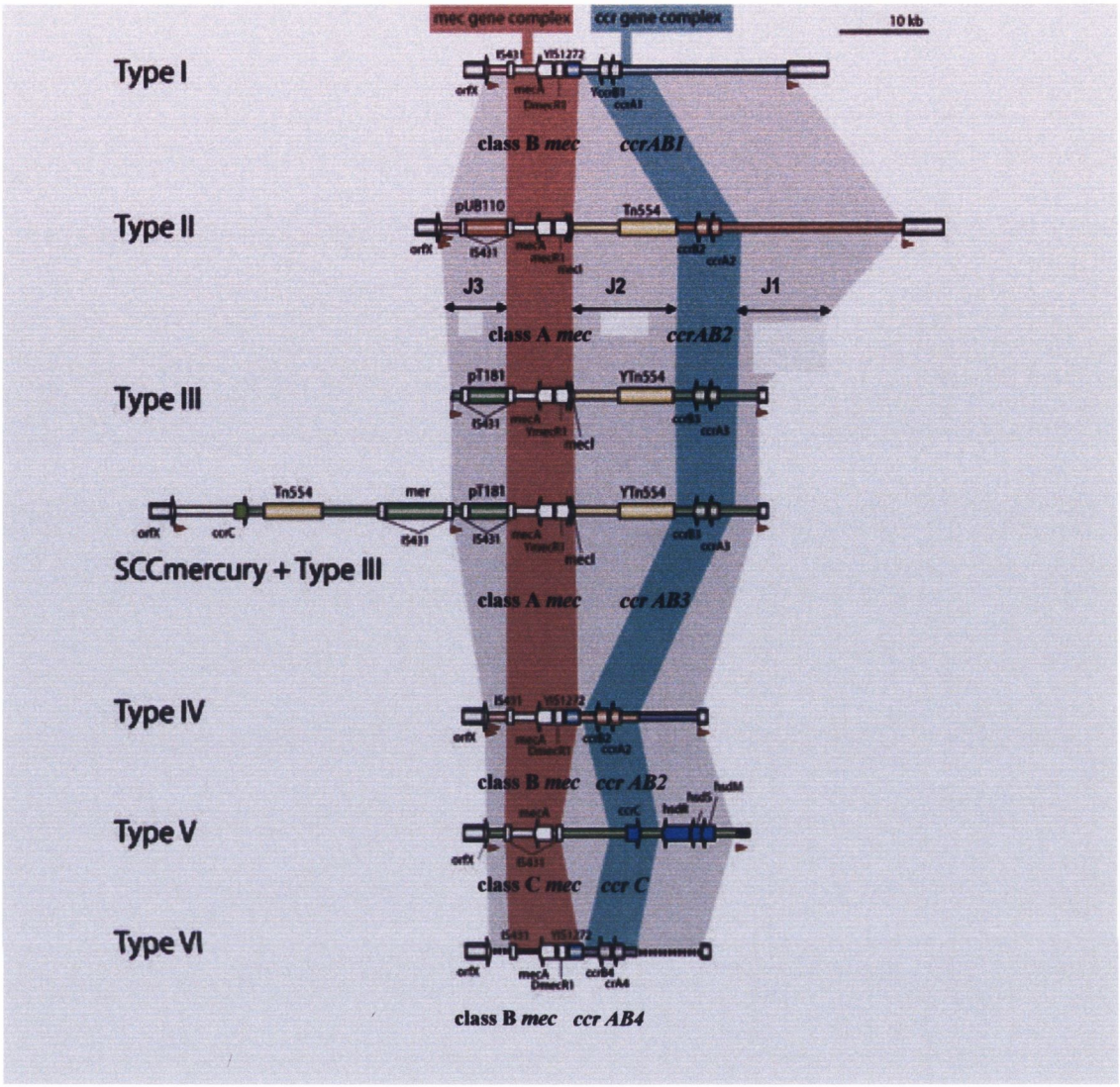


Figure 1.2. Structure of SCC*mec* types identified in *S. aureus*.

Each SCC*mec* type consists of a unique combination of the *ccr* and *mec* gene complexes and variants of each SCC*mec* type are defined by the junkyard-region (J-region).

SCC*mec* type I: type-1 *ccr* carrying *ccrAB1* and class B *mec*;

SCC*mec* type II: type-2 *ccr* carrying *ccrAB2*and class A *mec*;

SCC*mec* type III: type-3 *ccr* carrying *ccrAB3* and class A *mec*;

SCC*mec* type IV: type-2 *ccr* carrying *ccrAB2* and class B *mec*;

SCC*mec* type V: type-5 *ccr* carrying *ccrC* and class C *mec*;

SCC*mec* type VI: type-4 *ccr* carrying *ccrAB4* and class B *mec*.

{Adapted and printed with permission from Dr. T. Ito (www.staphylococcus.net)}

group of workers assigned SCC*mec* types by determining the *ccr* and *mec* gene complexes, others used a multiplex PCR assay to identify eight loci within the J-regions (Ito *et al.*, 2001; Ito *et al.*, 2004; Oliveira & de Lencastre, 2002). Assigning nomenclature to novel and variant SCC*mec* elements has also been inconsistent. A new nomenclature system for SCC*mec* elements has been proposed which includes descriptions of the *ccr* and *mec* gene complexes and the J-regions (Chongtrakool *et al.*, 2006).

Recently, SCC*mec* type VI has been described (Oliviera *et al.*, 2006). This SCC*mec* element had been previously described as SCC*mec* type IV but with a new *ccrAB* allotype (allotype 4) (Oliveira *et al.*, 2001a). Renaming this SCC*mec* element SCC*mec* type VI or type 4B according to the nomenclature by Chongtrakool *et al.* has resolved one of the nomenclature problems of SCC*mec* (Chongtrakool *et al.*, 2006; Oliviera *et al.*, 2006).

1.3.3 Other Factors influencing Meticillin Resistance

Genetic and environmental factors independent of SCC*mec* also influence meticillin resistance expression. Factors essential for meticillin resistance (*fem*) or auxillary (*aux*) genes are necessary for peptidoglycan precursor formation in *S. aureus* (Berger-Bachi *et al.*, 1992; Chambers, 1997). The two major global regulatory systems in *S. aureus* accessory gene regulator (*agr*) and staphylococcal accessory gene regulator (*sar*) that control the expression of cell wall and extracellular proteins, also play a role in expression of meticillin resistance (Piriz Duran *et al.*, 1996). Environmental factors such as temperature, osmolarity, availability of divalent cations and composition of growth medium influence the expression of meticillin resistance (Berger-Bachi & Rohrer, 2002). The inclusion of salt {sodium chloride (NaCl)} in growth medium and lowering the temperature of incubation have been incorporated into

routine susceptibility testing protocols to increase the detection of meticillin resistance among *S. aureus* isolates (Berger-Bachi & Rohrer, 2002).

1.4 Epidemiological Typing of MRSA

MRSA is associated with increased morbidity, mortality and length of hospital stay and represents a major financial burden on healthcare services (Craven & Shapiro, 2006). Epidemiological typing plays an important role in identifying and controlling nosocomial outbreaks of MRSA. Furthermore, with the increasing numbers of reports of CA-MRSA, strain typing has become an important tool to assist in the distinction between CA- and HA-MRSA isolates (Vandenesch *et al.*, 2003). One of the requirements for effective infection control is the ability to distinguish between different isolates of MRSA. In addition to differentiating isolates during outbreak investigations, epidemiological typing is also necessary for long-term surveillance of MRSA populations and assessment of worldwide spread and evolution of MRSA strains. In recent years, several global epidemiological studies have been conducted to investigate the emergence and evolution of MRSA and have provided insights into the global evolutionary pathways of MRSA (Enright *et al.*, 2002; Howe *et al.*, 2004; Vandenesch *et al.*, 2003).

1.4.1 Phenotypic Typing Methods

Phenotypic typing methods differentiate bacterial isolates by detecting expression of physical or morphological characteristics. However, phenotypic methods are often limited in reproducibility and reliability because phenotypic properties involve gene expression, which may vary depending on either environmental influences such as changes in growth conditions and growth phase or spontaneous mutation (Tenover *et al.*, 1997).

1.4.1.1 Phage Typing

Until relatively recently, *S. aureus* isolates were traditionally characterised by their differential susceptibility to an internationally recognised panel of typing phages (Blair & Williams, 1961). For over 30 years, the Centers for Disease Control and Prevention (CDC) in the USA used phage typing (a World Health Organization method) to distinguish between outbreak-related strains of *S. aureus* (Bannerman *et al.*, 1995). However, in 1995, the CDC adopted molecular methods for strain typing of *S. aureus* due to limitations of the phage typing technique. Limitations include the high proportion of non-typeable isolates, the necessity for the maintenance of stocks of typing phages and propagating strains and the training and expertise required. The availability of newer molecular methods provided an alternative epidemiological tool to phage typing (Bannerman *et al.*, 1995).

In the UK, the *S. aureus* Reference Service at Colindale (the *S. aureus* National Reference Laboratory for England and Wales) continues to use phage typing for epidemiological typing of MRSA and MSSA isolates (http://www.hpa.org.uk/cfi/lhcai/testing_staph.htm#phage) (Aucken *et al.*, 2002; Aucken & Westwell, 2002). Where phage typing fails to distinguish between new and epidemic MRSA strains and phage variants of epidemic MRSA strains, molecular methods are used to assist with the interpretation in epidemiological investigations (Aucken *et al.*, 2002; Aucken & Westwell, 2002; Walker *et al.*, 1999; Grady *et al.*, 1999). Studies from the UK have reported phage typing to be an invaluable and inexpensive method for typing large numbers of *S. aureus* isolates (Grady *et al.*, 1999; Wilcox *et al.*, 2000).

1.4.1.2 Antibiogram Typing

The routine antibiogram from the diagnostic laboratory can provide useful epidemiological information, if for example an isolate carries unusual resistance markers, but as an epidemiological typing method routine antimicrobial susceptibility patterns have poor discriminatory power (Tenover *et al.*, 1997). Bacteria are under constant antimicrobial selective pressure in the hospital environment. Overuse and misuse of antimicrobials has led to increased resistance among MRSA isolates. Antimicrobial resistance is often associated with mobile genetic elements such as transposons and plasmids, hence the antibiogram pattern may be unstable (Tenover *et al.*, 1997).

Antibiogram typing enhances the value of the antibiogram by determining the susceptibility patterns of isolates to a carefully selected panel of antimicrobial agents (Rossney *et al.*, 1994a; Rossney *et al.*, 1994b). In the method described by Rossney *et al.*, the antibiogram typing panel termed antibiogram-resistogram (AR) includes both clinically important antimicrobial agents and those that are not used clinically. The latter are included to minimise selective antimicrobial pressure in the hospital environment. Isolates are assigned an AR type based on the susceptibility pattern produced (Rossney *et al.*, 1994a; Rossney *et al.*, 1994b). This method of typing is a useful first-line technique in outbreak investigations but careful interpretation of antibiogram patterns and background knowledge of the local MRSA population is needed with this technique. Isolates that are epidemiologically related and genetically indistinguishable may manifest different antimicrobial susceptibility patterns due to the acquisition of new genetic material over time or the loss of plasmids (Locksley *et al.*, 1982). Similarly, unrelated isolates with indistinguishable resistant profiles may represent isolates that have acquired the same plasmid (Tenover *et al.*, 1997). Background knowledge

of the local MRSA population aids with interpretation of susceptibility patterns as it minimises the problems caused by the loss of labile resistant determinants (Tenover *et al.*, 1997).

1.4.2 Genotypic Typing Methods

Traditional phenotypic methods of isolate differentiation have been replaced by or supplemented with newer molecular methods in many laboratories. A study in 1992 found DNA-based techniques were the most effective typing methods in grouping outbreak-related strains of *S. aureus* (Tenover *et al.*, 1994). Molecular typing methods may be used to investigate local outbreaks or to compare the global epidemiology of strains from different geographical areas. Global epidemiological studies require procedures that are highly discriminatory and index genetic variation that accumulates slowly (Enright *et al.*, 2002).

1.4.2.1 Plasmid Analysis

Plasmid profile analysis was the first DNA-based typing method applied to *S. aureus* (McGowan *et al.*, 1979). Plasmids are extra-chromosomal DNA elements that are present in most clinical isolates and can be identified by subjecting plasmid DNA extracts to agarose gel electrophoresis. The number and size of the fragments generated are used to determine genetic relatedness. Plasmid analysis has been used successfully in nosocomial outbreaks of infection (Tenover *et al.*, 1997). But, because plasmids are not essential for host survival and can be gained or lost spontaneously, several studies have reported problems with reproducibility and the discriminatory power of this typing method (Goering, 2000). When determining clonality among isolates, careful interpretation of plasmid profiles and background knowledge of the local MRSA population is required due to the unstable nature of

plasmids. Unrelated isolates with indistinguishable plasmid profiles may represent isolates that have acquired the same plasmid (Tenover *et al.*, 1997).

*1.4.2.2 **Restriction Fragment Length Polymorphism (RFLP) Analysis***

Restriction digestion of chromosomal DNA has been at the core of several genotypic typing methods described. In this method, double-stranded chromosomal DNA is digested at specific recognition sites with restriction endonucleases and different types of gel electrophoresis are used to determine the number and size of the DNA fragments generated. Variations in the size and the number of fragments detected are referred to as restriction fragment length polymorphisms (RFLP) (Tenover *et al.*, 1997; Goering, 2000).

1.4.2.2.1 RFLP Analysis and Agarose Gel Electrophoresis

Conventional agarose gel electrophoresis uses restriction endonucleases that cut the chromosomal DNA into many fragments ranging in size from 0.5 to 50 kb in length. DNA fragments are separated by size and after electrophoresis, the gel is stained with an appropriate dye (usually ethidium bromide) and visualized under ultra-violet (UV) light (Tenover *et al.*, 1997).

1.4.2.2.2 RFLP Analysis using DNA Probes

Whilst agarose gel electrophoresis separates restriction fragments by size, Southern blot analysis detects the presence of DNA sequences in DNA samples. In this method, restriction fragments are separated by agarose gel electrophoresis, transferred (or blotted) onto a nitrocellulose membrane and hybridised with a labelled sequence of homologous DNA (a

probe). The probe DNA is either labelled by incorporating radioactivity or by tagging the molecule with a fluorescent or chromogenic dye. The specific sequences of the restriction fragments are detected when the probe binds to the complementary DNA sequence of the restriction fragments (Tenover *et al.*, 1997; Goering, 2000).

Ribosomal operons (ribotyping), insertion sequences (insertion sequence profiling) and multiple genetic loci have been used as probes for typing *S. aureus* isolates (Richardson *et al.*, 1994; Symms *et al.*, 1998; Tenover *et al.*, 1994). Apart from ribotyping, *S. aureus* typing methods utilising Southern blot analysis are not widely used due to their poor discriminatory power and lack of reproducibility (Tenover *et al.*, 1994).

1.4.2.3 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms (Schwartz & Cantor, 1984). Subsequently PFGE proved to be a highly effective molecular typing technique for bacterial species (Goering, 2000). Due to its excellent discriminatory power, typeability and reproducibility, along with its relative ease of use and interpretation, PFGE rapidly displaced other genotypic typing methods and became the typing method of choice for many species including *S. aureus* (Tenover *et al.*, 1997). In this method, the bacterial genome is digested with a restriction endonuclease (usually *Sma*I, in the case of *S. aureus*) which cleaves DNA infrequently and generates approximately 10 to 30 large fragments. These fragments are too big to be resolved by conventional agarose gel electrophoresis. In PFGE, fragments are separated in agarose gels positioned between banks of electrodes that surround the gel. During electrophoresis, the electric field is provided in pulses that alternate between these sets of electrodes causing the

DNA to continuously change direction and thereby improve separation (Tenover *et al.*, 1997). While PFGE has excellent discriminatory power, it is both labor-intensive and difficult to standardize among different laboratories and interpretation of PFGE patterns may often be subjective (Shopsin *et al.*, 1999).

The HARMONY group (comprising 12 laboratories in 10 European Union countries whose main aim is to harmonise typing methods of nosocomial pathogens) has described a European standardised protocol for PFGE typing of MRSA isolates. This standardised protocol facilitates the interlaboratory and international comparison of MRSA isolates (Murchan *et al.*, 2003). A recent study by the HARMONY group reported that PFGE followed by *Sma*I digestion is now generally accepted as the current gold standard for typing MRSA isolates in short-term epidemiological studies (Cookson *et al.*, 2007).

Interpretation of PFGE patterns can be performed by visualising banding patterns and applying the criteria described by Tenover *et al.* (Tenover *et al.*, 1995a). Digital images of gels stored electronically can be analysed using a number of software packages such as GelCompar or BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). Final interpretation requires visual inspection of PFGE patterns (Tenover *et al.*, 1995a).

1.4.2.4 PCR-based Typing Methods

Several PCR based methods have been described for typing *S. aureus* isolates. These include endonuclease digestion of PCR products (PCR-RFLP), repetitive-element PCR analysis and arbitrarily-primed PCR analysis (Hookey *et al.*, 1998; Deplano *et al.*, 2000; van Belkum *et al.*, 1995).

Arbitrarily-primed PCR {also referred to as randomly amplified polymorphic DNA (RAPD)-PCR} is the most widely used PCR based method for epidemiological typing (Tenover *et al.*, 1997). Under low annealing temperatures, short primer sequences (typically 10 bp) bind at multiple random chromosomal locations and the resulting PCR products of varying sizes are separated by agarose gel electrophoresis (Tenover *et al.*, 1997). Although PCR based methods are simple, provide rapid results and are less technically demanding than PFGE, they are less discriminatory, prone to artifacts and protocols are not yet standardised for these methods (Tenover *et al.*, 1997; Goering, 2000).

1.4.2.5 Multilocus Sequence Typing

Whilst PFGE following *Sma*I digestion has proved to be invaluable in short-term epidemiological studies of MRSA isolates, it is not suitable for global and evolutionary studies of MRSA due to difficulties with reproducibility and the complexity of PFGE pattern interpretation (Cookson *et al.*, 2007). Multilocus sequence typing (MLST) provides unambiguous and portable results that are suitable for global and evolutionary studies of MRSA (Enright *et al.*, 2002).

MLST involves DNA sequencing of internal fragments {450–500 base pairs (bp)} of seven housekeeping genes. The alleles at the seven loci provide an allelic profile that defines the sequence type (ST) of each isolate. Isolates with the same allelic profile are considered clonally related. Enright *et al.* developed and validated an MLST scheme for *S. aureus* (Enright *et al.*, 2000). MLST is more suited to the investigation of bacterial phylogeny and evolution of population lineages than for typing isolates in hospital outbreaks and epidemics

because it monitors the accumulation of change that occurs relatively slowly hence profiles are stable over time (Enright *et al.*, 2002). An advantage of MLST is that sequence data along with associated epidemiological information is stored in a central database that can be accessed via the Internet (www.mlst.net). An algorithm, named BURST (Based Upon Related Sequence Types) was devised to investigate relationships between groups of isolates among large MLST datasets (Enright *et al.*, 2002). Using this algorithm, allelic profiles of MRSA lineages can be visualised as clonal complexes (CC). STs that share five out of seven loci are placed in a common CC (Enright *et al.*, 2002). Using this algorithm, five pandemic MRSA clones have been described; CC5, CC8, CC22, CC30 and CC45 (Enright, 2003).

1.4.2.6 spa Typing

Although MLST is regarded as the reference method for long-term epidemiological studies of MRSA, the high cost of DNA sequencing is prohibitive for many laboratories. An alternative DNA sequencing technique is *spa* typing which is based on determining DNA sequence analysis of the chromosomally-encoded polymorphic X-region of the *S. aureus* protein A (*spa*) gene. The DNA sequence of the X-region of the *spa* gene is sufficiently stable for epidemiological typing but genetic polymorphisms in this region can be used to define epidemiological relatedness among MRSA isolates (Frenay *et al.*, 1996; Shopsin *et al.*, 1999). Although *spa* typing is less discriminatory than PFGE, it has several advantages in terms of ease of use, ease of interpretation, speed, standardisation and data management and dissemination (Shopsin *et al.*, 1999). It has been reported that the cost of sequencing compares favourably to that of PFGE and sequence data can be easily compared between laboratories using the *spa* sequence database accessed via the Internet (http://www.SpaServer.ridom.de) (Aires de Sousa *et al.*, 2006). In November 2004,

SeqNet.org, a European laboratory network for sequence-based typing of microbial pathogens, including *S. aureus* was founded (Friedrich *et al.*, 2006). The main objective of this network for *S. aureus* is to standardise the *spa* typing technique to ensure *spa* sequence data are easily compared between laboratories both nationally and internationally (Friedrich *et al.*, 2006). In a recent European evaluation of molecular typing methods for typing MRSA isolates, *spa* typing compared favorably with PFGE and MLST (Cookson *et al.*, 2007).

1.4.2.7 SCCmec Typing

It has been suggested that the complete characterisation of MRSA requires identification of the structural types of the *mec* element, SCC*mec* (Oliveira & de Lencastre, 2002). The six SCC*mec* types I-VI described to date in MRSA each carry a characteristic combination of *ccr* and *mec* genes as well as structural variations in other regions (Deurenberg *et al.*, 2007; Oliviera *et al.*, 2006). These differences can be exploited by amplifying the sequences that define each SCC*mec* type in order to identify the structural type of SCC*mec* in different MRSA isolates. Recently, a novel combination of multiplex PCR assays has been described for rapid identification of both *ccr* and *mec* genes as well as major differences in the J-regions (Kondo *et al.*, 2007).

1.4.2.8 DNA Microarrays

A DNA microarray is a collection of microscopic DNA spots, each representing target DNA regions attached to a solid surface such as glass, plastic or silicon chip. Each microarray comprises of thousands of attached DNA segments (or probes) placed in known locations. Microarray analysis involves labelling the target DNA with a fluorophore and hybridising the labelled DNA to the microarray. One of the applications of DNA microarrays is gene

expression profiling, where the expression level of thousands of genes can be monitored simultaneously. In addition, detection of single nucleotide polymorphisms within a population and comparative genomic hybridisation studies can be performed using DNA microarray analysis.

Recent publication of whole genome sequences of *S. aureus* isolates (N315, MRSA252 and MW2) and *S. aureus* Mu50 ATCC 700699 have shown that a large number of virulence-associated genes are carried by *S. aureus*. Studies have shown that among different strains of *S. aureus*, differences in genome sequences occur (Kuroda *et al.*, 2001; Holden *et al.*, 2004; Saunders *et al.*, 2004; Baba *et al.*, 2002; Lindsay & Holden, 2004). The information provided by genome sequences has served as a useful tool to compare extensive genotypic information in determining genetic relatedness among *S. aureus* strains (Dunman *et al.*, 2004; Lindsay & Holden, 2004). Several studies have used microarray analysis to investigate the underlying mechanisms of vancomycin-intermediate and vancomycin-resistant *S. aureus* isolates (Mongodin *et al.*, 2003; McAleese *et al.*, 2006; Fox *et al.*, 2007; Scherl *et al.*, 2006). Although these techniques are very expensive and time-consuming, they play a crucial role in characterisation of isolates at the genomic level (Lindsay & Holden, 2006).

1.5 Pandemic Clones of MRSA

Previously, epidemiological typing methods defined five pandemic MRSA clones; Iberian, Brazilian, Hungarian, New York/Japan and Paediatric (Oliveira *et al.*, 2002). The names assigned to each clone reflected either the geographic area in which they were first identified or an exclusive epidemiological feature (Oliveira *et al.*, 2002). The application of MLST and SCC*mec* types for MRSA nomenclature provides a rational and unambiguous system where

each clone is named in the format ST-resistance phenotype (MRSA, MSSA)-SCC*mec* type (Robinson & Enright, 2004). An overview of the major pandemic MRSA clones is shown in Table 1.1. Strains with the same ST harbor different SCC*mec* types and different SCC*mec* types have been acquired by *S. aureus* strains with different genetic backgrounds confirming that SCC*mec* was introduced several times into different *S. aureus* lineages (Enright *et al.*, 2002).

1.6 Financial Cost of MRSA

In addition to increased morbidity and mortality, increased prevalence of MRSA over the past decade has resulted in significant additional hospital costs (Kopp *et al.*, 2004; Shorr *et al.*, 2006; Chen *et al.*, 2005; Lepelletier *et al.*, 2004). The economic impact of MRSA infection in healthcare institutions including long-term-care facilities has been comprehensively investigated. Additional hospital costs have been associated with increased length of hospital stay, increased morbidity and costs relating to pharmacy and laboratory tests (Kopp *et al.*, 2004; Shorr *et al.*, 2006; Chen *et al.*, 2005; Lepelletier *et al.*, 2004; Watters *et al.*, 2004; Capitano *et al.*, 2003). Reports from the USA have compared costs associated with bacteraemia caused by MRSA and MSSA and found that increased length of hospital stay, additional hospital costs and increased mortality were all attributable to MRSA (Cosgrove *et al.*, 2005; Abramson & Sexton, 1999; Engemann *et al.*, 2003).

Shorr *et al.* retrospectively analysed a multi-hospital USA database to investigate the clinical and economic impact of early onset ventilator-associated pneumonia due to MRSA. These workers found that MRSA patients on average, consumed excess resources and had increased length of hospital stay with attributable additional costs of approximately $8,000 per case

(Shorr *et al.*, 2006). An Irish study of patients with head and neck cancer who became infected with MRSA post-operatively, found that the average length of hospital stay was three times longer for patients with MRSA than for patients who did not have MRSA (Watters *et al.*, 2004). Additional medical costs included antibiotic treatments and extra medical and nursing care (Watters *et al.*, 2004). A report from Spain in 2006 compared the costs of a screening programme to detect MRSA with the average cost of MRSA infection per patient (€2,730) and found the cost of the programme at €10,261 would be covered if it succeeded in preventing four MRSA infections per year (Gavalda *et al.*, 2006).

However, several studies have reported that MRSA screening on admission failed to reduce rates of nosocomial MRSA infection and were therefore not cost effective (Wenzel *et al.*, 2008; Harbarth *et al.*, 2008; Khan *et al.*, 2002). One large scale screening programme using a rapid molecular screening technique found that 57% of infected patients were MRSA-free on admission and acquired the MRSA infection during hospitalisation. In addition, positive results for 31% of patients in that study were only available after surgery because of time delays in notification of results (Harbarth *et al.*, 2008).

Multiple medical comorbidities (eg. diabetes mellitus, malignancy, chronic renal impairment, haemodialysis and endocarditis) have been associated with MRSA infection resulting in increased length of hospital stay and additional hospital costs (Rogers *et al.*, 2009). Chang *et al.* demonstrated that several medical comorbidities were associated with endocarditis caused by MRSA when compared to MSSA (Chang *et al.*, 2003a). Similarly, Fowler *et al.* reported that diabetes mellitus and immunosuppressive medication were independent risk factors for MRSA endocarditis (Fowler *et al.*, 2005).

Table 1.1. Overview of the major pandemic clones of MRSA.

CC	ST	SCC*mec* type	Clone
8	239	IIIA	Brazilian
8	239	III	Hungarian
8	247	IA	Iberian
8	250	I	Archaic
8	8	II	Irish-1
8	8	IV	EMRSA-2 and -6
5	5	II	New York/Japan
5	5	IV	Paediatric
22	22	IV	EMRSA-15
30	36	II	EMRSA-16
45	45	IV	Berlin

Abbreviations: CC, clonal complex; ST, sequence type (Enright, 2003; Oliveira *et al.*, 2001; Oliveira *et al.*, 2002).

The implementation of stringent policies to control MRSA in nosocomial settings has been shown to reduce health-care costs (Coskun & Aytac, 2006). In one institution, costs were reduced by $4,000,000 over a two-year period (Hacek *et al.*, 1999).

1.7 Glycopeptide Resistance

As mentioned in Section 1.1.2, the 1950s were characterised by an alarming increase in the prevalence of penicillinase-producing *S. aureus* strains that were multi-antibiotic resistant and spread easily in hospital environments (Shanson, 1981). A large scale programme aimed at discovering antibiotics with activity against these *S. aureus* strains was initiated by Eli Lilly (Griffith, 1981). During the course of this programme, a soil sample from Borneo was sent to the laboratories of Eli Lilly where an organism (*Nocardia orientalis* previously named *Streptomyces orientalis*) produced a substance ("Compound 05865") with a high degree of bactericidal activity against penicillinase-producing *S. aureus* (Geraci *et al.*, 1956; Griffith, 1981). Early laboratory attempts to induce resistance against "Compound 05865" failed and studies showed that after 20 serial passages of staphylococci in culture media containing the drug, resistance to penicillin increased 100,000-fold compared with only a 4–8 fold increase in resistance to "Compound 05865" (McGuire *et al.*, 1955). The US Food and Drug Administration (FDA) approved "Compound 05865" for clinical use and it was subsequently given the name "vancomycin" (derived from the word "vanquish") (Johnson *et al.*, 1990).

Initial preparations of vancomycin contained a number of impurities and, because of its brown colour, was nicknamed "Mississippi Mud" (Griffith, 1981). Early studies showed vancomycin to be ototoxic and nephrotoxic, hence it was relegated to a secondary role in the treatment of severe staphylococcal infections (Woodley & Hall, 1961; Geraci & Hermans, 1983). In the 1970s, the worldwide emergence of MRSA prompted renewed clinical interest in vancomycin (Griffith, 1981). Newer, purer preparations showed no ototoxicity and little nephrotoxicity (Farber & Moellering, 1983). By the 1980s, the use of vancomycin increased and when oral formulations became available in the mid-1980s, the rate of vancomycin usage further

accelerated (Kirst *et al.*, 1998). At this time, a new glycopeptide antibiotic, teicoplanin was developed in the Lepetit Research Laboratories (Milan, Italy) by the fermentation of *Actinoplanes teichomyceticus* (Parenti *et al.*, 1978). Since the early 1980s, teicoplanin has been used worldwide except in the USA where it was never licensed (Moellering, 2006). Vancomycin became the drug of choice for treating pseudomembranous colitis (caused by *Clostridium difficile* and *S. aureus*) and infections caused by MRSA and penicillin-resistant *Streptococcus pneumoniae* (Geraci & Hermans, 1983; Kirst *et al.*, 1998). With the increased use of both vancomycin and teicoplanin, it was perhaps inevitable that resistance to these antimicrobials would occur.

1.7.1 Emergence of Glycopeptide Resistance

1.7.1.1 Coagulase-Negative Staphylococci

Until 1975, coagulase-negative staphylococci (CoNS) were often grouped together as *S. albus* or *S. epidermidis* and, based on their inability to clot plasma, were often considered to be non-pathogenic staphylococci (Kloos & Schleifer, 1975; Fleer & Verhoef, 1984). To date, there are 32 species of coagulase-negative staphylococci of which 15 have been recovered from humans and the remainder from non-human sources (e.g. dust and soil samples) (Huebner & Goldmann, 1999). Most of the staphylococci recovered from humans belong to the *S. saprophyticus species* group or the *S. epidermidis species* group which include *S. epidermidis*, *S. capitis*, *S. hominis*, *S. haemolyticus*, *S. warneri*, *S. caprae*, *S. saccharolyticus* and *S. aucicularis* (Pfaller & Herwaldt, 1988).

Because certain species of CoNS normally inhabit the skin and mucous membranes of humans, it was previously assumed by many workers that infections caused by CoNS were

derived from patient's indigenous flora (Huebner & Goldmann, 1999). However, epidemiological typing studies of CoNS showed specific strains persisted in certain units of hospitals and it was believed that strains were transmitted among hospitalised patients (Huebner & Goldmann, 1999). Common infections associated with CoNS include bacteraemia, catheter-related infections, central nervous system shunt infections, endocarditis, urinary tract infections, surgical site infections and endophthalmitis (Huebner & Goldmann, 1999). CoNS have a particular affinity for foreign materials, such as indwelling medical devices. Advances in technology in modern medicine have led to increased usage of prosthetic devices, intravascular catheters and invasive technologies in immunosuppressed patients (such as intensive care patients, premature infants and cancer and transplant patients) (Kloos & Bannerman, 1994; Huebner & Goldmann, 1999). Because most infections due to CoNS are nosocomial in origin, they have become increasingly resistant to multiple antibiotics including glycopeptide antibiotics and have resulted in increased morbidity and excessive hospital costs (Kloos & Bannerman, 1994; Huebner & Goldmann, 1999).

In the early 1980s, several studies compared the activity of vancomycin and teicoplanin against clinical isolates of *S. aureus*, *S. epidermidis* and enterococci and found that the antimicrobial activity of both compounds was comparable (Cynamon & Granato, 1982; Pallanza *et al.*, 1983; Fainstein *et al.*, 1983). One group reported that teicoplanin was not as active as vancomycin against meticillin-resistant *S. epidermidis* (MRSE) strains with minimum inhibitory concentration (MIC) values ranging from 0.1–12.5 mg/L suggesting some teicoplanin resistance among MRSE strains (Fainstein *et al.*, 1983). In 1986, the first reports of teicoplanin resistance among clinical isolates of CoNS came from the UK and the USA (Wilson *et al.*, 1986). Two reports associated teicoplanin resistance with meticillin-resistant *S. haemolyticus* and MRSE, respectively (Wilson *et al.*, 1986; Grant *et al.*, 1986). By the late

1980s, several other studies had reported teicoplanin resistance among CoNS (Arioli & Pallanza, 1987; Moore & Speller, 1988; Goldstein *et al.*, 1990).

Vancomycin resistance among CoNS was first reported in the late 1980s (Schwalbe *et al.*, 1987; Froggatt *et al.*, 1989; Schwalbe *et al.*, 1990). Vancomycin-resistant meticillin-resistant *S. haemolyticus* was isolated from a patient undergoing continuous ambulatory peritoneal dialysis (CAPD) with *S. haemolyticus* peritonitis (Schwalbe *et al.*, 1987). Studies to determine the prevalence of glycopeptide resistance among CoNS reported prevalence rates varying from <1% to 5% but one study in 1989 reported that 42% of *S. haemolyticus* strains were intermediately resistant to vancomycin (Froggatt *et al.*, 1989; Herwaldt *et al.*, 1991; Gruneberg & Hryniewicz, 1998; Felmingham *et al.*, 1998; de Neeling *et al.*, 1998; Henwood *et al.*, 2000; Santos-Sanches *et al.*, 2000; Tacconelli *et al.*, 2001; Sieradzki *et al.*, 1998). In 1998, Sieradzki *et al.* reported heterogeneous resistance profiles for teicoplanin (but not for vancomycin) among several *S. epidermidis* and *S. haemolyticus* isolates collected between 1925 and 1964 (Sieradzki *et al.*, 1998). These isolates exhibited subpopulations capable of growth on agar containing >12 mg/L teicoplanin (Sieradzki *et al.*, 1998). More recently, four vancomycin-resistant CoNS were recovered from healthy carriers (two hospital staff members and two school personnel) who had not received prior antibiotic treatment (Palazzo *et al.*, 2005). All four CoNS (*S. haemolyticus*, *S. epidermidis* and two *S. capitis* isolates) exhibited unstable resistance to vancomycin and showed variable levels of resistance to several antimicrobial agents including oxacillin (Palazzo *et al.*, 2005).

The emergence of teicoplanin resistance in CoNS before vancomycin resistance correlates with the experimental observation that teicoplanin resistance is more readily selected *in vitro*

whereas vancomycin resistance is more difficult to induce (Watanakunakorn, 1984; Watanakunakorn, 1990; Mouton & Mulders, 1987). It has been postulated that staphylococci which have been exposed to vancomycin since the early 1950s have acquired genetic mutations and have advanced towards a preliminary stage of glycopeptide resistance which is phenotypically expressed as resistance to teicoplanin but not to vancomycin. One study which supports this hypothesis was carried out in Japan where between 1994 and 1995, 26 out of 96 *S. epidermidis* isolates were found to be resistant to teicoplanin even though teicoplanin was not approved for use in Japan at that time (Hiramatsu, 1998). The mechanisms of glycopeptide resistance among CoNS have not yet been elucidated. However, the fact that some strains exhibit resistance to vancomycin, some to teicoplanin and some to both suggests different mechanisms of resistance for these two antimicrobials (Tacconelli *et al.*, 2001).

1.7.1.2 Enterococci

Enterococci have been known to be important causes of infection such as urinary tract infection and endocarditis for almost a century but since the middle to late 1970s, they have emerged as important causes of nosocomial infection (Cetinkaya *et al.*, 2000). Before the late 1980s, *Enterococcus faecalis* was the predominant enterococcal species accounting for 80 to 90% of all clinical isolates followed by *E. faecium* at 5–15% (Murray, 1990; Lewis & Zervos, 1990; Moellering, 1992). Other enterococcal species which account for approximately 5% of clinical isolates include *E. gallinarum*, *E. casseliflavus*, *E. durans*, *E. avium* and *E. raffinosis* (Cetinkaya *et al.*, 2000).

Infections caused by enterococci include bacterial endocarditis, urinary tract infections (the most common), bacteraemia, intra-abdominal and pelvic infections and SSTIs (Murray, 1990).

In many instances, infections such as bacteraemia and SSTIs are polymicrobial with enterococci being only one of the organisms involved (Murray, 1990). Although enterococci are not particularly virulent, they have become increasingly important pathogens. Their intrinsic resistance to many commonly used antibiotics has been one reason why these organisms survive in the hospital environment. In addition, their ability to acquire additional antibiotic resistance determinants by mutation or by transfer of plasmids and/or transposons has also assisted their survival in nosocomial settings (Cetinkaya *et al.*, 2000). In 1994, Jett *et al.* noted that the incidence of naturally-occurring genetic exchange mechanisms was a significant element in the pathogenicity of enterococci (Jett *et al.*, 1994).

The first published reports of clinical isolates of *E. faecalis* and *E. faecium* exhibiting resistance to vancomycin came from Europe in 1988 and later from the USA (Leclercq *et al.*, 1988; Uttley *et al.*, 1988; Sahm *et al.*, 1989; Frieden *et al.*, 1993). To date six genes encoding glycopeptide resistance in enterococci (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) have been reported (Depardieu *et al.*, 2004). Table 1.2 details the types of vancomycin resistance in enterococci. The most common type of glycopeptide resistance in enterococci is *vanA*-type resistance which is associated with high-level resistance to both vancomycin and teicoplanin (Courvalin, 2006). Isolates exhibiting *vanB*-type resistance, the second most common type, demonstrate variable levels of resistance to vancomycin but are susceptible to teicoplanin. *VanD*-type resistance is characterised by moderate levels of resistance to vancomycin and teicoplanin whereas *vanC*, *vanE* and *vanG* isolates exhibit low levels of resistance to vancomycin only (Courvalin, 2006). Unlike other *van* genes, *vanC*-type resistance is intrinsic (i.e. it is not an acquired resistance determinant) and is specific to *E. gallinarum*, *E. casseliflavus* and *E. flavescens* (Courvalin, 2006).

Between 1989 and 1993, the CDC reported a 20-fold increase in the proportion of enterococci associated with nosocomial infection that were resistant to vancomycin (Anon., 1993). In diagnostic laboratories, several automated susceptibility testing methods and disk diffusion testing methods failed to detect enterococci exhibiting low-level resistance to vancomycin (Tenover *et al.*, 1993; Tenover *et al.*, 1995b). In 1992, new breakpoints for testing enterococci against vancomycin were published by the then National Committee for Clinical Laboratory Standards (NCCLS) {now the Clinical and Laboratory Standards Institute (CLSI)} and a standardised screening method for the detection of VRE was developed (Tenover *et al.*, 1993; Swenson *et al.*, 1994; Swenson *et al.*, 1995). Between 1988 and 1998, the proportion of VRE among enterococci rose from <1% to >20% in the USA (Malathum & Murray, 1999). In the UK, the Antibiotic Resistance Monitoring and Reference Laboratory (ARMRL) received over 300 isolates of glycopeptide-resistance enterococci from 30 different hospitals between 1988 and 1993 (Woodford *et al.*, 1995). In Europe, avoparcin, a glycopeptide antibiotic growth promoter added to animal feed was first licensed for use in 1975, but several studies showed that the use of avoparcin resulted in an increase in prevalence of VRE (Klare *et al.*, 1995; Aarestrup, 1995). Following the European Union ban on the use of avoparcin in animals in 1997, prevalence of VRE in both animals and humans decreased significantly across Europe (in Germany, Italy, Denmark and the Netherlands) (van den Bogaard *et al.*, 2000). However, extensive occurrence of VRE on broiler farms and in broiler flocks after the avoparcin ban have been reported from Norway, Denmark and New Zealand (Heuer *et al.*, 2002; Borgen *et al.*, 2000a; Manson *et al.*, 2004; Borgen *et al.*, 2000b). Avoparcin has never been used in the USA and it has been suggested that likely sources of VRE infection have been travel, tourists and imported food (Wegener, 1998). Vancomycin-resistant enterococci is a worldwide problem with reports from Australia, Belgium, Canada, Denmark, Germany, Ireland, Italy,

Table 1.2. Phenotypic and genotypic properties of vancomycin resistance genes in enterococci.

	Resistance		MIC values (mg/L)		Expression	Location	Modified Target
	Type	**Level**	**Vancomycin**	**Teicoplanin**			
Acquired	*vanA*	High	64–100	16–512	Inducible	Plasmid or Chromosome	D-alanine-D-lactate
	vanB	Variable	4–1000	0.5–1.0	Inducible	Plasmid or Chromosome	D-alanine-D-lactate
	vanD	Moderate	64–128	4–64	Constitutive	Chromosome	D-alanine-D-lactate
	vanG	Low	16	0.5	Inducible	Chromosome	D-alanine-D-serine
	vanE	Low	8–32	0.5	Inducible	Chromosome	D-alanine-D-serine
Intrinsic	*vanC*	Low	2–32	0.5–1.0	Constitutive or Inducible	Chromosome	D-alanine-D-serine

Malaysia, The Netherlands, New Zealand, Spain, Sweden and the USA (Cetinkaya *et al.*, 2000; Nourse *et al.*, 1998; Lavery *et al.*, 1997).

1.7.1.3 S. aureus

With the increasing incidence of VRE in the early 1990s, many workers believed that it was only a matter of time before vancomycin resistance appeared among *S. aureus* isolates. In 1990, teicoplanin resistance among clinical isolates of *S. aureus* was reported (Kaatz *et al.*, 1990; Brunet *et al.*, 1990). Two years later, Noble and co-workers demonstrated the *in vitro* transfer of *vanA* from *E. faecalis* to *S. aureus* and, following that report, the development of vancomycin resistance in *S. aureus* was monitored carefully (Shlaes & Shlaes, 1995; Noble *et al.*, 1992). Daum *et al.* reported increases in vancomycin and teicoplanin MIC values among "derivative isolates" (selected from two susceptible clinical isolates of *S. aureus*) following serial incubation in low-level vancomycin (Daum *et al.*, 1992). These "derivative isolates" had thicker cell walls when examined using transmission electron microscopy and exhibited changes in colonial morphology when grown on solid media (Daum *et al.*, 1992). The first report of *S. aureus* with reduced susceptibility to vancomycin came from Japan in 1997 followed by reports of two clinical isolates of *S. aureus* with reduced susceptibility to vancomycin from the USA (Hiramatsu *et al.*, 1997b; Anon., 1997a; Anon., 1997b).

1.7.2 Reduced Susceptibility to Glycopeptides among S. aureus

1.7.2.1 Definitions and Nomenclature

Since *S. aureus* with reduced susceptibility to glycopeptides was first reported in Japan in 1997, there has been considerable confusion regarding the nomenclature and definitions of

these isolates due to differences in glycopeptide MIC breakpoints (Hiramatsu *et al.*, 1997b; Brown *et al.*, 2005). *S. aureus* strains with vancomycin MICs of 8 mg/L have been considered resistant or intermediate in different countries depending on the susceptibility testing method used (Hiramatsu, 2001). The clinical MIC breakpoints for *S. aureus* used by three internationally recognised susceptibility testing methods are shown in Table 1.3.

To date, three types of reduced susceptibility to glycopeptides have been reported in clinical isolates of MRSA: vancomycin-resistant *S. aureus* {(VRSA), See Section 1.7.3.3}, glycopeptide-intermediate *S. aureus* {(GISA), See Section 1.7.3.1} and hetero-glycopeptide-intermediate *S. aureus* {(hGISA), See Section 1.7.3.2}. VRSA isolates exhibit vancomycin MIC values of ≥16 mg/L (Anon., 2008b; Anon., 2008a; Anon., 2008c). GISA isolates exhibit homogeneous intermediate resistance to glycopeptides (vancomycin and/or teicoplanin) whilst hGISA isolates exhibit heterogeneous intermediate resistance to glycopeptides. hGISA isolates display vancomycin or teicoplanin MIC values below the susceptible breakpoint but possess bacterial subpopulations occurring at frequencies as low as $1/10^6$ that exhibit reduced susceptibility (Hiramatsu *et al.*, 1997a; Tenover *et al.*, 2001; Brown *et al.*, 2005). The terms GISA and hGISA have been proposed to describe isolates with reduced susceptibility to glycopeptides but such isolates may show reduced susceptibility to vancomycin (vancomycin-intermediate *S. aureus*; VISA or hetero-vancomycin-intermediate *S. aureus*; hVISA) or to teicoplanin (teicoplanin-intermediate *S. aureus*; TISA or hetero-teicoplanin-intermediate *S. aureus*; hTISA) or to both vancomycin and teicoplanin (MacKenzie *et al.*, 2002; Tenover *et al.*, 2001; Brown *et al.*, 2005). Glycopeptide-susceptible *S. aureus* (GSSA) describe isolates that exhibit MIC values within the susceptible range to both vancomycin and teicoplanin and may be referred to as vancomycin-susceptible *S. aureus*; VSSA or teicoplanin-susceptible *S. aureus*; TSSA (Tenover *et al.*, 1998; Howden *et al.*, 2006; Bukhari *et al.*, 2004).

Table 1.3. Glycopeptide MIC (mg/L) breakpoint values for *S. aureus*.

	Vancomycin			Teicoplanin		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
CLSI [a]	**≤2**	**4–8**	**≥16**	**≤8**	**16**	**≥32**
[CLSI[b]]	[≤4]	[8–16]	[≥32]	[≤8]	[16]	[≥32]
BSAC[c]	≤4	8	>8	≤4	8	>8
EUCAST[d]	≤4	8	>8	≤4	8	>8

[a], Clinical and Laboratory Standards Institute (Anon., 2008b). [b], CLSI breakpoints prior to 2006. [c], British Society for Antimicrobial Chemotherapy (Anon., 2008a). [d], European Committee on Antimicrobial Susceptibility Testing (Anon., 2008c).

According to the 2006 revision of the CLSI guidelines, isolates are considered GSSA if they have vancomycin MICs of ≤2 mg/L and/or teicoplanin MICs of ≤8 mg/L; GISA isolates exhibit vancomycin MIC values between 4 and 8 mg/L and/or teicoplanin MIC values of 16 mg/L, whilst isolates for which vancomycin MIC ≥16 mg/L are termed VRSA (Table 1.2) (Anon., 2006a).

Although current British and European breakpoint criteria define VRSA as isolates with vancomycin MICs of ≥16 mg/L, isolates exhibiting vancomycin and/or teicoplanin MICs of 8 mg/L are considered GISA whilst GSSA isolates are defined as isolates exhibiting vancomycin and/or teicoplanin MICs of ≤4 mg/L (Anon., 2008a; Anon., 2008c).

In 2005, Wootton *et al.* determined the vancomycin MICs of an international collection of VSSA, VISA and hVISA strains using CLSI agar dilution techniques (Wootton *et al.*, 2005). Results from that study found that GSSA and VISA isolates exhibited vancomycin MICs of ≤2 mg/L and 4–8 mg/L, respectively. Vancomycin MICs of hVISA isolates ranged from 1–4 mg/L, with 80% and 18% exhibiting MICs of 2 mg/L and 4 mg/L, respectively (Wootton *et al.*, 2005). Reports of treatment failures associated with infections caused by GISA and hGISA prompted the CLSI to review glycopeptide breakpoints (Ariza *et al.*, 1999; Ward *et al.*, 2001; Woods *et al.*, 2004; Moore *et al.*, 2003; Wootton *et al.*, 2005; Tenover & Moellering, 2007). Previous recommendations issued by the CDC in the USA suggested isolates with vancomycin MICs of ≥4 mg/L should be retested using a reference MIC method and reported to health-care providers and local health authorities. In addition, all isolates should be sent to

the CDC for confirmation of VISA or VRSA status (Cosgrove *et al.*, 2004). In 2006, the CLSI published new vancomycin breakpoints (Table 1.2) (Anon., 2006a).

1.7.3 Reduced Susceptibility to Glycopeptides among Clinical Isolates of S. aureus

1.7.3.1 GISA

The first clinical isolate of *S. aureus* with reduced susceptibility to vancomycin was reported in Japan in 1997 (Hiramatsu *et al.*, 1997b). This isolate was recovered from an infected incision site of a four-month old baby who underwent heart surgery for pulmonary atresia; had a vancomycin MIC of 8 mg/L and was designated Mu50. According to CLSI criteria, this strain is GISA. The patient was treated with vancomycin for 29 days but the MRSA infection failed to resolve. Therapy was changed to a combination of vancomycin and arbekacin (an aminoglycoside approved for treatment of MRSA infection in Japan) and after 12 days, the wound began to heal. However 12 days after the cessation of antibiotic treatment, infection recurred. Complete resolution of infection required 23 days of therapy with arbekacin and ampicillin/sulbactam (Hiramatsu *et al.*, 1997b; Hiramatsu *et al.*, 1997a). The clinical course of the patient's infection clearly showed that it was the isolates's loss of susceptibility to vancomycin that determined therapeutic failure because other antibiotics were successful in combating the infection.

To date, reports of GISA have been relatively rare. GISA cases tend to be associated with patients who have had prolonged exposure to vancomycin and have been reported from Japan, France, Korea, UK, Brazil, Germany, Belgium, USA, Turkey and Ireland (Hiramatsu *et al.*, 1997b; Ploy *et al.*, 1998; Kim *et al.*, 2000; MacKenzie *et al.*, 2002; Manuel *et al.*, 2002; Oliveira *et al.*, 2001b; Bierbaum *et al.*, 1999; Denis *et al.*, 2002; Smith *et al.*, 1999; Turco *et*

al., 1998; Sieradzki *et al.*, 1999b; Rotun *et al.*, 1999; Anon., 2000b; Fridkin, 2001; Hageman *et al.*, 2001; Marlowe *et al.*, 2001; Cartolano *et al.*, 2004; Nakipoglu *et al.*, 2005; El Solh *et al.*, 2003; Hassan *et al.*, 2001; Elsaghier *et al.*, 2001; Robert *et al.*, 2006; Park *et al.*, 2001; Wilson *et al.*, 2006; Fridkin *et al.*, 2003; Pina *et al.*, 2000; de Lassence *et al.*, 2006; Dancer, 2002; Aucken *et al.*, 2000; Rossney *et al.*, 2006b).

1.7.3.2 hGISA

A second type of reduced susceptibility to vancomycin was reported in Japan in 1997. The initial isolate showed heterogeneous populations of cells with different levels of vancomycin susceptibility. This strain was termed heterogeneous GISA (hGISA) and was designated Mu3 (Hiramatsu *et al.*, 1997a). Mu3 was isolated in January 1996 from a 64 year-old Japanese man who developed MRSA pneumonia after an operation for primary lung cancer. Treatment with vancomycin for 12 days was ineffective. The patient was treated successfully with a 10-day course of ampicillin/sulbactam and arbekacin. Mu3 was isolated from purulent sputum before the first administration of ampicillin/sulbactam and arbekacin. When grown in a drug-free medium, Mu3 produced subpopulations of cells with varying degrees of vancomycin susceptibility (Hiramatsu *et al.*, 1997a).

Reports of hGISA have been more frequent than reports of GISA. Published prevalence rates range from 0–74%. However, criteria for identifying hGISA have not yet been standardised and reports of prevalence should be interpreted with caution as methods to detect hGISA vary widely from study to study (Brown *et al.*, 2005; Liu & Chambers, 2003). hGISA isolates have been reported from Japan, France, Germany, Korea, Thailand, Australia, China, The Netherlands, Greece, Ireland, Belgium, UK, USA, Turkey, Asia, South Africa, Taiwan,

Singapore, Hong Kong, Italy and Spain (Hiramatsu *et al.*, 1997a; Bert *et al.*, 2003; Mallaval *et al.*, 2004; Geisel *et al.*, 1999; Kim *et al.*, 2002; Trakulsomboon *et al.*, 2001; Charles *et al.*, 2004; Benquan *et al.*, 2002; Van Griethuysen *et al.*, 2003; Kantzanou *et al.*, 1999; Rossney & O'Connell, 2003; Denis *et al.*, 2002; Guerin *et al.*, 2000; Ward *et al.*, 2001; Lecaillon *et al.*, 2002; Howe *et al.*, 1998; El Solh *et al.*, 2003; Plipat *et al.*, 2005; Rybak *et al.*, 2005; Sancak *et al.*, 2005; Hubert *et al.*, 1999; Garnier *et al.*, 2006; Song *et al.*, 2004; Woodford *et al.*, 2000; Nonhoff *et al.*, 2005; Amod *et al.*, 2005; Wang *et al.*, 2004; Wong *et al.*, 1999; Sng *et al.*, 2005; Marchese *et al.*, 2000; Ariza *et al.*, 1999; Murray *et al.*, 2004).

1.7.3.3 VRSA

In July 2002, the first clinical VRSA isolate was reported from the USA (Sievert *et al.*, 2002). This isolate had a vancomycin MIC of >128 mg/L, carried both the *mecA* and *vanA* genes and was isolated from a patient in Michigan (Chang *et al.*, 2003b). A second VRSA isolate carrying both *mecA* and *vanA* was isolated from a patient in Pennsylvania in October, 2002. This isolate had a vancomycin MIC of 64 mg/L by the E-test™ method {AB bioMérieux (formerly AB Biodisk), Solna, Sweden}, 32 mg/L by the broth microdilution reference method, however automated susceptibility testing methods (MicroScan® and Vitek® systems) failed to detect this VRSA isolate (Miller *et al.*, 2002; Tenover *et al.*, 2004). To date, seven VRSA isolates have been recognised in the USA (five from Michigan and one each from Pennsylvania and New York). All seven VRSA isolates carried both *mecA* and *vanA* and were epidemiologically unrelated (Sievert *et al.*, 2008). Among five VRSA isolates, the *vanA* gene detected originated from VRE isolates that were also recovered from each patient (Sievert *et al.*, 2008). Two patients from whom VRSA was isolated and did not present with VRE

infection or colonization, had prior histories of enterococcal infection or colonisation (Sievert *et al.*, 2008).

In addition to the seven VRSA isolates from the USA, five VRSA isolates have been documented from a study in Zarqa, Jordan and two VRSA isolates have been reported from northern India (Bataineh, 2006; Tiwari & Sen, 2006). Bataineh *et al.* did not investigate the presence of *van* genes among the five VRSA isolates in Jordan and *van* genes were not detected among the two VRSA isolates from India (Bataineh, 2006; Tiwari & Sen, 2006). More recently, VRSA isolates have been reported from India and Iran; whilst the former VRSA isolate carried both *mecA* and *vanA*, the latter VRSA isolate carried *mecA* but was not investigated for the presence of *van* genes (Emaneini *et al.*, 2007; Saha *et al.*, 2008).

1.7.4 Mechanism of Resistance

1.7.4.1 Mode of Action of Vancomycin

Glycopeptides exert their antibacterial effects by inhibiting bacterial cell wall synthesis through high affinity binding of D-alanyl-D-alanine residues of the murein monomer. Binding of glycopeptides to D-alanyl-D-alanine in the completed peptidoglycan layer does not completely inhibit peptidoglycan synthesis though it may interfere with cross-bridge formation mediated by PBPs. However binding to murein monomers in the cytoplasmic membrane completely inhibits peptidoglycan synthesis. However if glycopeptides are to reach the cytoplasmic membrane, they have to pass through at least 20 layers of peptidoglycan. For effective clinical treatment with glycopeptides, surgical debridement of infected sites should be performed to reduce the patient's bacterial load. Decreasing bacterial cell numbers increases the chances of vancomycin reaching the cytoplasmic membrane and completely

inhibiting peptidoglycan synthesis thus preventing cell multiplication (Hiramatsu, 2001).

1.7.4.2 GISA/hGISA

The genetic mechanisms underlying GISA and hGISA isolates have not yet been elucidated (Howden *et al.*, 2006). GISA and hGISA isolates described to date do not carry any of the *van* determinants that are present in VRE, thus interspecies transfer of resistance genes is not responsible for reduced susceptibility to glycopeptides in *S. aureus*.

The mechanism of reduced susceptibility to vancomycin has been extensively studied using *S. aureus* Mu50 ATCC 700699 (prototype GISA) (Hanaki *et al.*, 1998a; Hanaki *et al.*, 1998b; Cui *et al.*, 2000; Cui *et al.*, 2003; Cui *et al.*, 2006). *S. aureus* Mu50 ATCC 700699 produces increased amounts of peptidoglycan relative to vancomycin-susceptible *S. aureus* (VSSA) isolates. Reports have suggested that *S. aureus* Mu50 ATCC 700699 may possess 30-40 layers of peptidoglycan compared to approximately 20 layers of peptidoglycan in VSSA isolates (Hiramatsu *et al.*, 2001; Pfeltz & Wilkinson, 2004). The thicker peptidoglycan layer "traps" more vancomycin molecules and prevents them from reaching the cytoplasmic membrane to prevent synthesis of peptidoglycan. This vancomycin trapping is termed "affinity trapping" (Hiramatsu, 2001). Studies by Cui *et al.* have shown that trapped vancomycin molecules in the outer peptidoglycan layers destroy the outer layer thus preventing any "untrapped" vancomycin molecules from reaching their target site at the cytoplasmic membrane. This phenomenon is described as "clogging" (Cui *et al.*, 2000; Cui *et al.*, 2006). Studies have shown that both GISA and hGISA strains have significantly thickened cell walls when examined by transmission electron microscopy (Cui *et al.*, 2000; Cui *et al.*, 2003).

Another factor associated with increased cell wall thickness is reduced activity of autolytic enzymes. Autolytic enzymes are involved in the shedding of outer peptidoglycan layers as new peptidoglycan is synthesised. Reduced autolytic activity results in the reduction of peptidoglycan turnover, resulting in a thickened cell wall (Hiramatsu, 2001). Initial studies on *S. aureus* Mu50 ATCC 700699 suggested that GISA strains had enhanced autolytic activity (Hanaki *et al.*, 1998a). However, subsequent studies with both clinical and laboratory-selected GISA isolates have reported reduced autolytic activity associated with GISA strains (Boyle-Vavra *et al.*, 2001; Boyle-Vavra *et al.*, 2003; Koehl *et al.*, 2004; Pfeltz *et al.*, 2000; Pfeltz & Wilkinson, 2004; Sieradzki & Tomasz, 1997; Sieradzki *et al.*, 1999b). A recent report reviewing the autolytic properties of *S. aureus* Mu50 ATCC 700699 found its autolytic activity to be reduced (Utaida *et al.*, 2006).

Other factors associated with vancomycin resistance in *S. aureus* Mu50 ATCC 700699 are decreased levels of intracellular glutamate resulting in increased synthesis of structurally altered non-amidated murein monomers (Cui *et al.*, 2000). These are inefficient substrates for cross-bridge formation by PBPs (Hanaki *et al.*, 1998b). This leads to an increase in D-alanyl-D-alanine residues in the peptidoglycan layer thus binding more vancomycin molecules. However, reduction in cross-linkage of peptidoglycan alone does not cause glycopeptide resistance (Cui *et al.*, 2000). Hanaki *et al.* have found that non-amidated murein monomers have a high affinity for binding vancomycin molecules (Hanaki *et al.*, 1998b). This in turn contributes to affinity trapping and clogging.

Structural and/or metabolic changes in cell wall teichoic acids have been reported to play a role in the expression of the GISA phenotype by reducing the rate of cell wall degradation,

thus maintaining a correlation between cell wall thickness and reduced susceptibility to vancomycin (Sieradzki & Tomasz, 2003).

The role of PBPs in vancomycin resistance is unclear. While some studies have reported an increased production of PBPs in GISA, others have shown that they are down-regulated (Hanaki *et al.*, 1998a; Moreira *et al.*, 1997; Sieradzki *et al.*, 1999a; Finan *et al.*, 2001). Many GISA isolates have reduced lysostaphin susceptibility, however increased susceptibilities have also been reported (Pfeltz & Wilkinson, 2004). An inverse relationship between GISA and MRSA resistance mechanisms has been reported (Pfeltz & Wilkinson, 2004; Sieradzki *et al.*, 1999c). In addition, decreased coagulase activity has been reported in GISA isolates (Moreira *et al.*, 1997). Altered expression of PBP2a has been observed among GISA and hGISA isolates with increased production in the prototype GISA and hGISA strains, *S. aureus* Mu50 ATCC 700699 and *S. aureus* Mu3 ATCC 700698 (Hiramatsu *et al.*, 1997a; Hanaki *et al.*, 1998a).

1.7.4.3 VRSA

Vancomycin resistance among the seven VRSA isolates reported from the USA was associated with acquisition of the *vanA* gene cluster (Sievert *et al.*, 2008). The high-level inducible resistance to both vancomycin and teicoplanin that characterises the vanA phenotype is mediated by the *vanA* gene which has been shown to reside on transposon, Tn*1546* (Arthur *et al.*, 1996). Acquisition of the *vanA* gene in VRSA was shown to occur via the horizontal transfer of an enterococcal plasmid carrying Tn*1546* from co-infecting VRE isolates (Chang *et al.*, 2003b; Clark *et al.*, 2005; Weigel *et al.*, 2003; Tenover *et al.*, 2004; Perichon & Courvalin, 2006; Weigel *et al.*, 2007). The *vanA* operon orchestrates the replacement of D-alanyl-D-

alanine dipeptide with D-alanyl-D-lactate depsipeptide in peptidoglycan synthesis, resulting in a decreased affinity of the molecule for glycopeptides (Arthur *et al.*, 1996).

In 1999, Sieradzki and Tomasz reported that in five vancomycin-resistant *S. aureus* isolates selected *in vitro* from the homogeneously MRSA strain, COL, increases in vancomycin resistance were accompanied by decreases in meticillin resistance. In addition, abnormalities in cell wall metabolism were noted including a reduction in the cross-linkage of peptidoglycan and decreased production of PBP2a and PBP4 (Sieradzki *et al.*, 1999c).

A report by Severin *et al.* demonstrated that when the plasmid encoding *vanA* from the Michigan VRSA isolate was transferred into MRSA COL, the transconjugant (COLVA) produced a cell wall peptidoglycan of unusual chemical composition when grown in the presence of vancomycin (Severin *et al.*, 2004a). It was also shown that the inactivation of *mecA* did not reduce the vancomycin MIC for strain COLVA implying that PBP2a is not needed for the biosynthesis of the cell wall produced in vancomycin-resistant cells (Severin *et al.*, 2004a). Another study by Severin *et al.* established that PBP2 is essential for the expression of high-level vancomycin resistance and cell wall synthesis in VRSA isolates carrying *vanA* (Severin *et al.*, 2004b).

Vancomycin-resistant *E. faecalis* emits a sex pheromone (cAM373) that promotes plasmid transfer. This pheromone is also produced by *S. aureus* and it has been postulated that emission of cAM373 by *S. aureus* cells in close proximity to VRE that carry plasmids encoding *van* genes could facilitate the transfer of these resistance genes to *S. aureus* (Showsh *et al.*, 2001).

1.7.5 Laboratory Detection Methods

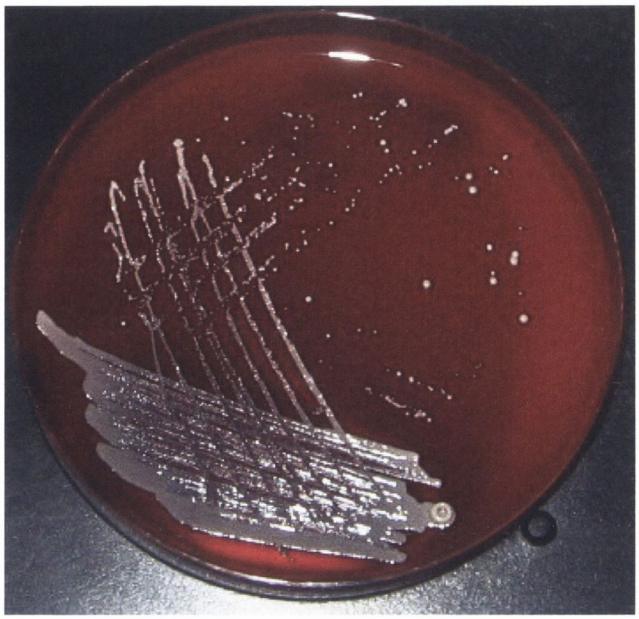
1.7.5.1 Phenotypic Detection Methods

Since the emergence of GISA, hGISA and VRSA, detection of isolates with reduced susceptibility to glycopeptides has been a challenge to diagnostic laboratories (Tenover *et al.*, 2001; Tenover, 2005). In MRSA, the vancomycin-resistant phenotype tends to be lost after subculture in the absence of selective antibiotic pressure (Boyle-Vavra *et al.*, 2000). The colonial morphology of GISA and hGISA isolates can initially appear mixed after 24 hours incubation at 35°C with isolates demonstrating two distinct colony types yielding identical susceptibility testing results (Figure 1.3) (Tenover *et al.*, 2001). Standard disk diffusion methods will not detect GISA and hGISA isolates (Hageman *et al.*, 2006). Automated susceptibility testing systems will not reliably detect VRSA and GISA and fail to detect hGISA (Hageman *et al.*, 2006). In addition, vancomycin agar screening media such as brain heart infusion (BHI) agar containing 6 mg/L vancomycin (BHIV6) detects VRSA but may not reliably detect GISA and fails to detect hGISA (Hageman *et al.*, 2006). Thus, depending on the method of susceptibility testing used in a diagnostic microbiology laboratory, clinical isolates of VRSA, GISA and hGISA may escape detection. A combination of phenotypic detection methods in conjunction with astute microbiology acumen is required to reliably detect VRSA, GISA and hGISA isolates. This requirement poses a serious challenge to clinical diagnostic microbiology laboratories.

1.7.5.1.1 GISA

Using CLSI methodology, reliable detection of GISA requires MIC measurement with a non-automated MIC method using an inoculum equivalent to a 0.5 McFarland turbidity standard

Panel A



Panel B

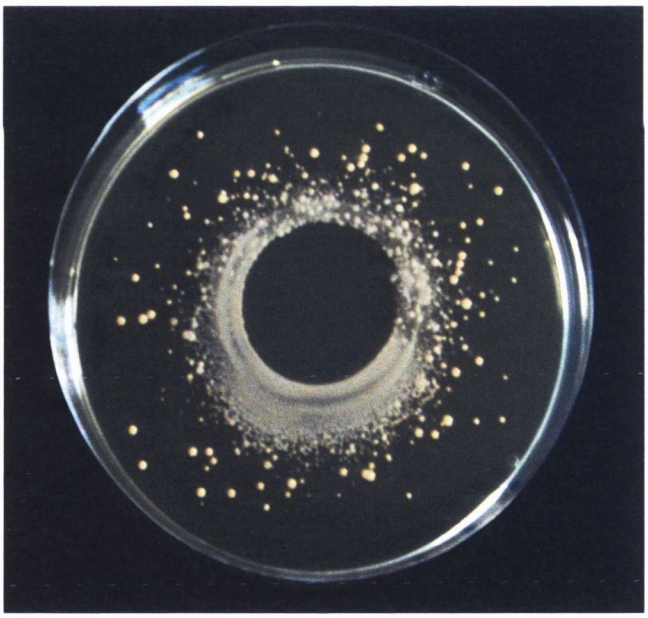


Figure 1.3. Heterogeneous colonial morphology exhibited by *S. aureus* Mu3 ATCC 700698.

Panels A and B present the colonial morphology exhibited by the prototype hGISA strain, *S. aureus* Mu3 ATCC 700698 when grown on blood agar and on brain heart infusion agar containing 4 mg/L vancomycin, respectively.

(Hageman *et al.*, 2006). Non-automated MIC methods include reference broth microdilution, the E-test™ system (AB bioMérieux, Solna, Sweden) and agar dilution MIC testing (Hageman *et al.*, 2006). Broth microdilution MIC testing involves inoculating a series of cation-adjusted Mueller-Hinton (MH) broths containing increasing vancomycin concentrations with a standard concentration of organism in a microtitre tray and incubating at 35°C for 24 hours (h). The MIC is defined as the lowest concentration of vancomycin that completely inhibits growth of the test organism (Anon., 2006a). The E-test™ MIC system is an agar diffusion test where an E-test™ strip (a plastic strip impregnated with a continuous gradient of antibiotic concentrations) is placed on MH agar inoculated with a suspension of the organism and incubated at 35°C for 24 h. The E-test™ MIC method has the advantage of showing small or isolated colonies within a zone of inhibition. Agar dilution MIC testing involves inoculating a series of MH agar plates containing increasing concentrations of vancomycin with a standard concentration of organism. The MIC is defined as the lowest concentration of vancomycin that completely inhibits growth of the test organism (Anon., 2006a).

Prior to the revision of the CLSI glycopeptide MIC breakpoints in 2006 (when vancomycin MICs 8–16 mg/L were used to define GISA), several agar screening methods were proposed to detect GISA isolates. The original screening method was growth on BHI agar containing 4 mg/L vancomycin (BHIV4) within 24 hours at 35°C with an initial inoculum of 10^6 colony forming units (CFU)/ml (Hiramatsu *et al.*, 1997a). However, Hubert *et al.* showed that a number of *S. aureus* isolates without elevated MICs grew on BHIV4 and recommended screening on MH agar containing 5 mg/L vancomycin (MHV5) (Hubert *et al.*, 1999). Commercially prepared BHI agar containing 6 mg/L vancomycin (BHIV6) with an inoculum concentration of 10^6 CFU/ml was shown to detect GISA (vancomycin MICs 8–16 mg/L) with

high sensitivity and specificity; however, the same medium when prepared in-house occasionally showed growth of the vancomycin-susceptible control organisms (Tenover *et al.*, 1998).

Prior to 2006, the CDC in the USA and the ARMRL in Colindale in the UK and the National MRSA Reference Laboratory (NMRSARL) in Ireland suggested the following criteria must be met before isolates are recognised as exhibiting reduced susceptibility to glycopeptides:

1. Growth of one or more colonies on BHIV6
2. Vancomycin E-test™ MIC of ≥6 mg/L on MH agar using an inoculum density equivalent to a 0.5 McFarland turbidity standard and a full 24 hour incubation period
3. Vancomycin broth microdilution MIC of 8–16 mg/L using CLSI methodology.

These guidelines permitted the detection of GISA (Tenover *et al.*, 2001).

Since 2006, isolates exhibiting vancomycin MICs between 4 and 8 mg/L are considered GISA. The CDC recommend that isolates exhibiting vancomycin MICs ≥4 mg/L with or without growth on BHIV6 should be considered as GISA (Anon., 2006e). BHIV6 reliably detected isolates with vancomycin MICs ranging between 8–16 mg/L with high sensitivity and specificity but may not detect GISA isolates exhibiting vancomycin MICs of 4 mg/L (Tenover *et al.*, 2001; Hageman *et al.*, 2006). Further studies are needed to define the level of sensitivity of BHIV6 with GISA isolates (Hageman *et al.*, 2006). Whilst automated susceptibility testing systems may not reliably detect GISA, disk diffusion studies will not detect GISA isolates (Hageman *et al.*, 2006).

1.7.5.1.2 hGISA

Detection of hGISA in clinical microbiology laboratories is very difficult. Standard disk diffusion, automated susceptibility testing methods, broth microdilution MIC and standard E-test™ MIC determinations fail to detect hGISA. It is believed that the initial inoculum (0.5 McFarland) used in these tests is too low to detect the resistant subpopulation, which is only present in $1/10^6$ cells (Walsh *et al.*, 2001). When first described by Hiramatsu *et al.*, subpopulations of hGISA grew in the presence of >4 mg/L vancomycin on an enriched medium, BHI (Hiramatsu *et al.*, 1997a). The population heterogeneity of hGISA isolates can be seen when isolates are investigated by population analysis (Hiramatsu *et al.*, 1997a). This procedure involves plating an overnight broth culture and its serial dilutions onto BHI agar plates containing increasing concentrations of vancomycin or teicoplanin. Figure 1.3 shows the heterogeneous population of cells exhibited by *S. aureus* Mu3 ATCC 700698 when grown on BHI agar containing 4 mg/L vancomycin. The colony counts at each concentration of antimicrobial give a measure of the resistant subpopulations in the test isolate (Hiramatsu *et al.*, 1997a; Nonhoff *et al.*, 2005). To confirm the presence of hGISA among *S. aureus* isolates, a complete population analysis should be performed to demonstrate heterogeneity (Hiramatsu *et al.*, 1997a). As heterogeneity is difficult to define precisely, a modified population analysis method termed modified population analysis profiling (PAP) has been described (Wootton *et al.*, 2001). In this method, the area under the population analysis curve (AUC) of the isolate is calculated and a ratio of the test AUC divided by the AUC of *S. aureus* Mu3 ATCC 700698 is calculated. This ratio is termed the PAP-AUC ratio. A PAP-AUC ratio of ≤0.89 defines GSSA, ≥0.9, hGISA, and ≥1.3, GISA (Wootton *et al.*, 2001). PAP-AUC ratio determination is a reliable and reproducible method of detecting hGISA (Walsh & Howe, 2002). However, the method is time-consuming and is unsuitable for clinical microbiology laboratories (Wootton *et al.*, 2001). The population analysis profiles of *S. aureus* Mu50

ATCC 700699 (prototype GISA), *S. aureus* Mu3 ATCC 700698 (prototype hGISA) along with a GSSA isolate (*S. aureus* ATCC 29213) is shown in Figure 1.4.

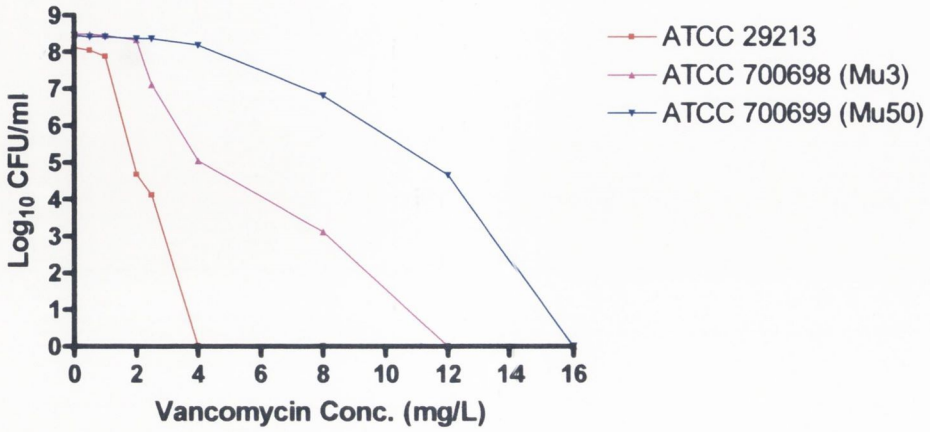
Walsh *et al.* have described a modified E-test™ method called the E-test™ macromethod that maximises detection of glycopeptide resistance. In this method, BHI agar, (a very nutritious medium) and a heavy inoculum (2.0 McFarland) are used to maximise detection of the resistant phenotype (Walsh *et al.*, 2001). The criteria for a *S. aureus* isolate to be classified as a possible hGISA or GISA are E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone. This modified procedure has been optimised for the detection of glycopeptide resistance but it is not a standard MIC determination. One study reported the sensitivity and specificity of this method to be 96% and 97%, respectively (Walsh *et al.*, 2001). More recently, single E-test™ strips containing both vancomycin and teicoplanin, termed E-test™ Glycopeptide Resistance Detection (GRD) strips have become available (Appelbaum, 2007; Yusof *et al.*, 2008). Although this method utilises MH agar plates and an inoculum density equivalent to a 0.5 McFarland turbidity standard, it is not a standard MIC method (Yusof *et al.*, 2008).

1.7.5.1.3 Agar Screening Methods for hGISA

There is a very great need for a reliable agar screening method for the detection of hGISA. Whilst BHIV6 is reliable for the detection of VRSA and GISA isolates exhibiting vancomycin MICs of 8 mg/L, it fails to detect hGISA (Hageman *et al.*, 2006). Several screening methods for hGISA have been advocated.

Hiramatsu *et al.* proposed a simplified method of population analysis as a method for

Panel A



Panel B

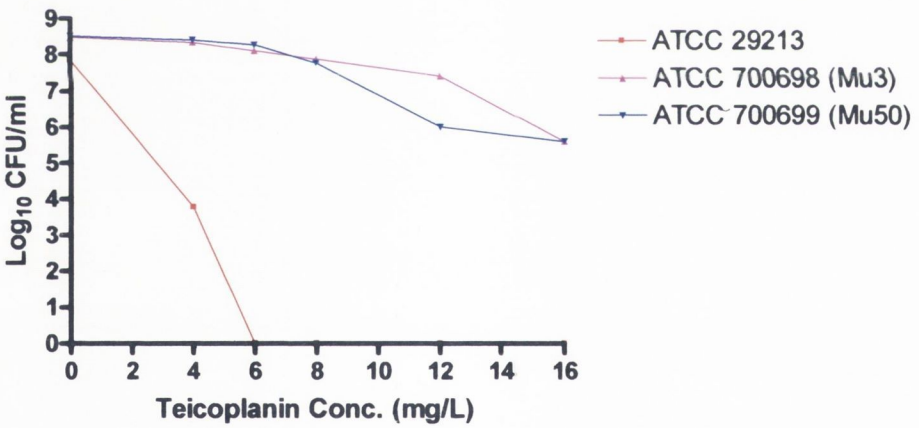


Figure 1.4. Population analysis profiles of *S. aureus* ATCC 29213, *S. aureus* Mu3 ATCC 700698 and *S. aureus* Mu50 ATCC 700699.

Panels A and B show the vancomycin and teicoplanin PAPs of *S. aureus* ATCC 29213 (GSSA), *S. aureus* Mu3 ATCC 700698 (prototype hGISA) and *S. aureus* Mu50 ATCC 700699 (prototype GISA), respectively.

screening for hGISA involving inoculation of 10^6 CFU/ml of organism onto BHIV4 and incubation at 35°C for 48 hours (Hiramatsu *et al.*, 1997a). A countable number of colonies after 48 hours indicated possible hGISA. However this method was not designed to detect all hGISA isolates and complete population analysis was recommended for confirmatory testing (Hiramatsu *et al.*, 1997a). One study has shown that *S. aureus* Mu3 ATCC 700698 was detected on only 80% of occasions using this screening method (Wootton *et al.*, 2001). Another study reported that the sensitivity and specificity of this method was 71% and 88%, respectively (Walsh *et al.*, 2001).

Voss *et al.* demonstrated that screening with MHV5 and BHIV6 fail to detect hGISA and that screening with MH containing 5 mg/L teicoplanin (MHT5) was a more reliable screening method (Voss *et al.*, 2003). Guerin *et al.* found that screening with BHI containing 6 mg/L teicoplanin (BHIT6) reliably detected hGISA. The Comité de l'Antibiogramme de la Société Française de Microbiologie (CASFM) found that screening results with BHIT6 were not reproducible from one batch of BHI to another and recommend screening with MHT5 (Guerin *et al.*, 2000; Anon., 2006d). The European Antimicrobial Resistance Surveillance System (EARSS) recommends MHT5 as a preliminary agar screen to detect isolates exhibiting reduced susceptibility to glycopeptides. Stationary-phase broth subcultures are inoculated onto MHT5 and incubated at 35°C for 48 hours (Anon., 2005b). Growth of one or more colonies is indicative of a positive result. EARSS recommends that isolates exhibiting positive results with MHT5 should subsequently be screened using the E-test™ macromethod (Anon., 2005b). Other screening methods have been described but clear evidence of their reliability and sensitivity has not been shown (Bernard *et al.*, 2004; Park *et al.*, 2000; Lecaillon *et al.*, 2002). A recent study by Wootton *et al.* compared three screening methods (BHIV6, MHT5 and the E-test™ macromethod) for detecting GISA and hGISA isolates and

found MHT5 and the E-test™ macromethod yielded sensitivity and specificity values of 86% and 76%, and 82% and 89%, respectively (Wootton *et al.*, 2007).

1.7.5.1.4 VRSA

Detection of VRSA isolates in clinical microbiology laboratories can be difficult. Three of the seven confirmed VRSA isolates in the USA were not reliably detected using automated susceptibility testing systems (Hageman *et al.*, 2006; Appelbaum, 2007). The CDC recommends that laboratories using automated susceptibility testing systems that are not validated for VRSA detection should also use a vancomycin agar screen plate, BHIV6, to ensure no VRSA isolates go undetected. BHIV6 reliably detects VRSA isolates (Hageman *et al.*, 2006). Although disk diffusion studies have been reported to detect VRSA, careful interpretation of results is needed. It has been reported that VRSA isolates produce subtle growth around a vancomycin 30-μg disk on MH agar after a 24 hour incubation period at 35°C (Tenover, 2005). MIC measurement using reference broth microdilution, E-test™ and agar dilution methods reliably detect VRSA isolates (Hageman *et al.*, 2006).

1.7.5.2 Genotypic Detection Methods

1.7.5.2.1 GISA/hGISA

The genetic basis for reduced susceptibility to glycopeptides in *S. aureus* has yet to be determined. Complex mechanisms producing changes in cell wall content and composition generate the GISA phenotype. It has been shown that the cell wall structure of *S. aureus* Mu50 ATCC 700699 is similar to that of the VSSA strain, *S. aureus* N315, except that the peptidoglycan chains in *S. aureus* Mu50 ATCC 700699 show significantly less cross-linking

and an increased content of pentapeptide chains (Hanaki *et al.*, 1998b). Sequence analysis of the genomes of *S. aureus* Mu50 ATCC 700699 and N315 revealed that there were 17 loss of putative function mutations specific for *S. aureus* Mu50 ATCC 700699 affecting important cell wall biosynthesis and intermediary metabolism genes (Avison *et al.*, 2002). A subsequent study investigating whether these 17 genes were disrupted in other GISA and hGISA isolates found that the sequences of only four genes were disrupted in both *S. aureus* Mu3 ATCC 700698 and *S. aureus* Mu50 ATCC 700699, and the remaining 13 genes were identical to those of the VSSA strain, N315. Furthermore, these four genes were not disrupted in the clinical hGISA and GISA isolates investigated in that study (Wootton *et al.*, 2004).

Several studies investigating the underlying genetic mechanisms of resistance in GISA isolates have been undertaken. Altered expression of several different genes such as *pbp2*, *pbp4*, *sigB*, *ddh*, *tcaA* and *vraSR* have been associated with GISA strains (Shlaes *et al.*, 1993; Sieradzki *et al.*, 1999a; Finan *et al.*, 2001; Bischoff *et al.*, 2001; Hanaki *et al.*, 1998a; Maki *et al.*, 2004; Kuroda *et al.*, 2000; Kuroda *et al.*, 2003; Boyle-Vavra *et al.*, 1997). Other studies have identified sets of up- and down-regulated genes in GISA strains (Kuroda *et al.*, 2000; Kuroda *et al.*, 2003; Mongodin *et al.*, 2003; Utaida *et al.*, 2003). Cui *et al.* compared six sets of transcriptional profiles between GISA and GSSA isolates and identified 17 genes (including eight novel genes) associated with glycopeptide resistance (Cui *et al.*, 2005). A report by Nelson *et al.* documented that 71% of VISA strains studied had impaired acetate catabolism compared with 8% of VSSA strains. As the implications of impaired acetate catabolism among *S. aureus* isolates are unknown, further studies are proceeding (Nelson *et al.*, 2007). Drummelsmith *et al.* observed that the expression of one particular gene (SAV2095) was markedly increased in VISA/hVISA isolates compared to MRSA isolates and that the expression of SAV2095 could be used as a biomarker for detecting VISA/hVISA isolates

(Drummelsmith *et al.*, 2007). Howden *et al.* reported that the up-regulation of *vraS* was not required for the development of the GISA/hGISA phenotype (Howden *et al.*, 2008). Another recent report has documented that the mutated response regulator *graR* was responsible for the conversion of isolates from the hGISA phenotype to the GISA phenotype (Neoh *et al.*, 2008).

Clinical case histories of several patients with GISA strains have revealed that most patients had infections originating from or involving biomedical devices (Hiramatsu *et al.*, 1997a; Hiramatsu *et al.*, 1997b; Fridkin, 2001). These findings suggest the possibility that these strains have increased ability to produce biofilm. Biofilms act as reservoirs for pathogenic organisms and may contribute to antimicrobial resistance (Donlan, 2002). A report by Vuong *et al.* showed that loss of function of *agr* enhanced the ability of *S. aureus* to adhere to polystyrene and form a biofilm (Vuong *et al.*, 2000). Sakoulas *et al.* studied the *agr* locus in geographically diverse *S. aureus* isolates including GISA, hGISA and GSSA and found that all GISA and hGISA isolates belonged to *agr* group II and all were defective in *agr* function (as measured by the production of delta-haemolysin) (Sakoulas *et al.*, 2002). A further study has shown that there is a strong relationship between vancomycin treatment failure and infection due to MRSA with the *agr* group II polymorphism (Moise-Broder *et al.*, 2004). Howe *et al.* studied a collection of GISA and hGISA isolates from the five pandemic clones of MRSA and found that all GISA and hGISA isolates belonged to *agr* group I or *agr* group II (Howe *et al.*, 2004).

1.7.5.2.2 VRSA

Extensive molecular studies (PCR and DNA sequence analysis) have been carried out on four of the seven VRSA isolates reported from the USA (Weigel *et al.*, 2003; Perichon &

Courvalin, 2004; Perichon & Courvalin, 2006; Tenover *et al.*, 2004; Bozdogan *et al.*, 2004; Weigel *et al.*, 2007). These isolates have been named as follows: MI-VRSA (Michigan VRSA, June 2002); PA-VRSA (Pennsylvania VRSA, September 2002); NY-VRSA-595 (New York VRSA, March 2004) and VRSA-5 (Michigan VRSA, March 2005) (Perichon & Courvalin, 2004; Perichon & Courvalin, 2006; Weigel *et al.*, 2007). Although all four isolates carry *vanA*, each exhibits distinct glycopeptide resistance phenotypes. MI-VRSA and VRSA-5 exhibit high-level resistance to glycopeptides (but are epidemiologically unrelated) and display stable vancomycin resistance (Weigel *et al.*, 2003; Perichon & Courvalin, 2004; Perichon & Courvalin, 2006). In contrast, PA-VRSA and NY-VRSA-595 exhibit low-level resistance to glycopeptides and the resistance phenotype is unstable as it was lost after a series of replica plating in the absence of vancomycin. In addition, vancomycin resistance in PA-VRSA and NY-VRSA-595 is inducible which is a significant concern as treatment with glycopeptides would inevitably lead to clinical failure (Perichon & Courvalin, 2004; Perichon & Courvalin, 2006; Weigel *et al.*, 2007). Subsequent isolates from the patient from whom NY-VRSA-595 was recovered have been studied recently (Weigel *et al.*, 2007). Three MRSA isolates recovered one month after NY-VRSA-595 was recognised exhibited vancomycin MICs between 2 and 4 mg/L but PCR analysis showed that all three MRSA isolates carried *vanA* (Weigel *et al.*, 2007).

1.8 Clinical Significance of hGISA

Whilst the clinical significance of GISA and VRSA is without question, the clinical role of hGISA has been debated since its emergence in 1997 (Hiramatsu *et al.*, 1997a; Ike *et al.*, 2001; Howe & Walsh, 2004; Arakawa *et al.*, 2004). It has been suggested that hGISA may be a laboratory-induced phenomenon and some workers have questioned whether it really exists

(Ike *et al.*, 2001; Arakawa *et al.*, 2004). Several studies have demonstrated the *in vitro* selection of GISA from hGISA suggesting hGISA is a precursor to GISA (Bobin-Dubreux *et al.*, 2001; Hussain *et al.*, 2002; Chesneau *et al.*, 2000; Cui *et al.*, 2003). Reversion of a GISA strain to hGISA has also been demonstrated (Boyle-Vavra *et al.*, 2000). In recent years, increasing numbers of reports of treatment failures associated with hGISA infections have been documented (Walsh & Howe, 2002; Liu & Chambers, 2003; Moore *et al.*, 2003; Rotun *et al.*, 1999; Ariza *et al.*, 1999; Murray *et al.*, 2004; Ward *et al.*, 2001).

A study comparing the clinical features of bacteraemia associated with GSSA and hGISA found that patients with hGISA presented with similar clinical findings: i.e. fever that persisted for >7 days after starting vancomycin therapy, high bacterial load infections and an initial low serum vancomycin level. These findings were considered to be useful clinical markers to predict suspect hGISA cases (Charles *et al.*, 2004). Howden *et al.* noted prolonged bacteraemia in patients with hGISA infection (Howden *et al.*, 2004). A report by Sakoulas *et al.* in 2004 suggested that as vancomycin MICs among MRSA isolates increased to ≥2 mg/L, the number of clinical failures for patients treated with vancomycin also increased (Sakoulas *et al.*, 2004). Reported cases of failed vancomycin therapy among *S. aureus* isolates exhibiting susceptible MICs prompted revision of the CLSI vancomycin MIC breakpoints. The vancomycin MICs were re-defined to increase the detection of heterogeneously resistant isolates of *S. aureus* (Tenover & Moellering, 2007).

1.9 MRSA in Ireland

1.9.1 Emergence of MRSA in Ireland

MRSA was first reported in Ireland in 1971 (Hone & Keane, 1974). The incidence of MRSA in eight Dublin hospitals between April 1971 and 1972 was 5% (Hone & Keane, 1974). From 1971 to 1975, MRSA caused sporadic infections in these eight Dublin hospitals (Cafferkey *et al.*, 1985). The first case of bacteraemia caused by high-level gentamicin-resistant MRSA (GMRSA) (gentamicin MIC >100 mg/L) was recorded in 1976 (Coleman *et al.*, 1985). This GMRSA strain (termed Phenotype I) predominated until 1978 when a second strain of GMRSA exhibiting low-level gentamicin-resistance (termed Phenotype II) emerged. Phenotype II GMRSA had a gentamicin MIC ≤30 mg/L and predominated among the MRSA population until 1985 (Coleman *et al.*, 1985; Cafferkey *et al.*, 1985). These two strains of MRSA spread rapidly throughout Dublin hospitals with bacteraemia rates reaching a peak between 1979 and 1982 (Morgan & Harte-Barry, 1989). Phenotype I GMRSA isolates have not been reported in the Irish MRSA population since 1982 (Carroll *et al.*, 1989). A third strain of GMRSA termed Phenotype III or the "Baghdad" strain was recognised in 1985 and spread rapidly from one Dublin hospital to another (Carroll *et al.*, 1989). Phenotype III isolates were similar to a strain isolated in Baghdad in 1984, and were first recovered in Dublin from a patient who had been hospitalised in Baghdad a short time earlier, indicating that Phenotype III MRSA had spread from Baghdad to Dublin (Carroll *et al.*, 1989).

1.9.2 Prevalence of MRSA in Ireland (1977-2007)

Between 1977 and 1986, prevalence of MRSA in one Dublin hospital changed dramatically with bacteraemia rates reaching a peak in 1979 (Morgan & Harte-Barry, 1989). The yearly

prevalence rates of MRSA from 1977 through to 1986 were 18%, 31%, 42%, 42%, 33%, 33%, 21%, 18%, 19% and 18%, respectively (Morgan & Harte-Barry, 1989). In another Dublin hospital, the incidence of MRSA began to rise in 1987 and prevalence was 20% in 1991. The prevalence of GMRSA rose sharply in 1992 (prevalence: 27.1%) and by Quarter 1 (Q1), 1993 the prevalence of MRSA was 47% compared to 16% in Q1, 1992 (Rossney *et al.*, 1994c).

In 1993, the first national survey of *S. aureus* in Ireland was conducted by questionnaire to determine the susceptibility of *S. aureus* to seven clinically useful antibiotics (meticillin, penicillin, gentamicin, erythromycin, ciprofloxacin, fusidic acid and mupirocin) (Moorhouse *et al.*, 1996). In nine hospitals, the average prevalence of MRSA was 15% but was as high as 29% to 38% in certain institutions. Rates of resistance to gentamicin and erythromycin were 11% and 20%, respectively (Moorhouse *et al.*, 1996).

A national questionnaire survey of MRSA was carried out over a two-week period in February 1995 to determine the extent of MRSA in Ireland before the introduction of national MRSA control guidelines (Johnson *et al.*, 1997). That survey reported an incidence of 15.9/100 000 population. The majority of MRSA-positive patients were elderly men and the largest proportion of isolates came from surgical and medical patients (Johnson *et al.*, 1997).

The North/South Study of MRSA in Ireland which was conducted during a two-week period in February 1999 was a joint investigation of the epidemiology of MRSA in both the North of Ireland (North) and in the South of Ireland (South). This study found prevalence rates per 100 000 population of 11 and 14 in the North and South, respectively (McDonald *et al.*, 2003).

In 1998, EARSS was established to collate antimicrobial resistance data for public health purposes in Europe. One objective of EARSS is to determine the proportion of MRSA among *S. aureus* bloodstream isolates in Europe. Ireland has participated in EARSS since 1999. In Ireland, in addition to collecting data, MRSA isolates are epidemiologically typed by AR typing and DNA macrorestriction digestion using *Sma*I followed by PFGE at the NMRSARL. In 1999 and 2000, isolates were also typed by phage typing. Between 1999 and 2003, the proportion of MRSA among invasive *S. aureus* isolates increased from 39% to 42% in Ireland (Murphy *et al.*, 2005). The proportions of MRSA varied widely across Europe with the highest proportions in southern and western Europe (>40%) and the lowest proportions in northern Europe (<1%) (Tiemersma *et al.*, 2004). Between 2002 and 2006, the yearly proportion of MRSA among invasive *S. aureus* isolates in Ireland was stable at approximately 42% but decreased to 38.5% in 2007 (Figure 1.5) (Anon., 2006c; Anon., 2007).

1.9.3 Community-acquired MRSA

In Ireland, the first report of PVL-positive CA-MRSA was documented in 2005 (Rossney *et al.*, 2005b; Rossney *et al.*, 2005a). In a recent study of CA-MRSA in Ireland, among 1,389 MRSA isolates investigated for carriage of *pvl* genes, 1.8% of isolates carried *pvl* (Rossney *et al.*, 2007). Six genotypes (ST30, ST8, ST22, ST80, ST5 and ST154) were exhibited by these PVL-positive isolates. Among isolates harboring *pvl*, 76% were CA-MRSA isolates; 52% were recovered from patients with SSTIs and 36% were from patients of non-Irish ethnic origin. This study reported that carriage of *pvl* cannot be used as the sole marker for CA-MRSA (Rossney *et al.*, 2007).

1.9.4 MRSA in Animals

In 2005, a study in Ireland documented the recovery of MRSA from 25 animals comprising 14 dogs, eight horses, one cat, one rabbit and a seal and also from 10 attendant veterinary personnel (O'Mahony *et al.*, 2005). Epidemiological typing confirmed two major strains among the MRSA isolates identified; one was indistinguishable from the predominant strain in Irish hospitals (ST22-MRSA-IV) but the strain from the eight horses was unlike any patterns previously seen in Irish hospitals (O'Mahony *et al.*, 2005; Shore *et al.*, 2005). A more detailed investigation of epidemiological types of MRSA isolates recovered from 27 dogs, nine horses, six cats and 22 veterinary staff in the UK and Ireland was reported by Moodley *et al.* in 2006. This study revealed that irrespective of geographical origin, MRSA isolates investigated clustered into two distinct CCs, CC8 and CC22 (Moodley *et al.*, 2006). Whilst the genotypes of all feline, 96% canine and 82% human MRSA isolates were related to CC22, 88% of equine isolates were related to CC8 (Moodley *et al.*, 2006). To date, MRSA isolated from pigs has not been reported in Ireland.

1.9.5 Epidemiological Typing of MRSA

During the 1970s and early 1980s, the three most common typing techniques used with MRSA in Ireland were diagnostic antibiogram typing, phage typing and plasmid profiling (Hone & Keane, 1974; Cafferkey *et al.*, 1985; Coleman *et al.*, 1985). By the late 1980s the value of phage typing had become limited as most isolates of MRSA belonged to phage lytic groups I or III and an increasing proportion of strains were non-typable using the International Set of Basic Typing Phages (Cafferkey *et al.*, 1983; Rossney *et al.*, 1994a). Plasmid profiling and antibiogram patterns were inherently variable because of the loss or gain of plasmids and in the case of plasmid typing, isolates that lacked plasmids could not be typed.

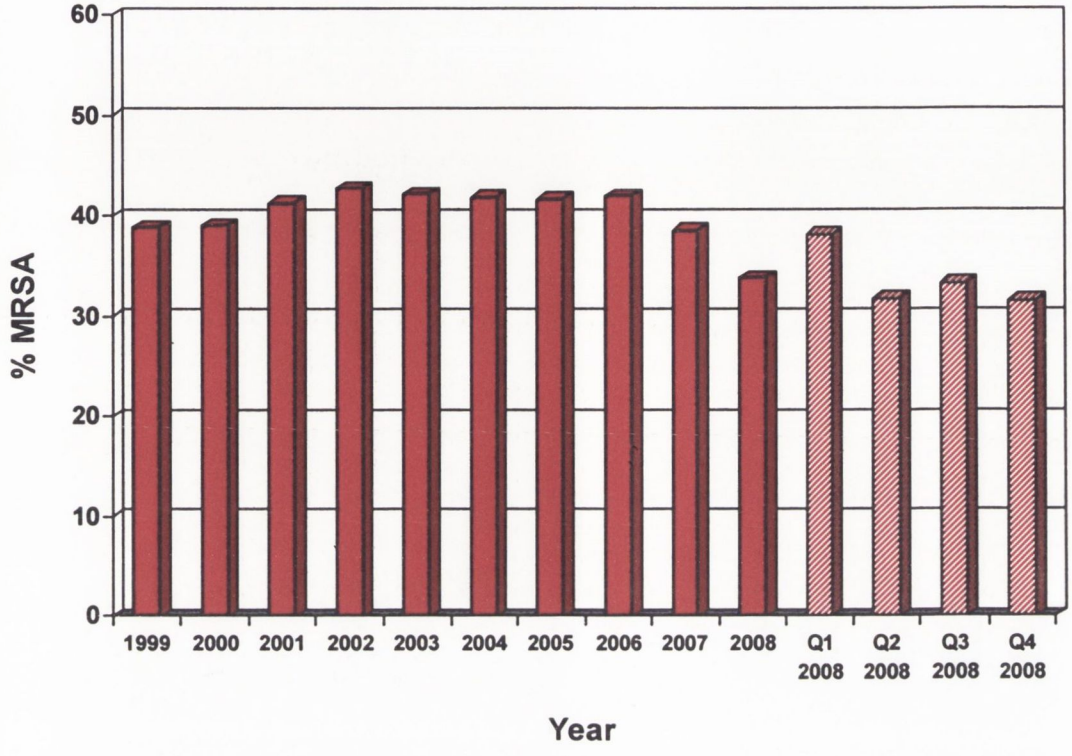


Figure 1.5. Proportion of MRSA among invasive *S. aureus* isolates in Ireland between 1999 and 2008.

In 1993, an antibiogram typing scheme for rapidly differentiating MRSA isolates was devised (Rossney *et al.*, 1994a). Susceptibility to 18 antibiotics and four chemicals were used to determine antibiogram types. Antibiogram subtypes were determined with reference to knowledge of the background MRSA population so that isolates were not misclassified. Antibiogram typing was found to be more discriminatory than phage typing or plasmid profiling.

1.9.5.1 Strain Variation Over a Ten-Year Period in One Dublin Hospital

Between 1989 and 1998 there was a fourfold increase in the number of patients carrying MRSA in one large tertiary-referral hospital in Dublin (Rossney & Keane, 2002). In 1989, the predominant AR types were AR01 and AR02, while phage typing showed that 65% of isolates were non-typable using the International Set of Basic Typing Phages. AR01 and AR02 have not been seen in the MRSA population since 1993. Between 1992 and 1993, the MRSA population exhibited 31 AR types with 90% of isolates belonging to just seven AR types (AR06, AR07, AR11, AR13, AR14, AR15 and AR22) with AR13 and AR14 predominating (Rossney *et al.*, 1994b). In 1994, AR13 and AR14 still dominated but by 1998 the prevalence of these types had declined and AR06 and AR07 became the dominant AR types among the MRSA population (prevalence: 32% and 24%, respectively) (Rossney & Keane, 2002).

1.9.5.2 North/South Study of MRSA in Ireland, 1999

AR typing of MRSA isolates from the North/South Study showed that 68% of isolates yielded previously familiar patterns. The most frequently occurring AR types among the MRSA population in the South were AR13, AR06, AR14 and AR07 (prevalence: 31%, 26%, 19% and

18%, respectively) and in the North, the predominant AR types were AR43, AR13 and AR06 (prevalence: 42%, 33% and 15%, respectively). Phage typing showed that 71% of isolates yielded non-typable, weak or inhibition reactions with the International Set of Basic Typing Phages (Rossney *et al.*, 2003b). Four new AR types were assigned to MRSA isolates during this study. One previous unfamiliar AR pattern (designated AR43) was the predominant AR type in the North whilst isolates exhibiting the other three new AR patterns were designated New01, New02 and New03, respectively. Some of the isolates yielding new AR patterns clustered with isolates of AR types, AR13, AR14 and AR43 when investigated by DNA macrorestriction analysis using *Sma*I followed by PFGE (Rossney *et al.*, 2003b).

1.9.5.3 EARSS 1999-2003

At the NMRSARL, MRSA isolates submitted to EARSS are investigated by AR typing, biotyping and DNA macrorestriction analysis using *Sma*I followed by PFGE. Five-digit pulsed-field type (PFT) numbers are assigned to PFGE patterns and PFTs of apparently related patterns are abbreviated to two-digit PFT groups (PFGs) (Rossney *et al.*, 2006a). Epidemiological typing results (AR typing and PFGE patterns) are combined to form AR-PFG types (Rossney *et al.*, 2006a). In 1999, four AR-PFG types predominated among the MRSA population in Ireland (AR-PFG 06-01, AR-PFG 07-02, AR-PFG 13-00 and AR-PFG 14-00) (Rossney *et al.*, 2006a). Among 1,580 blood culture MRSA isolates submitted to EARSS between 1999 and 2003, 57%, 7%, 14% and 22% of isolates exhibited AR-PFG types AR-PFG 06-01, AR-PFG 07-02, AR-PFG 13-00 and AR-PFG 14-00, respectively. The prevalence of AR-PFG 06-01 increased from 22% in 1999 to 80% in 2003 (Rossney *et al.*, 2006a). Resistance patterns of representative AR-PFG types are shown in Table 1.4.

Table 1.4. Resistance patterns of representative AR-PFG types.

AR-PFG type	AR type	Resistance pattern
06-01	AR06.1	Ap
	AR06.1c	ApCp
	AR06.3c	ApCdCp
	AR06.5	ApCdEr
	AR06.5c	ApCdCpEr
	AR06.6	ApEr
07-02	AR07.0c	Ak*ApCdCpErKnLnNmSpTb
	AR07.0cm	Ak*ApCdCpErKnLnMpNmSpTb
	AR07.2	Ak*ApCdCpErKnNmSpTb
	AR07.3	Ak*ApCpErKnNmSpTb
	AR07.4	Ak*ApCpErKnLnNmSpTb
	AR07.6	Ak*ApCpErKnLnNmSpRfTb
13-00	AR13.0	Ak*ApCdCpEbErGnKnLnMcNmPmaSmSpTb
	AR13.1	Ak*ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb
	AR13.1f	Ak*ApCdCpEbErFdGnKnLnMcMpNmPmaSmSpTb
	AR13.1t	Ak*ApCdCpEbErGnKnLnMcMpNmPmaSmSpTbTp
14-00	AR14.0	Ak*ApCdCpEbErGnKnMcNmPmaSmSpTb
	AR14.2	Ak*ApCdCpEbErGnKnMcMpNmPmaSmSpTb
	AR14.4	Ak*ApCdCpEbErGnKnMcNmPmaSmSpTbTp
	AR14.4m	Ak*ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp
43-00	AR43	ApCd*CpErKnNmSmSpTp
44-99.4	AR44	Ak*ApCdCpEbErGnKnLnMcMpNmPmaSmSpSuTbTeTp
New02-00	New02	AkApCdCpEbGnKnMcNmPmaRfSmSuTbTe

Abbreviations: Ak, amikacin; Ap, ampicillin; Cd, cadmium acetate; Cl, chloramphenicol; Cp, ciprofloxacin; Er, erythromycin; Eb, ethidium bromide; Fd, fusidic acid; Gn, gentamicin; Kn, kanamycin; Ln, lincomycin; Mc, mercuric chloride; Mp, mupirocin; Nm, neomycin; Pma, phenyl mercuric acetate; Rf, rifampicin; Sp, spectinomycin; Sm, streptomycin; Te, tetracycline; Tb, tobramycin; Tp, trimethoprim. Suffixes (lower case) after AR types indicate resistance to the following antimicrobials: c, ciprofloxacin; f, fusidic acid; m, mupirocin; t, trimethoprim. *, may be moderate or susceptible. Underlined AR type denotes the most frequently occurring pattern within each AR-PFG type (Rossney *et al.*, 2006; Rossney *et al.*, 2003).

1.9.5.4 New Strain of MRSA

In 2003, a new strain of MRSA was recognised at the NMRSARL. This multi-antibiotic resistant strain was recovered from a patient who had been hospitalised in Singapore a short time earlier and spread in one unit of a Dublin hospital causing a protracted outbreak. The AR pattern of this isolate was unlike any of the Irish MRSA strains and this pattern was assigned an AR type, AR44 (Rossney, 2003).

1.9.5.5 MLST and SCCmec Typing

A study investigating MLST and SCC*mec* types of representative MRSA isolates from Ireland between 1971 and 2002 showed that over 30 years, isolates belonging to the five major CCs and the major pandemic MRSA lineages were present among the MRSA population at different times (Shore *et al.*, 2005). The range of genotypes of representative MRSA isolates is shown in Table 1.5. Genotypes varied throughout the study period with ST250-MRSA-I (Phenotype I and Phenotype II) being present during the 1970s and early 1980s and ST239-MRSA-III (Phenotype III) during the late 1980s. Between 1992 and 1993, three of the major CCs were present among the MRSA population: CC8, ST239-MRSA-III (AR15), CC8, ST247-MRSA-I (AR22), CC8, ST8-MRSA-II (AR13 and AR14); CC22, ST22-MRSA-IV (AR06) and CC30, ST36-MRSA-II (AR07.0/07.2). During the 1990s, ST8-MRSA-II predominated among the MRSA population but by 2002 ST22-MRSA-IV was the dominant clone (Shore *et al.*, 2005).

1.9.6 Previous Glycopeptide Resistance Studies in Ireland

Vancomycin resistance was investigated among MRSA isolates collected in one Dublin

hospital between October and December 1998 and among isolates submitted to the North/South Study in 1999 (Anon., 2000a; Rossney & Keane, 2002). Isolates were screened on BHIV4 using a heavy inoculum (equivalent to a 2.0 McFarland turbidity standard), a modification of the method recommended for detection of glycopeptide resistance (Hiramatsu *et al.*, 1997a). Any isolate that grew on BHIV4 was sub-cultured onto BHI containing 8 mg/L vancomycin (BHIV8). No GISA or VRSA were detected but results suggested that 2.7% of isolates from the former study and 5% of isolates from the latter study may be hGISA (Rossney & Keane, 2002; Rossney *et al.*, 2003b). However, because these results were obtained following growth on BHIV4 and subsequent growth on BHIV8, the method may have selected for rather than detected the heteroresistant phenotype and possible hGISA isolates were not confirmed with PAP (Howe *et al.*, 2000). In both studies, none of the possible hGISA isolates yielded raised MICs (MIC >4 mg/L) when tested without prior exposure to vancomycin (Rossney & Keane, 2002; Rossney *et al.*, 2003b).

In addition to epidemiological typing, MRSA isolates submitted to the NMRSARL under the EARSS project are routinely screened using the E-test™ macromethod and BHIV6 agar screening media, which are the recommended screening methods for detection of VRSA, GISA and hGISA isolates (Brown *et al.*, 2005). Any isolate yielding preliminary screening results suggestive of reduced susceptibility to glycopeptides are further investigated by reference MIC methods (CLSI broth microdilution and E-test™ MIC methods) and by PAP methods using both vancomycin and teicoplanin to determine the resistant phenotype (Brown *et al.*, 2005; Nonhoff *et al.*, 2005). Prior to 2004, suspect hGISA isolates were sent to the Bristol Centre for Antimicrobial Research and Evaluation for confirmation of the hetero-glycopeptide resistant phenotype by PAP-AUC ratio determination.

Table 1.5. Genotypes of representative MRSA isolates (1971-2002).

CC	ST	AR type	SCC*mec* type	Clone
8	239	AR01, AR15, AR44, Ph III	III	Hungarian
8	250	Ph I, Ph II, AR02	I	Archaic
8	247	AR22, New02	I	Iberian
8	8	New01, New03, AR13, AR14	II	Irish-1
8	8	AR43	IV	EMRSA-2 and -6
5	5	AR07.3/07.4	II	New York/Japan
5	5	Unf 01	IV	Paediatric
22	22	AR06	IV	EMRSA-15
30	36	AR07.0/07.2	II	EMRSA-16
45	45	ARNT	IV	Berlin

Abbreviations: AR, antibiogram-resistogram; CC, clonal complex; NT, "no type"; Ph, phenotype; ST, sequence type; Unf, unfamiliar (Shore *et al.*, 2005).

1.10 Aims and Objectives

In Ireland, MRSA is a serious nosocomial problem and rates of meticillin-resistance in *S. aureus* isolates recovered from blood are among the highest in Europe (Rossney *et al.*, 2006a). Over the past two decades, vancomycin has served as the cornerstone of therapy against serious MRSA infections. Within the last decade, prevalence rates of MRSA have increased, GISA and hGISA isolates have emerged worldwide and have also been recovered among the MRSA population in Ireland (Appelbaum, 2006; Rossney *et al.*, 2006b). Whilst results from two previous glycopeptide resistance studies among MRSA isolates in Ireland suggest the hGISA phenotype was present in the MRSA population, hGISA isolates were not confirmed by PAP. To date, a comprehensive investigation of reduced susceptibility to glycopeptides among MRSA isolates recovered in Ireland has not been carried out.

The aims and objectives of the present study were:

- To determine the prevalence of VRSA, GISA and hGISA among the MRSA population in Ireland between 1998 and 2004. MRSA isolates selected for this study comprised of 3,189 isolates from 2,990 patients from six different collections of MRSA.
- To confirm the hetero-glycopeptide resistant phenotype among MRSA isolates using both vancomycin and teicoplanin PAP methods.
- To evaluate a number of agar screening techniques for the detection of GISA and hGISA and to establish a suitable agar screening method for reliable detection of hGISA for routine use in diagnostic microbiology laboratories.
- To apply this new agar screening method to MRSA isolated from all patients yielding MRSA during a prospective study between October and December 2004. In this phase

of the study, MRSA from patients on prolonged glycopeptide therapy were further investigated by collecting isolates at weekly intervals from any patient receiving >7 days glycopeptide therapy.

- To evaluate a number of phenotypic methods to enhance detection of the resistant phenotype for routine use in diagnostic microbiology laboratories.
- To perform epidemiological typing* analysis on isolates exhibiting reduced susceptibility to glycopeptides and to compare the prevalence of epidemiological types among these isolates with the epidemiological types of all isolates within each MRSA collection investigated in the study.
- To investigate whether epidemiological typing analysis can be used as a predictive marker for isolates that exhibit reduced susceptibility to glycopeptides.
- To perform molecular studies of GISA/hGISA isolates to determine if any characteristics could be used as suitable markers for identifying isolates exhibiting reduced susceptibility to glycopeptides.
- To investigate the presence of *van* genes among GISA/hGISA isolates to ensure no VRSA was present among the MRSA population.
- To investigate the ability of vancomycin to induce expression of the resistant phenotype among different MRSA strains and a GSSA control strain (*S. aureus* ATCC 29213) and to determine the stability of any such resistant phenotype by passaging isolates on drug-free medium for nine days.

*The term antibiogram-resistogram (AR) are used synonymously with antibiogram (A) and for the purpose of work presented in this thesis, antibiogram will be used.

CHAPTER II

Laboratory Detection of MRSA Isolates Exhibiting Reduced Susceptibility to Glycopeptides (1998 to 2004)

Chapter II Laboratory Detection of MRSA Isolates Exhibiting Reduced Susceptibility to Glycopeptides (1998 to 2004)

2.1 Introduction

Several large scale screening studies have been conducted worldwide to detect isolates exhibiting reduced susceptibility to glycopeptides (Walsh & Howe, 2002). Whilst reports of GISA have been relatively rare, the prevalence of hGISA reported has varied considerably due to the different methods employed for screening and confirmation (Walsh & Howe, 2002; Brown *et al.*, 2005; Liu & Chambers, 2003). Details of large scale screening studies and the detection methods used are shown in Table 2.1.

The simplified population analysis method described by Hiramatsu *et al.* has been adopted by many researchers to screen for isolates exhibiting reduced susceptibility to glycopeptides (Hiramatsu *et al.*, 1997a; Ike *et al.*, 2001; Hsueh *et al.*, 2004; Kim *et al.*, 2002; Song *et al.*, 2004; Lecaillon *et al.*, 2002; Aucken *et al.*, 2000; Wilcox & Fawley, 2001). A reported advantage of this method is its feasibility where isolates are screened by spotting bacterial suspensions of 10^6 CFU/ml on BHIV4 and incubating at 35°C for 48 h. Growth after 24 h indicates possible GISA whilst growth after 48 h indicates possible hGISA isolates (Hiramatsu *et al.*, 1997a). However, studies have shown this method to be unreliable (sensitivity and specificity values of 71% and 88%, respectively) in detecting isolates with reduced susceptibility to glycopeptides (Walsh *et al.*, 2001; Wootton *et al.*, 2001; Park *et al.*, 2001).

A group of researchers in Korea found initial screening on Mueller-Hinton agar containing 8 mg/L teicoplanin (MHT8) to be a reliable method for detecting staphylococcal isolates exhibiting reduced susceptibility to teicoplanin (Park *et al.*, 2000). The French Society for Microbiology recommends preliminary screening on MHT5 to detect all isolates exhibiting

both intermediate-resistance and heterogeneous-intermediate-resistance to teicoplanin (El Solh *et al.*, 2003).

An evaluation of methods for detecting reduced susceptibility to glycopeptides showed that screening with the E-test™ macromethod yielded sensitivity and specificity values of 96% and 97%, respectively (Walsh *et al.*, 2001). EARSS recommend a screening algorithm of preliminary screening on MHT5 with all screen-positive isolates being investigated with the E-test™ macromethod. Any isolate yielding E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone should be confirmed by PAP (Anon., 2005b). Several studies have reported a higher prevalence of isolates exhibiting reduced susceptibility to teicoplanin than to vancomycin and it has been suggested that both vancomycin and teicoplanin PAP methods should be used when confirming isolates suspected of having reduced susceptibility to glycopeptides (Hassan *et al.*, 2001; MacKenzie *et al.*, 2002; El Solh *et al.*, 2003; Nonhoff *et al.*, 2005).

Although several large scale screening programmes investigating reduced susceptibility to glycopeptides among *S. aureus* isolates have been performed, few evaluation studies of detection techniques have been undertaken (Walsh *et al.*, 2001). To date, no completely reliable screening method incorporating an antibiotic in agar medium has yet been described for the detection of hGISA isolates. The E-test™ macromethod can be used to screen for isolates with reduced susceptibility to glycopeptides but PAP methods remain the reference method for confirmation of the hetero-resistant phenotype. However, these methods are not suitable for routine clinical laboratory use (Walsh *et al.*, 2001; Wootton *et al.*, 2001; Nonhoff *et al.*, 2005).

Table 2.1. Large scale screening studies for detecting isolates exhibiting reduced susceptibility to glycopeptides.

Country	Isolates tested (*n*=)	Detection Method used	% hGISA	% GISA	Reference
Japan	1,149	BHIV4	1–26	0	Hiramatsu *et al.*, 1997
Japan	6,625	BHIV4 + PAP	0	0	Ike *et al.*, 2001
Taiwan	5,500	BHIV4	0	0	Hsueh *et al.*, 2004
South Korea	4,483	BHIV4 + PAP	0.5	0	Kim *et al.*, 2002
South Korea	1,357	BHIV4 + PAP	4	0	Song *et al.*, 2004
South Korea	1,519	MHT8 + MIC methods	8	0	Park *et al.*, 2001
France	858	BHIV4 + PAP	1	0	Lecaillon *et al.*, 2002
France	883	MHT5 + PAP	5	0	Mallaval *et al.*, 2004
France	2,300	BHIT4 + PAP	11	0	Garnier *et al.*, 2006
France	1,445	MIC methods	0	2	Robert *et al.*, 2006
Belgium	2,145	BHIV6 + PAP	0.1	0.1	Denis *et al.*, 2002
UK	41,567	ISAV4, BHIV4, BHIV6	0	0	Aucken *et al.*, 2000
UK	3,173	BHIV2, BHIV4, BHIV6, BHIV8	0.2	0	Wilcox *et al.*, 2001
USA	630	MHV5	0	0	Hubert *et al.*, 1999

Abbreviations: BHIV2, BHIV4, BHIV6, BHIV8, BHIT4, brain heart infusion agar containing 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L vancomycin or 4 mg/L teicoplanin, respectively; MHT5, MHT8, MHV5, Mueller-Hinton agar containing 5 mg/L, 8 mg/L teicoplanin or 5 mg/L vancomycin, respectively; ISAV4, Isosensitest agar containing 4 mg/L vancomycin; MIC, minimum inhibitory concentration; PAP, population analysis profiling.

Several phenotypic properties of both clinical and laboratory-selected isolates of *S. aureus* exhibiting reduced susceptibility to glycopeptides have been identified. Activated cell wall synthesis resulting in a thickened cell wall is the most common feature associated with GISA/hGISA isolates (Hanaki *et al.*, 1998a; Geisel *et al.*, 2001; Cui *et al.*, 2000; Hiramatsu *et al.*, 1997b; Sieradzki *et al.*, 1998; Sieradzki & Tomasz, 1997; Sieradzki & Tomasz, 1998; Moreira *et al.*, 1997; Sieradzki & Tomasz, 1999; Daum *et al.*, 1992; Cui *et al.*, 2006; Pfeltz *et al.*, 2000; Sieradzki & Tomasz, 2003; McCallum *et al.*, 2006). Reduced peptidoglycan cross-linking in addition to alterations in PBP2 and PBP4 expression have also been documented (Sieradzki & Tomasz, 1997; Sieradzki & Tomasz, 1998; Sieradzki & Tomasz, 1999; Finan *et al.*, 2001; Hanaki *et al.*, 1998b; Koehl *et al.*, 2004; Moreira *et al.*, 1997; Sieradzki *et al.*, 1999c; Hanaki *et al.*, 1998a). Changes in colonial morphology, a slower growth rate, decreased coagulase activity, reduced susceptibility to lysostaphin and decreased autolytic activity have also been reported (Hiramatsu *et al.*, 1997b; Sieradzki & Tomasz, 1997; Pfeltz *et al.*, 2000; McCallum *et al.*, 2006; Daum *et al.*, 1992; Moreira *et al.*, 1997; Koehl *et al.*, 2004; Sieradzki *et al.*, 1998; Sieradzki & Tomasz, 2006; Sieradzki *et al.*, 1999b; Utaida *et al.*, 2006; Boyle-Vavra *et al.*, 2001; Boyle-Vavra *et al.*, 2003; Pfeltz & Wilkinson, 2004). Although several phenotypic characteristics have been reported to be associated with the GISA/hGISA phenotype, expression has varied considerably and no single property is definitive of the GISA/hGISA phenotype.

In 1999, Wong *et al.* reported that the addition of salt (4% NaCl) to MH agar containing 4 mg/L vancomycin (MHV4) induced expression of reduced susceptibility in hGISA isolates and that an interaction (antagonistic effect) between vancomycin and beta-lactam antibiotics was observed when disks containing a beta-lactam antibiotic were placed on MHV4 (Wong *et*

al., 1999). The concomitant use of low concentration beta-lactam antibiotics antagonises the activity of vancomycin against some strains of MRSA. Subsequent studies used these characteristics to screen for isolates exhibiting reduced susceptibility to glycopeptides (Chesneau *et al.*, 2000; Wong *et al.*, 1999; Woodford *et al.*, 2000). However, not all GISA/hGISA isolates investigated demonstrated this interaction effect to beta-lactams, thus beta-lactam/vancomycin interaction was subsequently reported to be characteristic of some but not all isolates exhibiting reduced susceptibility to glycopeptides (Chesneau *et al.*, 2000; Woodford *et al.*, 2000; Cui *et al.*, 2003).

2.2 Aims and Objectives

The aims and objectives were:

- To investigate isolates exhibiting reduced susceptibility to glycopeptides among 3,189 MRSA isolates recovered from six collections of MRSA in Ireland between 1998 and 2004 and to determine the prevalence of VRSA, GISA and hGISA amongst these MRSA populations.
- To evaluate a number of agar screening methods for detecting isolates exhibiting reduced susceptibility to glycopeptides and to define a suitable agar screen to reliably detect hGISA isolates.
- To apply this new agar screen prospectively to all MRSA isolates collected in one 936-bed tertiary-referral adult university hospital (H1) during Q4, 2004. One isolate per patient was collected unless routine susceptibility tests in the diagnostic laboratory suggested the patient was carrying more than one strain of MRSA. Patients receiving >7 days glycopeptide therapy had isolates collected at weekly intervals as long as therapy continued.

- To evaluate phenotypic methods to enhance detection of the resistant phenotype by investigating:
    1. Disk diffusion susceptibility testing using low concentration vancomycin and teicoplanin antimicrobial disks.
    2. Agar screening media containing vancomycin and the effect of the addition of 4% NaCl to vancomycin agar screening media.
    3. Beta-lactam/vancomycin interaction on vancomycin agar screening media with and without the addition of 4% NaCl.

2.3 Materials and Methods

2.3.1 Bacterial Isolates

MRSA isolates (n=3,189 isolates from 2,990 patients) from six different collections of MRSA recovered between 1998 and 2004 were investigated for reduced susceptibility to glycopeptides. In general, one isolate per patient was included unless routine susceptibility tests in the diagnostic laboratory suggested the patient was carrying more than one strain of MRSA. Isolates were collected from the following sources:

1. All MRSA isolates recovered in one 936-bed tertiary-referral adult university hospital (H1) during Q4, 1998 (n=188).
2. All MRSA isolates recovered during a two-week study period in 1999 from all hospitals that participated in the North/South Study of MRSA in Ireland (n=714).
3. MRSA isolates from blood cultures (one isolate per patient per quarter) submitted to the NMRSARL under the EARSS project between 1999 and 2003 (n=1,580).
4. All MRSA isolates recovered from H1 during Q2, 2003 (n=330).
5. All MRSA isolates submitted to the NMRSARL between 2000 and 2003 for investigation of reduced susceptibility to glycopeptides (n=97).
6. All MRSA isolates recovered from H1 during a prospective study in Q4, 2004 (n=280). In addition, isolates collected at weekly intervals from patients receiving >7 days glycopeptide therapy as long as therapy continued were also investigated (n=30).

Isolates in Collections 1 and 2 were screened for growth on BHIV4 using a heavy inoculum (200-µl volumes of bacterial suspensions with densities equivalent to a 2.0 McFarland turbidity standard) during two previous studies of MRSA in Ireland (Anon., 2000a; Rossney & Keane, 2002). All 84 of 188 isolates from Collection 1 and 489 of 714 isolates from

Collection 2 that grew on BHIV4 were included in the present study.

2.3.2 Isolate Storage

All isolates were stored at -70°C on cryoprotective beads (Protect Bacterial Preservers, Technical Service Consultant Ltd., Hayward, UK).

2.3.3 Identification of MRSA

Bacterial isolates were identified as *S. aureus* by testing for staphylocoagulase production using the tube coagulase test (Normal Human Control Plasma, Technoclone Ltd., Dorking, Surrey, UK) (Rossney *et al.*, 1990). Isolates that tested coagulase-negative were investigated further using a *S. aureus* identification kit (Pastorex Staph-Plus, BioRad, Marnes-la-Coquette, France) (Davies & Zadik, 1997). Isolates showing equivocal staphylocoagulase and/or Pastorex Staph-Plus results were tested for the production of thermostable DNase (Lachica *et al.*, 1971). Further identification using the API Staph system (bioMeriéux sa, Marcy l'Étoile, France) was performed when necessary.

Meticillin susceptibility was detected on Colombia agar (LabM, Lab 1, International Diagnostics Group plc, Bury, Lancashire, UK), containing 7% horse blood (CBA) using a heavy inoculum and meticillin disk (10 µg) (Oxoid Limited, Basingstoke, UK), with incubation at 30°C for 24 h (Annear, 1968; Hewitt *et al.*, 1969). Control strains used were *S. aureus* ATCC 25923 as a susceptible control and *S. aureus* ATCC 43300 as a low-level meticillin-resistant control. Isolates, which did not appear fully resistant to meticillin were retested using oxacillin {1 µg and 5 µg/disk, (Oxoid)}. Isolates showing equivocal results

were tested for the presence of PBP2a using the Mastalex-MRSA kit (MAST Diagnostics, Merseyside, UK).

2.3.4 Preliminary Agar Screening

Details of the control strains used with each agar screening method are described in Table 2.2.

2.3.4.1 Vancomycin Agar Screen

2.3.4.1.1 Brain Heart Infusion Agar containing 6 mg/L Vancomycin

BHI agar (Oxoid, CM 0375) containing 6 mg/L vancomycin (vancomycin hydrochloride, Sigma-Aldrich Limited, Tallaght, Ireland, V2002) (BHIV6) was prepared in-house. Colonies from an overnight culture on CBA were suspended in sterile saline to a density equivalent to a 0.5 McFarland turbidity standard (Tenover *et al.*, 1998). The inoculum was delivered using 10-µl loops and four isolates were inoculated per plate. Plates were incubated for 48 h at 35°C and growth was observed after 24 h and 48 h. Colonies were counted to a maximum count of 100 but if >100 colonies were obtained, growth was graded as + (>100 colonies); ++ (semi-confluent growth); +++ (confluent growth). Growth of ≥1 colony after 48 h indicated a positive result.

2.3.4.2 Teicoplanin Agar Screen

2.3.4.2.1 Mueller-Hinton Agar containing 5 mg/L Teicoplanin

MH agar (Becton, Dickinson and Company (BD) Maryland, USA; 225250) containing 5 mg/L teicoplanin (teicoplanin sodium salt, Sanofi-Aventis, Paris, France) (MHT5) was prepared in-house. Colonies from an overnight culture on CBA were inoculated into BHI broth (Oxoid,

Table 2.2. Quality control strains used with agar screening media and MIC methods and expected results for each strain.

	Screening Medium		MIC Method[a]	
Control Strain	BHIV6	MHT5, MHT8, BHIT5	V[b]	T[c]
S. aureus ATCC 25923	NG	NG	…	…
S. aureus ATCC 29213	NG	NG	0.5–2.0	0.25–1.0
S. aureus Mu3 ATCC 700698	NG	G	…	…
S. aureus Mu50 ATCC 700699	G	G	…	…
E. faecalis ATCC 29212	NG	NG	1.0–4.0	0.125–0.5
E. faecalis ATCC 51299	G	NG	…	…

[a], E-test™ and CLSI broth microdilution MIC methods (Anon., 2006b).

[b], Vancomycin quality control range (mg/L) (Anon., 2006a).

[c], Teicoplanin quality control range (mg/L) (Anon., 2006a).

Abbreviations: BHIV6, BHIT5, brain heart infusion agar containing 6 mg/L vancomycin and 5 mg/L teicoplanin, respectively; MHT5, MHT8, Mueller-Hinton agar containing 5 mg/L and 8 mg/L teicoplanin respectively; G, growth; NG, no growth.

CM 225) and incubated at 35°C overnight (Anon., 2005b). Suspensions of stationary phase broth subcultures were inoculated onto MHT5 plates using 10-µl loops and four isolates were inoculated per plate. Plates were incubated and read as described in Section 2.3.4.1.1 above.

2.3.4.2.2 Mueller-Hinton Agar containing 8 mg/L Teicoplanin

MH agar containing 8 mg/L teicoplanin (MHT8) was prepared in-house. Colonies from an overnight culture on CBA were suspended in sterile saline to a density equivalent to a 0.5 McFarland turbidity standard (Park *et al.*, 2000). Plates were inoculated, incubated and read as described in Section 2.3.4.1.1 above.

2.3.4.3 Overview of Agar Screening

Details of the isolates screened and the screening techniques used are summarised in Table 2.3. A schematic representation of the numbers of isolates screened by preliminary agar screening methods is shown in Figure 2.1. All isolates in the present study were screened with BHIV6. All isolates in Collections 1, 2 and 4 were screened with MHT5. Between 1999 and 2002, isolates in Collection 3 that yielded E-test™ macromethod values >4 mg/L for vancomycin and/or teicoplanin were also screened with MHT5 (n=87). In addition to screening with MHT5, isolates in Collection 4 were screened with MHT8.

2.3.5 E-test™ Macromethod

E-test™ macromethod values were determined according to the manufacturer's instructions using vancomycin and teicoplanin E-test™ strips (AB bioMérieux, Solna, Sweden) (Anon., 2004b). Colonies from an overnight culture on CBA were suspended in sterile saline to a

density equivalent to a 2.0 McFarland turbidity standard. Two hundred microlitre volumes of bacterial suspensions were pipetted onto each BHI agar plate and spread evenly with a swab. Plates were dried at room temperature (RT), E-test™ strips were applied and plates were incubated at 35°C for 48 h. The interpretive criteria for reduced susceptibility were values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone (Walsh *et al.*, 2001).

All isolates from Collections 1 and 2 that grew on BHIV4, all isolates in Collections 3 and 5 and any isolates from Collections 4 and 6 that grew on any screening media were investigated by the E-test™ macromethod. In total, 2,348 isolates were investigated. Details of the isolates investigated are summarised in Table 2.3 and shown in Figure 2.1.

2.3.6 MIC Methods

2.3.6.1 E-test™

Isolates were grown overnight on CBA and suspensions were prepared in sterile saline to a density equivalent to a 0.5 McFarland turbidity standard. Bacterial suspensions were inoculated onto MH agar and left to dry at RT. Vancomycin and teicoplanin E-test™ strips were applied according to the manufacturer's instructions and plates were incubated at 35°C for a full 24 h (Anon., 2004a). Isolates were classified as susceptible, intermediate or resistant according to CLSI breakpoints as follows: vancomycin MIC, ≤2 mg/L (susceptible), 4–8 mg/L (intermediate), ≥16 mg/L (resistant); teicoplanin MIC, ≤8 mg/L (susceptible), 16 mg/L (intermediate), ≥32 mg/L (resistant) (Anon., 2006a). Details of the control strains used and expected values are shown in Table 2.2.

Table 2.3. Numbers of isolates investigated by preliminary agar screening and by MIC methods.

	Screening Methods						MIC	
MRSA Collection	(BHIV4[a])[b]	BHIV6[a]	MHT5[a]	MHT8[a]	BHIT5[a]	E-test[c]	E-test[d]	CLSI[e]
1. H1 Q4, 1998 (*n*=188)	(188)[b]	84	84	…	…	84	7	84
2. N/S Study 1999 (*n*=714)	(714)[b]	489	489	…	…	489	74	489
3. EARSS 1999-2003 (*n*=1,580)	…	1,580	87	…	…	1,580	169	87
4. H1 Q2, 2003 (*n*=330)	…	330	330	330	330	37[f] [36][g]	18	…
5. NMRSARL 2000-2003 (*n*=97)	…	97	…	…	…	97	19	…
6. H1 Q4, 2004 (*n*=280)	…	280	…	…	280[30][h]	25	6	…
Total (*n*=3,189)	(902)[b]	2,860	990	330	640	2,348	293	660

[a], Growth of ≥1 colony indicated a positive result. [b], Screening carried out in previous studies (Rossney & Keane, 2002; Anon., 2000). [c], E-test™ macromethod. [d], E-test™ MIC. [e], CLSI broth microdilution. [f], Isolates investigated following screening with MHT5. [g], Isolates in Collection 4 were also screened using BHIT5 and an additional 36 suspect isolates requiring E-test™ macromethod investigation were found. [h], Isolates in Collection 6 (*n*=280 plus an additional 30 isolates from patients on >7 days glycopeptide therapy) were screened with BHIT5 only and 25 isolates required investigation by the E-test™ macromethod. Abbreviations: BHIV4, BHIV6, brain heart infusion agar containing 4 mg/L and 6 mg/L vancomycin, respectively; BHIT5, brain heart infusion agar containing 5 mg/L teicoplanin; EARSS, European Antimicrobial Resistance Surveillance System; H1, Hospital 1; MHT5, MHT8, Mueller-Hinton agar containing 5 mg/L and 8 mg/L teicoplanin, respectively; NMRSARL, National MRSA Reference Laboratory; N/S, North/South Study of MRSA in Ireland 1999; Q, quarter; …, not done.

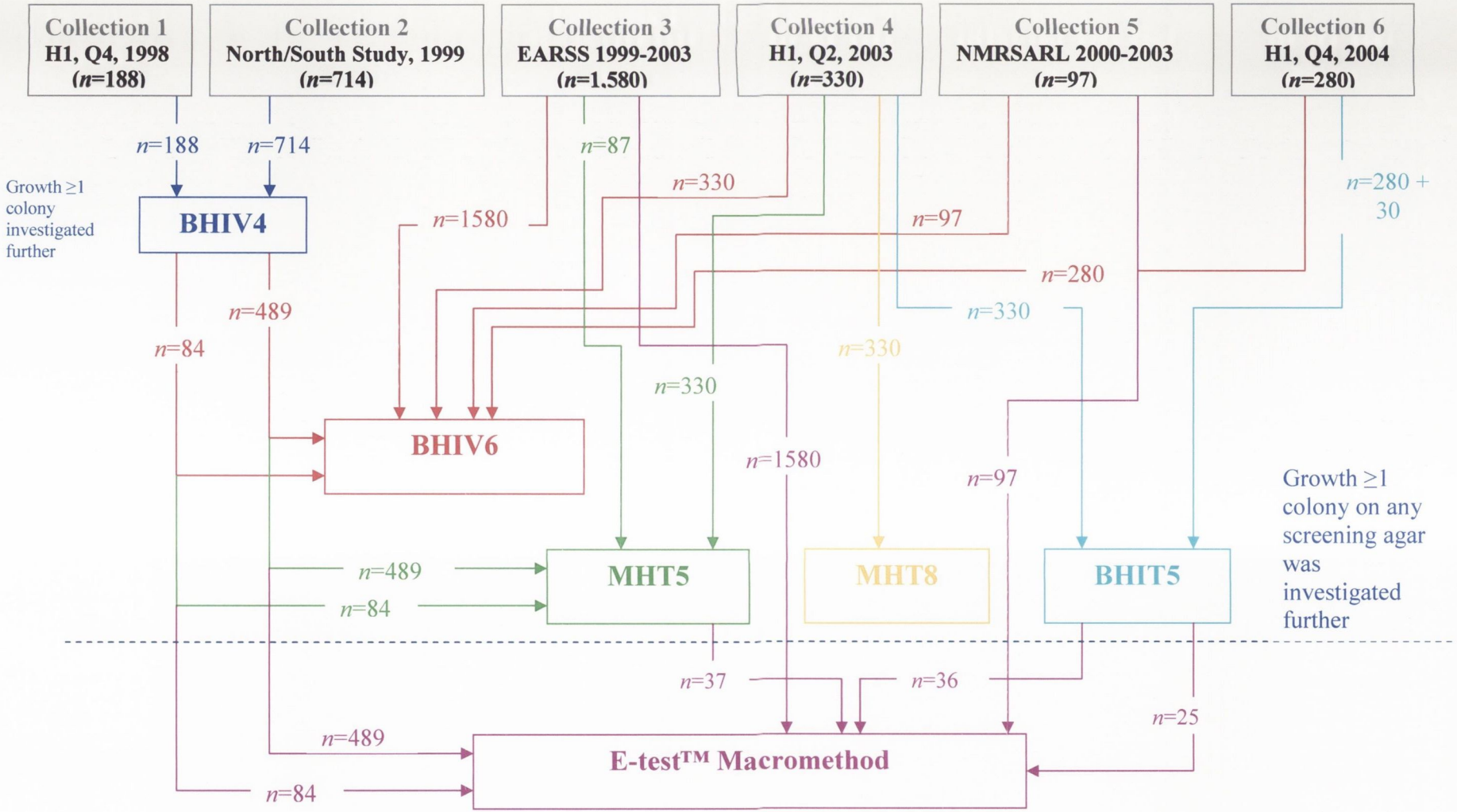


Figure 2.1. Numbers of isolates screened by preliminary agar screening methods and the E-test™ macromethod.

E-test™ MICs for vancomycin and teicoplanin were performed on all isolates exhibiting E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone (*n*=139) and on isolates from Collection 3 that yielded E-test™ macromethod values >4 mg/L for both vancomycin and/or teicoplanin (*n*=169). In total, 293 isolates were investigated of which the details are shown in Table 2.3.

2.3.6.2 Broth Microdilution

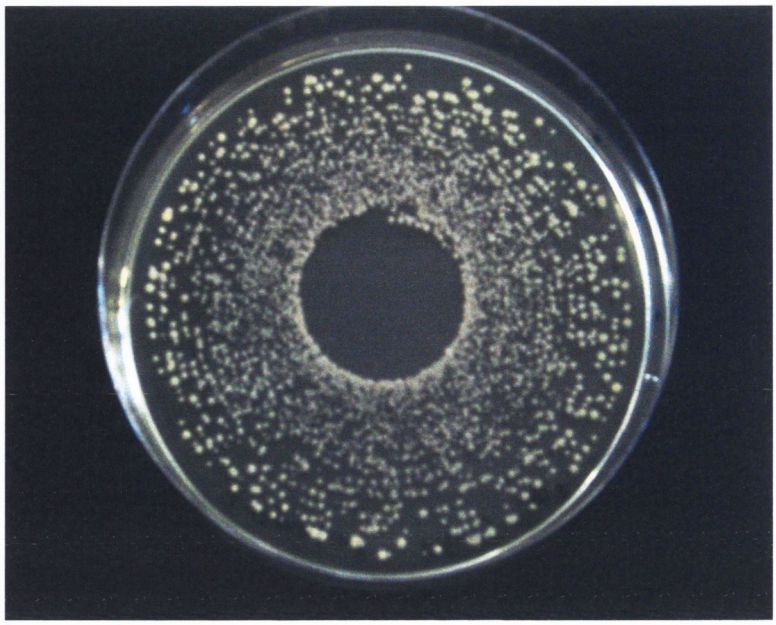
Broth microdilution MICs for vancomycin and teicoplanin were undertaken as described in CLSI M7-A7 (Anon., 2006b). Microtitre trays containing antibiotic in 50-µl volumes were prepared in-house and frozen at -20°C. Bacteria from an overnight culture on CBA were suspended in sterile saline to a density equivalent to a 0.5 McFarland turbidity standard (10^8 CFU/ml). Bacterial suspensions were diluted (1/100) in cation-adjusted Mueller-Hinton Broth {BD, 212322, (CAMHB)} and 50-µl volumes were inoculated into each well resulting in approximately 5 x 10^5 CFU/ml in each well. Microtitre trays were incubated at 35°C for 24 h. Isolates were classified as susceptible, intermediate or resistant according to CLSI breakpoints as described in Section 2.3.6.1. The control strains used are detailed in Table 2.2.

MICs were determined on all isolates requiring further investigation from Collections 1 and 2 (*n*=84 and 489, respectively) and on all isolates in Collection 3 recovered between 1999 and 2002 that yielded E-test™ macromethod values >4 mg/L for both vancomycin and/or teicoplanin (*n*=87). Details of the isolates investigated are shown in Table 2.3.

2.3.7 Population Analysis Profiling

Colonies from an overnight culture on CBA were inoculated into tryptic soy broth (TSB) (BD, 211825). After 24 h incubation, cultures were diluted in saline to 10^{-3} (10^5 CFU/ml) and 10^{-6} (10^2 CFU/ml) and 50-µl volumes were inoculated onto BHI agar plates containing 0, 0.5, 1.0, 2.0, 2.5, 4.0, 8.0, 12.0 and 16.0 mg/L vancomycin and 0, 4.0, 6.0, 8.0, 12.0 and 16.0 mg/L teicoplanin, respectively, using a spiral plater (Don Whitley Scientific Limited, Shipley, West Yorkshire UK) (Wootton *et al.*, 2001; Nonhoff *et al.*, 2005). Colonies were counted after 48 h incubation at 35°C manually using the counting grid (S00113) supplied with the spiral plater and also using an automated colony counting system, ProtoCol (Synoptics Ltd., Cambridge, UK). The log of the CFU/ml was plotted against the antibiotic concentration using GraphPad Prism software (GraphPad Software™, Inc., San Diego, California, USA) to obtain vancomycin and teicoplanin PAPs. The vancomycin PAP was used to calculate the AUC of each test isolate. To distinguish between GISA, hGISA and GSSA isolates, a ratio of the AUC of the test strain to the AUC of *S. aureus* Mu3 ATCC 700698 was calculated (Wootton *et al.*, 2001). Vancomycin PAP-AUC (vPAP-AUC) ratio criteria for the determination of reduced susceptibility to glycopeptides were: GSSA, <0.9, hGISA, 0.9–1.29; GISA, ≥1.3. Using teicoplanin PAP (tPAP), the criterion for defining hTISA isolates was any isolate with resistant subpopulations that grew at concentrations above the susceptible breakpoint (>8 mg/L teicoplanin) (Nonhoff *et al.*, 2005). Control strains *S. aureus* Mu3 ATCC 700698, *S. aureus* Mu50 ATCC 700699 and *S. aureus* ATCC 29213 (GSSA) were included with each batch of isolates tested. Figure 2.2 shows the colonial morphology exhibited by a hTISA isolate when grown on BHI agar containing 4 mg/L and 12 mg/L teicoplanin, respectively.

Panel A



Panel B

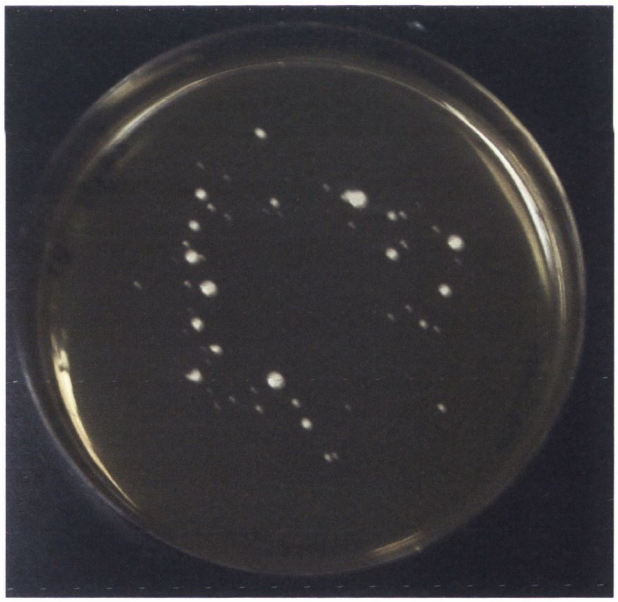


Figure 2.2. Heterogeneous colonial morphology exhibited by a hTISA isolate on brain heart infusion agar containing teicoplanin.

Panels A and B present the colonial morphology exhibited by a hTISA isolate when grown on brain heart infusion agar containing 4 mg/L and 12 mg/L teicoplanin, respectively.

PAPs were performed on all isolates exhibiting E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone (n=139) as recommended by Walsh *et al.* (Walsh & Howe, 2002). In addition, any isolate exhibiting an E-test™ macromethod teicoplanin value of 8 mg/L (n=119) was also investigated by vPAP-AUC and tPAP. In a pilot study, 36 isolates yielding teicoplanin E-test™ macromethod values of 6 mg/L were investigated by PAPs. The numbers of isolates requiring investigation by PAP methods following screening with the E-test™ macromethod are shown in Table 2.4.

2.3.8 Evaluation of Agar Screening Methods

2.3.8.1 Bacterial Isolates

Six agar screening methods were investigated using 110 hGISA and 68 GSSA isolates detected during the present study. All hGISA isolates were confirmed by PAP using both vancomycin and teicoplanin. The 68 GSSA isolates comprised 24 isolates that yielded E-test™ macromethod ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone and were negative by vPAP-AUC and tPAP methods and 44 isolates that yielded negative screening results with the E-test™ macromethod. The GSSA isolates included 10 with E-test™ macromethod values of ≥4 mg/L and ≤8 mg/L for both vancomycin and teicoplanin and 34 isolates with E-test™ macromethod values ≤2 mg/L for both vancomycin and teicoplanin.

2.3.8.2 Agar Screening Media

The media evaluated were MHT5 and BHI agar containing 5 mg/L teicoplanin (BHIT5). Each medium was evaluated using 10-µl volumes from three inoculum preparations consisting of

bacterial suspensions prepared in saline to densities equivalent to 0.5 and 2.0 McFarland turbidity standards, respectively, ($MHT5_{0.5}$, $BHIT5_{0.5}$, $MHT5_{2.0}$ and $BHIT5_{2.0}$) and from stationary phase BHI broth subcultures (10^8 CFU/ml) ($MHT5_S$ and $BHIT5_S$). Twelve isolates were inoculated by pipette onto each plate and screening tests were performed in duplicate. Plates were incubated at 35°C and growth was observed after 24 h and 48 h. Colonies were counted and quantified as described in Section 2.3.4.1.1 above. Growth of ≥1 colony after 48 h indicated a positive result. The control strains used are detailed in Table 2.2.

During preliminary agar screening, it had been noted that 51 isolates shown subsequently to be hGISA by vPAP-AUC and tPAP methods failed to grow on MHT5 when the inoculum was delivered by 10-µl loop. Therefore, when evaluating the six agar screening methods, the inoculum was delivered by pipette. Isolates from Collection 4 were re-screened using the screening medium that yielded the best overall performance in this evaluation ($BHIT5_{0.5}$) because preliminary screening of these isolates on MHT5 was performed by inoculating with a 10-µl loop.

2.3.9 Prospective Study

MRSA isolates recovered in H1 during Q4, 2004 were collected daily, identified and stored as described in Sections 2.3.3 and 2.3.2, respectively. One isolate per patient was collected unless routine susceptibility tests in the diagnostic laboratory suggested the patient was carrying more than one strain of MRSA. In collecting MRSA isolates, no distinction was made between colonised or infected patients. Computer records of patient details were utilised to generate a list of patients receiving glycopeptide therapy. Patients receiving >7 days glycopeptide therapy had isolates collected at weekly intervals as long as therapy continued.

Table 2.4. Numbers of isolates yielding positive results by agar screening and the E-test™ macromethod.

MRSA Collection	Screening Methods						
	(BHIV4[a])[b]	BHIV6[a]	MHT5[a]	MHT8[a]	$BHIT5_{0.5}$[a]	E-test™ Macromethod	
						V & T ≥8 or T ≥12[c]	T 8[d]
1. H1 Q4, 1998 (*n*=188)	(84)[b]	0	33	…	…	7	8
2. N/S Study 1999 (*n*=714)	(489)[b]	0	178	…	…	74	62
3. EARSS 1999-2003 (*n*=1,580)	…	0	30	…	…	15	31
4. H1 Q2, 2003 (*n*=330)	…	0	37	0	73	18	7
5. NMRSARL 2000-2003 (*n*=97)	…	0	…	…	…	19	11
6. H1 Q4, 2004 (*n*=280)	…	0	…	…	25	6	0
Total (*n*=3,189)	(573)[b]	0	278	0	98	139	119

[a], Growth of ≥1 colony indicated a positive result.

[b], Screening carried out in previous studies (Rossney & Keane, 2002; Anon., 2000).

[c], Cut-off values: ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone.

[d], Cut-off value was 8 mg/L for teicoplanin alone.

Abbreviations. See Table 2.3.

$BHIT5_{0.5}$, brain heart infusion agar containing 5 mg/L teicoplanin with an inoculum density equivalent to a 0.5 McFarland turbidity standard.

All isolates were screened with $BHIT5_{0.5}$, which was prepared in-house at weekly intervals. Colonies from overnight cultures on CBA were suspended in sterile saline to a density equivalent to a 0.5 McFarland turbidity standard. Plates were inoculated, incubated and read as described in Section 2.3.8.2 above. Any isolate yielding growth on $BHIT5_{0.5}$ was investigated by the E-test™ macromethod and PAPs were determined where necessary. In total, 310 isolates recovered from 270 patients were collected and screened on $BHIT5_{0.5}$. Details of isolates screened are shown in Table 2.3 and Figure 2.1.

2.3.10 Evaluation of Phenotypic Methods to Enhance Detection

2.3.10.1 Bacterial Isolates

Twelve hGISA isolates comprising 10 hVISA & hTISA and two hVISA isolates were randomly selected to investigate phenotypic methods to enhance detection of the resistant phenotype. These methods were further evaluated using 205 MRSA isolates comprising 117 hGISA and 88 MR-GSSA isolates, described in detail in Section 2.3.10.3.

2.3.10.2 Pilot Study

2.3.10.2.1 Disk Diffusion Screening

Methods of disk diffusion susceptibility testing evaluating antimicrobial disks containing 1 µg, 2 µg, 5 µg and 30 µg vancomycin and teicoplanin, respectively, per disk were investigated using 12 hGISA isolates described in Section 2.3.10.1. Vancomycin 5 µg (CT0188B), vancomycin 30 µg (CT0058B) and teicoplanin 30 µg (CT06477B) antimicrobial disks were purchased from Oxoid. Disks containing vancomycin at concentrations of 1 µg/disk and 2

µg/disk and teicoplanin at concentrations of 1 µg/disk, 2 µg/disk and 5 µg/disk were prepared in-house using blank disks (Oxoid). In addition, MH agar and MH agar containing 2% NaCl (Merck 1.06400) (MHS) were prepared in-house.

Overnight cultures on CBA suspended in sterile saline to densities equivalent to 0.5 McFarland turbidity standard (10^8 CFU/ml) were swabbed onto MH and MHS agar plates, respectively, and four antimicrobial disks were applied per plate. Plates were inverted and incubated at 35°C for 18 h. Positive control reference strains were *S. aureus* Mu3 ATCC 700698 and *S. aureus* Mu50 ATCC 700699 whilst *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 were included as negative control strains. Following incubation, zone sizes were measured with callipers.

2.3.10.2.2 Vancomycin Agar Screening

Nine vancomycin agar screening media were investigated using 12 hGISA isolates prepared at three different inoculum densities. Nine agar screen media comprising MHV4, MHV4 containing 4% NaCl, MHV5, MHV5 containing 4% NaCl, BHIV4, BHIV4 containing 4% NaCl, BHI agar containing 5 mg/L vancomycin (BHIV5), BHIV5 containing 4% NaCl and BHIV6 containing 4% NaCl were prepared in-house. Each medium was evaluated using 10-µl volumes from three inoculum preparations consisting of bacterial suspensions prepared in saline to densities equivalent to 0.5 (1 X 10^8 CFU/ml), 1.0 (3 X 10^8 CFU/ml) and 2.0 (6 X 10^8 CFU/ml) McFarland turbidity standards. Four isolates were inoculated by pipette onto each plate and screening tests were performed in duplicate. Plates were incubated at 35°C and growth was observed after 24 h and 48 h. Colonies were counted and quantified as described in Section 2.3.4.1.1. Growth of ≥1 colony after 48 h indicated a positive result. Positive

control reference strains were *S. aureus* Mu3 ATCC 700698, *S. aureus* Mu50 ATCC 700699 and *E. faecalis* ATCC 51299 whilst *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 were negative control strains.

2.3.10.2.3 Beta-lactam/Vancomycin Interaction

Interaction between beta-lactams and vancomycin was investigated among 12 hGISA isolates using the nine vancomycin agar screening methods described in Section 2.3.10.2.2. Each medium was evaluated using suspensions of organism prepared in sterile saline to densities equivalent to 0.5, 1.0 and 2.0 McFarland turbidity standards. Bacterial suspensions were swabbed onto quadrants of each respective agar plate and a 30-µg aztreonam disk (Oxoid, CT0264B) was placed at the centre of each inoculum. Plates were inverted, incubated at 35°C and growth was observed after 24 h and 48 h. Positive and negative control strains used with each method evaluated were *S. aureus* Mu3 ATCC 700698 and *S. aureus* Mu50 ATCC 700699, respectively.

2.3.10.3 Evaluation Study

Methods that yielded the best overall performance in the pilot study were evaluated using 205 MRSA isolates comprising 117 hGISA and 88 MR-GSSA isolates. All 117 hGISA isolates were confirmed by PAP using both vancomycin and teicoplanin as described in Section 2.3.7. The 88 MR-GSSA isolates comprised 31 isolates that yielded E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone and were negative by vPAP-AUC and tPAP methods and 57 isolates that yielded negative screening results with the E-test™ macromethod. The 57 MR-GSSA isolates comprised 10 isolates with E-test™ macromethod values of ≥4 mg/L and ≤8 mg/L for both vancomycin and teicoplanin,

13 isolates with E-test™ macromethod values of 4 mg/L for both vancomycin and teicoplanin and 34 isolates with E-test™ macromethod values ≤2 mg/L for both vancomycin and teicoplanin.

Further evaluation of teicoplanin 1 µg/disk and 2 µg/disk was performed on MH agar as described in Section 2.3.10.2.1. Beta-lactam/vancomycin interaction was investigated on BHIV4 containing 4% NaCl as described in Section 2.3.10.2.3.

2.3.11 Sensitivity and Specificity

The performance of each method in detecting isolates exhibiting reduced susceptibility to glycopeptides (GISA or hGISA) was evaluated by comparison with vancomycin and/or teicoplanin PAP methods. Each method was assessed for its sensitivity and specificity in discriminating GISA or hGISA isolates from MRSA isolates. The sensitivity is based on the number of GISA or hGISA isolates that were correctly identified. The specificity is based on the number of correct negative results, i.e., the true number of MRSA isolates that were correctly identified.

2.4 Results

2.4.1 Preliminary Agar Screening

The numbers of isolates yielding positive results by preliminary agar screening methods are shown in Table 2.4. No isolate grew on BHIV6 and MHT8 agar screen plates. Of 990 isolates screened with MHT5 when the inoculum was delivered by 10-µl loop, 278 isolates yielded growth. Among these 278 isolates, 81 isolates were confirmed as hGISA by vancomycin and teicoplanin PAP methods but an additional 51 isolates subsequently confirmed as hGISA did not grow on this medium.

2.4.2 E-test™ Macromethod

Of 2,348 isolates screened using the E-test™ macromethod, 139 isolates yielded values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone. When a cut-off value of 8 mg/L for teicoplanin alone was accepted as a positive result, an additional 119 isolates required further investigation by PAP methods. Details of the numbers of isolates yielding positive macromethod values among each collection are shown in Table 2.4.

2.4.3 MIC Determination

All 293 isolates investigated by E-test™ MIC determination (Table 2.3) for both vancomycin and teicoplanin exhibited susceptible results as no isolate had a vancomycin or teicoplanin MIC of >2 mg/L or >8 mg/L, respectively. Similarly, all 660 isolates tested using the broth microdilution method (Table 2.3) yielded MICs of ≤2 mg/L for vancomycin and ≤8 mg/L for teicoplanin. Hence there were no VRSA or GISA among these MRSA isolates.

2.4.4 Population Analysis Profiling

Population analysis profiling was performed on 294 isolates comprising 139 isolates exhibiting E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone (Table 2.4), 119 isolates yielding teicoplanin E-test™ macromethod values of 8 mg/L (Table 2.4) and 36 isolates yielding teicoplanin E-test™ macromethod values of 6 mg/L. In total, 178 isolates were confirmed as hGISA by vancomycin and/or teicoplanin PAP methods. Among 36 isolates yielding teicoplanin E-test™ macromethod values of 6 mg/L, no hGISA were detected. Details of hGISA isolates detected using vPAP-AUC and/or tPAP methods and the breakdown of hGISA isolates into hVISA, hTISA and hVISA & hTISA isolates are shown in Table 2.5. The vancomycin and teicoplanin PAPs of four hGISA isolates and three control strains are shown in Figure 2.3.

Of 139 isolates yielding E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone, 73 isolates were confirmed as hGISA by vPAP-AUC ratio determination, 95 by tPAP and 108 by both methods. These 108 hGISA isolates comprised of 13 hVISA, 35 hTISA and 60 hVISA & hTISA isolates. An additional 70 hGISA isolates were detected following investigation of 119 isolates yielding teicoplanin E-test™ macromethod values of 8 mg/L. Of these 70 hGISA isolates, 53 were confirmed as hTISA by tPAP and 17 as hVISA & hTISA by both vPAP-AUC and tPAP methods. In total, 178 hGISA isolates were detected comprising of 13 hVISA, 88 hTISA and 77 hVISA & hTISA isolates.

Table 2.5. Numbers of hGISA isolates detected using vancomycin and/or teicoplanin population analysis profiling and breakdown into hVISA, hTISA and hVISA & hTISA isolates.

Isolates investigated by PAP	vPAP-AUC[a]	tPAP[b]	Total hGISA	hVISA[c]	hTISA[d]	hVISA & hTISA
139[e]	73	95	108	13	35	60
119[f]	17	70	70	0	53	17
258	**90**	**165**	**178**	**13**	**88**	**77**

[a], vPAP-AUC, vancomycin population analysis profile-area under the curve ratio determination.

[b], tPAP, teicoplanin population analysis profile.

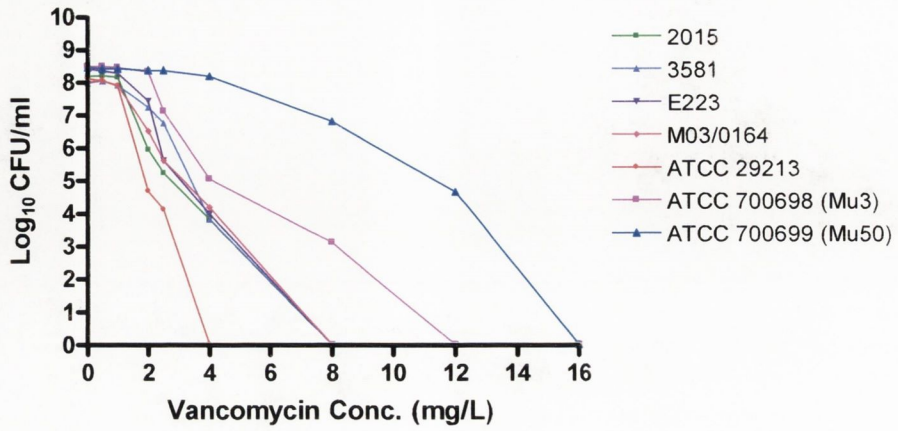
[c], hVISA defined as isolates with vPAP-AUC ratios ranging from 0.9–1.29.

[d], hTISA defined as isolates with resistant subpopulations capable of growth at >8 mg/L teicoplanin.

[e], Isolates exhibiting E-test™ macromethod values of ≥8 mg/L for vancomycin & teicoplanin or ≥12 mg/L for teicoplanin alone.

[f], Additional isolates requiring PAP when the teicoplanin E-test™ macromethod cut-off value was decreased to 8 mg/L.

Panel A



Panel B

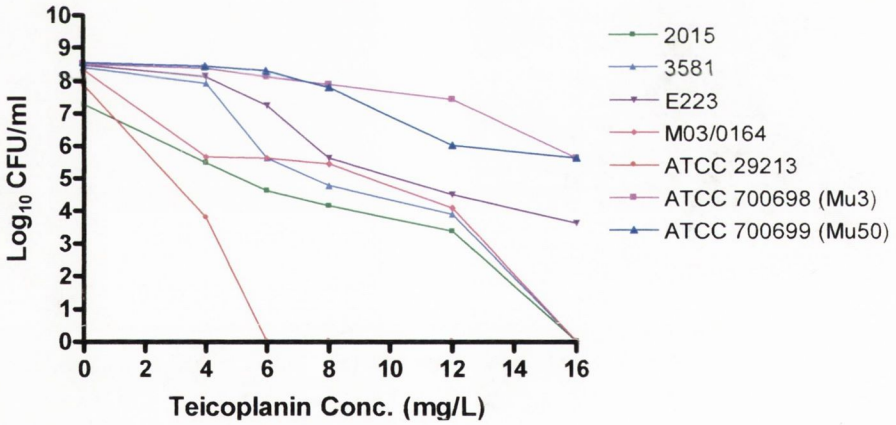


Figure 2.3. Vancomycin and teicoplanin population analysis profiles of four hGISA isolates and three control strains.

Panels A and B present the vancomycin and teicoplanin population analysis profiles of four hGISA isolates and *S. aureus* ATCC 29213 (GSSA), *S. aureus* Mu3 ATCC 700698 (prototype hGISA strain) and *S. aureus* Mu50 ATCC 700699 (prototype GISA strain), respectively.

As shown in Figure 2.4, vPAP-AUC ratios ranged from 0.5–1.25 among 178 hGISA isolates. Among hVISA, hTISA and hVISA & hTISA isolates vPAP-AUC ratios ranged from 0.9–1.15, 0.46–0.89 and 0.9–1.25, respectively.

2.4.4.1 Vancomycin and Teicoplanin PAP

Of 139 isolates exhibiting E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone, 2.3% (73/3189) were confirmed as hGISA by vPAP-AUC, 3% (95/3189) by tPAP and 3.4% (108/3189) by both PAP methods. Lowering the teicoplanin E-test™ macromethod cut-off value to 8 mg/L detected a further 2.2% (70/3189) of isolates as hGISA bringing the overall proportion of hGISA detected among six collections of MRSA to 5.6% (178/3189). Using vPAP-AUC and tPAP alone, the proportions of hGISA detected were 2.8% (90/3189) and 5.2% (165/3189), respectively whilst both PAP methods detected 5.6% hGISA among six collections of MRSA. Details of the numbers of hGISA isolates detected by each PAP method among each collection are shown in Table 2.6.

Among 178 hGISA isolates, 3%, 24% and 73% exhibited vancomycin MICs of 0.5, 1.0 and 2.0 mg/L, respectively. The majority (76%) of hGISA isolates had teicoplanin MICs of 2.0 mg/L whilst 0.5%, 5%, 18% and 0.5% exhibited teicoplanin MICs of 0.5, 1.0, 4.0 and 8.0 mg/L, respectively.

2.4.5 Evaluation of Screening Methods

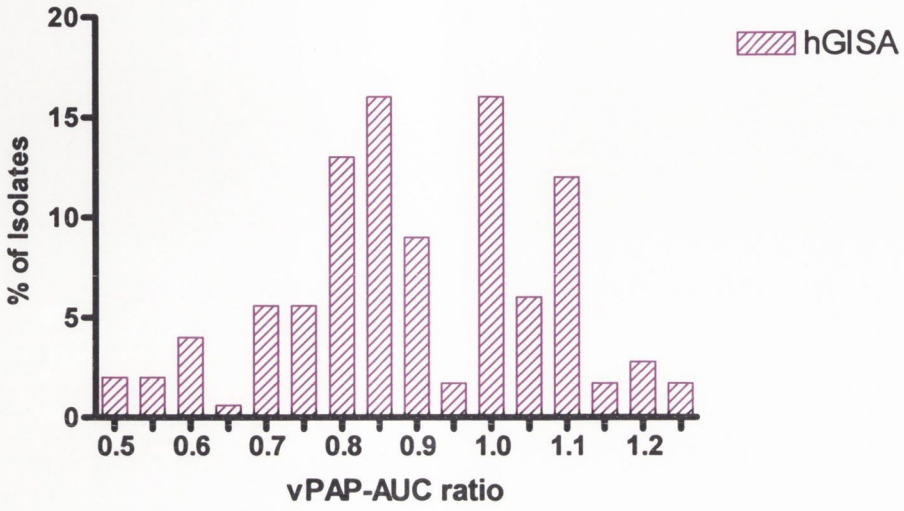
Evaluation of $MHT5_{0.5}$, $MHT5_{2.0}$, $MHT5_{S}$, $BHIT5_{0.5}$, $BHIT5_{2.0}$ and $BHIT5_{S}$ agar screening media showed that the sensitivity of all methods except $MHT5_{0.5}$ was ≥98% (the sensitivity of

$MHT5_{0.5}$ was 66%) (Table 2.7). However, the specificity for methods exhibiting 100% sensitivity ranged from 4–57%. Sensitivity, specificity, positive and negative predictive values of each agar screening method for the detection of hGISA isolates and isolates yielding positive E-test™ macromethod values are detailed in Table 2.7. During preliminary screening with MHT5 when the inoculum was delivered by 10-µl loop, the sensitivity and specificity of MHT5 was 61% and 77%, respectively. When the inoculum was delivered by pipette, the sensitivity of this agar screening method increased to 100% but the specificity decreased to 6%.

The ability of these screening methods to detect isolates yielding E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone varied. Sensitivity values ranged from 59–100% and specificity values ranged from 6–85%. $BHIT5_{0.5}$ and $MHT5_{2.0}$ yielded the best overall performance with sensitivities of 98% and 95% and specificities of 70% and 60%, respectively. Lowering the E-test™ macromethod cut-off value to 8 mg/L teicoplanin allowed detection of a further 70 hGISA isolates. Using the criterion of a teicoplanin E-test™ macromethod value of ≥8 mg/L, sensitivity and specificity values of $BHIT5_{0.5}$ and $MHT5_{2.0}$ were 99% and 96% and 84% and 73%, respectively. Two isolates with positive E-test™ macromethod values failed to grow on $BHIT5_{0.5}$. However, vPAP-AUC and tPAP analysis showed these two isolates to be GSSA isolates.

$BHIT5_{0.5}$ was used to re-screen all isolates in Collection 4. Of 330 isolates screened, 73 isolates yielded ≥1 colony, were subsequently investigated by the E-test™ macromethod and 25 isolates yielded positive E-test™ macromethod results (Table 2.4). Of these 25 isolates, 16 hGISA were confirmed by vPAP-AUC and tPAP methods (Table 2.6).

Panel A



Panel B

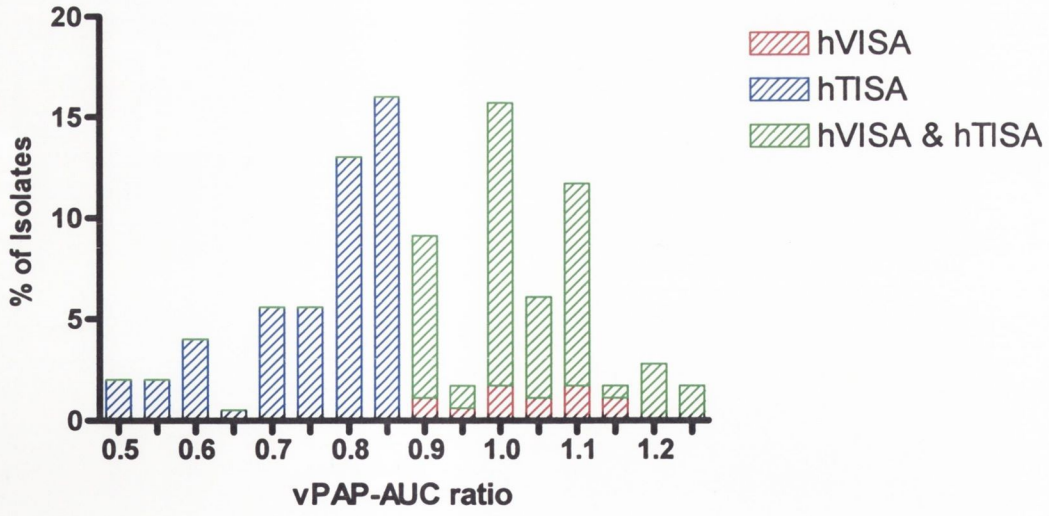


Figure 2.4. Range of vPAP-AUC ratios among hGISA isolates.

Panels A and B present the range of vPAP-AUC ratios among hGISA and hVISA, hTISA and hVISA & hTISA isolates, respectively.

Table 2.6. Numbers of hGISA isolates detected by vancomycin and/or teicoplanin population analysis profiling [figures in parentheses indicate the percentage hGISA among each collection].

	E-test™ Macromethod Cut-off Values								
	V & T ≥8 or T ≥12[a] (*n*=139)			T 8[b] (*n*=119)			T ≥8[c] (*n*=258)		
MRSA Collection	**vPAP-AUC[d]**	**tPAP[e]**	**Total**	**vPAP-AUC**	**tPAP**	**Total**	**vPAP-AUC**	**tPAP**	**Total**
1. H1 Q4, 1998 (*n*=188)	3 [1.6]	5 [2.7]	**5 [2.7]**	0 [0]	5 [2.7]	**5 [3]**	3 [1.6]	10 [5.3]	**10 [5.3]**
2. N/S Study 1999 (*n*=714)	47 [6.6]	46 [6.4]	**58 [8]**	4 [0.6]	23 [3.2]	**23 [3.4]**	51 [7]	69 [9.7]	**81 [11]**
3. EARSS 1999-2003 (*n*=1,580)	4 [0.25]	11 [0.7]	**11[0.7]**	6 [0.4]	28 [1.8]	**28 [1.9]**	10 [0.6]	39 [2.5]	**39 [2.5]**
4. H1 Q2, 2003 (*n*=330)	4 [1.2]	10 [3]	**11 [3.3]**	0 [0]	5 [1.5]	**5 [1.7]**	4 [1.2]	15 [4.5]	**16 [4.9]**
5. NMRSARL 2000-2003 (*n*=97)	15 [15]	19 [20]	**19 [20]**	7 [7]	9 [9]	**9 [9]**	22 [23]	28 [29]	**28 [29]**
6. H1 Q4, 2004 (*n*=280)	0 [0]	4 [1.4]	**4 [1.4]**	0 [0]	0 [0]	**0 [0]**	0 [0]	4 [1.4]	**4 [1.4]**
Total (*n*=3,189)	**73 [2.3]**	**95 [3]**	**108 [3.4]**	**17 [0.5]**	**70 [2.2]**	**70 [2.2]**	90 [2.8]	165 [5.2]	**178 [5.6]**
N/S Study 1999[f]									
North 1999 (*n*=206)	23 [11]	15 [7.3]	**24 [12]**	0 [0]	2 [1]	**2 [1]**	23 [11]	17 [8]	**26 [13]**
South 1999 (*n*=508)	24 [4.7]	31 [6]	**34 [6.7]**	4 [0.8]	21 [4]	**21 [4]**	28 [5.5]	52 [10]	**55 [11]**

[a], Isolates exhibiting E-test™ macromethod values of ≥8 mg/L for vancomycin & teicoplanin or ≥12 mg/L for teicoplanin alone. [b], Isolates requiring PAP when the teicoplanin E-test™ macromethod cut-off value was decreased to 8 mg/L. [c], Isolates exhibiting E-test™ macromethod values of ≥8 mg/L for teicoplanin. [d], vPAP-AUC, vancomycin population analysis profile-area under the curve ratio determination. hGISA defined as isolates with vPAP-AUC ratios ranging from 0.9–1.29. [e], tPAP, teicoplanin population analysis profile. hGISA defined as isolates with resistant subpopulations capable of growth at >8 mg/L teicoplanin. [f], Breakdown of North/South Study of MRSA in Ireland 1999 into North and South.
Abbreviations. See Table 2.3.

Table 2.7. Evaluation of six agar screen methods to detect hGISA isolates and isolates yielding positive E-test™ macromethod values.

	hGISA				E-test™ Macromethod[a]				E-test™ Macromethod[b]			
Method	**Sens**	**Spec**	**PPV**	**NPV**	**Sens**	**Spec**	**PPV**	**NPV**	**Sens**	**Spec**	**PPV**	**NPV**
$MHT5_{0.5}$	66	82	89	61	59	85	95	47	58	91	95	42
$MHT5_{2.0}$	98	53	77	95	95	60	85	84	96	73	91	84
$MHT5_{S}$	100	6	63	100	99	6	71	75	99	7	76	75
$BHIT5_{0.5}$	100	57	79	100	98	70	88	95	99	84	95	95
$BHIT5_{2.0}$	100	18	66	100	100	23	75	100	100	27	80	100
$BHIT5_{S}$	100	4	63	100	100	6	71	100	100	7	77	100

[a], Values for detecting isolates with E-test™ macromethod values of ≥8 mg/L for vancomycin & teicoplanin or ≥12 mg/L for teicoplanin alone. [b], Values for detecting isolates with E-test™ macromethod values of ≥8 mg/L for teicoplanin alone.

Abbreviations: $MHT5_{0.5}$, $MHT5_{2.0}$, $MHT5_{S}$, Mueller-Hinton agar containing 5 mg/L teicoplanin with inoculum densities equivalent to a 0.5 or 2.0 McFarland turbidity standards or from a stationary phase broth culture. $BHIT5_{0.5}$, $BHIT5_{2.0}$, $BHIT5_{S}$, brain heart infusion agar containing 5 mg/L teicoplanin with inoculum densities equivalent to 0.5 or 2.0 McFarland turbidity standards or from a stationary phase broth culture. Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value; expressed as percentages.

2.4.6 Prospective Study

Three hundred and ten isolates recovered from 270 patients were screened with $BHIT5_{0.5}$ (Table 2.3). These isolates comprised 244 isolates from 238 patients and 66 isolates from 32 patients receiving >7 days glycopeptide therapy. The 66 isolates comprised one isolate from 12 patients (n=12), two isolates from 11 patients (n=22), three isolates from four patients (n=12) and four isolates from five patients (n=20).

Twenty-five isolates yielded growth of ≥1 colony on $BHIT5_{0.5}$ and were investigated by the E-test™ macromethod of which six isolates (recovered from five patients) yielded positive E-test™ macromethod results (Table 2.4). No hVISA isolates were detected but four isolates were confirmed as hTISA by tPAP (Table 2.6).

Demographic and clinical details of patients from whom isolates yielding positive E-test™ macromethod results were recovered are shown in Table 2.8. Five patients yielded isolates exhibiting positive E-test™ macromethod values. Isolates recovered from three patients (Patients 1, 2 and 3) were confirmed as hTISA isolates. Two of these three patients (Patients 1 and 2) had received >7 days glycopeptide therapy but Patient 3 had not received glycopeptide therapy during Q4, 2004. The hTISA phenotype was identified from the fourth isolate recovered from Patient 1 whilst both isolates recovered from Patient 2 exhibited the hTISA phenotype. Among the patients (Patients 4 and 5) from whom GSSA isolates were identified, glycopeptide therapy was administered to only one patient (Patient 5) during Q4, 2004.

2.4.7 Evaluation of Phenotypic Methods to Enhance Detection

2.4.7.1 Pilot Study

2.4.7.1.1 Disk Diffusion Screening

The sizes of zones of inhibition obtained for hGISA isolates and GSSA control strains (*S. aureus* ATCC 25923 and *S. aureus* ATCC 29213) investigated on MH and MHS agar plates using vancomycin 1 µg, 2 µg, 5 µg and 30 µg per disk and teicoplanin 5 µg and 30 µg per disk overlapped (shown in Table 2.9). Hence, these antimicrobial disks were unsuitable for screening for hGISA isolates. On MHS agar plates, teicoplanin 1 µg/disk and 2 µg/disk were unsuitable for screening for hGISA isolates but on MH agar these antimicrobial disks appeared promising and were investigated further in the evaluation study described in Section 2.3.10.3.

2.4.7.1.2 Vancomycin Agar Screening

The sensitivity of vancomycin agar screening methods for detecting hGISA isolates are shown in Table 2.10. Among 27 different vancomycin agar screening methods investigated, the sensitivity ranged from 0 to 100%. At each inoculum density, all hGISA isolates yielded growth on BHIV4 and BHIV4 + 4% NaCl. The addition of 4% NaCl to BHIV5 increased the detection of hGISA by 140% (7/5) and 150% (3/2) at inoculum densities of 0.5 and 1.0 McFarland turbidity standards, respectively. However, none of these agar screening methods were considered for further evaluation as (i) agar screening media containing salt fail to detect salt intolerant strains of MRSA thus eliminating BHIV4 + 4% NaCl and BHIV5 + 4% NaCl, (ii) an inoculum density equivalent to a 0.5 McFarland turbidity standard is most suitable for use in clinical diagnostic microbiology laboratories, and (iii) a previous study showed the

Table 2.8. Demographic and clinical details of patients from whom MRSA isolates that yielded positive E-test™ macromethod values were recovered during Q4, 2004 in H1.

Patient	Age, years	Sex	Ward	Vanc (days)[a]	Isolates (*n*=)[b]	Date isolated	Site	BHIT5	E-test[c] V	E-test[c] T	vPAP	tPAP	Comment	A-PFG Type	Diagnosis/ Underlying illness
1	83	M	A	25	4	13/12/'04	Blood	NG	…		…	…		06-01	Surgical, bacteraemic,
						21/12/'04	Blood	NG	…		…	…		06-01	Renal impairment,
						28/12/'04	Blood	+	6.0	2.0	…	…		06-01	Hepatic impairment,
						10/01/'05	Blood	+++	12.0	12.0	0.74	Pos	hTISA	06-01	No history of MRSA
2	72	F	B	8	2	07/10/'04	Sputum	+++	8.0	8.0	0.54	Pos	hTISA	07-02	Surgical, renal impairment,
						01/11/'04	Throat	+++	8.0	8.0	0.54	Pos	hTISA	07-02	History of MRSA
3	32	M	C	Nil	2	08/11/'04	Groin	30 cols	6.0	12.0	0.82	Pos	hTISA	14-00	Surgical, hepatic impairment,
						10/11/'04	Pressure sore	15 cols	6.0	6.0	…	…		14-00	No history of MRSA
4	67	F	C	Nil	1	10/11/'04	Nose	9 cols	8.0	8.0	0.83	Neg	GSSA	14-00	Gastro-intestinal bleed post-stenting, History of MRSA
5	28	M	A	8	4	06/10/'04	Throat	NG	…		…	…		NT-01	Tracheostomy,
						23/11/'04	BAL	1 col	12.0	8.0	0.73	Neg	GSSA	NT-01	History of MRSA
						14/12/'04	Nose	NG	…		…	…		NT-01	
						11/01/'05	Nose	1 col	6.0	4.0	…	Neg		NT-01	

[a], Duration of vancomycin exposure. [b], No. of isolates recovered from each patient during Q4, 2004. [c], E-test™ macromethod values.

Abbreviations: A-PFG, antibiogram-pulsed-field group type; BAL, Broncho alveolar lavage; BHIT5, brain heart infusion agar containing 5 mg/L teicoplanin; col, colony; cols, colonies; F, female; M, male; Neg, negative; NG, no growth; NT, "no type" {Isolates were assigned no antibiogram type (NT) as the susceptibility pattern produced suggested possible loss of resistance determinants}; Pos, positive; T, teicoplanin; tPAP, teicoplanin population analysis profile; V, vancomycin; vPAP, vancomycin population analysis profile-area under the curve ratio determination; …, not done.

Table 2.9. Disk diffusion zone diameters (mm) of hGISA isolates and *S. aureus* control strains obtained with varying concentrations of vancomycin and teicoplanin antimicrobial disks on Mueller-Hinton (MH) agar [figures in parentheses indicate zone diameters obtained when MH containing 2% NaCl was used].

	Vancomycin							
	1 µg		2 µg		5 µg		30 µg	
hGISA (*n*=12)	9–11	[6–11]	11–13	[8–12.5]	14–17	[14–17]	19–23.5	[20.5–23]
S. aureus ATCC 25923	11	[11]	12	[12]	14	[14]	21	[21]
S. aureus ATCC 29213	11.5	[11.5]	13	[13]	14	[14]	21	[21]
S. aureus Mu3 ATCC 700698	9	[9]	11.5	[11]	15.5	[17]	22	[23]
S. aureus Mu50 ATCC 700699	6	[6]	7	[6]	12	[11]	20	[21]
	Teicoplanin							
	1 µg		2 µg		5 µg		30 µg	
hGISA (*n*=12)	6–9	[6–11]	6–10	[6–13]	10–14	[13–15.5]	15–17	[16.5–20]
S. aureus ATCC 25923	10	[11]	12	[13]	13.5	[14]	17	[17]
S. aureus ATCC 29213	10.5	[12.5]	12	[14]	15	[15]	18	[17]
S. aureus Mu3 ATCC 700698	6	[6]	6	[6]	7	[8]	15	[16]
S. aureus Mu50 ATCC 700699	6	[6]	7	[6]	10	[12.5]	16	[17]
	Teicoplanin							
Evaluation Study	1 µg		2 µg					
hGISA (*n*=117)	6–11	…	6–13	…	…	…	…	…
GSSA (*n*=31)	6–10	…	6–12	…	…	…	…	…
High E-test™ macromethod values (*n*=10)[a]	9–11	…	9–13	…	…	…	…	…
Intermediate E-test™ macromethod values (*n*=13)[b]	9–13	…	11–14	…	…	…	…	…
Low E-test™ macromethod values (*n*=34)[c]	10–13	…	11–14	…	…	…	…	…

[a], E-test™ macromethod values of ≥4 mg/L and ≤8 mg/L for both vancomycin and teicoplanin. [b], E-test™ macromethod values of 4 mg/L for both vancomycin and teicoplanin. [c], E-test™ macromethod values ≤2 mg/L for both vancomycin and teicoplanin. …, not done.

Table 2.10. Sensitivity (%) of vancomycin agar screening methods to detect hGISA isolates (n=12) and to demonstrate beta-lactam/vancomycin interaction exhibited by hGISA isolates.

	Inoculum Density[c]	MHV4		MHV5		BHIV4		BHIV5		BHIV6
			+ NaCl		+ NaCl		+ NaCl		+ NaCl	+ NaCl
Vancomycin Agar Screening[a]	**1 X 10^8 CFU/ml**	0	50	0	25	100	100	42	100	50
	3 X 10^8 CFU/ml	0	20	0	20	100	100	40	100	60
	6 X 10^8 CFU/ml	40	100	0	40	100	100	100	100	100
Beta-Lactam/ Vancomycin Interaction[b]	**1 X 10^8 CFU/ml**	0	33	0	0	83	100	17	83	42
	3 X 10^8 CFU/ml	0	60	0	0	80	100	20	40	60
	6 X 10^8 CFU/ml	40	80	0	0	100	100	20	100	100

[a], Sensitivity (%) of vancomycin agar screening methods to detect hGISA isolates.

[b], Sensitivity (%) of vancomycin agar screening methods to demonstrate beta-lactam/vancomycin interaction exhibited by hGISA isolates.

[c], Three inoculum preparations equivalent to 0.5 (1 X 10^8 CFU/ml), 1.0 (3 X 10^8 CFU/ml) and 2.0 (6 X 10^8 CFU/ml) McFarland turbidity standards were evaluated with each method.

Abbreviations: MHV4, MHV5, Mueller-Hinton agar containing 4 mg/L and 5 mg/L vancomycin, respectively; BHIV4, BHIV5, BHIV6, brain heart infusion agar containing 4 mg/L, 5 mg/L and 6 mg/L vancomycin, respectively; NaCl, sodium chloride.

sensitivity and specificity of screening for hGISA isolates with BHIV4 using an inoculum density equivalent to a 0.5 McFarland turbidity standard was 71% and 88%, respectively, (Walsh *et al.*, 2001).

2.4.7.1.3 Beta-lactam/Vancomycin Interaction

Details of the sensitivity of vancomycin agar screening methods to demonstrate beta-lactam/vancomycin interaction exhibited by hGISA isolates are shown in Table 2.10. Overall, BHIV4 + 4% NaCl yielded the best performance and was further evaluated as described in Section 2.3.10.3.

2.4.7.2 Evaluation Study

2.4.7.2.1 Disk Diffusion Susceptibility Testing

Disk diffusion zone diameters of 117 hGISA and 88 MR-GSSA isolates obtained with teicoplanin 1 µg/disk and 2 µg/disk on MH agar are detailed in Table 2.9. The sizes of zones of inhibition obtained for (i) hGISA isolates (n=117), (ii) GSSA isolates yielding E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone and negative vPAP-AUC and tPAP results (n=31), (iii) GSSA isolates yielding E-test™ macromethod values of ≥4 mg/L and ≤8 mg/L for both vancomycin and teicoplanin (n=10), (iv) GSSA isolates yielding E-test™ macromethod values of 4 mg/L for both vancomycin and teicoplanin (n=13) and (v) GSSA isolates yielding E-test™ macromethod values ≤2 mg/L for both vancomycin and teicoplanin (n=34) overlapped with GSSA control strains *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213, respectively. Thus, teicoplanin 1 µg/disk and 2 µg/disk are not suitable for screening for hGISA isolates.

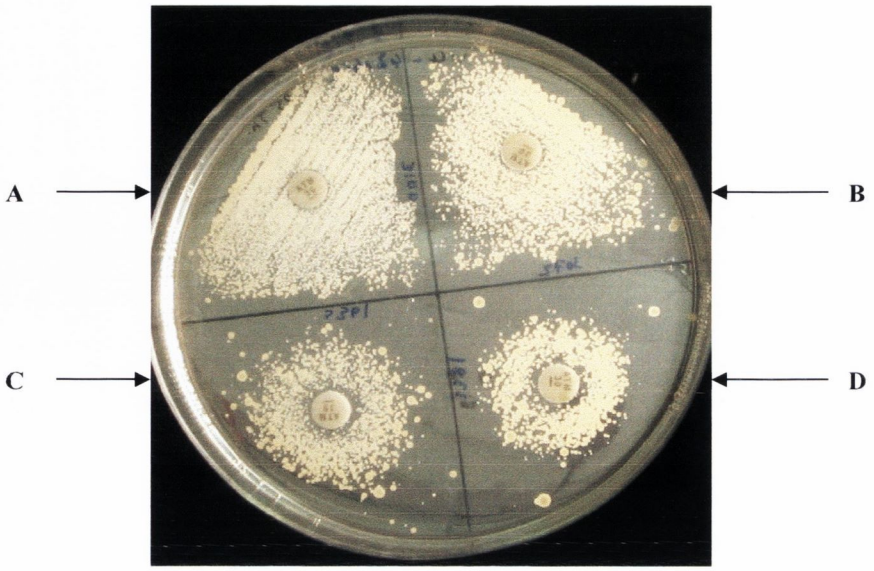
2.4.7.2.2 Beta-lactam/Vancomycin Interaction

Interaction between beta-lactams and vancomycin on BHIV4 containing 4% NaCl was exhibited by only 22% (26/117) hGISA isolates and 10% (3/31) of GSSA isolates yielding E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone. Hence, this method is not suitable for screening for hGISA isolates. No detectable interaction was observed among isolates from collections (iii), (iv) and (v) described above. Beta-lactam/vancomycin interaction exhibited by representative hGISA isolates on BHIV4 containing 4% NaCl is shown in Figure 2.5.

2.4.8 Prevalence of Reduced Susceptibility to Glycopeptides in Ireland

No VRSA or GISA isolates were detected. The proportion of hGISA isolates among the 3,189 isolates investigated was 5.6% (178/3189) from 5.8% of patients (172/2990) (Table 2.11). Between Q4, 1998 and Q2, 2003, the proportion of MRSA-positive patients with hGISA from H1 was similar at 5.9% (10/169) and 5.3% (16/303), respectively, but during the prospective study in Q4, 2004, the proportion fell to 1.5% (4/270). Among patients with blood culture isolates investigated under the EARSS project, the proportion was 2.6% (39/1507) but prevalence was 12% (81/682) among isolates investigated during the North/South Study of MRSA in Ireland, 1999 where there was a higher rate in the North (14%, 26/192) compared to the South (11%, 55/490). The highest proportion of hGISA was among isolates sent to the NMRSARL for investigation of reduced susceptibility to glycopeptides {29% (28/97)}. These isolates comprised of 27 hGISA isolates from 22 patients and one isolate from an environmental source. Single isolates were recovered from 19 patients; two isolates were

Panel A



Panel B

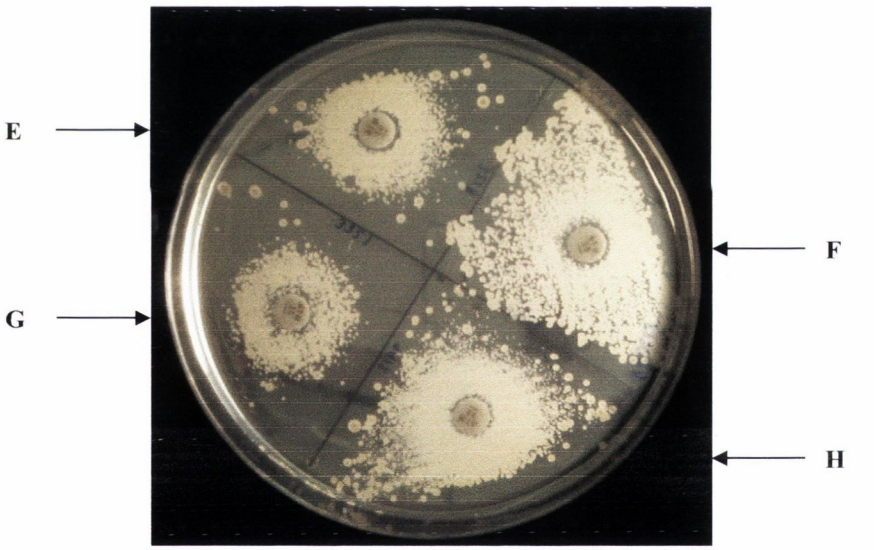


Figure 2.5. Beta-lactam/vancomycin interaction.

Panels A and B demonstrate an interaction between beta-lactams and vancomycin exhibited by representative hGISA isolates on BHIV4 containing 4% NaCl. Isolates demonstrating this interaction (C, D, E and G) are inhibited by vancomycin but are capable of growing around the aztreonam 30 µg disk (ATM 30) forming a zone of confluent growth. No detectable interaction was observed for isolates A, B, F and H. Isolates A and C represent control strains, *S. aureus* Mu50 ATCC 700699 (negative) and *S. aureus* Mu3 ATCC 700698 (positive), respectively.

Table 2.11. Numbers of hGISA isolates detected by vancomycin and/or teicoplanin PAP methods and the proportions of hGISA among each collection.

	hGISA	
MRSA Collection (Isolates/Patients)	**Isolates (%)**	**Patients (%)**
1. H1 Q4, 1998 (*n*=188/169)	10 (5.3)	10 (5.9)
2. N/S Study 1999 (*n*=714/682)	81 (11)	81 (12)
3. EARSS 1999-2003 (1,580/1,507)	39 (2.5)	39 (2.6)
4. H1 Q2, 2003 (*n*=330/303)	16 (4.9)	16 (5.3)
5. NMRSARL 2000-2003 (*n*=97/59)	28[a] (29)	22[a] (37)
6. H1 Q4, 2004 (*n*=280/270)	4 (1.4)	4 (1.5)
Total (*n*=3,189/2,990)	178 (5.6)	172 (5.8)
N/S Study 1999[b]		
North 1999 (*n*=206/192)	26 (13)	26 (14)
South 1999 (*n*=508/490)	55 (11)	55 (11)

[a], hGISA isolates comprised of 27 hGISA from 22 patients and one isolate from an environmental source. Single isolates were recovered from 19 patients; two isolates were recovered from one patient and three isolates were recovered from two patients.

[b], Breakdown of North/South Study of MRSA in Ireland 1999 into the North and South.

Abbreviations. See Table 2.3.

recovered from one patient and three isolates were recovered from two patients. Overall the proportion of hGISA among this patient population was 37% (22/59).

Whilst the inclusion of tPAP increased the proportions of hGISA detected among all six collections of MRSA, significant increases were detected among two collections (Table 2.6). Although the proportions of hGISA isolates detected among isolates submitted from the North and South of Ireland to the North/South Study of MRSA in Ireland, 1999 were 13% and 11%, respectively, an increase of 5.5% was detected among isolates submitted from the South when tPAP was included for confirmation of hGISA phenotype. Among isolates recovered from H1 during Q4, 2004, the proportion of hGISA increased from 0 to 1.4% when tPAP was included.

2.5 Discussion

Since the emergence of hGISA in 1997, reported prevalence rates have varied due to the different methods adopted to screen for and confirm isolates exhibiting reduced susceptibility to glycopeptides (Hiramatsu *et al.*, 1997a; Kim *et al.*, 2000; Ike *et al.*, 2001; Park *et al.*, 2000; Franchi *et al.*, 1999; Hubert *et al.*, 1999; Kantzanou *et al.*, 1999). Whilst it has been suggested that hGISA may be a laboratory induced-phenomenon, hGISA isolates have been associated with treatment failure and have been shown to be precursors of GISA (Ariza *et al.*, 1999; Hiramatsu *et al.*, 1997a; Sieradzki *et al.*, 2003; Ward *et al.*, 2001). To accurately assess the clinical significance of hGISA isolates, reliable screening and confirmatory methods are required (Liu & Chambers, 2003). Where other studies have shown that the original method described by Hiramatsu *et al.* (10^6 CFU/ml inoculated onto BHIV4) did not reliably detect hGISA isolates, a modification of this method was used in two previous studies of MRSA in Ireland (Hiramatsu *et al.*, 1997a; Walsh *et al.*, 2001; Wootton *et al.*, 2001; Anon., 2000a; Rossney & Keane, 2002). Isolates from Collections 1 and 2 were screened on BHIV4 using an inoculum concentration of 10^8 CFU/ml to ensure no hGISA isolates went undetected (Anon., 2000a; Rossney & Keane, 2002).

In the clinical laboratory, it is likely that many hGISA isolates go undetected as the initial inoculum (10^6 CFU/ml) used in routine laboratory methods (such as disk diffusion testing, automated susceptibility testing methods and MIC measurement) is too low to detect the resistant subpopulation exhibited by hGISA isolates (Walsh *et al.*, 2001). According to British and European breakpoints, a *S. aureus* isolate exhibiting a vancomycin MIC of 4 mg/L is considered susceptible (Anon., 2008a; Anon., 2008c). In 2006, revised vancomycin breakpoints were issued by the CLSI to detect heterogeneously resistant isolates of *S. aureus*

(Tenover & Moellering, 2007). But results from the present study show that all hGISA isolates detected had vancomycin MICs within the susceptible range (≤2 mg/L vancomycin) according to all three breakpoint criteria. Hence, screening methods such as $BHIT5_{0.5}$ and the E-test™ macromethod are required to detect hGISA isolates.

Previous studies have shown the E-test™ macromethod to be a reliable agar screening method but it is too expensive for routine use on all *S. aureus* isolates in diagnostic laboratories (Walsh *et al.*, 2001). Confirmatory testing with population analysis remains the reference method but it is labour-intensive, time-consuming and unsuitable for routine diagnostic use (Wootton *et al.*, 2001; Nonhoff *et al.*, 2005). EARSS guidelines for detection of reduced susceptibility to glycopeptides among *S. aureus* isolates suggest preliminary screening with MHT5 using a stationary phase broth subculture inoculum ($MHT5_S$) and investigating any growth with the E-test™ macromethod (Anon., 2005b). During the present study, $MHT5_S$ showed poor sensitivity but $BHIT5_{0.5}$ was shown to reliably detect hGISA isolates and isolates exhibiting E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone or isolates yielding teicoplanin E-test™ macromethod values of 8 mg/L. The method of inoculum delivery was found to be crucial when screening for isolates exhibiting reduced susceptibility to glycopeptides as 51 (39%) hGISA were missed when a 10-µl loop was used to inoculate MHT5 agar screen plates. Although results from the present evaluation study showed the sensitivity of $MHT5_S$ and $BHIT5_{0.5}$ was similar, $BHIT5_{0.5}$ exhibited better specificity (57% for detecting hGISA and 84% for detecting isolates exhibiting teicoplanin E-test™ macromethod values of 8 mg/L, respectively). The poor specificity of $MHT5_S$ (6% for detecting hGISA and 7% for detecting isolates exhibiting teicoplanin E-test™ macromethod values of 8 mg/L, respectively) makes this method

unsuitable for routine diagnostic use. $BHIT5_{0.5}$ is a suitable agar screen where screen-positive isolates are investigated by the E-test™ macromethod and confirmed by PAP (Fitzgibbon *et al.*, 2007). In a subsequent evaluation of $BHIT5_{0.5}$ performed at the NMRSARL, 492 blood culture MRSA isolates were screened with $BHIT5_{0.5}$ and the E-test™ macromethod. Results from that study showed that when the criterion for a positive result was growth of >1 colony, the sensitivity and specificity values for the detection of (i) hGISA and (ii) isolates exhibiting E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone, and (iii) isolates exhibiting teicoplanin E-test™ macromethod values of ≥8 mg/L were 100% and 92% for categories (i) and (ii) and 95% and 94% for category (iii), respectively (Fitzgibbon *et al.*, 2007).

In the present study, when the E-test™ manufacturer's criteria of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone and vPAP-AUC only was used, the proportion of hGISA isolates detected was 2.3% (73/3189). Decreasing the teicoplanin E-test™ macromethod cut-off value to 8 mg/L and using vPAP-AUC as a confirmatory method increased the number of hGISA detected by 23% (17/73). The inclusion of tPAP to confirm the hGISA phenotype among isolates exhibiting E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone and isolates exhibiting teicoplanin E-test™ macromethod values of 8 mg/L increased the numbers of hGISA detected by 32% (35/108) and 76% (53/70), respectively. Overall, decreasing the teicoplanin E-test™ macromethod cut-off value to 8 mg/L and including tPAP to confirm the hGISA phenotype increased the number of hGISA detected by 144% (105/73) bringing the overall proportion of hGISA to 5.6% (178/3189).

Disk diffusion studies incorporating low concentration vancomycin and teicoplanin antimicrobial disks and vancomycin agar screening methods with and without the addition of 4% NaCl all proved unsatisfactory methods for screening for hGISA isolates in the present study. A recent report documented that among isolates exhibiting reduced susceptibility to glycopeptides, only VRSA isolates are reliably detected using disk diffusion susceptibility testing methodology (Gould, 2007). In the present study, beta-lactam/vancomycin interaction was also shown to be unreliable for detecting hGISA isolates. Whilst results from the pilot study showed that the addition of salt (4% NaCl) to BHIV4 and BHIV5 increased detection of hGISA by 20% (2/10) and 400% (8/2), respectively, further analysis of BHIV4 containing 4% NaCl (using 117 hGISA isolates and 88 MR-GSSA isolates) yielded sensitivity and specificity values of 22% and 97%, respectively. Hence, as previously documented, beta-lactam/vancomycin interaction is not suitable for screening for isolates exhibiting reduced susceptibility to glycopeptides.

Clinical features associated with hGISA/GISA infection include high bacterial load infection, recurrent MRSA infection, prolonged vancomycin therapy (>7 days), bacteraemia, renal and hepatic impairment, ongoing or recent dialysis, diabetes mellitus, infections associated with prosthetic devices and immunosuppression (Fridkin, 2001; Fridkin *et al.*, 2003; Charles *et al.*, 2004; Howden *et al.*, 2004; Howden, 2005; Walsh & Howe, 2002). Some of these clinical details were present among the three patients from whom hGISA isolates were recovered in the present study during Q4, 2004 in H1. Patient 1, an 83-year-old male, surgical patient with both renal and hepatic impairment was bacteraemic for at least four weeks, received 25 days vancomycin therapy but the MRSA infection failed to resolve. The progression from GSSA to hGISA following prolonged glycopeptide exposure is likely here as both the hTISA isolate

(recovered 13 days after the GSSA isolate) and the earlier GSSA isolates exhibited indistinguishable antibiogram-pulsed-field-group (A-PFG) types (A-PFG 06-01).

Patient 2 was a 72-year-old female surgical patient with renal impairment who had received vancomycin therapy for eight days following isolation of MRSA from a sputum specimen. Although this first isolate exhibited the hTISA phenotype, there was no evidence that the patient had received glycopeptide therapy in H1 but she did have a history of MRSA and may have received glycopeptide therapy in another institution. Subsequent glycopeptide therapy in H1 failed to eradicate the MRSA but it may have provided sufficient antimicrobial selective pressure to have the later MRSA retain reduced susceptibility. Both isolates exhibited indistinguishable A-PFG types (A-PFG 07-02). Why the first isolate recovered from this patient exhibited the hTISA phenotype is unknown but it is unlikely that hospital transmission occurred as all MRSA isolates recovered from patients in H1 during Q4, 2004 were screened.

A hTISA isolate was recovered from a groin swab from Patient 3, a 32-year-old male surgical patient with hepatic impairment, a history of MRSA infection but with no previous history of glycopeptide therapy. A second isolate with an indistinguishable A-PFG type, A-PFG 14-00 recovered from this patient from a different site did not exhibit reduced susceptibility to glycopeptides. Although the overall prevalence of hGISA among MRSA isolates recovered in H1 during Q4, 2004 was low, it is very difficult to draw a conclusion of clinical significance as hGISA isolates were recovered from only three patients. A significant finding in the prospective study was the emergence of a hTISA isolate (rather than hVISA or hGISA) recovered from Patient 1 following 25 days vancomycin therapy. In a previous study, Vaudaux *et al.* reported the emergence of subpopulations of vancomycin and/ or teicoplanin

resistance phenotypes in a GSSA strain following exposure to glycopeptides with significant increases in teicoplanin MIC values observed (Vaudaux *et al.*, 2001).

Although the overall prevalence was 5.8% (172/2990), rates varied from 1.5–37% depending on the patient population studied. The highest proportion of hGISA (37%) was found among isolates submitted to the NMRSARL specifically requesting glycopeptide investigation studies. These isolates caused specific problems in different institutions where patients with persistent MRSA infection were not responding to glycopeptide therapy and further investigations were required. hGISA isolates were identified in four institutions, H3, H4, H7 and H8 and represented 21%, 43%, 32% and 4% of hGISA isolates, respectively. Single incidents involving nine and one hGISA isolates were identified in H7 and H8, respectively. Two incidents involving six hGISA isolates were identified in H3 whilst four separate incidents involving 12 hGISA isolates were identified in H4. Epidemiological typing analysis of hGISA isolates recovered from NMRSARL isolates will be described in Section 3.4.1.1.

A prevalence of 2.6% was found among blood culture isolates submitted to NMRSARL under the EARSS project where the protocol required data on the first isolate per patient per quarter. Since the hGISA phenotype tends to be associated with prolonged exposure to glycopeptides, a lower prevalence amongst this population might be expected (Walsh & Howe, 2002). In a clinical evaluation of four patients from whom hGISA were recovered from blood culture MRSA isolates, three had received glycopeptide therapy for ≥14 days (Rossney *et al.*, 2005c). The lowest prevalence was observed in H1 during the prospective study in 2004 when isolates from all specimen sites were investigated. Four hTISA isolates were recovered from three patients but only one hTISA isolate was confirmed among additional isolates collected at weekly intervals from patients on prolonged glycopeptide therapy. Interestingly, whilst the

prevalence of hGISA in H1 decreased between 1998 and 2004 (5.9% to 1.5%, respectively), there was a 220% increase in glycopeptide usage in this institution during this period. Whilst previous studies have documented that the risk of developing hGISA is strongly associated with exposure to glycopeptides, these finding suggest that this is not the only factor involved in the development of the hGISA phenotype (Walsh & Howe, 2002). Epidemiological typing analysis may provide an explanation for the decrease in prevalence of hGISA despite an increase in glycopeptide usage as previous studies have reported that some strains may be pre-disposed to develop the hGISA phenotype. In Ireland, the MRSA population changed between 1999 and 2003, where the prevalence of one strain (AR-PFG 06-01) increased by 880% (Sieradzki *et al.*, 2003; Rossney *et al.*, 2006a). Epidemiological typing analysis of isolates recovered from H1 during Q4, 1998, Q2, 2003 and Q4, 2004 is described in Section 3.4.1.1.

A prevalence of 11% was found among isolates submitted to the North/South Study of MRSA in Ireland, 1999. Using vPAP-AUC analysis, the proportion of hGISA among isolates exhibiting E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone from the North appeared much higher than the South {12% and 6.7%, respectively (Table 2.6)}. Using the criterion of a teicoplanin E-test™ macromethod cut-off value of 8 mg/L, an additional 4% hGISA were confirmed by tPAP from the South compared to 1% from the North. Overall, there was a greater disparity between the North and South when vPAP-AUC was used alone to confirm hGISAs (11% and 5.5%, respectively). Using both vancomycin and teicoplanin PAP methods the proportion of hGISA among the isolates investigated were 13% and 11% from the North and South of Ireland, respectively. In a previous study, glycopeptide resistance was sought among isolates

submitted to the North/South Study of MRSA in Ireland, 1999 and a prevalence of 5% hGISA was reported (Anon., 2000a). However, in that study, vancomycin only was used and the hetero-resistant phenotype may have been selected rather than detected as only 21% (8/38) of the original suspect hGISA isolates were confirmed as hGISA in this study (Anon., 2000a). The difference in prevalence rates found between the North and South may reflect differences in epidemiological types as discussed in Chapter 3.

CHAPTER III

Epidemiological Typing of hGISA Isolates

Chapter III Epidemiological Typing of hGISA Isolates

3.1 Introduction

Early studies of GISA and hGISA isolates suggested isolates exhibiting reduced susceptibility to glycopeptides were represented by a single MRSA clone with a potential to develop vancomycin resistance more readily than MRSA isolates of different clonal origins (Hiramatsu *et al.*, 1997a). Epidemiological typing studies of *S. aureus* Mu50 ATCC 700699 (prototype GISA strain) and *S. aureus* Mu3 ATCC 700698 (prototype hGISA strain) reported that these isolates belonged to clonotype II-A (New York/Japan clone) (Hiramatsu *et al.*, 1997a). When *Sma*I digested genomic DNA from eight GISA isolates recovered from patients in the USA were analysed by PFGE and compared to the PFGE patterns of *S. aureus* Mu3 ATCC 700698 and *S. aureus* Mu50 ATCC 700699, closely related PFGE profiles were observed among GISA isolates from the USA but these patterns differed by more than six bands from Japanese isolates suggesting GISA isolates from the USA and Japan did not represent a single clone (Tenover *et al.*, 1998). Nevertheless, MLST and SCC*mec* typing revealed that the first six GISA isolates reported (from Japan, USA and Scotland) all belonged to CC5, carried SCC*mec* type II and exhibited MLST, ST5 (Enright *et al.*, 2002). According to the nomenclature proposed by Enright *et al.*, these isolates are named ST5-MRSA-II or ST5-GISA-II (Enright *et al.*, 2002).

While initial molecular studies focussed mainly on GISA from the USA and Japan, Howe *et al.* studied a global collection of MRSA isolates exhibiting reduced susceptibility to glycopeptides by MLST and SCC*mec* typing (Howe *et al.*, 2004). Representative GISA and hGISA isolates from France, China, Japan, Norway, Poland, Sweden, UK and USA were among the isolates studied. MLST typing yielded nine genotypes of MRSA representing all five major pandemic clones of MRSA (Howe *et al.*, 2004). hGISA have arisen from all five

pandemic clones whilst GISA have emerged in only two of the five pandemic clones, CC5 and CC8 (Howe *et al.*, 2004).

In many countries (such as France, Germany, Belgium, Singapore, Japan, Greece, UK, Korea, Taiwan, Brazil, Thailand and Spain) GISA and hGISA isolates have been shown to represent a restricted range of epidemic MRSA strains (Bertrand *et al.*, 2003; Bierbaum *et al.*, 1999; Cartolano *et al.*, 2004; Denis *et al.*, 2002; Garnier *et al.*, 2006; Geisel *et al.*, 1999; Hiramatsu *et al.*, 1997a; Kantzanou *et al.*, 1999; Kim *et al.*, 2002; Lecaillon *et al.*, 2002; Mallaval *et al.*, 2004; Nonhoff *et al.*, 2005; Wang *et al.*, 2004; Oliveira *et al.*, 2001c; Ploy *et al.*, 1998; Trakulsomboon *et al.*, 2001; Ariza *et al.*, 1999; de Lassence *et al.*, 2006; Sng *et al.*, 2005; Guerin *et al.*, 2000; Howden *et al.*, 2004; dos Santos Soares *et al.*, 2000; MacKenzie *et al.*, 2002). However, clonally diverse GISA and hGISA MRSA strains have also been reported from France, UK and Australia (Woodford *et al.*, 2000; Manuel *et al.*, 2002; Chesneau *et al.*, 2000; Aucken *et al.*, 2002; Pina *et al.*, 2000; Murray *et al.*, 2004). Many isolates exhibiting reduced susceptibility to glycopeptides tend to be multi-antibiotic resistant and related to epidemic gentamicin-resistant MRSA clones that have disseminated in hospitals throughout Europe, Brazil and Japan (Bierbaum *et al.*, 1999; Geisel *et al.*, 1999; Hiramatsu *et al.*, 1997a; Kantzanou *et al.*, 1999; Chesneau *et al.*, 2000; Heym *et al.*, 2002; Bert *et al.*, 2003; Guerin *et al.*, 2000; El Solh *et al.*, 2003; dos Santos Soares *et al.*, 2000; Ariza *et al.*, 1999). The majority of GISA isolates have emerged from MRSA strains previously infecting the patient, suggesting the resistant phenotype has developed *in vivo* during treatment rather than as a result of acquisition of a new strain (Wang *et al.*, 2004; Smith *et al.*, 1999; Fridkin, 2001; Bierbaum *et al.*, 1999; Ploy *et al.*, 1998; Rotun *et al.*, 1999). In 1992, Noble and co-workers described vancomycin resistance in *S. aureus* due to the acquisition of *vanA* from *E. faecalis* to *S. aureus* (Noble *et al.*, 1992). To date, all seven VRSA isolates recognised in the USA

carried both *mecA* and *vanA* and were epidemiologically unrelated (Sievert *et al.*, 2008). Following the isolation of the first clinical VRSA isolate, analysis of PFGE patterns of VRSA and VSSA isolates subsequently recovered from this patient yielded indistinguishable PFGE profiles (Chang *et al.*, 2003b).

In Ireland, whilst all five pandemic clones of MRSA have been present at some time among the MRSA population, ST5-MRSA-II representing the New York/Japan clone has not predominated during any period (Shore *et al.*, 2005). The predominant strains in Ireland since the 1990s have been two multi-antibiotic resistant strains AR-PFG 13-00 (ST8-MRSA-II) and AR-PFG 14-00 (ST8-MRSA-II) but since 1999 prevalence of these strains has decreased whilst the prevalence of a non-multi-antibiotic resistant strain AR-PFG 06-01 (ST22-MRSA-IV) has increased (Rossney *et al.*, 2006a). Whilst MLST and SCC*mec* typing analysis are used for international comparisons among MRSA GISA and hGISA isolates, no reports of large epidemiological typing studies of GISA/hGISA isolates has yet been described (Howe *et al.*, 2004).

3.1.1 Epidemiological Typing of MRSA in Ireland

As mentioned in Section 1.9.2, Ireland has participated in EARSS since 1999. MRSA isolates from blood cultures (one isolate per patient per quarter) submitted to the NMRSARL under the EARSS project are monitored using a combination of both phenotypic and genotypic epidemiological typing methods. Phenotypic methods include AR typing and a simple biotyping technique whilst the genotypic typing method is DNA macrorestriction digestion using *Sma*I followed by PFGE (Rossney *et al.*, 2006a). A previous study has shown that a

combination of both phenotypic and genotypic epidemiological typing is required to accurately distinguish among strains of MRSA circulating in Ireland (Rossney *et al.*, 2006a).

3.1.1.1 Antibiogram Typing

AR typing was developed in 1994 using the modified Stokes' disk diffusion technique (Stokes & Ridgway, 1980; Rossney *et al.*, 1994a). To comply with the decision made by the Academy of Medical Laboratory Science and the Irish Society of Clinical Microbiologists in 2001 to adopt the CLSI susceptibility testing method in Ireland, the feasability of transferring AR typing to CLSI methodology was investigated at the NMRSARL. AR typing was performed in duplicate using both CLSI and the modified Stokes' disk diffusion techniques to determine the susceptibility patterns and compare results of 1,381 MRSA isolates. As the AR typing panel included many antimicrobial agents for which no CLSI breakpoints existed, breakpoints for these antimicrobials were determined at the NMRSARL. During the transition from Stokes' to CLSI disk diffusion susceptibility testing, the value of using a higher concentration of ethidium bromide (EB) disks (60 µg instead of 20 µg) and using cadmium acetate instead of cadmium nitrate in the AR typing panel were evaluated (Rossney *et al.*, 2006a). Zones sizes for cadmium acetate and EB60 proved easier to read and were more accurately determined and hence were substituted for cadmium nitrate and EB20, respectively, in the AR typing panel (Rossney *et al.*, 2006a). From September 2004, CLSI susceptibility testing was adopted for AR typing MRSA isolates at the NMRSARL (Rossney *et al.*, 2007). The term antibiogram-resistogram (AR) are used synonymously with antibiogram (A) and for the purpose of work presented in this thesis, antibiogram will be used.

3.1.1.2 Biotyping

During the 1960s and 1970s, biotyping methods proved to be a useful additional tool in epidemiological typing studies although they lack reproducibility and have poor discriminatory power (Tenover *et al.*, 1997). In 1990, Coia *et al.* reported that a simple biotyping method (using gentamicin susceptibility, pigment production and hydrolysis of urea and Tween 80) was useful as a preliminary epidemiological typing method for MRSA isolates if representative isolates were typed using a different typing technique (Coia *et al.*, 1990). A previous study of Irish MRSA isolates evaluated this biotyping method alongside AR typing and DNA macrorestriction analysis and found that the inclusion of biotyping results (particularly hydrolysis of urea) was necessary to accurately identify the predominant non-multi-antibiotic resistant strain AR-PFG 06-01 (Rossney *et al.*, 2006a). The majority of isolates that exhibit the AR06 AR pattern are urease-negative but isolates exhibiting the AR06 AR pattern and urease activity require DNA macrorestriction analysis for confirmation. Hence such isolates are described as "No Type" (NT) pending further investigation.

3.1.1.3 DNA Macrorestriction Digestion Analysis

In a previous epidemiological typing study of Irish MRSA isolates, two PFGE protocols (PFGE Method I and II) described by de Lencastre *et al.* and MacKenzie *et al.*, respectively, were compared using 1,580 MRSA isolates (Rossney *et al.*, 2006a; de Lencastre *et al.*, 1994; MacKenzie *et al.*, 2002). Cluster analysis of MRSA isolates based on PFGE patterns yielded similar clusters for both protocols (Rossney *et al.*, 2006a). PFGE Method II yielded better separation of high-molecular-weight bands, hence all later studies of DNA macrorestriction digestion analysis of Irish MRSA isolates at the NMRSARL were conducted using this PFGE protocol (Rossney *et al.*, 2006a; Rossney *et al.*, 2007).

3.1.1.3.1 PFGE Pattern Analysis and Nomenclature

According to the criteria of Tenover *et al.*, PFGE banding patterns are interpreted as (i) indistinguishable if they contain the same number and size of bands; (ii) closely related if they differ by one to three bands; (iii) possibly related if they differ by four to six bands and (iv) unrelated if there is a difference of seven or more bands (Tenover *et al.*, 1995a). In a previous epidemiological typing study of Irish MRSA isolates, PFGE patterns were grouped into PFGE types (according to criteria of Tenover *et al.*) and assigned five-digit PFT numbers following visual inspection of gel images (Rossney *et al.*, 2006a). Blocks of 1,000 numbers were assigned to apparently related PFTs and the first two digits of the PFT were used to describe the PFGE group (PFG) (Rossney *et al.*, 2006a). For example, different groups of PFTs were assigned the series of numbers 00001-00999, 01000-01999, 02000-02999 and 03000-03999 which were abbreviated to PFG-00, PFG-01, PFG-02, PFG-03, respectively. A designated series of numbers 99000-99999 were assigned to sporadic patterns where blocks of 100 numbers were assigned to each sporadically occurring pattern e.g. the series of numbers 99400-99499 (PFG-99.4) were assigned to a new strain of MRSA imported from Singapore in 2002 (Rossney *et al.*, 2006a).

3.1.1.4 MLST and SCCmec Typing

A previous study has shown that a combination of epidemiological typing results (AR typing and PFGE patterns) yielding AR-PFG types is a suitable method for monitoring the MRSA population in Ireland (Rossney *et al.*, 2006a). Representative MRSA isolates typed by MLST and SCC*mec* typing methods in previous studies have revealed that the ST and SCC*mec* type of isolates exhibiting AR-PFG 06-01 was ST22-MRSA-IV and isolates exhibiting AR-PFG

13-00 and AR-PFG 14-00 were ST8-MRSA-II but variants of SCC*mec* type II, SCC*mec* type IID and SCC*mec* type IIE were observed among these isolates (Shore *et al.*, 2005; Rossney *et al.*, 2006a). Phenotypically, isolates exhibiting AR-PFG types, AR-PFG 07-02 and AR-PFG 07-03 were distinguished by their susceptibility to cadmium acetate where the former was resistant and the latter was susceptible but MLST and SCC*mec* typing analysis revealed substantial genetic difference among these isolates where AR-PFG 07-02 was ST36-MRSA-II and AR-PFG 07-03 was ST5-MRSA-II (Shore *et al.*, 2005; Rossney *et al.*, 2006a). Similarly, isolates exhibiting the AR06 AR pattern and urease activity yielded a variety of PFGE patterns and were shown to exhibit either ST30, ST12 or ST45 (Rossney *et al.*, 2006a).

3.2 Aims and Objectives

The aims and objectives were:

- To determine the epidemiological types of all hGISA isolates (n=178) detected during the present study using antibiogram typing and DNA macrorestriction digestion using *Sma*I.
- To investigate the epidemiological types of all MRSA isolates recovered during the prospective study in H1 during Q4, 2004 using antibiogram typing and a biotyping technique.
- To compare the prevalence of epidemiological types of hGISA isolates with the epidemiological types of all isolates within each MRSA collection in the study.
- To compare epidemiological typing results of hGISA isolates from the six collections of MRSA, with particular reference to isolates from the North/South Study of MRSA in Ireland, 1999.
- To investigate whether epidemiological typing is a useful marker to predict isolates exhibiting reduced susceptibility to glycopeptides.

3.3 Materials and Methods

3.3.1 Bacterial Isolates

All isolates in Collections 1–5 (described in Table 2.3) were typed using antibiogram typing in previous studies (Rossney & Keane, 2002; Rossney *et al.*, 2006a; Anon., 2000a; Rossney *et al.*, 2007). The antibiogram types of all isolates in Collection 6 (all MRSA isolates recovered in H1, during Q4, 2004 as described in Section 2.3.1) were determined during the present study. One isolate per patient was investigated unless routine susceptibility tests in the diagnostic laboratory suggested the patient was carrying more than one strain of MRSA. This criterion was also applied to isolates recovered at weekly intervals from patients receiving glycopeptide therapy.

DNA macrorestriction analysis using *Sma*I followed by PFGE was performed on all 178 hGISA isolates detected during the present study (as described in Section 2.4.4). In previous studies, PFGE typing of digested chromosomal DNA was performed on representative isolates from Collections 1 and 2 and on all isolates from Collections 3 and 6 (Anon., 2000a; Rossney & Keane, 2002; Rossney *et al.*, 2006a; Rossney *et al.*, 2007). In the current study, DNA macrorestriction digestion analysis was also performed on representative isolates from Collections 4 and 5 (to provide a match for hGISA isolates as described in Section 4.3.1).

3.3.2 Antibiogram Typing

Isolates from Collections 1–5 were investigated previously by antibiogram typing using a panel of 23 antimicrobials as described previously (Rossney *et al.*, 1994a; Rossney & Keane, 2002; Rossney *et al.*, 2006a; Anon., 2000a). In the present study, susceptibility to a panel of 23 antimicrobials (shown in Table 3.1) was determined using CLSI disk diffusion method as

Table 3.1. Antimicrobial panel for antibiogram typing using CLSI susceptibility testing and zone diameter interpretative criteria.

	Antimicrobial	Concentration	Zone Diameter (mm)		
		(µg)	R (≤)	I	S (≥)
Panel A[a]	Gentamicin	10	12	13–14	15
	Kanamycin	30	13	14–17	18
	Ampicillin	10	28	–	29
	Erythromycin	15	13	14–22	23
	Tetracycline	30	14	15–18	19
	Vancomycin	30	14	–	15
	Rifampicin	5	16	17–19	20
	Ciprofloxacin	5	15	16–20	21
	Chloramphenicol	30	12	13–17	18
	Sulphonamide	300	12	13–16	17
	Trimethoprim	5	10	11–15	16
Panel B[b]	Amikacin	30	14	15–19	20
	Neomycin	30	15	16–17	18
	Streptomycin	25	13	14–15	16
	Tobramycin	10	17	18	19
	Lincomycin	2	14	15–16	17
	Fusidic Acid	10	23	24–26	27
	Mupirocin	5	12	13–19	20
	Mupirocin	200	15	16–29	30
	Spectinomycin	500	13	14–19	20
	Ethidium Bromide	60	13	14	15
	Mercuric Chloride	10	13	14	15
	Phenyl Mercuric Acetate	10	24	25–28	29
	Cadmium Acetate	130	10	11–15	16

[a], Panel A exhibits zone diameter interpretive standards according to CLSI M100-S16 (Anon., 2006a).
[b], Panel B exhibits zone diameters established at the NMRSARL.
Abbreviations: CLSI, Clinical and Laboratory Standards Institute; R, resistant; I, intermediate; S, susceptible.

described previously (Rossney *et al.*, 2007). Briefly, colonies from an overnight culture on CBA were suspended in sterile saline to a density equivalent to a 0.5 McFarland turbidity standard. Bacterial suspensions were inoculated onto MH agar plates (four plates per isolate) and antimicrobial disks were applied. All antimicrobial disks were purchased from Oxoid except for spectinomycin, ethidium bromide, cadmium acetate, mercuric chloride and phenyl mercuric acetate disks, which were purchased from Abtek Biologicals (Liverpool, UK). Plates were incubated at 35°C for 18 h. Following incubation, zones of inhibition were measured using the automated zone reader ProtoZone (Synoptics Ltd.) and results were recorded in a MicroSoft Access database which was used to facilitate pattern recognition by comparing the patterns obtained with the test isolates with a collection of previously recognised antibiogram patterns (Rossney *et al.*, 1994a; Rossney *et al.*, 1994b; Rossney *et al.*, 2006a). Isolates were assigned an antibiogram (A) type, A type variant, unfamiliar pattern or NT based on the susceptibility pattern produced. Isolates were assigned NT pending results of PFGE as the susceptibility pattern produced suggested possible loss of resistance determinants.

3.3.3 Biotyping

Isolates from Collection 6 were investigated for (i) hydrolysis of urea in Christensen's urea medium {Urea Agar Base, Oxoid CM0053 with 2% (w/v) urea (Merck 108487; Merck, Darmstadt, Germany)}, (ii) pigment production on nutrient agar (Oxoid CM0003) containing 1% (v/v) Tween 80 (Merck 822187) {nutrient agar Tween, NUT} and (iii) hydrolysis of Tween 80 in NUT (Coia *et al.*, 1990). Urea agar slopes and NUT plates were incubated at 35°C for 48 h.

3.3.4 DNA Macrorestriction Digestion Analysis

PFGE typing of digested chromosomal DNA was performed by a modification of a previously described method (MacKenzie *et al.*, 2002). Control strain *S. aureus* NCTC 8325 was included with each batch of isolates tested. DNA extraction is described in Appendix II (AII.1.1). Use of heavy suspensions of bacterial cells of hGISA isolates proved satisfactory for DNA extraction. DNA was digested with the restriction endonuclease (RE) *Sma*I (Promega R6121; Promega Corporation, Madison W1, USA) at a concentration of 20 units (U) according to the manufacturer's instructions. DNA in agarose plug aliquots of approximately 2 mm was transferred to microcentrifuge tubes for digestion in a total reaction volume of 150-µl containing 20U *Sma*I in 1X RE J buffer. Digestion was allowed to proceed for 3 h at 22°C (Thermomixer Comfort 5355; Eppendorf – Netheler – Hinz GmbH, Hamburg, Germany). During digestion, a 1% weight/volume (w/v) agarose gel {Pulsed Field Certified Agarose, (Bio-Rad 162-0137)} in 0.5X Tris-borate-EDTA (TBE) was prepared as outlined in AII.3.1 and following digestion, plug slices were trimmed and loaded into the wells of the gel. Electrophoresis was performed in a CHEF DRIII PFGE apparatus (Bio-Rad 170-3913) with a 120° angle and ramped linearly with switch times of 6.8–63.8 s over 23 h as outlined in AII.4.1. After electrophoresis, gels were stained with ethidium bromide (0.5 mg/L) (Sigma E-4391) for 30 minutes (min), destained in RO water and photographed under UV light as described in AII.5.

3.3.4.1 PFGE Pattern Analysis

PFGE banding patterns were analysed using the software package Gel Compar (Applied Maths, Sint-Martens-Latem, Belgium). Similarity was determined by the unweighted pair group method using arithmetic averages (UPGMA) using the Dice coefficient with 1% band

tolerance and 0.5% optimisation. Final analysis of PFGE patterns was performed by visual inspection of gel images and clusters were distinguished using the criteria of Tenover *et al.* (Tenover *et al.*, 1995a). Five-digit PFTs were assigned to PFGE patterns and PFTs of apparently related patterns were abbreviated to two-digit PFT groups (Rossney *et al.*, 2006a).

3.4 Results

3.4.1 Antibiogram Typing

3.4.1.1 Antibiogram Type Distribution among hGISA Isolates

Amongst hGISA isolates identified in this study, 97% exhibited seven antibiogram types (A06, A07, A13, A14, A43, A44 and New02). Isolates exhibiting the antibiogram pattern New02 were first recognised during the North/South Study of MRSA in Ireland, 1999 and had been tentatively designated New02 pending further investigation (Rossney *et al.*, 2003b). The distribution of antibiogram types among hGISA isolates within each collection is shown in Table 3.2. The predominant antibiogram types among the hGISA population were A13 and A14 (prevalence: 52% and 20%, respectively) whilst the prevalence rates of antibiogram types A06, A07, A43, A44 and New02 were 3%, 14%, 2%, 3% and 3%, respectively. Six isolates representing 3% of the overall hGISA population yielded antibiogram patterns to which either (i) no antibiogram type (NT), (ii) an unfamiliar (Unf) antibiogram type or (iii) an antibiogram type variant, were assigned.

A13 was the predominant antibiogram type amongst hVISA, hTISA and hVISA & hTISA isolates representing 85%, 42% and 58% of isolates, respectively (Figure 3.1). Amongst hTISA isolates, the proportion of isolates exhibiting A07 and A14 was similar (26%), whereas among hVISA & hTISA isolates, the proportions were 3% and 16%, respectively. A06 and A07 were not present among hVISA isolates whilst A14 accounted for 8% of these isolates.

Nineteen hGISA isolates (11%) exhibited antibiogram types other than A06, A07, A13 and A14 (details are shown in Table 3.2 and Figure 3.1). Of these hGISA isolates, 89% (17/19)

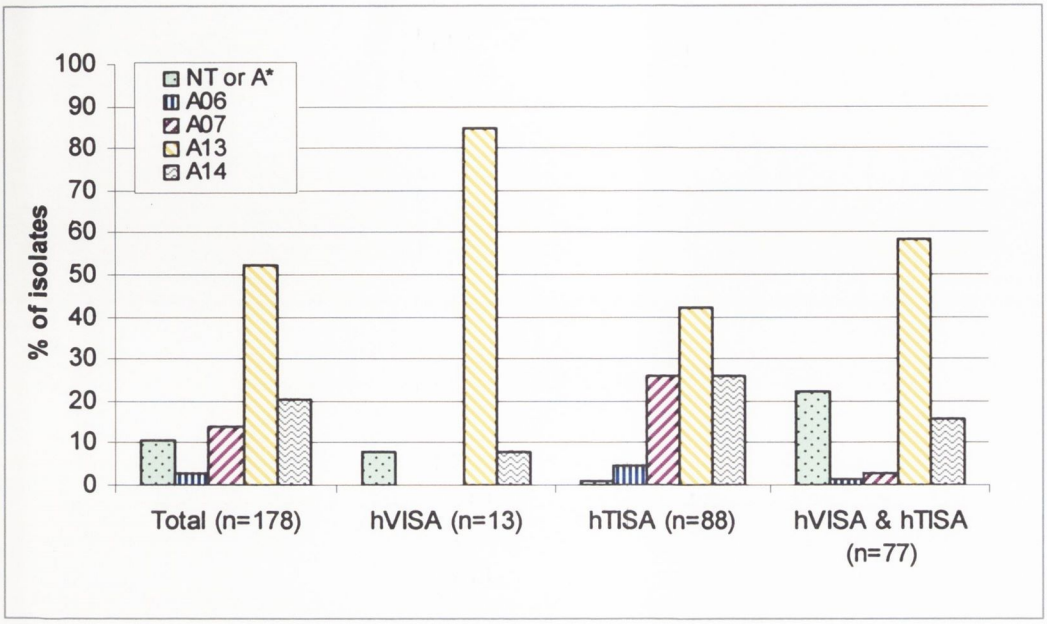
Table 3.2. Numbers and antibiogram type distribution of hGISA isolates among each collection [figures in parentheses indicate the percentage of isolates exhibiting representative antibiogram types].

MRSA Collection	hGISA (*n*=)	Antibiogram Type Distribution							
		A06	A07	A13	A14	A43	A44	New02	Other
1. H1 Q4, 1998 (*n*=188)	10	1 [10]	1 [10]	5 [50]	3 [30]	…	…	…	…
2. N/S Study 1999 (*n*=714)	81	…	4 [5]	53 [65]	13 [16]	3 [4]	…	5 [6]	3 [4]
3. EARSS 1999-2003 (*n*=1580)	39	2 [5]	7 [18]	19 [49]	9 [23]	…	1 [3]	…	1 [3]
4. H1 Q2, 2003 (*n*=330)	16	…	7 [44]	1 [6]	4 [25]	…	4 [25]	…	…
5. NMRSARL 2000-2003 (*n*=97)	28	1 [4]	4 [14]	15 [54]	6 [21]	…	…	…	2 [7]
6. H1 Q4, 2004 (*n*=280)	4	1 [25]	2 [50]	…	1 [25]	…	…	…	…
Total (*n*=3189)	**178**	**5 [3]**	**25 [14]**	**93 [52]**	**36 [20]**	**3 [2]**	**5 [3]**	**5 [3]**	**6 [3]**
N/S Study 1999[a]									
North 1999 (*n*=206)	26	…	…	17 [65]	…	3 [12]	…	5 [19]	1 [4]
South 1999 (*n*=508)	55	…	4 [7]	36 [65]	13 [24]	…	…	…	2 [4]

[a], Breakdown of North/South Study of MRSA in Ireland 1999 into North and South.
Abbreviations. A, antibiogram type. See also Table 2.3.

Resistance patterns of antibiogram types: **A06**, ApCdCpEr; **A07**, Ak*ApCdCpErKnLnNmSpTb; **A13**, Ak*ApCdCpEbErGnKnLnMcMpNmPmaSmSpTb; **A14**, Ak*ApCdCpEbErGnKnMcMpNmPmaSmSpTbTp; **A43**, ApCd*CpErKnNmSmSpTp; **A44**, Ak*ApCdCpEbErGnKnLnMcMpNmPmaSmSpSuTbTeTp; **New02**, AkApCdCpEbGnKnMcNmPmaRfSmSuTbTe.
Abbreviations. See Table 1.4.

Panel A



Panel B

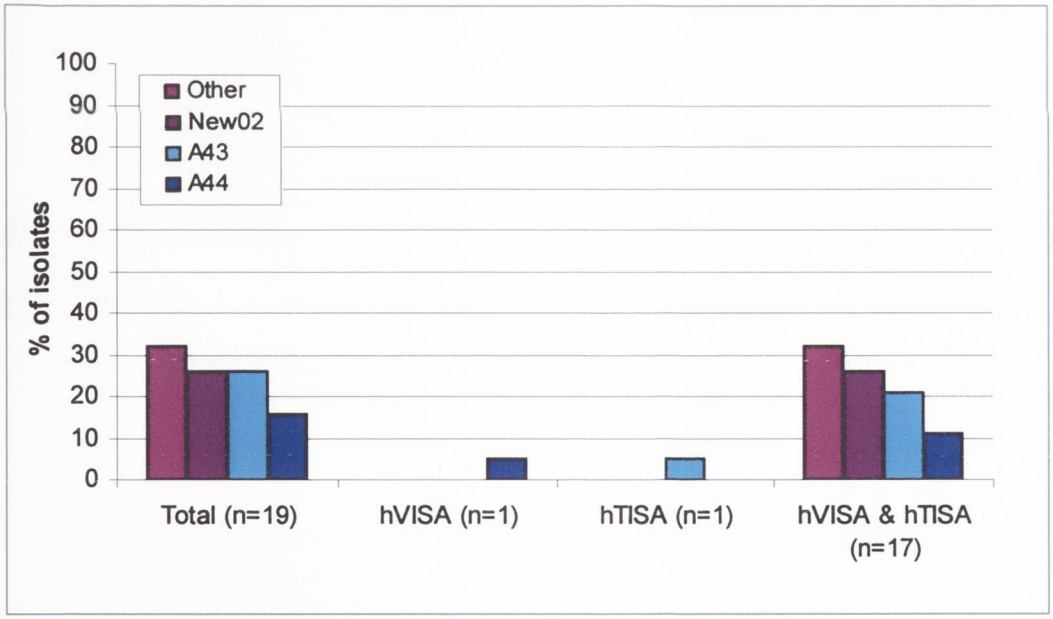


Figure 3.1. Antibiogram type distribution among hVISA, hTISA and hVISA & hTISA isolates.

Panel A shows the antibiogram (A) type distribution among hVISA, hTISA and both hVISA & hTISA isolates. NT or A* represent isolates that exhibit A type NT, unfamiliar A types, A type variants and additional A types (A43, A44 and New02). Panel B shows the distribution of these additional A types and "other" A types among hGISA isolates. The "other" group comprise isolates that exhibit NT (n=2), unfamiliar A types (n=2) and A type variants (n=2).

were represented among hVISA & hTISA isolates whilst 5% and 5% were represented among hVISA and hTISA isolates, respectively. New02 and isolates exhibiting NT or antibiogram type variants were found among hVISA & hTISA isolates only and represented 26% and 32% of isolates, respectively.

3.4.1.2 Prospective Study

The predominant antibiogram type among the MRSA population recovered from patients during the prospective study in H1 during Q4, 2004 was A06 (prevalence: 86%) whilst the prevalence rates of antibiogram types A07, A13, A14 and A43 were 2.5%, <1%, 1.8% and <1%, respectively. Twenty-three isolates (8%) yielded antibiogram patterns to which no antibiogram type was assigned and were designated NT pending results of PFGE analysis. Of these 23 isolates, 96% (22/23) yielded non-multi-antibiotic resistant patterns.

3.4.2 Comparison of Antibiogram Types between hGISA and all MRSA Isolates

The antibiogram type distribution among hGISA isolates and among all MRSA within each collection is shown in Figure 3.2. A13 and A14 predominated among the hGISA isolates within four collections and represented 52% and 20%, respectively of the total hGISA isolates. A07 predominated among hGISA isolates identified in H1 during Q2, 2003 and Q4, 2004 (prevalence: 44% and 50%, respectively) whilst A13 and A14 represented 6% and zero and 25% and 25% of isolates, respectively. Among all collections, A06 predominated among the background MRSA population (prevalence: 51%) followed by A13 and A14 representing 16% and 11% of the overall MRSA isolates, respectively. However, in H1 during Q2, 2003 and Q4, 2004, A13 and A14 represented <1% and <1% of isolates, respectively, whilst A07 accounted for 4% of isolates in the former study and 2.5% of isolates in the latter study.

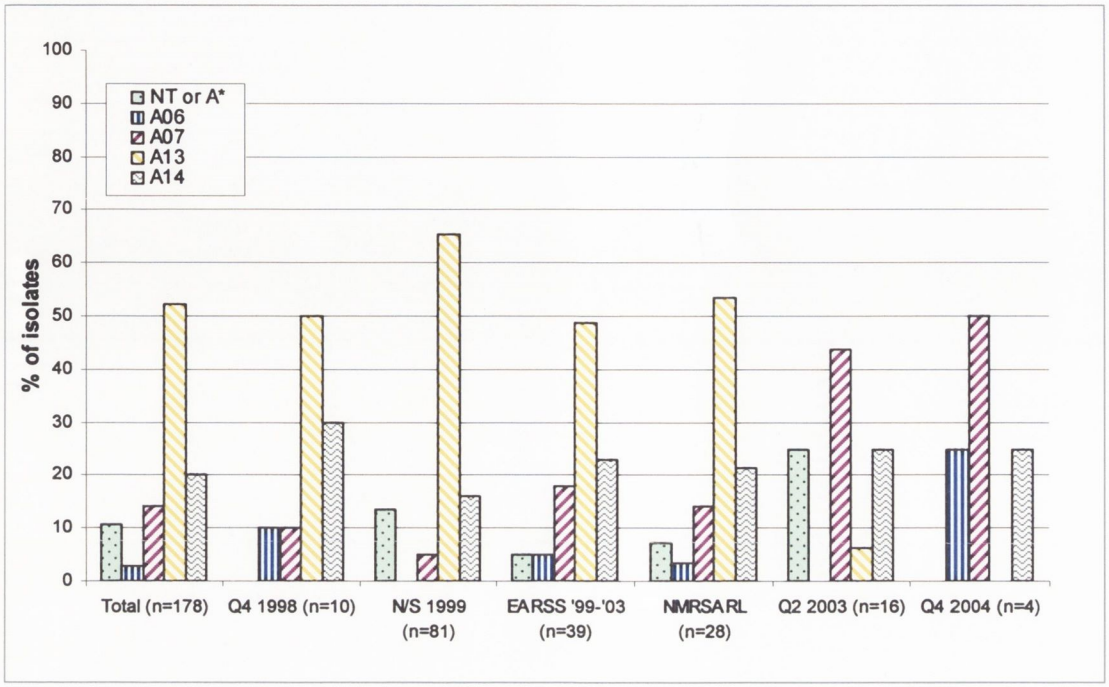
3.4.2.1 Comparison within the North/South Study of MRSA in Ireland

A breakdown of the antibiogram type distribution of hGISA isolates recovered from the North and South of Ireland during the North/South Study of MRSA in Ireland 1999 is detailed in Table 3.2 and Figure 3.3. Overall, A13 predominated among hGISA isolates recovered during the North/South Study of MRSA in Ireland (prevalence: 65%). Among hGISA isolates from the North three antibiogram types, A13, A43 and New02 predominated (prevalence: 65%, 12% and 19%, respectively) whilst one isolate exhibited an NT antibiogram pattern. In the South, A13, A14 and A07 accounted for 65%, 24% and 7% of hGISA isolates, respectively. In addition, two isolates from the South exhibited antibiogram patterns to which NT were assigned. Also shown in Figure 3.3 is the antibiogram type distribution among all MRSA isolates recovered during the North/South Study of MRSA in Ireland and the breakdown of isolates recovered from the North and South of Ireland. Isolates exhibiting A43 and New02 were first recognised during the North/South Study of MRSA in Ireland and were only identified in the North, representing 42% and 4% of isolates, respectively (Rossney *et al.*, 2003b). A43 and New02 represented 12% and 19% of hGISA isolates, respectively, identified in the North during the North/South Study of MRSA in Ireland.

3.4.3 Biotyping

Among 280 isolates investigated during the prospective study in H1 during Q4, 2004, 4% (12/280) hydrolysed urease of which 67% (8/12) and 33% (4/12) of isolates exhibited A07 and NT antibiogram patterns, respectively. Whilst 69% (195/280) of isolates hydrolysed Tween 80, no correlation was observed between hydrolysis of Tween 80 and/or pigment production

Panel A



Panel B

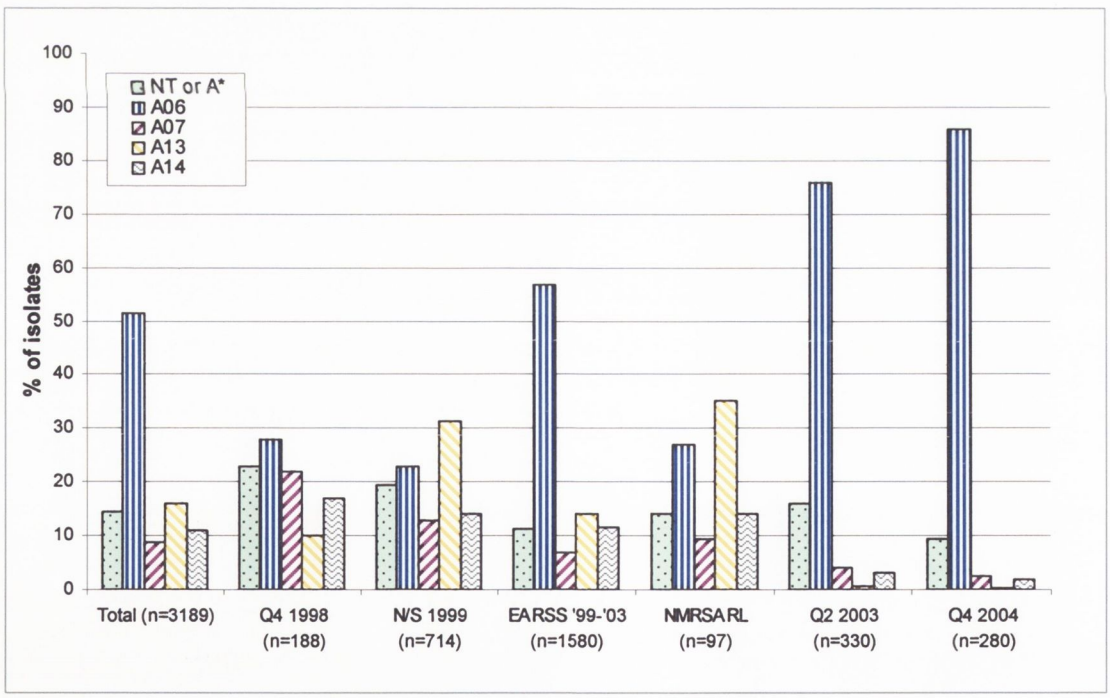
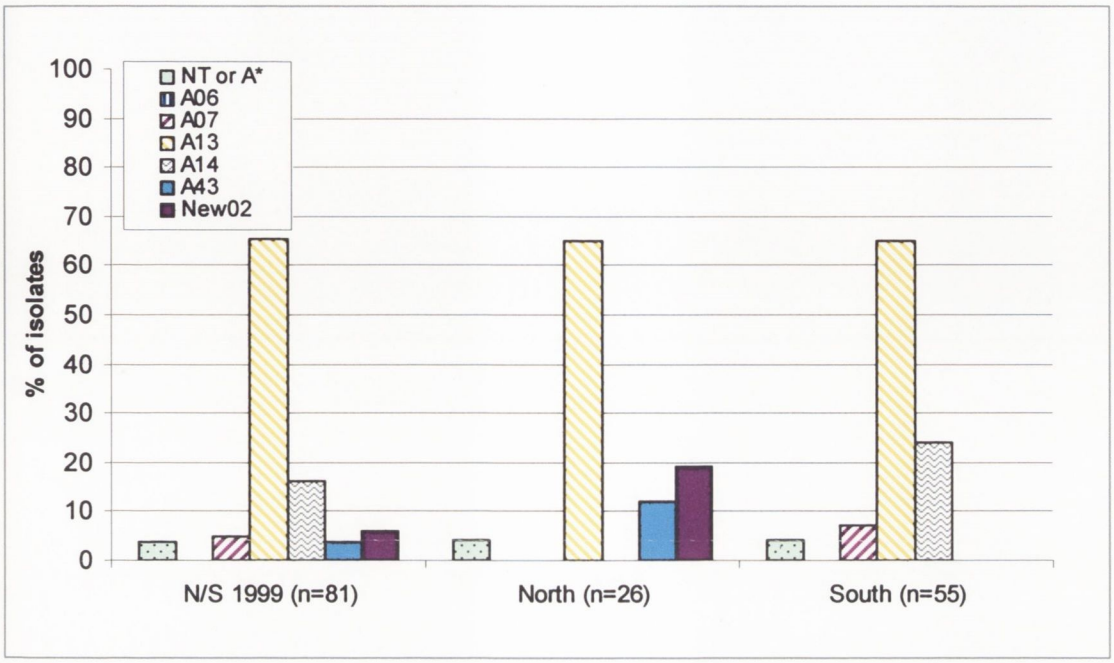


Figure 3.2. Antibiogram type distribution among hGISA and among all MRSA isolates.

Panels A and B show the antibiogram (A) type distribution among hGISA isolates and among all MRSA isolates within each collection, respectively. NT or A* represent isolates that exhibit A type NT, unfamiliar A types, A type variants and additional A types (A43, A44 and New02).

Panel A



Panel B

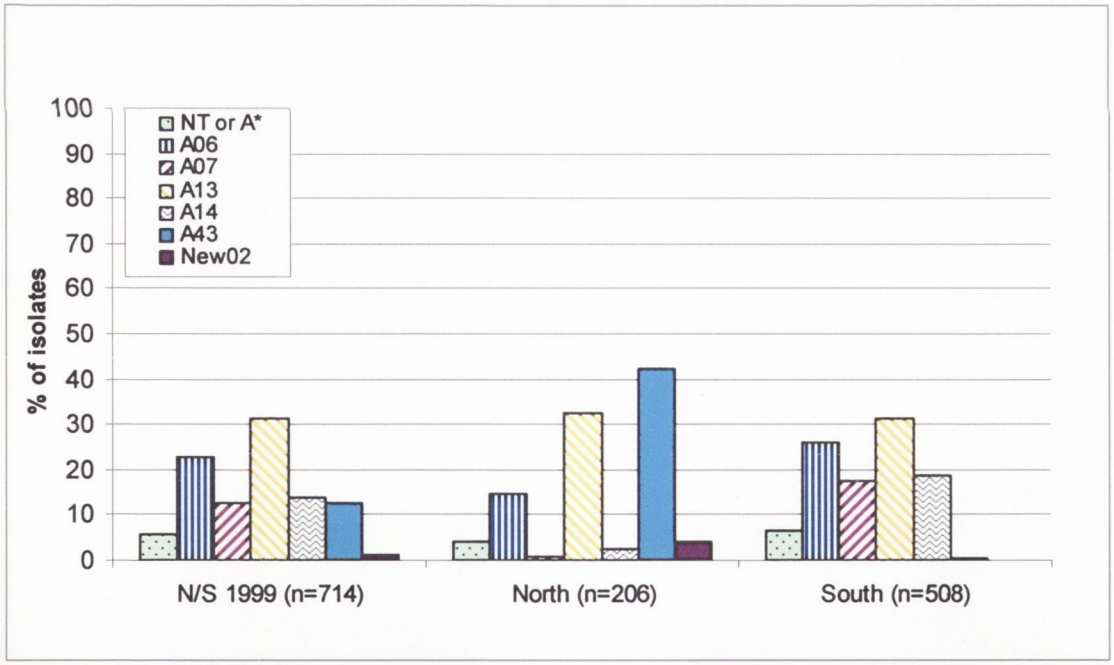


Figure 3.3. Antibiogram type distribution among hGISA isolates and all MRSA isolates recovered during the North/South Study of MRSA in Ireland 1999.

Panel A shows the antibiogram (A) type distribution among hGISA isolates. Panel B shows the A type distribution among all MRSA isolates recovered during the North/South Study of MRSA in Ireland alongside the breakdown of isolates recovered from both the North and South of Ireland. NT or A* represent isolates that exhibit A type NT, unfamiliar A types and A type variants.

and any one antibiogram type. Overall, 75% (210/280), 24% (66/280) and 1% (4/280) of isolates yielded yellow, cream and white colonies on NUT plates, respectively.

3.4.4 DNA Macrorestriction Digestion Analysis

Analysis of the PFGE patterns of 178 hGISA isolates investigated showed that 77% (138/178) of isolates belonged to PFG-00 whilst 3%, 15%, <1% and 4% of isolates belonged to PFG-01, PFG-02, PFG-03 and PFG-99.4, respectively. Among isolates belonging to PFG-00, 51 PFTs were identified of which 39%, 39% and 22% were interpreted as closely related, possibly related and unrelated, respectively, according to the criteria of Tenover *et al.* (Tenover *et al.*, 1995a). Furthermore, 28% (39/138) of isolates belonging to PFG-00 exhibited a single PFT. Four PFTs that differed by between one and three bands (interpreted as closely related) were identified among isolates belonging to PFG-01. Five PFTs were identified among isolates belonging to PFG-02, three PFTs differed by four to six bands (interpreted as possibly related) whilst two PFTs differed by greater than six bands (interpreted as unrelated). One PFT was identified among isolates belonging to PFG-99.4.

Antibiogram types and PFGs of hGISA isolates were combined to form A-PFG types. The most frequently occurring A-PFGs among hGISA isolates were A-PFG 13-00 and A-PFG 14-00 accounting for 52% (93/178) and 20% (36/178) of hGISA isolates, respectively whilst A-PFGs, A-PFG 06-01, A-PFG 07-02, A-PFG 43-00, A-PFG 44-99.4 and A-PFG New02-00 represented 3% (5/178), 14% (25/178), 2% (3/178), 3% (5/178) and 3% (5/178) of isolates, respectively. Four PFGs were identified among five hGISA isolates that exhibited either NT or Unf antibiogram patterns or antibiogram type variants (A-PFG NT-00, A-PFG NT-02, A-PFG 07-03, A-PFG Unf-99 and A-PFG 13-99). Three attempts to extract DNA from one

hGISA isolate that exhibited an unfamiliar antibiogram pattern were unsuccessful, thus no PFGE pattern was obtained for this isolate.

The dendrograms depicted in Figures 3.4 and 3.5 show the PFGE banding patterns of the predominant PFTs of hGISA isolates recovered between 1998 and 2004. Two clusters were identified among hGISA isolates recovered in H1 during Q4, 1998 and during the North/South Study of MRSA in Ireland, 1999 (Figure 3.4). Cluster I consisted of isolates exhibiting PFG-00 and shared <50% similarity with Cluster II. Isolates within Cluster I exhibited A-PFG 13-00, A-PFG 14-00 and New02-00 whilst isolates within Cluster II exhibited A-PFG 07-02. From the dendrogram showing the PFGE banding patterns among representative hGISA isolates recovered from Collections 3 (EARSS), 4 (H1 Q2, 2003) and 5 (NMRSARL), two clusters sharing <50% similarity were identified (shown in Figure 3.5). Cluster I contained two subgroups: the first contained isolates exhibiting A-PFG 13-00 and A-PFG 14-00 and the second contained isolates exhibiting A-PFG 44-99.4 whilst Cluster II contained isolates exhibiting A-PFG 07-02. Figure 3.6 presents ethidium-bromide stained agarose gels showing PFGE patterns of representative hGISA isolates from selected hospitals.

Among 52% (93/178) hGISA isolates that exhibited A-PFG 13-00, 37% (34/93) exhibited a single PFT (00002). Between 1998 and 2004, hGISA isolates exhibiting this PFT were identified in 14 hospitals whilst clusters of hGISA isolates {representing 74% (25/34) of isolates} exhibiting this PFT were identified in five hospitals, two in the North (H5 and H9) and three in the South (H2, H3 and H4) of Ireland, respectively. Representative isolates from clusters of hGISA isolates exhibiting this PFT from H2, H3, H5 and H4 are shown in Figures 3.4 and 3.5.

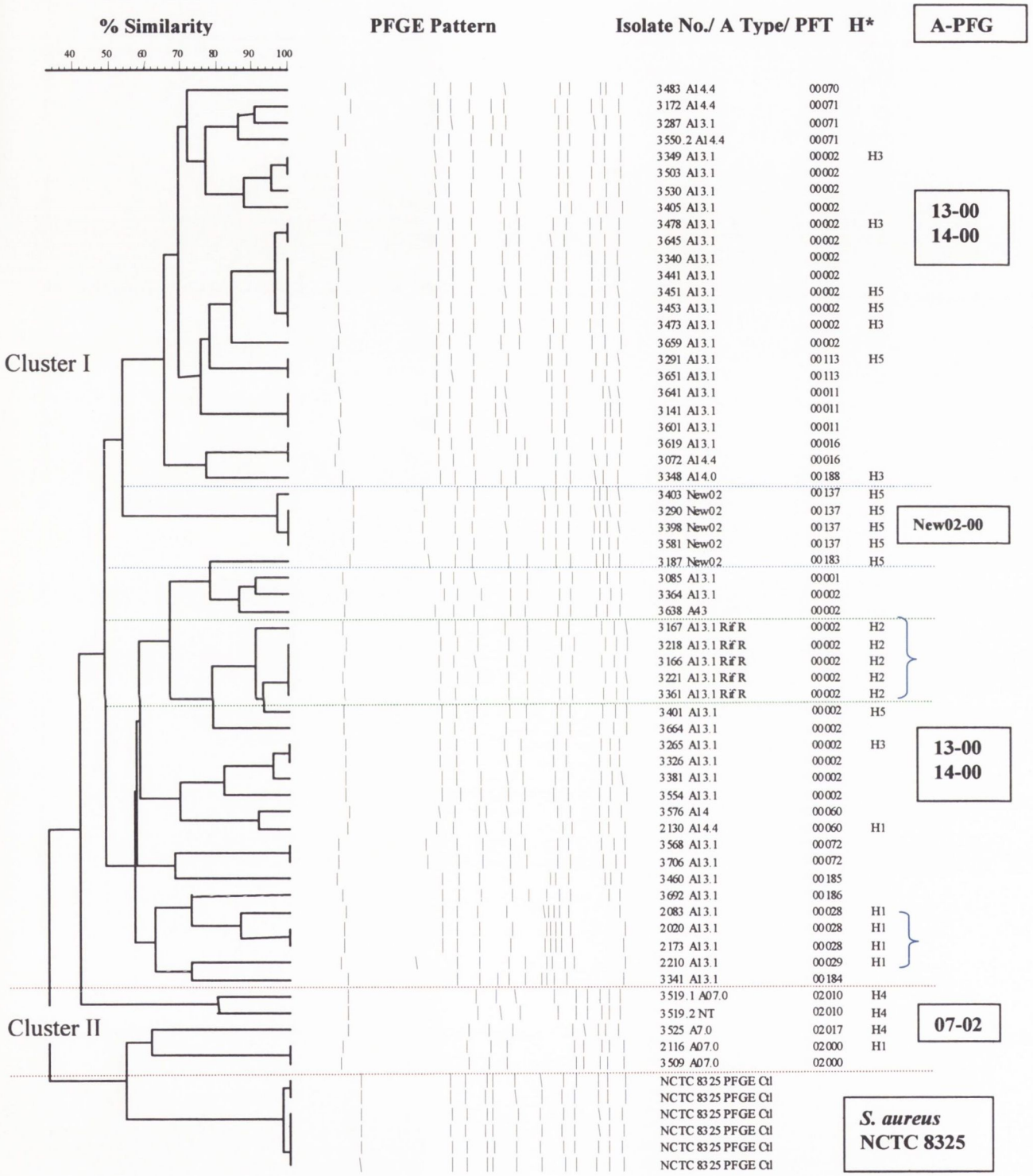


Figure 3.4. Dendrogram depicting % similarity among representative hGISA isolates (recovered between 1998 and 1999) investigated by *Sma*I digestion of chromosomal DNA followed by PFGE.

PFGE banding patterns of representative hGISA isolates recovered in H1 during Q4, 1998 and during the North/South Study of MRSA in Ireland, 1999 were analysed using Gel Compar with percent similarity determined using UPGMA with Dice coefficient (1% tolerance, 0.5% optimisation).

Abbreviations. A, antibiogram type; PFT, pulsed-field group type; A-PFG, antibiogram-pulsed-field group type; *, selected hospitals.

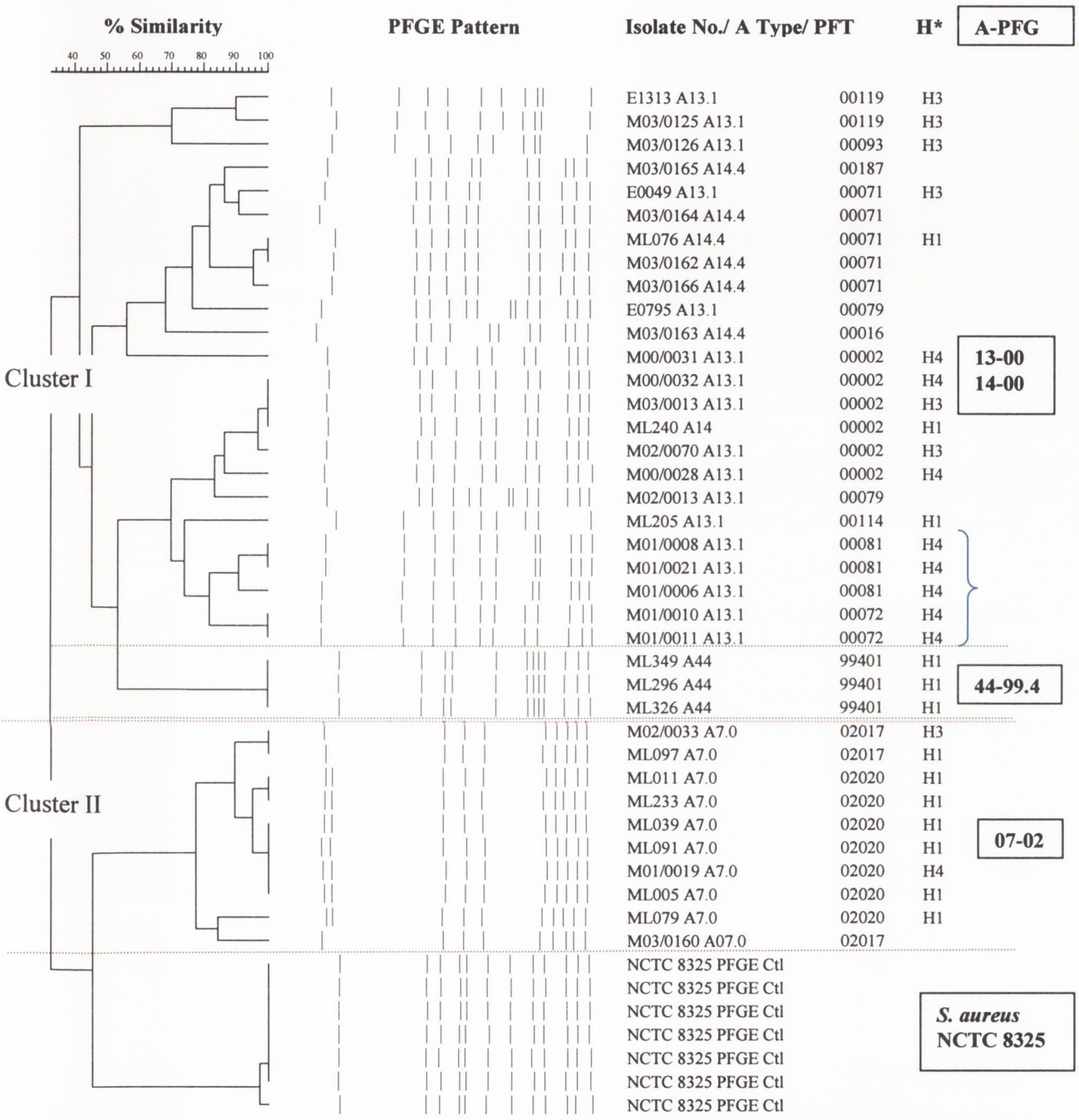


Figure 3.5. Dendrogram depicting % similarity among representative hGISA isolates (recovered between 1999 and 2004) investigated by *Sma*I digestion of chromosomal DNA followed by PFGE.

PFGE banding patterns of representative hGISA isolates recovered from Collections 3 (EARSS), 4 (H1, Q2, 2003) and 5 (NMRSARL) were analysed using Gel Compar with percent similarity determined using UPGMA with Dice coefficient (1% tolerance, 0.5% optimisation).

Abbreviations. A, antibiogram type; PFT, pulsed-field group type; A-PFG, antibiogram-pulsed-field group type; *, selected hospitals.

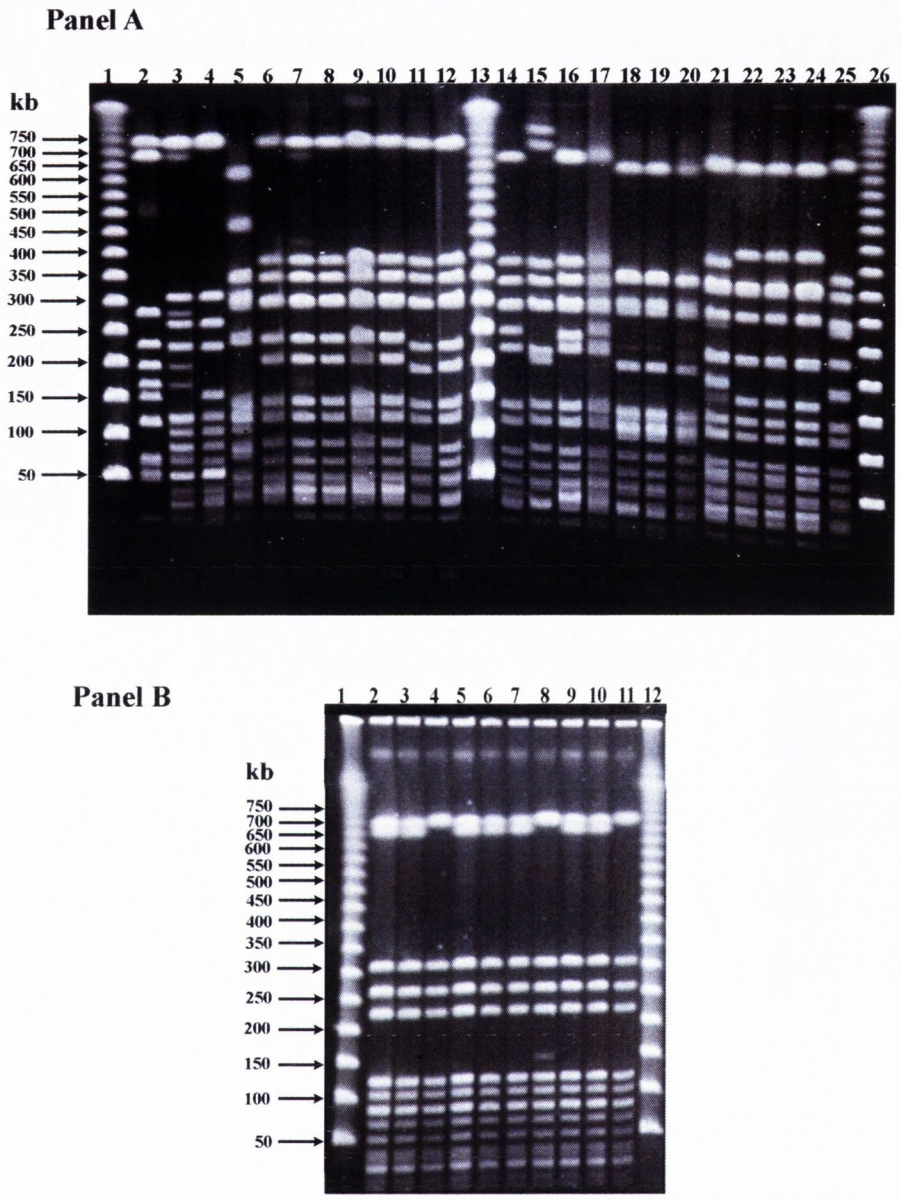


Figure 3.6. Ethidium-bromide stained agarose gels showing PFGE patterns of representative hGISA isolates from selected hospitals.

Molecular size reference marker (Lambda Ladder PFG) is located in lanes 1, 13 and 26 in Panel A and 1 and 12 in Panel B. *S. aureus* NCTC 8325 is located in lane 25 in Panel A.

Panel A: DNA from hGISA isolates exhibiting specific A-PFG types from selected hospitals (H) is located in lanes as follows: 2, A-PFG 06-01 (H1); 3–4, A-PFG 07-02 (H4); 5, A-PFG 07-03 (H4); 6–10, A-PFG 13-00 (H2);11–12, A-PFG 43-00 (H6); 14–17, A-PFG 14-00 (H7); 18–20, A-PFG 44-99.4 (H1); 21–24, A-PFG New02-00 (H5).

Panel B: DNA from hTISA isolates exhibiting A-PFG 07-02 from selected hospitals are located in lanes as follows: 2, (H1); 3, (H4); 4, (H3); 5–10, (H1); 11, (H4).

3.4.5 Clusters of hGISA Isolates

During Q4, 1998, a cluster of hGISA isolates exhibiting A-PFG 13-00 (and PFT 00028) was identified in H1 amongst an MRSA population where A-PFG 13-00 represented only 10% of isolates (details are shown in Figures 3.2 and 3.4). Of isolates submitted to the North/South Study of MRSA in Ireland 1999, clusters of hGISA were identified in different institutions. In the South, a cluster of hGISA isolates exhibiting A-PFG 13-00 with indistinguishable PFGE patterns (PFT 00002) was identified in one institution (H2). PFGE patterns of representative hGISA isolates from this cluster are shown in Figure 3.6 (Panel A, lanes 6–10). All these isolates were resistant to rifampicin and represented 100% of isolates exhibiting A-PFG 13-00 from that institution (shown in Figure 3.4). Although A-PFG 13-00 was exhibited by 65% (53/81) of hGISA isolates recovered during the North/South Study of MRSA in Ireland 1999 and 53% (28/53) exhibited PFT 00002, isolates in the hGISA cluster from H2 were all resistant to rifampicin whereas rates of rifampicin resistance among all MRSA isolates recovered during this period were low (3.5%) (Anon., 2000a).

In a second institution in the South (H3), nine hGISA isolates were identified of which 78% (7/9) exhibited A-PFG 13-00 that yielded an indistinguishable PFT (00002). In that institution, 64% (30/47) of isolates submitted to the North/South Study of MRSA in Ireland exhibited A-PFG 13-00 and of these 23% (7/30) were confirmed as hGISA. During this period, A-PFG 13-00 predominated amongst the MRSA population in that hospital.

Amongst isolates submitted to the NMRSARL for glycopeptide investigation studies from H4 in 2001, a cluster of hGISA isolates exhibiting A-PFG 13-00 with closely related PFGE banding patterns (PFTs 00072, 00081) were identified (shown in Figure 3.5). Although 75%

(15/20) of isolates submitted exhibited A-PFG 13-00, A-PFG 06-01 predominated (prevalence: 49%) amongst the MRSA population in H4 during this period. Clinical data regarding this incident revealed that a patient was transferred from H3 (where A-PFG 13-00 predominated) to H4 and received glycopeptide therapy in this institution.

In the North, a cluster of hGISA isolates yielding a previously unfamiliar antibiogram pattern, designated New02 was identified in one institution (H5) during the North/South Study of MRSA in Ireland 1999 (Rossney *et al.*, 2003a). These isolates yielded PFGE banding patterns that belonged to PFG-00 and were designated A-PFG New02-00 (shown in Figure 3.4). PFGE patterns of representative hGISA isolates from this cluster are shown in Figure 3.6 (Panel A, lanes 21–24). Because the North/South Study of MRSA in Ireland was conducted over a two-week study period it is difficult to ascertain whether these isolates represented an underlying problem with hGISA or whether they represent a single isolate that spread in this institution. Clinical details of the patients from whom these isolates were recovered were unavailable. Overall, among isolates submitted to the North/South Study of MRSA in Ireland from the North, A-PFG New02-00 accounted for 4% (8/206) of isolates but 75% (6/8) of these isolates were identified in H5 where they accounted for 21% (6/28) of all MRSA isolates. Strikingly, the hGISA phenotype was identified among 83% (5/6) of these New02-00 isolates.

Among the overall hGISA isolates exhibiting A-PFG 07-02, 92% (23/25) were confirmed as hTISA. A cluster of hTISA isolates exhibiting A-PFG 07-02 yielding an indistinguishable PFGE pattern (PFT 02020), was identified in H1 during Q2, 2003 (Figure 3.5). The PFGE banding patterns of representative hTISA isolates from this cluster are shown in Figure 3.6 (Panel B, lanes 2 and 5–10). In addition, a cluster of hTISA isolates exhibiting A-PFG 07-02 (PFTs 02010, 02017) was identified in H4 (South) during the North/South Study of MRSA in

Ireland amongst an MRSA population where A-PFG 07-02 represented only 19% of isolates (Figure 3.4). PFGE banding patterns of representative hTISA isolates from this cluster are shown in Figure 3.6 (Panel B, lanes 3 and 11).

A cluster of hGISA isolates exhibiting A-PFG 44-99.4 was recovered in H1 during Q2, 2003 (Figure 3.5). A-PFG 44-99.4, a multi-antibiotic resistant strain was imported to Ireland from Singapore and caused an outbreak in H1 (Rossney, 2003). This strain exhibited a previously unfamiliar antibiogram pattern and was assigned a new antibiogram type, A44 (Rossney, 2003). The index isolate was susceptible to glycopeptides but later isolates of this strain developed reduced susceptibility to glycopeptides. This strain accounted for 4% (12/330) of isolates recovered in H1 during Q2, 2003. Four isolates {33% (4/12)} were confirmed as hGISA. The PFGE banding patterns of these hGISA isolates exhibiting A-PFG 44-99.4 are shown in Figure 3.6 (Panel A, lanes 18–20). Among the eight GSSA isolates exhibiting A-PFG 44-99.4, 62% (5/8) yielded growth on BHIT5 and 60% (3/5) of these isolates yielded positive E-test™ macromethod values but were confirmed as GSSA isolates by vancomycin and teicoplanin PAP methods.

3.5 Discussion

Among the hGISA isolates identified from six collections of MRSA recovered in Ireland between 1998 and 2004, the predominant A-PFG types were A-PFG 13-00 (ST8-MRSA-II) and A-PFG 14-00 (ST8-MRSA-II) (Rossney *et al.*, 2006a). During the 1990s, these two multi-resistant strains predominated among the MRSA population but since 1998 the prevalence of a non-multi-antibiotic resistant strain, A-PFG 06-01 (ST22-MRSA-IV) has increased and accounted for 80% of the MRSA in Ireland submitted to the EARSS project in 2003 (Rossney *et al.*, 2006a). Other workers in Europe have also documented the replacement of older multi-resistant MRSA strains with newer more susceptible strains of MRSA (Donnio *et al.*, 2004; Denis *et al.*, 2004; Witte *et al.*, 2000). However, although results from this study show that the majority of hGISA isolates (80%) exhibit a multi-resistant phenotype, five hGISA isolates (3%) in this study exhibited a non-multi-antibiotic resistant phenotype, A-PFG 06-01 (ST22-MRSA-IV). Furthermore, another study investigating MLST and SCC*mec* types among a global collection of GISA and hGISA isolates has identified seven hGISA isolates exhibiting ST22-MRSA-IV (Howe *et al.*, 2004). Thus, epidemiological typing analysis based on susceptibility testing results alone cannot be used as a marker to predict isolates exhibiting reduced susceptibility to glycopeptides.

Although DNA macrorestriction analysis using *Sma*I followed by PFGE typing is currently the gold standard method for short-term epidemiological typing of MRSA, 77% of hGISA isolates in the present study belonged to a single PFG, PFG-00. Among these isolates, five A-PFGs {A-PFG 13-00 (67%), A-PFG 14-00 (26%), A-PFG 43-00 (2%), A-PFG New02-00 (4%) and A-PFG NT-00 (1%)} were identified. A difficulty encountered when typing MRSA is that MRSA isolates represent a subset of *S. aureus* and thus exhibit limited genetic diversity. This

difficulty is exacberated when typing hGISA isolates which represent a subset of MRSA. A further problem with PFGE is that when using the criteria of Tenover *et al.*, the criteria were designed for studying discrete sets of isolates recovered over short time-periods in for example outbreak investigations. An added challenge is that ideally isolates should be typed over short periods of time. In the present study, PFGE was used to investigate a large population of MRSA, collected and tested over a prolonged period. Hence, although pattern recognition was confirmed by visual inspection of gels, when dendrograms were drawn with Gel Compar, indistinguishable PFGE patterns did not always yield 100% similarity (Tenover *et al.*, 1995a). For long-term epidemiological typing of MRSA, MLST is superior to PFGE as it involves DNA sequencing of internal fragments of seven housekeeping genes that provides unambiguous, portable results suitable for global and evolutionary studies of MRSA (Enright *et al.*, 2002).

In this study, a combination of antibiogram typing results and DNA macrorestriction digestion analysis showed that among hGISA isolates, four out of five pandemic clones were represented, CC8, CC30, CC22 and CC5. CC8 was represented by isolates exhibiting A-PFG 13-00 and A-PFG 14-00 (ST8-MRSA-II), A-PFG 44-99.4 (ST239-MRSA-III), A-PFG New02-00 (ST247-MRSA-I) and A-PFG 43-00 (ST8-MRSA-IV) whilst CC30 and CC22 were represented by isolates exhibiting A-PFG 07-02 (ST36-MRSA-II) and A-PFG 06-01 (ST22-MRSA-IV), respectively. As illustrated in Figures 3.4 and 3.5, all CC8 isolates exhibiting A-PFG 13-00 and A-PFG 14-00 clustered with isolates exhibiting A-PFG New02-00 (CC8) and A-PFG 44-99.4 (CC8) whilst isolates exhibiting A-PFG 07-02 (CC30) shared <50% similarity with these isolates.

CC5, ST5-MRSA-II representing the New York/Japan clone was represented by only one isolate that exhibited A-PFG 07-03. During initial studies of GISA and hGISA isolates, it was postulated that isolates exhibiting reduced susceptibility to glycopeptides descended from this MRSA clone and were designated ST5-GISA-II (Hiramatsu *et al.*, 1997a; Enright *et al.*, 2002). A previous study of Irish MRSA isolates reported that ST5-MRSA-II was present among the MRSA population between 1992 and 1993, but its prevalence was low (Shore *et al.*, 2005).

A previous study has reported that all these genotypes except ST239-MRSA-III were represented among a collection of GISA and hGISA isolates from around the world (Howe *et al.*, 2004). The ST239-MRSA-III strain has however been associated with reduced susceptibility to glycopeptides in other studies (Hsu *et al.*, 2005; Okuma *et al.*, 2002; Murray *et al.*, 2004).

Some workers have reported the Iberian clone (ST247-MRSA-I) to be associated with reduced susceptibility to teicoplanin (Heym *et al.*, 2002; Aucken *et al.*, 2002; Robinson & Enright, 2004). Heym *et al.* reported the spread of the Iberian clone to France where it acquired reduced susceptibility to teicoplanin (Heym *et al.*, 2002). In 2002, Aucken *et al.* reported borderline teicoplanin resistance among a new epidemic strain of MRSA in the UK, termed EMRSA-17 subsequently shown to be ST247-MRSA-I (Aucken *et al.*, 2002; Robinson & Enright, 2004). In the present study, ST247-MRSA-I was represented by hGISA isolates exhibiting A-PFG New02-00 but these isolates showed reduced susceptibility to both vancomycin and teicoplanin.

An interesting finding of the present study was that 92% (23/25) of hGISA isolates exhibiting A-PFG 07-02 were confirmed as hTISA isolates. Isolates exhibiting A-PFG 07-02 (ST36-MRSA-II) are similar to EMRSA-16 isolates in the UK and reduced susceptibility to glycopeptides has previously been documented among EMRSA-16 isolates (Shore *et al.*, 2005; Howe *et al.*, 2004). Whilst the present study noted an association between A-PFG 07-02 (ST36-MRSA-II) and reduced susceptibility to teicoplanin, other workers have not reported similar findings. Although 92% of isolates exhibiting A-PFG 07-02 were confirmed as hTISA, these isolates represented only 26% (23/88) of hGISA isolates showing reduced susceptibility to teicoplanin only whilst A-PFG 13-00 was the predominant A-PFG type among hTISA isolates (42%).

The question remains as to whether clusters of hGISA isolates identified in this study represent "real" clusters (i.e. isolates of particular MRSA strains that have the ability to develop reduced susceptibility in response to exposure to glycopeptides) or whether one isolate among each cluster exhibiting reduced susceptibility to glycopeptides spread within each institution. Previous studies have documented spread not only in individual institutions but between countries also (Cartolano *et al.*, 2004; Garnier *et al.*, 2006; Bert *et al.*, 2003; de Lassence *et al.*, 2006; Heym *et al.*, 2002). Although unstable heteroresistance to vancomycin and/ or teicoplanin is common among clinical isolates of MRSA, stable heteroresistance is associated with spread (Plipat *et al.*, 2005). Analysis of epidemiological typing results in the present study revealed that among clusters of hGISA isolates recognised in selected institutions, different resistant phenotypes were identified. For example: (i) two hVISA & hTISA isolates and three hTISA isolates were confirmed among a cluster of hGISA isolates that exhibited A-PFG 13-00 with indistinguishable PFGE patterns in H1 during Q4, 1998; (ii) among six hGISA isolates that exhibited A-PFG 13-00 with indistinguishable PFGE patterns

in H3, two isolates were hVISA & hTISA, three were hTISA and one was hVISA; (iii) among 25 isolates that exhibited A-PFG 13-00 and the single PFT (00002) identified in five hospitals (H2, H3, H4, H5 and H9), six hVISA isolates, four hTISA isolates and 15 hVISA & hTISA isolates were confirmed.

A cluster of hVISA & hTISA isolates exhibiting A-PFG 13-00 with closely related PFGE banding patterns (PFTs 00072, 00081) was identified in H4 amongst an MRSA population where A-PFG 06-01 predominated (prevalence: 49%). Clinical data confirmed that a patient was transferred from H3 to H4. But, among hGISA isolates exhibiting A-PFG 13-00 in H3, PFT 00002 predominated whereas PFTs 00072 and 00081 were not present among hGISA isolates identified in H3. These findings suggest that the hGISA isolate (exhibiting A-PFG 13-00 and PFTs 00072, 00081) was acquired in H4. However, these results should be interpreted with caution as PFT 00072 and PFT 00081 differ from PFT 00002 by only one and two bands, respectively.

Among clusters of hGISA isolates identified in this study, clusters of isolates exhibiting A-PFG New02-00 (ST247-MRSA-I) and A-PFG 44-99.4 (ST239-MRSA-III), respectively, yielded particularly interesting results. A-PFG New02-00 was first recognised in the North during the North/South Study of MRSA in Ireland (Rossney *et al.*, 2003b). All isolates in this hGISA cluster were confirmed as hVISA & hTISA isolates. It is not known when A-PFG New02-00 was first introduced to H5 because the North/South Study was a "snapshot" study conducted over a two-week period. In addition, whether this strain exhibited reduced susceptibility to glycopeptides when introduced or whether hGISA developed following exposure to glycopeptides in H5 with subsequent spread in this institution, is not known.

A-PFG 44-99.4 (ST239-MRSA-III) was imported to Ireland from Singapore, and caused an outbreak in H1 (Rossney, 2003). Although confined to the original unit and recognised as a new strain with 48 hours of isolation, a worrying aspect was how quickly this strain spread following its introduction into H1 (Rossney, 2003). The index isolate was susceptible to glycopeptides but, within three months it had developed reduced susceptibility and the hGISA strain also spread within the unit. Among the cluster of hGISA isolates recovered from four patients, three hVISA & hTISA isolates and one hVISA isolate were confirmed. The pooled AUC values calculated for *S. aureus* Mu3 ATCC 700698 (mean of 100 ± 5) and *S. aureus* ATCC 29213 (mean of 15 ± 5) on 10 separate occasions suggested good reproducibility for tPAP. Because the index strain in this case can be identified, it can be postulated that isolates exhibiting reduced susceptibility to glycopeptides can spread rapidly within institutions and individual isolates can manifest different hGISA phenotypes.

An interesting finding in the current work was the differences in the predominant A-PFG types among hGISA isolates identified in H1 between 1998 and 2004. Whilst A-PFG 13-00 predominated among hGISA isolates in 1998, A-PFG 07-02 was the predominant A-PFG type identified among hGISA isolates in H1 during Q2, 2003 (Figure 3.2). During the prospective study in Q4, 2004, hGISA isolates recovered from three patients each exhibited a different A-PFG type (Figure 3.2). Among 92% of hTISA isolates exhibiting A-PFG 07-02, 56% (13/23) were identified in H1 between 1998 and 2004 whilst the remaining 44% were identified in six other institutions, H4 (19%), H11 (9%), H3 (4%), H8 (4%), H10 (4%) and H12 (4%), respectively. Among the overall MRSA population (n=3189), A-PFG 07-02 accounted for only 9% (279/3189) of isolates (Figure 3.2). The majority {40%, (113/279)} were identified in H1 while 38% were reported from these six institutions (prevalence was as follows: H4, 10%; H11, 1%; H3, 10%; H8, 6%; H10, 3% and H12, 8%). Among the hTISA isolates

exhibiting A-PFG 07-02 in H1, 54% (7/13) of isolates were identified during Q2, 2003 when the prevalence of A-PFG 07-02 among all MRSA isolates was 4% (Figure 3.2).

Among hGISA isolates (20%) exhibiting A-PFG 14-00 in the present study, 64%, 33% and 3% were confirmed as hTISA, hVISA & hTISA and hVISA isolates, respectively. No clusters of hGISA isolates were identified but all nine isolates that exhibited A-PFG 14-00 identified in H1 were confirmed as hTISA isolates. Among these isolates, 44% (4/9) were identified during Q2, 2003. Interestingly, during the prospective study (Q4, 2004) in H1 one hTISA isolate and one GSSA isolate that exhibited A-PFG 14-00 were identified from two patients, Patient 3 and 4, who shared the same ward but neither patient had received glycopeptide therapy. Whilst Patient 3 had no previous history of MRSA infection, the first isolate recovered from this patient exhibited the hTISA phenotype (Table 2.8). Patient 4 had a documented history of MRSA infection. However, both isolates exhibited PFTs that differed by greater than six bands (interpreted as unrelated) suggesting that transmission between these two patients had not occurred.

Whilst epidemiological typing is not a suitable marker for detecting isolates exhibiting reduced susceptibility to glycopeptides, it is an important investigational tool for monitoring changes in the MRSA population. Recognising outbreaks of MRSA exhibiting reduced susceptibility to glycopeptides and determining the source in addition to recognising new strains of MRSA, are important aspects of understanding and ultimately controlling these infections. The threat associated with glycopeptide resistance among MRSA isolates serves as a strong incentive to continually monitor the MRSA population.

CHAPTER IV

Molecular and Experimental Studies of hGISA Isolates

Chapter IV Molecular and Experimental Studies of hGISA Isolates

4.1 Introduction

Whilst the underlying genetic mechanisms for reduced susceptibility to glycopeptides in *S. aureus* have not yet been determined, several studies have investigated whether certain characteristics (both phenotypic and genotypic) are associated with GISA and hGISA isolates (Cui *et al.*, 2006; Hanaki *et al.*, 1998a; Pfeltz *et al.*, 2000; Sieradzki & Tomasz, 1997; Sieradzki *et al.*, 1998; Sieradzki *et al.*, 1999b; Sieradzki & Tomasz, 2006; Sieradzki & Tomasz, 2003; Sieradzki *et al.*, 2003; Kuroda *et al.*, 2003). Following the initial report of reduced susceptibility to glycopeptides among *S. aureus* isolates, early studies focussed mainly on phenotypic characteristics (Hanaki *et al.*, 1998a; Sieradzki *et al.*, 1998; Sieradzki & Tomasz, 1997; Sieradzki & Tomasz, 1998; Pfeltz *et al.*, 2000; Sieradzki & Tomasz, 1999). Hiramatsu *et al.* reported that *S. aureus* Mu50 ATCC 700699 (the first clinical GISA isolate) had an abnormally thickened cell wall when viewed under electron microscopy compared to a GSSA strain and a three-fold increase in the production of cell wall murein precursors and in PBP2 and PBP2a (Hiramatsu *et al.*, 1997b). Although the first clinical *S. aureus* isolate exhibiting reduced susceptibility to glycopeptides was reported in 1997, resistance to glycopeptides among *S. aureus* isolates had been investigated since the early 1990s using laboratory-derived resistant mutants of *S. aureus* (Daum *et al.*, 1992; Kaatz *et al.*, 1990; Shlaes *et al.*, 1993; Biavasco *et al.*, 1991). Following the *in vitro* transfer of *vanA* from *E. faecalis* to *S. aureus* demonstrated by Noble and co-workers, the development of vancomycin resistance in *S. aureus* was monitored carefully (Shlaes & Shlaes, 1995; Noble *et al.*, 1992). Daum *et al.* reported increases in vancomycin and teicoplanin MIC values among "derivative isolates" (selected from two susceptible clinical isolates of *S. aureus*) following serial incubation in low-level vancomycin (Daum *et al.*, 1992). These "derivative isolates" had thicker cell walls when examined using transmission electron microscopy (Daum *et al.*, 1992).

4.1.1 Molecular Studies of GISA/hGISA Isolates

Advances in molecular technology such as genome sequencing have allowed comparisons of the prototype GISA strain *S. aureus* Mu50 ATCC 700699 with other clinical GISA and hGISA isolates (Wootton *et al.*, 2004; Avison *et al.*, 2002). In a study by Cui *et al.*, 17 genes (including eight genes with no known previous function) involved in cell wall biosynthesis were identified and reported to be associated with reduced susceptibility to glycopeptides among *S. aureus* isolates (Cui *et al.*, 2005). In a recent study investigating comparative proteomics of clinical MRSA and VISA isolates conducted by Drummelsmith *et al.*, it was observed that the expression of one particular gene (SAV2095) was markedly increased in VISA/hVISA isolates and could be used as a biomarker for detecting VISA/hVISA isolates (Drummelsmith *et al.*, 2007). Alterations in gene expression resulting in down-regulation of *tcaA*, inactivation of *mprF* and over-expression of the global regulator *sigB* and the two-component sensor transducer *VraSR* have also been observed in *S. aureus* isolates exhibiting reduced susceptibility to glycopeptides (Maki *et al.*, 2004; Nishi *et al.*, 2004; Ruzin *et al.*, 2003; Bischoff *et al.*, 2001; Kuroda *et al.*, 2003; Sieradzki *et al.*, 2003; Sieradzki & Tomasz, 2003; Singh *et al.*, 2003). Recently, Howden *et al.* noted that the up-regulation of *vraS* was not required for the development of the GISA/hGISA phenotype (Howden *et al.*, 2008). Another recent report documented that the mutated response regulator *graR* was responsible for the conversion of isolates from the hGISA phenotype to the GISA phenotype (Neoh *et al.*, 2008). Although several loss of function mutations affecting cell wall biosynthesis have been observed in isolates exhibiting the GISA/hGISA phenotype, the underlying genetic mechanism has not yet been established and it is postulated that several mechanisms are involved in the development of the GISA/hGISA phenotype.

Recent studies have reported that certain genotypic characteristics are associated with GISA and hGISA isolates (Sakoulas *et al.*, 2002; Sakoulas *et al.*, 2003; Sakoulas *et al.*, 2005; Sakoulas *et al.*, 2006; Howe *et al.*, 2004; Howden *et al.*, 2006; Moise-Broder *et al.*, 2004). The *agr* locus in *S. aureus* has been central to several investigations of genotypic properties of GISA and hGISA isolates (Verdier *et al.*, 2004; Sakoulas *et al.*, 2002; Fowler *et al.*, 2004; Sakoulas *et al.*, 2006; Howe *et al.*, 2004; Moise-Broder *et al.*, 2004). In *S. aureus*, delta-haemolysin is the only translated product of RNAIII (the effector molecule of the *agr* response) and thus serves as a measure of *agr* function. Several studies have investigated the production of delta-haemolysin among isolates exhibiting reduced susceptibility to glycopeptides (Sakoulas *et al.*, 2002; Sakoulas *et al.*, 2006; Fowler *et al.*, 2004; Howe *et al.*, 2004). Loss of function of the *agr* locus has been associated with increased biofilm formation (Vuong *et al.*, 2000). Biofilm formation has been suggested as a contributing factor in GISA and hGISA infections (Vuong *et al.*, 2000; Sakoulas *et al.*, 2002). Additional molecular studies of isolates exhibiting reduced susceptibility to glycopeptides have included the investigation of vancomycin resistance genes (described in Section 1.7.1.2) (Howden *et al.*, 2006). To date, no clinical GISA or hGISA isolate carrying a vancomycin resistance gene has been reported.

4.1.1.1 agr Locus

The *agr* locus in *S. aureus* encodes a two-component regulatory system that co-ordinates expression of a number of virulence genes (Ji *et al.*, 1997; Lyon *et al.*, 2000; Novick *et al.*, 1993). The *agr* locus comprises a quorum-sensing system that, during the transition from exponential to stationary phase of growth, up-regulates the production of secreted virulence

factors such as haemolysins (alpha-, beta- and delta-haemolysins), proteases, exoproteins and exotoxins, and down-regulates the production of cell-associated virulence factors such as adhesins (Ji *et al.*, 1997; Novick *et al.*, 1993; Peng *et al.*, 1988; Recsei *et al.*, 1986).

A schematic diagram of the *agr* locus in *S. aureus* is shown in Figure 4.1. The *agr* locus comprises two divergent transcriptional units RNAII and RNAIII, which are under the control of two promoters P2 and P3, respectively. The P2 operon consists of a propeptide AgrD and a membrane protein AgrB, both required for the synthesis of an autoinducing peptide (AIP) which activates *agr* expression in *S. aureus*. A two-component signal transduction system, AgrC (a membrane sensor) and AgrA (a response regulator) responsible for sensing and responding to the AIP, also forms part of the P2 operon. RNAIII, a regulatory RNA molecule is the effector molecule of the *agr* response that up-regulates transcription of secreted toxins and extracellular enzymes and down-regulates production of cell-surface associated proteins and adhesins (Dufour *et al.*, 2002b).

DNA sequence polymorphisms at the *agr* locus define four distinct genetic groups, *agr* groups I–IV that have been associated with distinct clinical conditions (Jarraud *et al.*, 2002; Ji *et al.*, 1997). Previous reports have documented that the majority of *S. aureus* isolates belong to *agr* group I (Moore & Lindsay, 2001; van Leeuwen *et al.*, 2000). Initial studies with GISA and hGISA isolates documented that *agr* group II polymorphisms were associated with isolates exhibiting reduced susceptibility to glycopeptides and may be predictive of vancomycin therapy failure (Sakoulas *et al.*, 2002; Moise-Broder *et al.*, 2004). However, subsequent studies have shown that GISA and hGISA isolates belong to both *agr* group I and II (Howe *et al.*, 2004; Verdier *et al.*, 2004). CA-MRSA strains have been associated with *agr* group III

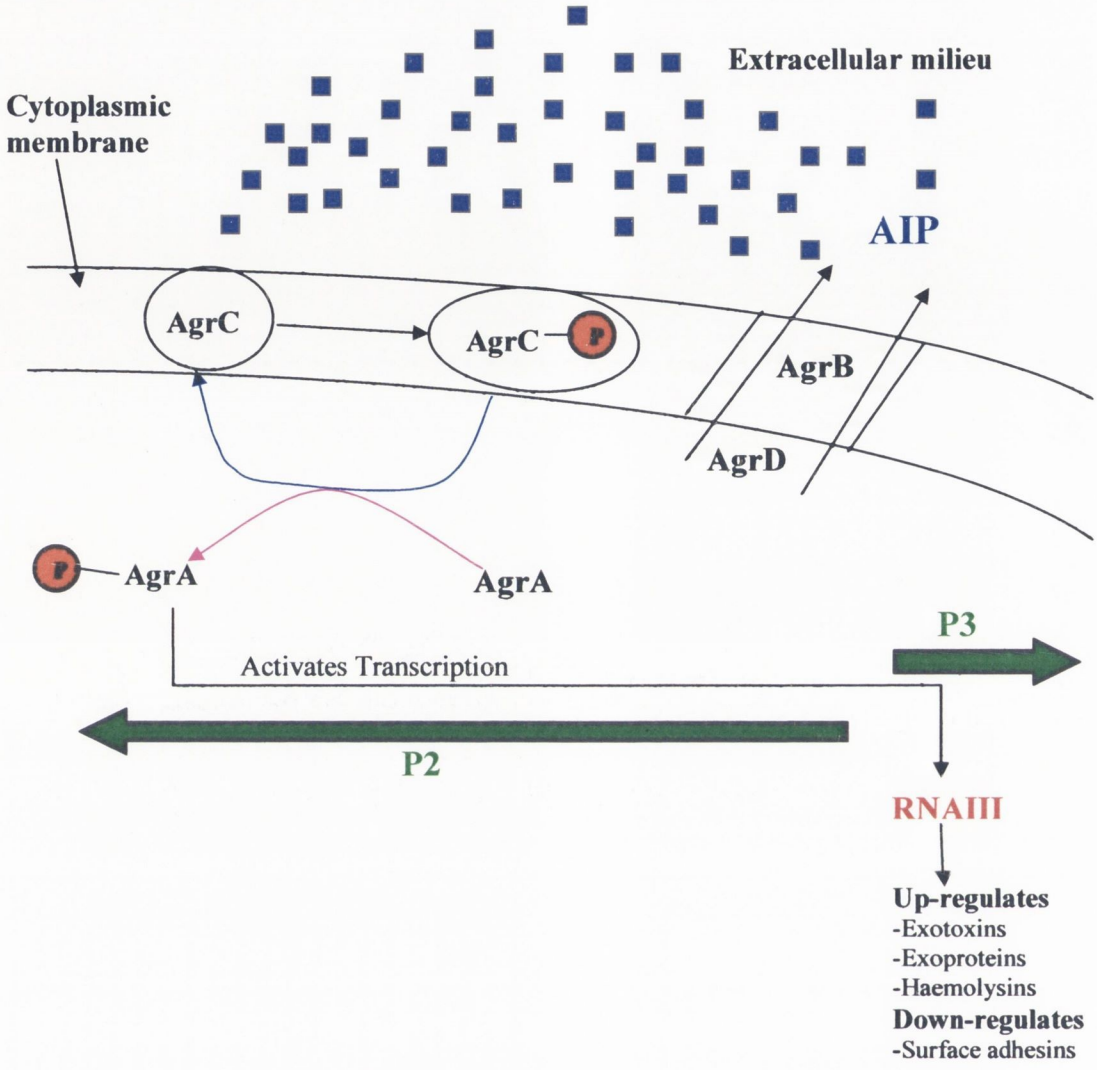


Figure 4.1. The *agr* locus in *S. aureus.*

The *agr* system comprises two divergent transcriptional units (RNAII and RNAIII) under the control of two promoters, P2 and P3, respectively. During bacterial growth, AgrD is secreted by AgrB (a membrane protein) and cleaved to produce an autoinducing peptide (AIP). When the concentration of the AIP in the extracellular milieu reaches a threshold level, AIP interacts with the membrane sensor AgrC causing phosphorylation of AgrC which in turn causes AgrA to be phosphorylated. Phosphorylated AgrA activates transcription of RNAIII resulting in up-regulation of the production of secreted virulence factors and down-regulation of the production of cell-associated virulence factors.

whilst exfoliatin-producing strains of *S. aureus* have been reported to belong to *agr* group IV (Dufour *et al.*, 2002a; Naimi *et al.*, 2003; Jarraud *et al.*, 2002).

4.1.1.1.1 Delta-Haemolysin

Delta-haemolysin is one of four classes of cytotoxins produced by *S. aureus* (Bohach *et al.*, 1997). Other cytotoxins include alpha- and beta-haemolysins as well as the two-component toxins gamma-haemolysin and PVL (Bohach *et al.*, 1997). Delta-haemolysin comprises 26 amino acids and is produced by approximately 97% of *S. aureus* isolates and by 50–70% of CoNS (Bohach *et al.*, 1997). A feature that distinguishes delta-haemolysin from other staphylococcal haemolysins is its ability to lyse both horse and human red blood cells (RBCs) and to act synergistically with beta-haemolysin in the lysis of sheep RBCs (Williams & Harper, 1947; Kreger *et al.*, 1971). Although the precise role of delta-haemolysin in pathogenesis remains unclear, it produces a range of cytolytic effects and causes membrane damage to a variety of mammalian cells and organelles including erythrocytes, spheroplasts, protoplasts, tissue culture cells, leukocytes, lysosomes and mitochondria (Bohach *et al.*, 1997). Whilst delta-haemolysin has limited antimicrobial activity, when produced in large amounts it can be lethal to bacterial cells (Bohach *et al.*, 1997). In previous studies of isolates exhibiting reduced susceptibility to glycopeptides, compromised *agr* function resulting in lack of delta-haemolysin production has been associated with both GISA and hGISA isolates (Sakoulas *et al.*, 2002; Renzoni *et al.*, 2004).

4.1.1.1.2 Surface Adhesins

Surface adhesins, one of the many virulence factors produced by *S. aureus*, are attached to peptidoglycan and exposed on the cell surface. During the early stages of bacterial growth,

surface adhesins are expressed but when bacteria enter the late exponential phase of growth and reach a high population density, adhesin production is decreased and exoprotein production is increased under the control of the *agr* locus as described in Section 4.1.1.1 above. Surface adhesins of *S. aureus* mediate attachement to blood proteins such as immunoglobulin G (IgG), fibrinogen and prothrombin or to extracellular matrix proteins such as fibronectin and collagen (Lowy, 1998). One surface adhesin, a polysaccharide molecule, poly-*N*-succinyl β-1-6 glucosamine (PNSG) has been reported to be the major adhesin involved in mediating attachement to biomedical devices among CoNS and *S. aureus* (McKenney *et al.*, 2000). Several studies have investigated adherence in coagulase-negative staphylococcal infections associated with biomedical devices (Christensen *et al.*, 1985). In 1985, Christensen *et al.* described a quantitative method by which adherence of CoNS to plastic tissue culture plates was measured (Christensen *et al.*, 1985). As several infections associated with GISA and hGISA isolates have involved biomedical devices, Sakoulas *et al.* used a modification of this method to show that all GISA and hGISA isolates studied were defective in *agr* function, suggesting these isolates had a greater ability to adhere to polystyrene (Christensen *et al.*, 1985; Sakoulas *et al.*, 2002).

4.1.1.2 Vancomycin Resistance Genes

As mentioned in Section 1.7.1.2, six vancomycin resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) characterised by both phenotypic and genotypic properties have been described in enterococci (Depardieu *et al.*, 2004). Resistance to vancomycin occurs when the vancomycin-binding target (D-alanyl-D-alanine residues in the murein monomer) is modified by the presence of operons that encode enzymes for synthesis of low-affinity peptidoglycan precursors, in which D-alanyl-D-alanine is replaced by D-alanyl-D-lactate in the *vanA*, *vanB*

and *vanD* resistance types or D-alanyl-D-serine in the *vanC*, *vanE* and *vanG* resistance types (Arthur *et al.*, 1996). Phenotypically, varying levels of resistance to both vancomycin and teicoplanin are exhibited by each resistant phenotype whilst the location of the gene and mode of regulation of gene expression determines genotypic characteristics (Courvalin, 2006). Whilst *vanA* and *vanB* operons may have either a plasmid or chromosomal location, *vanC*, *vanD*, *vanE* and *vanG* have been reported to be located only on the chromosome (Courvalin, 2006). *vanA*-type resistance exhibits inducible high-level resistance to both vancomycin and teicoplanin, is mediated by transposon Tn*1546* and is the most frequently encountered type of glycopeptide resistance amongst enterococci. *vanA* is the only reported glycopeptide resistance gene to be successfully transferred from *Enterococcus species* to *S. aureus in vivo* and *in vitro* (Courvalin, 2006). To date, seven VRSA isolates carrying both *mecA* and *vanA* genes have been reported in the USA. It is postulated that the *vanA* gene detected in all seven VRSA isolates was donated from VRE isolates within polymicrobial biofilms in each patient (Sievert *et al.*, 2008). As VRE, MRSA and *S. epidermidis* are major causes of nosocomial infection, a multiplex PCR-based assay designed to detect all six vancomycin resistance genes and to identify *Enterococcus species*, *S. aureus* and *S. epidermidis* has been described (Depardieu *et al.*, 2004).

4.1.2 Experimental Studies of GISA/hGISA Isolates

Whilst studies on laboratory-derived mutants of *S. aureus* have documented the *in vitro* selection of GISA from GSSA, it has been observed that the ability to acquire the resistant phenotype may vary among different *S. aureus* strains (Pfeltz *et al.*, 2000; Turner *et al.*, 2001; Sieradzki *et al.*, 2003). Hiramatsu *et al.* reported that following selection with vancomycin, hGISA isolates remain stable following nine days passage in drug-free medium (Hiramatsu *et*

al., 1997a). However, Boyle-Vavra *et al.* observed the reversion of GISA to GSSA following 15 days passage on non-selective medium (Boyle-Vavra *et al.*, 2000). A study by Bobin-Dubreux *et al.* reported that following 21 days passage on non-selective medium, no reversion of the GISA phenotype was seen and concluded that reversion does not occur for all GISA isolates (Bobin-Dubreux *et al.*, 2001). Instability of the GISA and hGISA phenotype has also been reported in other studies (Cui *et al.*, 2003; Hussain *et al.*, 2002; Geisel *et al.*, 1999).

4.2 Aims and Objectives

The aims and objectives were:

- To investigate the *agr* locus among hGISA isolates (n=178) and a control group of meticillin-resistant (MR)-GSSA isolates (n=176) exhibiting comparable A-PFG types by determining:
    - *agr* groups using DNA amplification studies.
    - Delta-haemolysin production using sheep RBCs.
    - Adherence properties using measurement of the ability to bind to polystyrene.
- To investigate the presence of vancomycin resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) among hGISA isolates.
- To investigate the ability of vancomycin to induce the resistant phenotype among different MRSA strains and a GSSA control strain by:
    - Exposing GSSA isolates to increasing concentrations of vancomycin.
    - Exposing hGISA isolates to increasing concentrations of vancomycin.
- To determine the stability of the resistant phenotype by passaging selected GISA/hGISA isolates in drug-free medium for nine consecutive days.

4.3 Materials and Methods

4.3.1 Bacterial Isolates

All hGISA isolates (n=178) detected during the present study (as described in Section 2.4.4) were matched with a control group of MR-GSSA isolates (n=176) exhibiting similar A-PFG types. MR-GSSA isolates were selected from the same collection of isolates from which the corresponding hGISA isolates were detected. These hGISA and MR-GSSA isolates were investigated for *agr* group, delta-haemolysin production and ability to bind to polystyrene. All hGISA isolates were investigated for the presence of vancomycin resistance genes.

Bacterial isolates used in vancomycin selection studies are described in Section 4.3.4.1.

4.3.2 agr Locus

4.3.2.1 agr Typing

Genomic DNA was extracted from hGISA (n=178) and MR-GSSA (n=176) subcultures grown overnight in TSB using the Wizard™ Genomic DNA Purification Kit protocol for isolation of genomic DNA from Gram-positive bacteria (Promega #A1120, Promega Corporation, Madison W1, USA). Details of the extraction protocol used are described in Appendix II (AII.1.2.1).

agr groups were determined using a previously described *agr* group–specific multiplex PCR assay (Gilot *et al.*, 2002). The following primers were used to amplify *agr* sequences: Pan (5' –ATG CAC ATG GTG CAC ATG C –3'); *agr* I (5' –GTC ACA AGT ACT ATA AGC TGC GAT –3'); *agr* II (5' –TAT TAC TAA TTG AAA AGT GGC CAT AGC –3'); *agr* III (5' –

GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G –3') and *agr* IV (5' –CGA TAA TGC CGT AAT ACC CG –3') (Sigma-Genosys, Sigma-Aldrich House, Haverhill, Suffolk, UK) (Gilot *et al.*, 2002). Genomic DNA (1 µl) was added to a total volume of 24 µl containing 1X PCR Master Mix {50 U/ml *Taq* DNA polymerase, 400 µM each of dATP, dGTP, dCTP, dTTP and 3 mM $MgCl_2$ (Promega M 7502)}, 2 mM $MgCl_2$ and 0.3 µM concentration of each *agr* primer. Amplification was carried out in a Hybaid Px2 Research Thermal Cycler (Thermo Electron Corporation, Thermo Fischer Scientific, Inc., Basingstoke, UK) using the following temperature programme: one cycle of 5 min at 94°C; 26 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C; and a final cycle of 72°C for 10 min. With each batch of isolates tested, four *agr* group–specific *S. aureus* control strains were included as well as a negative (non-template) control. Details of *agr* group–specific *S. aureus* control strains are shown in Table 4.1. Further details of the PCR amplification technique for *agr* typing are described in Appendix II (AII.2.1).

Amplification products were electrophoresed in 2% (w/v) agarose gels at 90 volts (V) for 90 min in 1X TBE running buffer. DNA reference size marker {50 bp DNA Step Ladder (Promega #G4521)} was included on each gel. Gels were stained with ethidium bromide (0.5 mg/L) and photographed under UV light. Further details of electrophoresis procedures are described in Appendix II (AII.3.2 and AII.4.2). The expected product sizes of *agr* sequences were as follows: *agr* group I strains (441 bp DNA fragment), *agr* group II strains (575 bp DNA fragment), *agr* group III strains (323 bp DNA fragment) and *agr* group IV strains (659 bp DNA fragment) (Gilot *et al.*, 2002). *agr* groups were not confirmed using DNA sequencing in the present study.

Table 4.1. Control reference strains used with techniques for molecular studies of hGISA isolates.

Method	Control Strain	Reference
***agr* Typing**		
agr group I	*S. aureus* NCTC 8325	Jarraud *et al.*, 2000
agr group II	*S. aureus* Mu3 ATCC 700698	Sakoulas *et al.*, 2002
agr group III	*S. aureus* RN8465	Jarraud *et al.*, 2000
agr group IV	*S. aureus* RN4850	Jarraud *et al.*, 2000
Delta-Haemolysin Assay	*S. aureus* RN4220	Sakoulas *et al.*, 2002
	S. aureus RN6607	Sakoulas *et al.*, 2002
	S. aureus Mu3 ATCC 700698	Sakoulas *et al.*, 2002
	S. aureus Mu50 ATCC 700699	Sakoulas *et al.*, 2002
Adherence Assay	*S. aureus* RN9120	Sakoulas *et al.*, 2002
	S. aureus RN6607	Sakoulas *et al.*, 2002
	S. aureus Mu3 ATCC 700698	Sakoulas *et al.*, 2002
	S. aureus Mu50 ATCC 700699	Sakoulas *et al.*, 2002
***van* Assay**		
vanA	*E. faecalis* E206	Udo *et al.*, 2003
vanB	*E. faecalis* ATCC 51299	Tenover *et al.*, 1993
		Swenson *et al.*, 1995
vanC	*E. casseliflavus* ATCC 25788	Depardieu *et al.*, 2004
vanD	*E. faecium* BM4339	Depardieu *et al.*, 2004
vanE	*E. faecalis* BM4405	Depardieu *et al.*, 2004
vanG	*E. faecalis* BM4518	Depardieu *et al.*, 2004
nuc	*S. aureus* ATCC 25923	Merlino *et al.*, 2002

4.3.2.2 Delta-Haemolysin Assay

Delta-haemolysin expression for hGISA (n=178) and MR-GSSA isolates (n=176) was determined using a previously described method (Sakoulas *et al.*, 2002). An indicator strain *S. aureus* RN4220 capable of producing a large zone of beta-haemolysis without interference of alpha- or delta-haemolysins was used to determine delta-haemolysin expression of test isolates on sheep blood agar. As delta- and beta-haemolysins act synergistically in the lysis of sheep RBCs, an enhanced zone of haemolysis in the area overlapping between the indicator strain *S. aureus* RN4220 and test isolate was a measure of delta-haemolysin expression.

BHI agar (Oxoid, CM 0375) containing 7% sheep blood (Oxoid, SR0051) was prepared in-house. Using a 1-µl loop, overnight BHI broth (Oxoid, CM 225) subcultures were streaked onto sheep blood agar plates as described in Figure 4.2. *S. aureus* RN4220, the indicator strain was streaked vertically whilst test isolates were streaked horizontally at either side of *S. aureus* RN4220 allowing a gap of 3–4 mm between the indicator strain and test isolates to ensure a zone of haemolysis could be observed. Four test isolates were inoculated onto each plate. With each batch of isolates tested, *S. aureus* RN6607 was included as a positive control and *S. aureus* Mu3 ATCC 700698 and *S. aureus* Mu50 ATCC 700699 were included as negative controls. Plates were incubated at 35°C overnight and following incubation, delta-haemolysin expression was graded as +, weak/small zone of haemolysis or ++, large zone of haemolysis.

4.3.2.3 Adherence Assay

The ability of hGISA (n=178) and MR-GSSA isolates (n=176) to adhere to plastic microtitre plates was determined using a modification of the original quantitative method of measuring

adherence of CoNS to plastic tissue culture plates described by Christensen *et al.* (Christensen *et al.*, 1985; Sakoulas *et al.*, 2002).

Colonies from an overnight culture on CBA were inoculated into TSB and incubated overnight at 35°C. These subcultures were diluted 1/200 in fresh TSB and 200-µl volumes were dispensed in duplicate into sterile 96-well flat-bottomed microtitre plates (Sterilin Sero Wel Microtitration Plates, 612F96, Barloworld Scientific, Stone, Staffordshire, UK). Plates were stacked in batches of three, placed in plastic bags and incubated overnight at 35°C. Following incubation, plates were examined for bacterial growth in each well. The supernatant was discarded and each well was washed four times with 300-µl volumes of sterile phosphate–buffered saline (Oxoid, BR 0014a). Adherent cells were fixed by heating at 65°C for one hour. Cells were stained with 200-µl volumes of Gram's crystal violet (Merck, 1.01408.0100) and residual stain was removed with tap water. Plates were air-dried and the optical density at 540 nm (OD_{540nm}) was measured using a microplate photometer (Multiskan Ascent, Thermo Electron Corporation, Thermo Fisher Scientific, Inc., Basingstoke, UK). Each isolate was classified as follows: OD_{540nm} <0.12, non-adherent; 0.12–0.24, weakly-adherent; >0.24, strongly-adherent (Christensen *et al.*, 1985; Sakoulas *et al.*, 2002). With each batch of isolates tested, *S. aureus* RN9120, *S. aureus* Mu3 ATCC 700698 and *S. aureus* Mu50 ATCC 700699 were included as positive controls and *S. aureus* RN6607 and an uninoculated TSB were included as negative controls.

4.3.3 van Assay

Genomic DNA was extracted from hGISA (n=178) isolates from overnight TSB subcultures using the Wizard™ Genomic DNA Purification Kit protocol as described in Appendix II

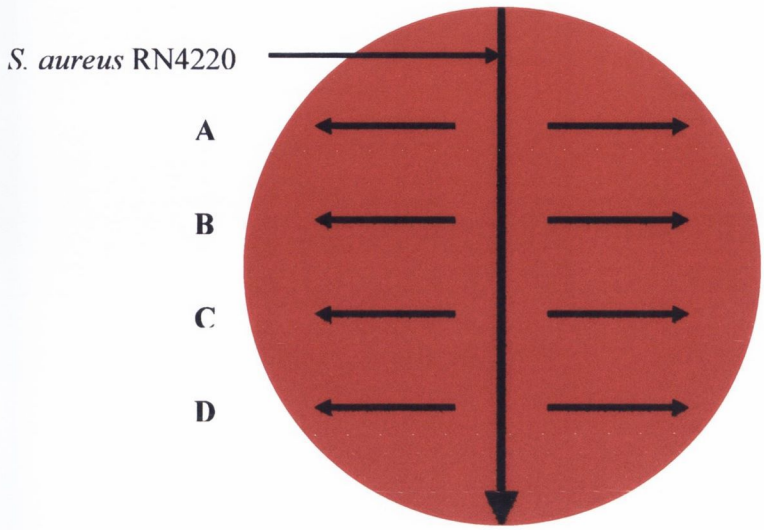


Figure 4.2. Delta-haemolysin assay.

S. aureus RN4220, the indicator strain was streaked vertically along the centre of the sheep blood agar. Horizontal streaks at either side of *S. aureus* RN4220 labelled A, B, C and D represent test isolates.

(AII.1.2.1). A previously described multiplex PCR-based assay was performed to detect six glycopeptide resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) characterised in *Enterococcus species* and to identify *S. aureus* to the species level (Depardieu *et al.*, 2004). Expression of *vanA*, *vanB*, *vanC*, *vanE* and *vanG* in *Enterococcus species* are inducible. Primers targeting the genes *vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG* and primers complementary to the *S. aureus* thermonuclease *nuc* gene were purchased from Sigma-Genosys and are detailed in Table 4.2.

Purified genomic DNA (1 µl) was added to a total volume of 99 µl containing 1X PCR Master Mix and 40 pmol of each primer pair except for *vanE* where 200 pmol of *vanE* primers was used. Amplification was performed in a Hybaid Px2 Research Thermal Cycler with the following thermal cycling profile: one cycle of 3 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 54°C and 1 min at 72°C; and a final cycle of 72°C for 7 min. Control strains included with each batch of isolates tested are detailed in Table 4.1. A negative (non-template) control was also included with each batch of isolates tested. Further details of the PCR technique for amplification of *van* and *nuc* genes are described in Appendix II (AII.2.2).

Amplified DNA was electrophoresed in 2% (w/v) agarose gels at 70 V for 180 min in 1X TBE running buffer. DNA reference size marker (50 bp DNA Step Ladder) was included on each gel. Gels were stained and photographed as described in Appendix II (AII.5). The expected amplimer sizes for *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG* and *nuc* genes are detailed in Table 4.2.

4.3.4 Vancomycin Selection Studies

4.3.4.1 Bacterial Isolates

Eight isolates comprising two hGISA isolates {E223 (A-PFG 13-00) and *S. aureus* Mu3 ATCC 700698}, two hTISA isolates {MF096 (A-PFG 07-02) and E1033 (A-PFG 13-00)} and four GSSA isolates {ML175 (A-PFG 44-99.4), MF301 (A-PFG 06-01), E1168 (A-PFG 13-00) and *S. aureus* ATCC 29213} were used to investigate ability to develop resistance to vancomycin and stability of the resistant phenotype among *S. aureus* isolates in a series of vancomycin selection studies. Among the GSSA isolates, ML175 and MF301 were the first in a series of MRSA isolates recovered from each of two patients from whom hGISA and hTISA isolates, respectively, were subsequently recovered.

4.3.4.2 Vancomycin Population Analysis Profiling

Vancomycin PAPs were performed on all eight isolates as described in Section 2.3.7. Following vancomycin PAPs, one colony was picked from the agar plate containing the highest concentration of vancomycin yielding a countable number of colonies. This colony was inoculated into TSB and incubated overnight at 35°C. Vancomycin PAPs were performed on this first resistant derivative of each isolate. The above procedure was repeated and vancomycin PAPs were performed on the second and third step resistant derivatives of each isolate. To determine the stability of these resistant derivatives, the third step resistant derivative of each isolate was passaged for nine days on CBA (non-selective medium), inoculated into TSB and vancomycin PAPs were performed.

For each isolate and respective vancomycin step resistant derivative, the vancomycin PAP was used to calculate the AUC and vPAP-AUC ratio was determined. As described in Section

Table 4.2. Oligonucleotide primers used to investigate vancomycin resistance genes and *S. aureus* thermostable nuclease gene among hGISA isolates.

Gene	Primer Sequence (5' – 3')	Amplimer Size (bp)
vanA	GGG AAA ACG ACA ATT GC GTA CAA TGC GGC CGT TA	732
vanB	ACG GAA TGG GAA GCC GA TGC ACC CGA TTT CGT TC	647
vanC	ATG GAT TGG TAC TTG TAT TAG CGG GAG TGA CTC GTA A	827
vanD	TGT GGG ATG CGA TAT TCA A TGC AGC CAA GTA TCC GGT AA	500
vanE	TGT GGT ATC GGA GCT GCA G ATA GTT TAG CTG GTA AC	430
vanG	CGG CAT CCG CTG TTT TTG A GAA CGA TAG ACC AAT GCC TT	941
nuc	GAC TAT TAT TGG TTG ATA CAC CTG GCC TTG ACG AAC TAA AGC TTC G	218

2.3.7, vPAP-AUC ratio criteria for reduced susceptibility to glycopeptides were: GSSA, <0.9, hGISA, 0.9–1.29; GISA, ≥1.3. Control reference strains *S. aureus* Mu3 ATCC 700698, *S. aureus* Mu50 ATCC 700699 and *S. aureus* ATCC 29213 (GSSA) were included with each batch of isolates tested.

4.3.4.3 Agar Screening Methods

Vancomycin step resistant derivatives of each isolate (n=24) were screened for growth on BHIV6 and $BHIT5_{0.5}$ as described in Sections 2.3.4.1.1 and 2.3.8.2, respectively. Twelve isolates were inoculated by pipette onto each plate and screening tests were performed in duplicate.

4.3.4.4 Broth Microdilution MIC Methods

Vancomycin and teicoplanin broth microdilution MICs were determined for vancomycin step resistant derivatives of each isolate (n=24) as described in Section 2.3.6.2. Isolates were classified as susceptible, intermediate or resistant according to CLSI breakpoints as described in Section 2.3.6.1.

4.4 Results

4.4.1 agr Locus

4.4.1.1 agr Typing

agr groups among hGISA and MR-GSSA isolates investigated are shown in Table 4.3. Of 178 hGISA isolates, 85% (151/178) belonged to *agr* group I whilst 1% (2/178) and 14% (25/178) belonged to *agr* groups II and III, respectively. Similarly, among 176 MR-GSSA isolates, the majority (84%; 148/176) belonged to *agr* group I whilst 3.5% (6/176) and 12.5% (22/176) belonged to *agr* groups II and III, respectively. No isolate in this study belonged to *agr* group IV. All hGISA isolates (n=25) and MR-GSSA isolates (n=21) exhibiting A-PFG 07-02 belonged to *agr* group III. hGISA isolates (n=2) that belonged to *agr* group II exhibited A-PFG 13-99 and A-PFG 07-03, respectively. PCR amplicons of representative hGISA isolates and *agr* control strains *S. aureus* NCTC 8325 (*agr* group I), *S. aureus* Mu3 ATCC 700698 (*agr* group II), *S. aureus* RN8465 (*agr* group III) and *S. aureus* RN4850 (*agr* group IV) are shown in Figure 4.3.

4.4.1.2 Delta-Haemolysin Assay

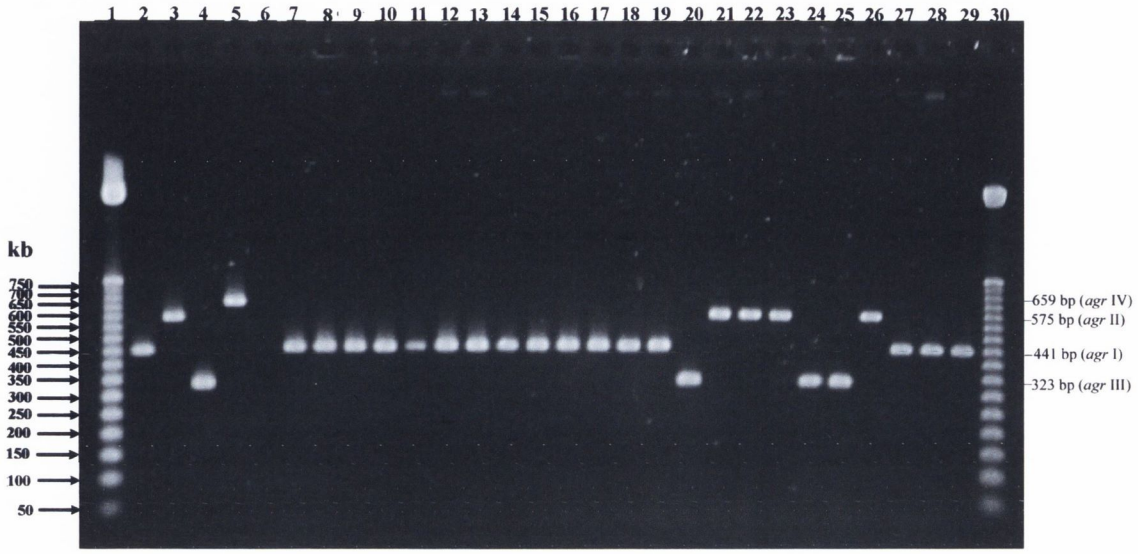
Sixty percent (107/178) of hGISA isolates and 67% (118/176) of MR-GSSA isolates yielded positive results (shown in Table 4.3). hGISA isolates exhibiting A-PFG 13-00, A-PFG 07-02 and A-PFG 14-00 accounted for 48% (34/71), 34% (24/71) and 11% (8/71) of delta-haemolysin negative isolates, respectively. Interestingly, 96% (24/25) and 100% (21/21) of hGISA and MR-GSSA isolates exhibiting A-PFG 07-02, respectively did not produce delta-

Table 4.3. Comparison of *agr* groups, delta-haemolysin production and adherence ability among hGISA (*n*=178) and MR-GSSA (*n*=176) isolates. Numbers indicate percentages within each group. [Figures in parentheses indicate MR-GSSA isolates].

A-PFG Type	Isolates (*n*=)		*agr* Group I		*agr* Group II		*agr* Group III		Delta-Haemolysin Production		Adherence Ability	
	178	**[176]**	**85**	**[84]**	**1**	**[3.5]**	**14**	**[12.5]**	**60**	**[67]**	**70**	**[45]**
A-PFG 06-01	5	[5]	100	[100]	0	[0]	0	[0]	60	[80]	40	[20]
A-PFG 07-02	25	[21][a]	0	[0]	0	[0]	100	[100]	4	[0]	32	[24]
A-PFG 13-00	93	[93]	100	[100]	0	[0]	0	[0]	63	[73]	87	[55]
A-PFG 14-00	36	[36]	100	[100]	0	[0]	0	[0]	78	[75]	61	[36]
A-PFG 43-00	3	[3]	100	[100]	0	[0]	0	[0]	67	[100]	100	[67]
A-PFG 44-99.4	5	[5]	100	[100]	0	[0]	0	[0]	100	[100]	60	[20]
A-PFG New02-00	5	[3][b]	100	[100]	0	[0]	0	[0]	100	[100]	20	[0]
Others	6	[10]	67	[30]	33	[60]	0	[10]	67	[70]	83	[70]

[a, b], No matching MR-GSSA isolates were found for four and two hGISA isolates exhibiting A-PFG 07-02 and A-PFG New02-00, respectively.
Abbreviations: A-PFG, antibiogram-pulsed-field group type.

Panel A



Panel B

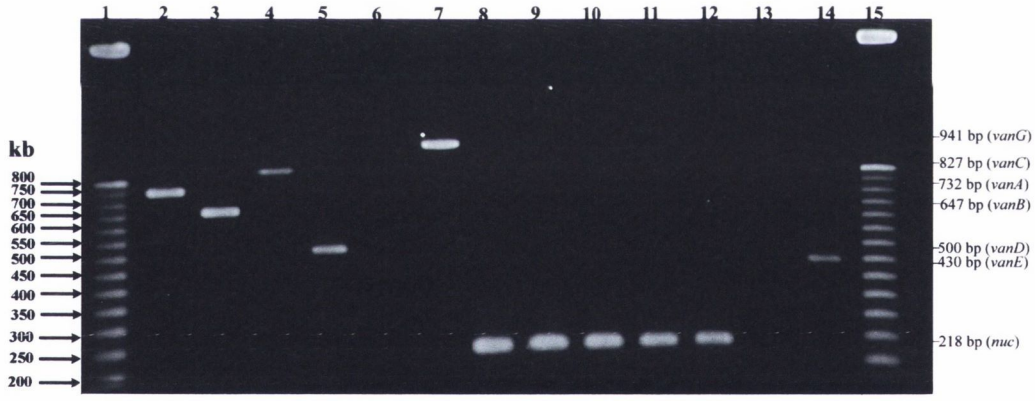


Figure 4.3. Ethidium-bromide stained agarose gels showing amplified DNA products of representative hGISA isolates using *agr* and *van*-specific primers.

Panels A and B present PCR amplicons obtained when control strains and representative hGISA isolates were amplified using *agr* and *van*-specific primers, respectively. Molecular size marker (50 bp DNA Step Ladder) is located in lanes 1 and 30 in Panel A and 1 and 15 in Panel B. Amplified DNA from the following isolates are located in lanes as follows:
Panel A. Lanes: 2, *S. aureus* NCTC 8325 (*agr* I); 3, *S. aureus* Mu3 ATCC 700698 (*agr* II); 4, *S. aureus* RN8465 (*agr* III); 5, *S. aureus* RN4850 (*agr* IV); 6, non-template control; 7–19, hGISA (*agr* I); 20, hGISA (*agr* III); 21–23, hGISA (*agr* II); 24–25, hGISA (*agr* III); 26, hGISA (*agr* II); 27–29, hGISA (*agr* I).
Panel B. Lanes: 2, *E. faecalis* E206 (*vanA*); 3, *E. faecalis* ATCC 51299 (*vanB*); 4, *E. casseliflavus* ATCC 25788 (*vanC*); 5, *E. faecium* BM4339 (*vanD*); 6, *E. faecalis* BM4405 (*vanE*); 7, *E. faecalis* BM4518 (*vanG*); 8, *S. aureus* ATCC 25923 (*nuc*); 9–12, hGISA isolates; 13, non-template control; 14, *E. faecalis* BM4405 (*vanE*). Amplimer for *E. faecalis* BM4405 appeared very weak when photographed but was visible to the naked eye under UV light (lane 6). Hence, the PCR amplicon shown in lane 14 was obtained when *E. faecalis* BM4405 was amplified with *vanE* primers alone in a separate PCR reaction.

haemolysin. Figure 4.4 demonstrates delta-haemolysin expression among representative hGISA isolates and *S. aureus* control strains on sheep blood agar.

4.4.1.3 Adherence Assay

Overall, 70% (125/178) of hGISA isolates and 45% (80/176) of MR-GSSA isolates were classified as adherent to polystyrene according to the criteria of Christensen *et al.* (Table 4.3) (Christensen *et al.*, 1985). A comparison of the classification of adherence (non-adherent, weakly-adherent or strongly-adherent) among hGISA and MR-GSSA isolates is shown in Figure 4.5. Whilst there was little difference between the mean OD_{540nm} values exhibited by non-adherent and weakly-adherent hGISA and MR-GSSA isolates, respectively, strongly-adherent hGISA isolates yielded a higher mean OD_{540nm} value (OD_{540nm}= 0.8) compared to adherent MR-GSSA isolates (OD_{540nm}= 0.65). The A-PFG type distribution and the mean OD_{540nm} values among adherent hGISA and MR-GSSA isolates, respectively is also presented in Figure 4.5. Isolates exhibiting A-PFG 13-00 yielded the highest mean OD_{540nm} values among both hGISA and MR-GSSA isolates. Among adherent hGISA isolates, 6% (8/125) were isolated from sites involving biomedical devices. Whilst A-PFG 13-00 was exhibited by the majority (62.5%; 5/8) of these isolates, A-PFG types, A-PFG 14-00 and A-PFG 44-99.4 represented 25% (2/8) and 12.5% (1/8) of isolates, respectively.

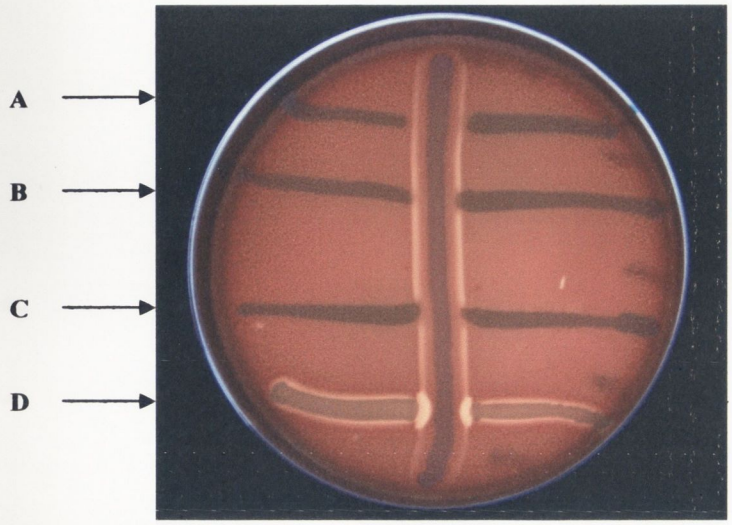
4.4.1.4 Correlation between agr Types, Delta-Haemolysin and Adherence

The differences in *agr* types, delta-haemolysin production and adherence properties between hGISA (n=178) and MR-GSSA (n=176) isolates were not statistically significant (p=0.55).

Among 85% (151/178) of hGISA isolates that belonged to *agr* group I, 77% (116/151) were classified as adherent whilst 30% (46/151) did not produce delta-haemolysin. However, among MR-GSSA isolates that belonged to *agr* group I {84% (148/176)}, 47% (70/148) were classified as adherent whilst 24% (36/148) were delta-haemolysin negative (p=0.30). Although hGISA isolates that belonged to *agr* group II (n=2) differed from each other in both the ability to produce delta-haemolysin and to adhere to polystyrene, among the six MR-GSSA isolates that belonged to *agr* group II, 67% (4/6) were classified as adherent and all produced delta-haemolysin (p=0.45). Whilst 96% (24/25) of hGISA isolates that belonged to *agr* group III were delta-haemolysin negative, only 32% (8/25) were classified as adherent. Similarly, among MR-GSSA isolates that belonged to *agr* group III, 100% (22/22) did not produce delta-haemolysin and 27% (6/22) were classified as adherent (p=0.78).

Among 71 delta-haemolysin negative hGISA isolates, 65% (46/71), 1% (1/71) and 34% (24/71) belonged to *agr* groups I, II and III, respectively. But, among 58 MR-GSSA isolates 62% (36/58) and 38% (22/58) belonged to *agr* groups I and III, respectively (p=0.5). Whilst all delta-haemolysin negative isolates that belonged to *agr* group III (n=24) exhibited A-PFG 07-02, 74% (34/46) of delta-haemolysin negative isolates that belonged to *agr* group I exhibited A-PFG 13-00. One delta-haemolysin negative hGISA isolate belonged to *agr* group II and exhibited A-PFG 13-99. Sixty-five percent (46/71) of delta-haemolysin negative hGISA isolates were classified as adherent of which 80% (37/46), 18% (8/46) and 2% belonged to *agr* groups I, II and III, respectively. Among 23 delta-haemolysin negative MR-GSSA isolates that were classified as adherent, 74% and 26% belonged to *agr* groups I and III, respectively (p=0.6).

Panel A



Panel B

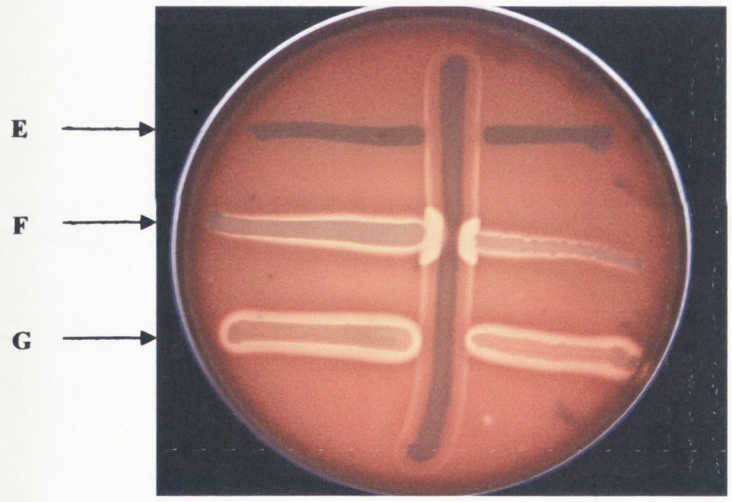
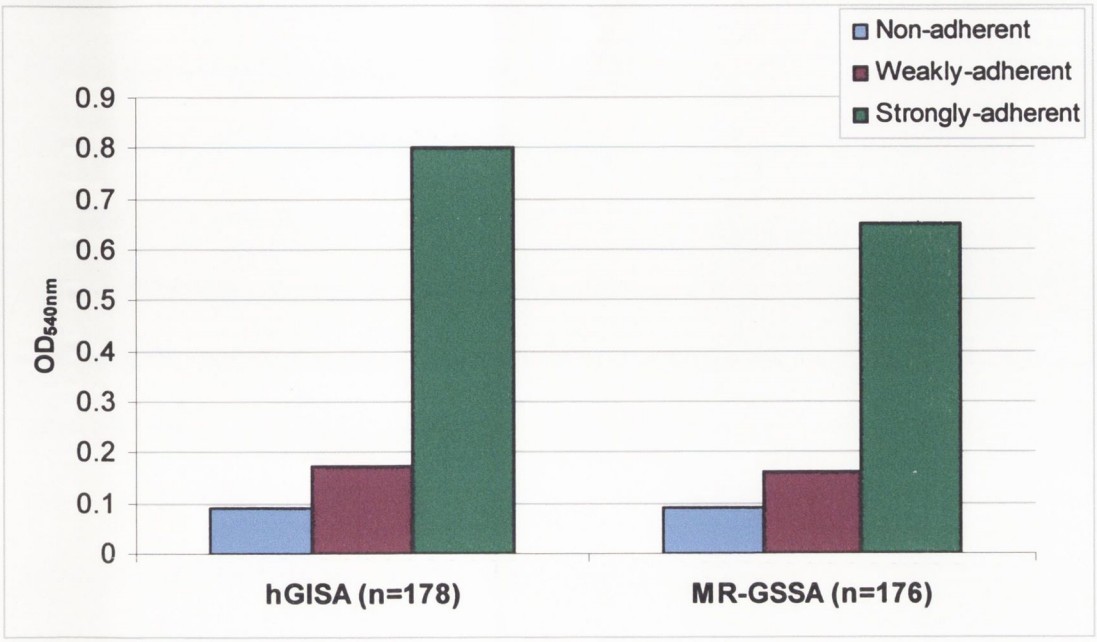


Figure 4.4. Delta-haemolysin expression among selected hGISA isolates.

Panels A and B present delta-haemolysin expression among selected hGISA isolates on sheep blood agar. The vertical streak denotes indicator strain *S. aureus* RN4220 and an enhanced zone of haemolysis in the area overlapping between *S. aureus* RN4220 and test isolate was a measure of delta-haemolysin expression. Horizontal streaks labelled A to G represent the following isolates: (A), hGISA isolate; (B), *S. aureus* Mu3 ATCC 700698; (C), *S. aureus* Mu50 ATCC 700699; (D), *S. aureus* RN6607; (E–G), hGISA isolates.

Beta- and delta-haemolysin expression was observed with isolates D and F, respectively, whilst isolate G produced beta-haemolysin only.

Panel A



Panel B

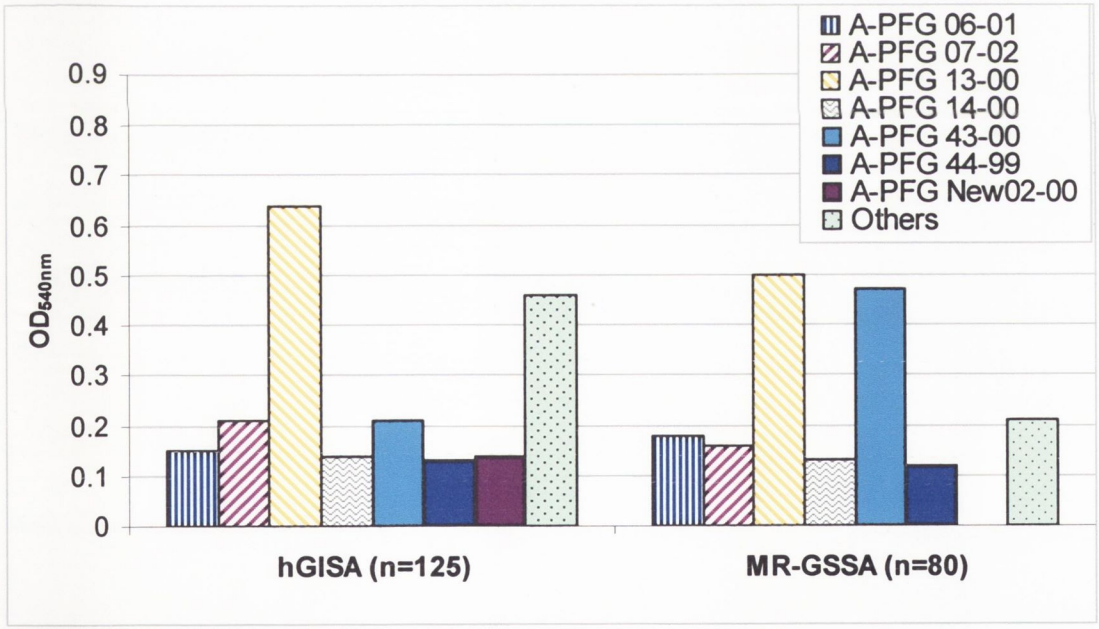


Figure 4.5. Adherence assay.

Panel A presents the mean OD_{540nm} values among hGISA and MR-GSSA isolates.

Panel B shows the A-PFG type distribution and the mean OD_{540nm} values among adherent hGISA and MR-GSSA isolates, respectively.

Ninety-three percent (116/125) of adherent hGISA isolates belonged to *agr* group I whilst 1% (1/125) and 6% (8/125) belonged to *agr* groups II and III, respectively. Eighty-seven percent (70/80) of adherent MR-GSSA isolates belonged to *agr* group I whilst 5% and 8% belonged to *agr* groups II and III, respectively (p=0.4). Among adherent isolates, 37% (46/125) were delta-haemolysin negative of which 80% (37/46), 2% (1/46) and 18% (8/46) belonged to a*gr* groups I, II and III, respectively. Similarly, among MR-GSSA isolates the majority of adherent isolates that were delta-haemolysin negative belonged to *agr* group I {74%, (17/23)} whilst the remaining isolates (n=6) belonged to *agr* group III (p=0.6). Although all eight adherent isolates originating from sites involving biomedical devices belonged to *agr* group I, 88% (7/8) of these isolates produced delta-haemolysin.

4.4.2 van Assay

No vancomycin resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) were detected among 178 hGISA isolates investigated. PCR amplicons obtained when control strains and representative hGISA isolates were amplified with *van*-specific primers are shown in Figure 4.3.

4.4.3 Vancomycin Selection Studies

4.4.3.1 Vancomycin Population Analysis Profiling

The distribution of vPAP-AUC ratios among eight isolates and their respective vancomycin step resistant derivatives are shown in Table 4.4. Following the initial selection with vancomycin, vPAP-AUC ratios increased for all eight isolates. Significant increases were observed for GSSA and hTISA isolates which exhibited vPAP-AUC ratios between 0.9 and

1.29, and thus met the criteria for hGISA classification. Upon the second exposure to vancomycin, vPAP-AUC ratios increased slightly for all isolates but remained between 0.9 and 1.29 following the third exposure to vancomycin. Hence, no isolate developed the GISA phenotype according to vPAP-AUC ratio criteria. Following nine days passage on CBA, vPAP-AUC ratios for the third step resistant derivative of each isolate remained between 0.9 and 1.29, thus the hGISA phenotype was not lost for any isolate. A schematic presentation of the vPAPs of each isolate and respective resistant derivatives is shown in Figures 4.6 to 4.9.

Figure 4.6 presents the vancomycin PAPs of GSSA isolates, ML175 and E1168 and their respective vancomycin step resistant derivatives. Following selection with vancomycin, increases in vPAP-AUC ratios paralled changes in the vPAPs for both isolates. In contrast, although vPAP-AUC ratios for GSSA isolates, MF301 and *S. aureus* ATCC 29213 increased to 1.09 and 1.23, respectively, significant changes were not observed in the vPAPs of these isolates as neither isolate grew on BHI agar containing >4 mg/L vancomycin (shown in Figure 4.7).

Following exposure to vancomycin, the vancomycin PAPs of hTISA isolates, MF096 and E1033 (shown in Figure 4.8) and hGISA isolates, E223 and *S. aureus* Mu3 ATCC 700698 (shown in Figure 4.9) and their respective vancomycin step resistant derivatives fell between the prototype hGISA strain, *S. aureus* Mu3 ATCC 700698 and the prototype GISA strain, *S. aureus* Mu50 ATCC 700699. Following nine days passage on non-selective medium, the third step resistant derivative of each isolate remained stable.

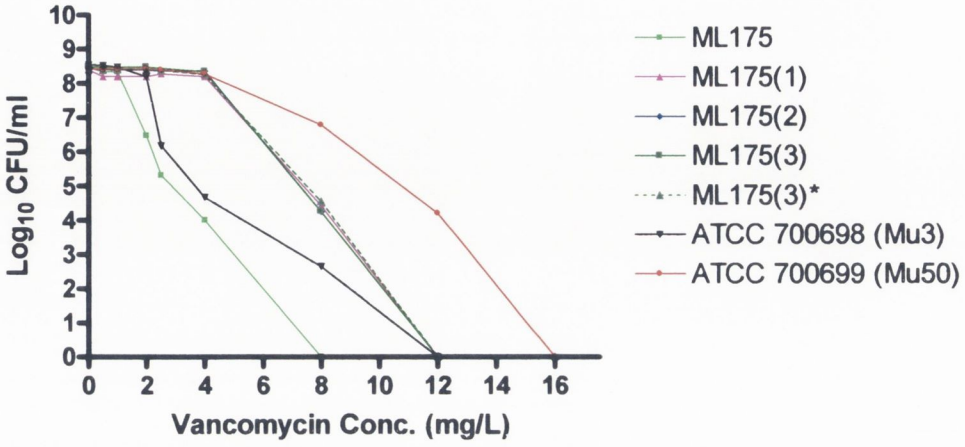
Table 4.4. vPAP-AUC ratios and agar screen results among isolates and respective step resistant derivatives following selection with vancomycin.

Isolate	A-PFG	Phenotype	vPAP-AUC[a]	Agar Screen	
				BHIV6	BHIT5
ML175	44-99.4	GSSA	0.88	NG	3 cols
ML175 (1)[b]		hGISA	1.2	NG	+++
ML175 (2)[c]		hGISA	1.27	NG	+++
ML175 (3)[d]		hGISA	1.24	30 cols	+++
ML175 (3)*[e]		hGISA	1.27	...	...
MF301	06-01	GSSA	0.52	NG	NG
MF301 (1)[b]		hGISA	0.95	NG	2 cols
MF301 (2)[c]		hGISA	0.99	NG	+
MF301 (3)[d]		hGISA	0.9	NG	6 cols
MF301 (3)*[e]		hGISA	1.09	...	...
MF096	07-02	hTISA[f]	0.6	NG	+++
MF096 (1)[b]		hGISA	1.19	NG	+++
MF096 (2)[c]		hGISA	1.2	+++	+++
MF096 (3)[d]		hGISA	1.2	+++	+++
MF096 (3)*[e]		hGISA	1.19	...	...
E1033	13-00	hTISA	0.89	NG	+++
E1033 (1)[b]		hGISA	1.16	NG	+++
E1033 (2)[c]		hGISA	1.24	+++	+++
E1033 (3)[d]		hGISA	1.19	+++	+++
E1033 (3)*[e]		hGISA	1.24	...	...
E223	13-00	hGISA[g]	0.92	NG	+++
E223 (1)[b]		hGISA	1.21	NG	+++
E223 (2)[c]		hGISA	1.25	NG	+++
E223 (3)[d]		hGISA	1.22	+++	+++
E223 (3)*[e]		hGISA	1.26	...	...
E1168	13-00	GSSA	0.79	NG	NG
E1168 (1)[b]		hGISA	1.17	NG	+++
E1168 (2)[c]		hGISA	1.24	NG	+++
E1168 (3)[d]		hGISA	1.22	+++	+++
E1168 (3)*[e]		hGISA	1.23	...	...
***S. aureus* ATCC 29213**		GSSA	0.59	NG	NG
S. aureus ATCC 29213 (1)[b]		hGISA	1.16	NG	+
S. aureus ATCC 29213 (2)[c]		hGISA	1.21	NG	9 cols
S. aureus ATCC 29213 (3)[d]		hGISA	1.13	NG	2 cols
S. aureus ATCC 29213 (3)*[e]		hGISA	1.23	...	...
***S. aureus* Mu3 ATCC 700698**		hGISA	1.0	NG	+++
S. aureus Mu3 ATCC 700698 (1)[b]		hGISA	1.2	NG	+++
S. aureus Mu3 ATCC 700698 (2)[c]		hGISA	1.25	+++	+++
S. aureus Mu3 ATCC 700698 (3)[d]		hGISA	1.23	+++	+++
S. aureus Mu3 ATCC 700698 (3)*[e]		hGISA	1.26	...	...

[a], vPAP-AUC, Vancomycin population analysis profile-area under the curve ratio determination. [b-d], First, second and third step resistant derivatives of each isolate. [e], Third step resistant derivatives following nine days passage on non-selective medium. [f], hTISA defined as isolates with resistant subpopulations capable of growth at >8 mg/L teicoplanin but with vPAP-AUC ratios <0.9. [g], hGISA defined as isolates with vPAP-AUC ratios ranging from 0.9–1.29.

Abbreviations. A-PFG, antibiogram-pulsed-field group type; BHIV6, BHIT5, brain heart infusion agar containing 6 mg/L vancomycin and 5 mg/L teicoplanin, respectively; cols, colonies; …, not done.

Panel A



Panel B

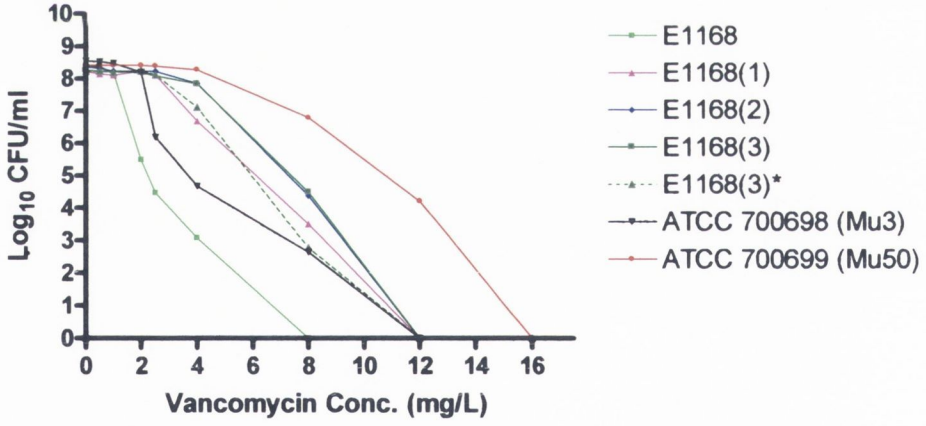
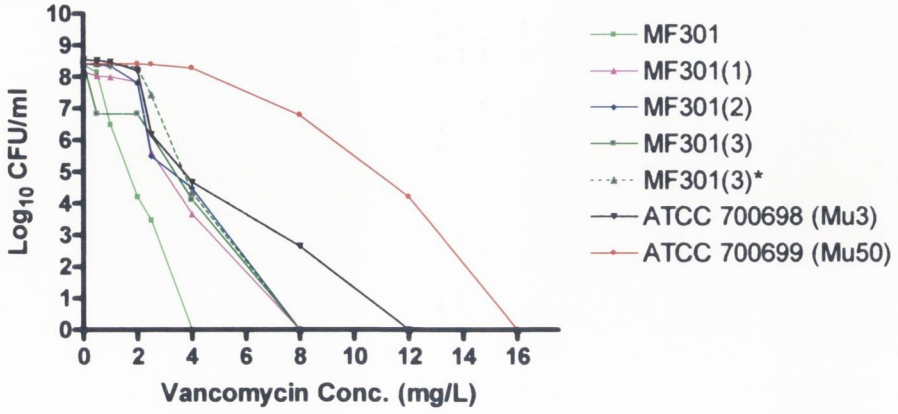


Figure 4.6. Vancomycin population analysis profiles of GSSA isolates (ML175 and E1168) and respective vancomycin step resistant derivatives.

Panels A and B show vancomycin population analysis profiles of ML175 and E1168, respectively and the first, second and third step resistant derivatives of each GSSA isolate. Also presented in both panels are vancomycin PAPs of the third step resistant derivative of each isolate following nine days passage in drug-free medium {(3)*}.

Panel A



Panel B

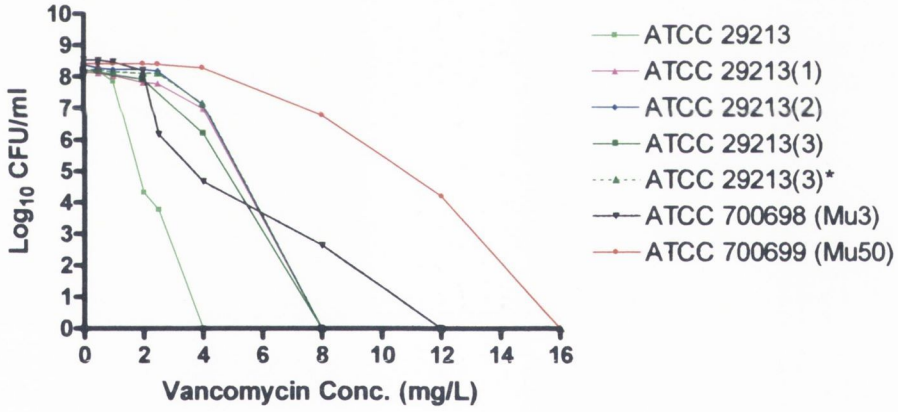
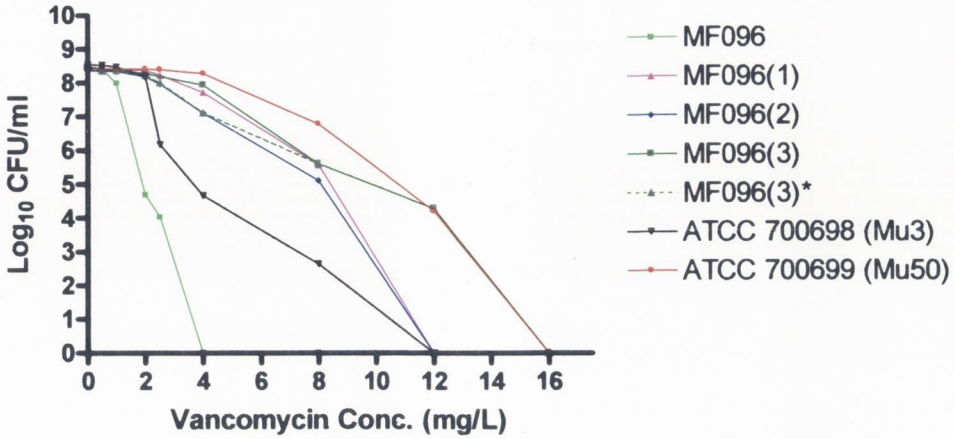


Figure 4.7. Vancomycin population analysis profiles of GSSA isolates (MF301 and *S. aureus* ATCC 29213) and respective vancomycin step resistant derivatives.

Panels A and B show vancomycin population analysis profiles of MF301 and *S. aureus* ATCC 29213, respectively and the first, second and third step resistant derivatives of each GSSA isolate. Also presented in both panels are vancomycin PAPs of the third step resistant derivatives of each isolate following nine days passage in drug-free medium {(3)*}.

Panel A



Panel B

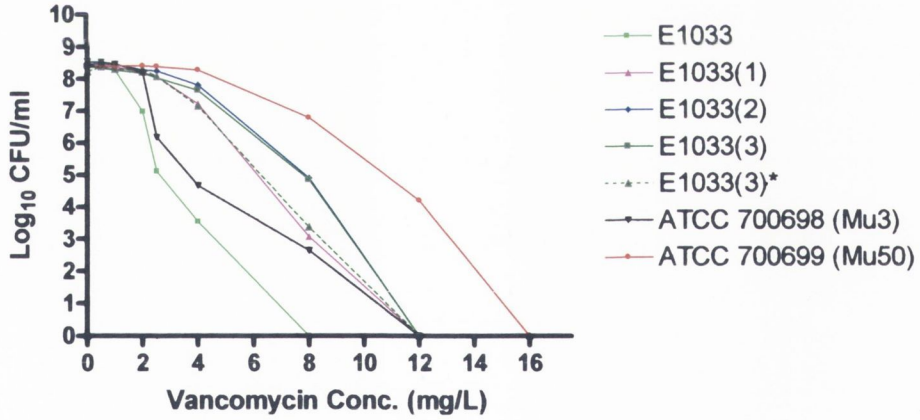
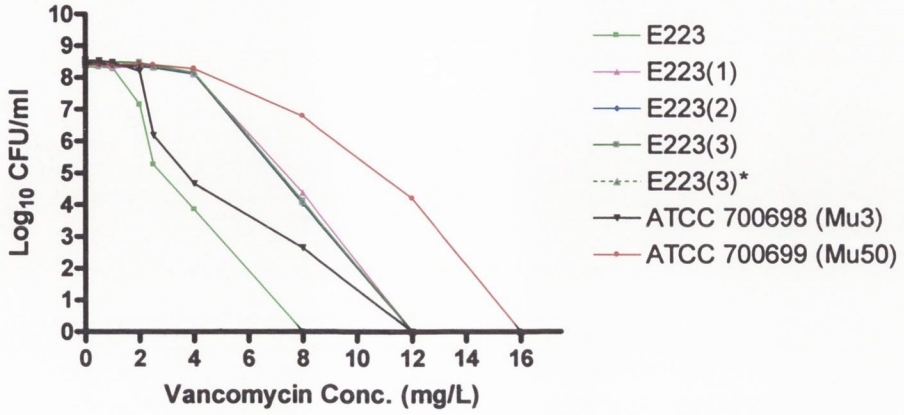


Figure 4.8. Vancomycin population analysis profiles of two hTISA isolates and respective vancomycin step resistant derivatives.

Panels A and B show vancomycin population analysis profiles of isolates MF096 and E1033, respectively and the first, second and third step resistant derivatives of each hTISA isolate. Also demonstrated in both panels are vancomycin PAPs of the third step resistant derivatives of each isolate following nine days passage in drug-free medium {(3)*}.

Panel A



Panel B

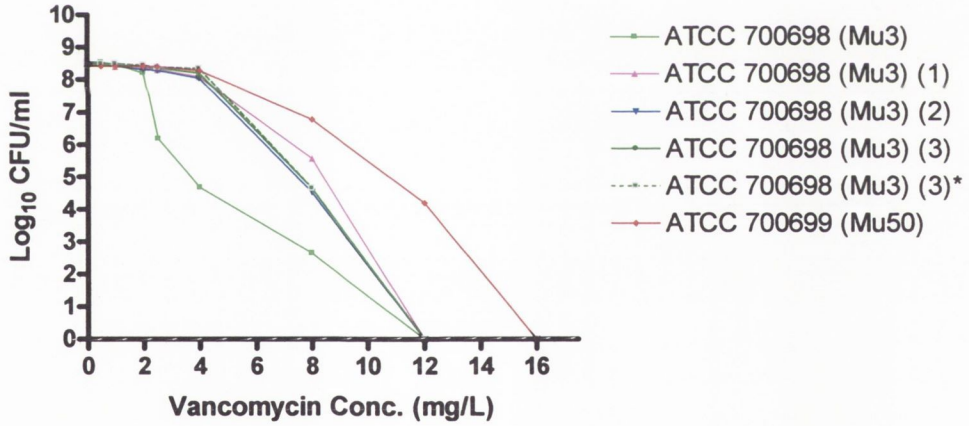


Figure 4.9. Vancomycin population analysis profiles of two hGISA isolates and respective vancomycin step resistant derivatives.

Panels A and B present vancomycin population analysis profiles of hGISA isolates, E223 and *S. aureus* Mu3 ATCC 700698, respectively and the first, second and third step resistant derivatives of each hGISA isolate. Also shown in both panels are vancomycin PAPs of the third step resistant derivatives of each isolate following nine days passage in drug-free medium {(3)*}.

4.4.3.2 Agar Screening Methods

4.4.3.2.1 Vancomycin Agar Screen

None of the first vancomycin selected resistant derivatives yielded growth on BHIV6 but three of the second vancomycin selected resistant derivatives screened with BHIV6 yielded growth {MF096 (2), E1033 (2) and *S. aureus* Mu3 ATCC 700698 (2)}. Details are shown in Table 4.4. MF301 (3) and *S. aureus* 29213 (3) were the only isolates among the third vancomycin selected resistant derivatives not to yield growth on BHIV6. Interestingly, these were the only isolates whose vancomycin-selected resistant derivatives did not grow on BHI agar containing >4 mg/L vancomycin (Figure 4.7).

4.4.3.2.2 Teicoplanin Agar Screen

All vancomycin selected resistant derivatives screened with $BHIT5_{0.5}$ yielded growth. The maximum yield of growth (+++) was observed for all resistant derivatives except for selected resistant derivatives of MF301 and *S. aureus* 29213 (Table 4.4). Under vancomycin selection, the yield of growth on BHIT5 for selected resistant derivatives of MF301 increased from two colonies to "+" (>100 colonies) but decreased to six colonies at the third step selection with vancomycin. Similarly, a decrease in the yield of growth on BHIT5 was observed with vancomycin selected resistant derivatives of *S. aureus* 29213.

4.4.3.3 Brothmicrodilution MIC Determination

All eight isolates and respective vancomycin selected resistant derivatives tested using the broth microdilution method yielded MICs of ≤2 mg/L for vancomycin and ≤8 mg/L for teicoplanin. Hence there were no GISA among these MRSA isolates.

4.5 Discussion

Whereas previous studies have reported that genotypic characteristics such as *agr* typing, delta-haemolysin production and adherence ability could be used as markers to identify isolates exhibiting reduced susceptibility to glycopeptides, results from the present study showed no statistically significant difference between hGISA and MR-GSSA isolates investigated for these characteristics (Sakoulas *et al.*, 2002; Sakoulas *et al.*, 2005). In the present study, *agr* typing results reflect findings of other studies where isolates exhibiting reduced susceptibility to glycopeptides have been shown to belong to *agr* groups I and II (Sakoulas *et al.*, 2002; Verdier *et al.*, 2004; Howe *et al.*, 2004). However, inclusion of a control group of MR-GSSA isolates matched for genetic background showed that whilst the majority of hGISA isolates (85%) belonged to *agr* group I, so too did the majority of the matched MR-GSSA isolates (84%). Furthermore, only a minority of both hGISA (1%) and matched MR-GSSA (3.5%) isolates belonged to *agr* group II. This observation is contradictory to the findings of Moise-Broder *et al.* who reported that *agr* group II polymorphisms among MRSA isolates was predictive of vancomycin treatment failure (Moise-Broder *et al.*, 2004). If that criteria alone was used to identify hGISA isolates in the present study, 99% (176/178) of isolates would have been missed.

Another interesting finding among *agr* typing results in the present study was that 14% and 12.5% of hGISA and MR-GSSA isolates, respectively, belonged to *agr* group III. No report to date has documented an association between *agr* group III and isolates exhibiting reduced susceptibility to glycopeptides but several reports have shown a strong association between CA-MRSA isolates and *agr* group III (Vandenesch *et al.*, 2003; Naimi *et al.*, 2003; Rossney *et al.*, 2007). In Ireland, a previous study investigating *agr* types among 25 *pvl*-positive MRSA isolates reported that 56% (14/25) belonged to *agr* group III whilst 36% (9/25) and 4% (1/25)

belonged to *agr* groups I and II, respectively (Rossney *et al.*, 2007). Among the 1,389 isolates investigated in that study were 42 isolates that were subsequently confirmed as hGISA in the present study. These 42 hGISA isolates belonged to *agr* groups I (67%) and III (33%), respectively, but all were negative for the carriage of *pvl*. Although, 12.5% of MR-GSSA isolates in the present study belonged to *agr* group III, these isolates were chosen from the same collections of isolates from which hGISA were detected but CA-MRSA status had not been investigated among these isolates.

Several genetic backgrounds have been associated with GISA and hGISA isolates (Luczak-Kadlubowska *et al.*, 2006; Howe *et al.*, 2004). MLST combined with SCC*mec* typing revealed that *S. aureus* Mu3 ATCC 700698 (prototype hGISA strain) and *S. aureus* Mu50 ATCC 700699 (prototype GISA strain) exhibited ST5-MRSA-II, whilst both isolates belonged to *agr* group II (Enright *et al.*, 2002; Sakoulas *et al.*, 2002). Howe *et al.* reported a diverse range of genotypes among hGISA isolates that belonged to *agr* groups I and II, respectively (Howe *et al.*, 2004). In a previous study of MRSA in Ireland, isolates that belonged to *agr* group I exhibited ST22-MRSA-IV and ST8-MRSA-IV, respectively; one isolate belonged to *agr* group II and was associated with ST5-MRSA-IV whilst isolates that belonged to *agr* group III exhibited ST30-MRSA-IV and ST80-MRSA-IV, respectively (Rossney *et al.*, 2007). In the present study, both hGISA isolates and MR-GSSA isolates that exhibited A-PFG 07-02 (ST36-MRSA-II) belonged to *agr* group III. In addition, one MR-GSSA isolate that exhibited A-PFG NT-02 belonged to *agr* group III. A range of genotypes were associated with both hGISA and MR-GSSA isolates that belonged to *agr* group I including ST22-MRSA-IV (A-PFG 06-01); ST8-MRSA-II (A-PFGs 13-00 and 14-00); ST8-MRSA-IV (A-PFG 43-00); ST239-MRSA-III (A-PFG 44-99.4) and ST247-MRSA-I (A-PFG New02-00) (Rossney *et al.*,

2006a). Thus, *agr* typing alone and *agr* typing combined with MLST results are not satisfactory methods to distinguish among hGISA and MR-GSSA isolates.

Previous studies reported that isolates defective in *agr* function (delta-haemolysin negative) had an increased ability to bind to polystyrene (Vuong *et al.*, 2000; Sakoulas *et al.*, 2002). However, the present study showed no correlation between a lack of delta-haemolysin production and an increased ability to bind to polystyrene among both hGISA and MR-GSSA isolates. Furthermore, although 70% of hGISA isolates were classified as adherent, only 6% (8/125) of these isolates originated from sites involving biomedical devices and seven out of eight of these hGISA isolates expressed delta-haemolysin. Among 178 hGISA isolates, only one hGISA isolate was delta-haemolysin negative, belonged to *agr* group II and was classified as adherent. This isolate was recovered from a blood culture and exhibited A-PFG 13-99. As mentioned in Section 3.1.1.3.1, isolates exhibiting sporadic PFGE patterns were assigned PFGs in the series of numbers 99000-99999.

As expected, no vancomycin resistance genes (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) were detected among 178 hGISA isolates investigated. Previous studies have shown that the GISA/hGISA phenotype derives from changes in cell wall synthesis whilst VRSA isolates have arisen from the exchange of genetic material between VRE and *S. aureus* isolates (Appelbaum, 2007; Sievert *et al.*, 2008). To date, no vancomycin resistance genes have been identified among GISA or hGISA isolates.

A diverse range of genotypes were present among hGISA and GSSA isolates chosen for vancomycin selection studies. hGISA isolates were represented by ST36-MRSA-II, ST8-

MRSA-II and ST5-MRSA-II whilst GSSA isolates were represented by ST239-MRSA-III, ST8-MRSA-II, ST22-MRSA-IV and *S. aureus* ATCC 29213.

Under vancomycin selection, respective resistant derivatives of all four hGISA isolates showed ability to develop reduced susceptibility to vancomycin by growing on BHI agar containing ≥8 mg/L vancomycin. Following passage on drug-free medium for nine days, these resistant derivatives remained stable. The third step resistant derivative of MF096 {(MF096(3)*} was the only isolate to yield growth on BHI agar containing 12 mg/L vancomycin and the phenotype of this isolate remained stable following nine days passage in drug-free medium (Figure 4.8). GSSA isolates, ML175 and E1168 also showed the ability to develop resistance to vancomycin following the first vancomycin step selection (Figure 4.6). However, although vancomycin resistant derivatives of the other GSSA isolates, MF301 and *S. aureus* ATCC 29213 did not yield growth on BHI agar containing >4 mg/L vancomycin, vPAP-AUC ratios ranged between 0.9 and 1.29 for these isolates and furthermore, all vancomycin resistant derivatives yielded growth on $BHIT5_{0.5}$.

Initial agar screening using BHIV6 failed to detect all eight isolates investigated for vancomycin selection studies. Following the second step selection with vancomycin, only three of eight vancomycin resistant derivatives (MF096, E1033 and *S. aureus* Mu3 ATCC 700698) yielded growth on BHIV6. Furthermore, BHIV6 failed to detect two isolates (MF301 and *S. aureus* ATCC 29213) following the third step selection with vancomycin. In contrast, screening using $BHIT5_{0.5}$ detected all of the third step selected resistant derivatives. Overall, only three isolates (MF301, E1168 and *S. aureus* ATCC 29213) failed to grow on $BHIT5_{0.5}$; these isolates were confirmed GSSA isolates and were screened prior to vancomycin selection.

MF301 (ST22-MRSA-IV; A-PFG 06-01) and ML175 (ST239-MRSA-III; A-PFG 44-99.4) were the first in a series of isolates recovered from two patients in H1. MF301 exhibited a susceptible antibiogram and following selection with vancomycin, vPAP-AUC ratios increased and ranged between 0.9 and 0.99. However, vancomycin resistant derivatives failed to yield growth on BHIV6 agar screening medium and on BHI agar containing >4 mg/L vancomycin but all vancomycin resistant derivatives yielded growth on $BHIT5_{0.5}$. Subsequently, the hTISA phenotype was confirmed from a later isolate (that exhibited A-PFG 06-01) recovered from this patient. ML175 exhibited a more resistant antibiogram and following the first vancomycin step selection, this isolate showed the ability to develop reduced susceptibility to vancomycin (Table 4.4). Among later isolates recovered from this patient, the hGISA phenotype was identified. These hGISA isolates exhibited an indistinguishable A-PFG type from the first isolate recovered from this patient, A-PFG 44-99.4. The potential importance of hGISA is that it may be associated with treatment failure and it may be a precursor of GISA (Hiramatsu *et al.*, 1997a). Previous reports have linked hGISA with clinical failure and evidence of the evolution of hGISA to GISA during an infection has also been reported (Ward *et al.*, 2001; Ariza *et al.*, 1999; Sieradzki *et al.*, 2003). Whether the genetic background of isolates contributes to the potential to develop reduced susceptibility to glycopeptides more readily has yet to be determined. In the present study, the resistant phenotype was induced in all isolates investigated, irrespective of their genotype and was detected by screening with $BHIT5_{0.5}$.

In the present study, investigations of both phenotypic and genotypic properties among hGISA isolates did not reveal any distinctive characteristics that can be used to identify hGISA isolates. Although previous studies have shown certain characteristics could be used as markers for screening for isolates exhibiting reduced susceptibility to glycopeptides, this study

showed that when compared to a control group of MR-GSSA isolates matched for genetic background, these characteristics are unsuitable for predicting potential hGISA isolates.

CHAPTER V

General Discussion

5.1 General Discussion

Since the emergence of reduced susceptibility to glycopeptides among clinical MRSA isolates in 1997, several studies have been conducted to determine the prevalence of GISA/hGISA isolates among MRSA populations (Walsh & Howe, 2002). But partly because of the difficulty in detecting and accurately defining hGISA isolates, reported prevalence rates have varied widely and some workers have questioned whether hGISA isolates may be a laboratory-induced phenomenon (Walsh & Howe, 2002; Ike *et al.*, 2001; Arakawa *et al.*, 2004). In the present study, the overall proportion of hGISA isolates among six collections of MRSA was 5.6% (178/3189) but rates varied when different methods for detecting and confirming hGISA isolates were utilised. Using the recommended E-test™ macromethod cut-off values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone and using vPAP-AUC alone, the proportion of hGISA isolates detected was 2.3% (73/3189) whilst the inclusion of tPAP increased the numbers of hGISA detected by 32% (35/108). Decreasing the teicoplanin E-test™ macromethod cut-off value to ≥8 mg/L and using both vPAP-AUC and tPAP increased the numbers of hGISA detected by a further 65% (70/108). Overall, decreasing the teicoplanin E-test™ macromethod cut-off value to ≥8 mg/L and the inclusion of tPAP increased the numbers of hGISA detected by 144% (105/73).

Because of the lack of recommended methods to define hGISA isolates, it is difficult to compare prevalence rates obtained in the present study with previously reported studies of isolates exhibiting reduced susceptibility to glycopeptides. There are few reports where both vancomycin and teicoplanin PAP methods were used to confirm hGISA isolates (Nonhoff *et al.*, 2005; Luczak-Kadlubowska *et al.*, 2006). In addition, many studies investigate prevalence among selected patient groups but as the present study showed prevalence rates vary

considerably (1.5% to 37%) depending on the patient population studied. Thus, to accurately determine prevalence, assess clinical significance and respond to the development of antimicrobial resistance among MRSA isolates exhibiting reduced susceptibility to glycopeptides, a reliable method of detection for hGISA isolates is required for routine use in clinical microbiology laboratories. A recent report documented that BHIV6 reliably detected isolates exhibiting vancomycin MICs of >6 mg/L with high sensitivity and specificity but may not detect GISA isolates exhibiting vancomycin MICs of 4 mg/L (Appelbaum, 2007). That report identified the need for a reliable agar screening method to detect GISA and hGISA isolates exhibiting low vancomycin MICs (Appelbaum, 2007). In a recent report, Goldstein also emphasised the clinical importance of detecting low-level glycopeptide resistance among *S. aureus* isolates (Goldstein, 2007). The screening method developed in the present study ($BHIT5_{0.5}$) is suitable for use in routine clinical microbiology laboratories and shows sensitivity and specificity values of 100% and 57% for detecting hGISA isolates and 100% and 84% for detecting isolates yielding teicoplanin E-test™ macromethod values of ≥8 mg/L, respectively (Fitzgibbon *et al.*, 2007).

Results from the present study suggest that the development of reduced susceptibility to glycopeptides among MRSA isolates is a multifactorial process. Of the several phenotypic and genotypic characteristics explored in order to define a suitable marker for isolates exhibiting reduced susceptibility to glycopeptides, no one characteristic definitively identified hGISA isolates. Previous studies reported that *agr* typing, delta-haemolysin production, MLST and SCC*mec* analysis could be used to predict isolates exhibiting reduced susceptibility to glycopeptides, but the present study showed that the process involved in the development of hGISA isolates was much more complicated and that no one characteristic investigated to date could be used to identify these isolates. Whilst genome sequencing and gene expression

analyses have not yet identified a suitable marker for identifying GISA/hGISA isolates, alterations in gene expression (down-regulation of *tcaA*, inactivation of *mprF* and over-expression of the global regulator *sigB* and the two-component sensor transducer *VraSR*) have been observed among GISA/hGISA isolates (Maki *et al.*, 2004; Nishi *et al.*, 2004; Ruzin *et al.*, 2003; Bischoff *et al.*, 2001; Kuroda *et al.*, 2003; Sieradzki *et al.*, 2003; Sieradzki & Tomasz, 2003; Singh *et al.*, 2003). However, further studies are required to identify whether all GISA/hGISA isolates share such gene expression markers. Comparative proteomic analyses on GISA/hGISA isolates have identified increased expression of a particular gene (SAV2095) in VISA/hVISA isolates that could be used as a possible biomarker. Further characterisation of SAV2095 is in progress (Drummelsmith *et al.*, 2007). In a recent study, genomic sequencing identified a loss-of-function mutation in the *agr* locus among *S. aureus* isolates exhibiting reduced susceptibility to vancomycin (Mwangi *et al.*, 2007). But whilst these workers and others believe that the *agr* locus plays a key role in the development of reduced susceptibility to glycopeptides, results from the present study show that there is no statistically significant difference between *agr* types of hGISA and MR-GSSA isolates (Mwangi *et al.*, 2007; Rose *et al.*, 2008).

Although 178 hGISA isolates comprising of 13 hVISA, 88 hTISA and 77 hVISA & hTISA isolates were detected in the present study, the significance of the breakdown of hGISA isolates is not known. As the present study was based on retrospective analysis of MRSA isolates, clinical details such as exposure to glycopeptides in individual institutions were not available, thus the numbers of hVISA, hTISA and hVISA & hTISA isolates detected could not directly be attributed to exposure to either vancomycin and/or teicoplanin. Although both vancomycin and teicoplanin share the same mode of action (inhibition of cell wall synthesis through high affinity binding of D-alanyl-D-alanine residues of the murein monomer),

different mechanisms of target binding for each glycopeptide have been described thus explaining the emergence of reduced susceptibility to vancomycin (hVISA) and teicoplanin (hTISA), respectively (Hiramatsu, 1998). It has also been proposed that individual *S. aureus* cells may express resistance mechanisms on the cell membrane e.g. over-production of PBP2 has been observed among the prototype GISA strain, *S. aureus* Mu50 ATCC 700699 and the prototype hGISA strain, *S. aureus* Mu3 ATCC 700698 resulting in reduced susceptibility to teicoplanin (Hiramatsu, 1998; Hanaki *et al.*, 1998a). An interaction between beta-lactams and vancomycin has been observed among some GISA/hGISA isolates reported but no interaction has been shown between beta-lactams and teicoplanin (Hiramatsu, 2001; Cui *et al.*, 2003).

Initial reports of GISA/hGISA isolates suggested that the genetic background of isolates may be a predisposing factor for the potential to develop reduced susceptibility to vancomycin and/or teicoplanin (Hiramatsu *et al.*, 1997a). It was believed that all GISA/hGISA isolates belonged to the NewYork/Japan clone (CC5, ST5-MRSA-II) (Hiramatsu *et al.*, 1997a; Enright *et al.*, 2002). But Howe *et al.* showed among a global collection of GISA and hGISA isolates investigated, that all five pandemic clones of MRSA (CC5, CC8, CC22, CC30 and CC45) were represented among hGISA isolates whilst CC5 and CC8 were represented among GISA isolates (Howe *et al.*, 2004). In the present study, hGISA isolates belonged to CC8 (80%), CC30 (14%), CC22 (3%) and CC5 (<1%) suggesting that the genetic background of an isolate is not predictive of the hGISA phenotype. The correlation between epidemiological typing results and the numbers of hVISA, hTISA and hVISA & hTISA obtained in the present study were difficult to compare to other studies as no other reported study has detected such a large number of hGISA isolates.

Among all clusters of hGISA isolates identified, epidemiological typing results correlated with results of *agr* typing, delta-haemolysin production and adherence ability, except in two institutions, H2 and H3, respectively. In H3, among six hGISA isolates (two hVISA & hTISA isolates, three hTISA isolates and one hVISA isolate) that exhibited A-PFG 13-00 and yielded indistinguishable PFGE patterns, delta-haemolysin was produced by one of the hVISA & hTISA isolates, one hTISA isolate and the hVISA isolate. All six isolates belonged to *agr* group I and were classified as adherent. In the second institution (H2), seven hGISA isolates that exhibited A-PFG 13-00, were rifampicin resistant and yielded indistinguishable PFGE banding patterns. All seven hGISA isolates were classified as adherent and belonged to *agr* group I, but only 43% (3/7) of these isolates produced delta-haemolysin. Is the difference in expression of delta-haemolysin a significant marker to distinguish among the hGISA isolates identified in both these institutions? How significant is the production of delta-haemolysin among *S. aureus* isolates?

As mentioned in Section 4.1.1.1.1, the role of delta-haemolysin in *S. aureus* pathogenesis is unclear but as delta-haemolysin activity is a useful indicator of a functional *agr* operon, it is likely that the differences if any, between these hGISA isolates resides at a genotypic level and involves a complex process. Interestingly, among four hGISA isolates (three hVISA & hTISA isolates and one hVISA isolate) that exhibited A-PFG 44-99.4, all four isolates belonged to *agr* group I, expressed delta-haemolysin and were classified as adherent. Thus, it is possible that hGISA isolates (representing one strain) that are indistinguishable by A-PFG typing, *agr* typing, delta-haemolysin production and adherence ability may exhibit different resistant phenotypes i.e. hVISA or hTISA or hVISA & hTISA. Hence, clusters of hGISA isolates that exhibit indistinguishable A-PFG types and different resistant phenotypes may or may not represent the same strain. Until the underlying mechanism of resistance has been fully

elucidated, the reason why some *S. aureus* isolates exhibit reduced susceptibility to vancomycin and/or to teicoplanin will not be fully understood. In recent years, with advances in molecular technology, studies exploring reduced susceptibility to glycopeptides in *S. aureus* have increased. Gene expression studies, combined proteomic and transcriptomic approaches have identified shared molecular markers among GISA/hGISA isolates. However, global comparative studies are required to determine whether all reported isolates exhibiting reduced susceptibility to vancomycin and/or teicoplanin share the same molecular markers. Because of the difficulty in detecting GISA/hGISA isolates phenotypically, a reliable molecular marker for the detection of isolates exhibiting reduced susceptibility to vancomycin and/or teicoplanin is required to ensure successful antimicrobial therapy for these isolates and to prevent the development of high-level resistance.

Due to the increasing prevalence of MRSA in the USA during the 1980s, vancomycin was widely used to treat staphylococcal infections. At the same time, vancomycin was being used more frequently to treat serious MRSA infections as it was one of the few antibiotics to which resistance had not developed and had become the cornerstone of therapy. Increasing use of vancomycin increased the selective presssure in staphylococci. By the late 1980s, reports of vancomycin resistance among CoNS were emerging but serious concerns were not raised about the spread of vancomycin-resistant bacteria until 1994 when the yearly usage of vancomycin in the USA had doubled since the late 1980s (Kirst *et al.*, 1998).

Whilst there was heightened awareness in the USA regarding the emergence of reduced susceptibility to glycopeptides among staphylococci, it was quite unexpected that the first report of a clinical *S. aureus* isolate exhibiting reduced susceptibility to vancomycin should come from Japan (Noble *et al.*, 1992; Hiramatsu *et al.*, 1997a). Although MRSA was a

serious nosocomial problem in Japan and had increased during the 1980s, vancomycin was not available in Japan until 1991, hence this report of the first clinical *S. aureus* isolate exhibiting reduced susceptibility to vancomycin was surprising (Hiramatsu, 1998). Prior to 1997, reports of vancomycin clinical failures asssociated with MRSA pneumonia were documented in Japan but at the time it was believed that these therapeutic failures were a result of vancomycin's poor bactericidal activity and low tissue penetration (Hiramatsu, 1998).

Why the first clinical *S. aureus* isolate exhibiting a vancomycin MIC of 8 mg/L was identified in Japan is unknown but it has been suggested that widespread use of third-generation cephalosporins during the 1980s to treat MRSA infections in Japan may have created pressure for the selection of resistance among *S. aureus* isolates and this resistance was expressed as heterogeneous resistance to vancomycin (Hiramatsu, 1998). It has previously been shown that over-use of beta-lactam antibiotics raised the level of meticillin resistance among MRSA strains in Japan where the prevalence of one MRSA strain termed clonotype II-A (New York/Japan clone or ST5-MRSA-II) increased and became the dominant clone in the 1990s (Hiramatsu, 1998; Enright *et al.*, 2002). ST5-MRSA-II was initially believed to have a predisposition to develop reduced susceptibility to glycopeptides but it is more likely that this clone developed in Japan as a result of over-exposure to beta-lactam antibiotics rather than this genotype having an intrinsic ability that allows the GISA/hGISA phenotype to develop (Hiramatsu, 1998). In the present study, ST5-MRSA-II was represented by a single isolate whilst ST8-MRSA-II predominated among hVISA (85%), hTISA (42%) and hVISA & hTISA (58%) isolates, respectively.

In Europe, avoparcin, a glycopeptide antibiotic growth promoter added to animal feed was first licensed for use in 1975, but several studies showed that the use of avoparcin resulted in

an increase in prevalence of VRE (Klare *et al.*, 1995; Aarestrup, 1995). Following the European Union ban on the use of avoparcin in animals in 1997, prevalence of VRE in both animals and humans decreased significantly across Europe (in Germany, Italy, Denmark and the Netherlands) (van den Bogaard *et al.*, 2000). Avoparcin has never been used in the USA and it has been suggested that likely sources of VRE infection have been travel, tourists and imported food (Wegener, 1998). Furthermore, why the first VRSA isolate was recognised in the USA where avoparcin had not been licenced for use is not known. In addition, why five of the seven VRSA isolates recognised in the USA were identified in south-eastern Michigan is not quite understood. But a recent report has identified some factors that may provide some explanations (Sievert *et al.*, 2008). Firstly, because the first VRSA isolate was recognised in south-eastern Michigan, there has been heightened awareness in that State of *S. aureus* isolates exhibiting reduced susceptibility to glycopeptides. Secondly, Michigan has a large population of patients with diabetes and end-stage renal disease, conditions frequently associated with both MRSA and VRE infections. And thirdly, Michigan may have been exposed to increased vancomycin selective pressure during the 1980s as it was one of the first locations where MRSA was identified outside the hospital environment (Sievert *et al.*, 2008).

Under the North American-Free Trade Agreement, factory-farmed animals (animals that are fed antibiotics and hormones) such as pigs, poultry and cows are allowed to move freely between Canada and the USA. In North America, whilst MRSA has previously been identified among horses, dogs and cats, it was only recently identified among pigs and pig farmers in Ontario, Canada where the prevalence of MRSA was 25% and 20%, respectively (Khanna *et al.*, 2008). Interestingly, Ontario, Canada borders the State of Michigan in the USA. Most worryingly, the south-east Michigan/south-west Ontario land border crossings are the busiest international crossings in the North American continent and represent 50% of the

traffic volume crossing the USA/Canada border. Five of the seven VRSA isolates in the USA were identified in south-eastern Michigan (Sievert *et al.*, 2008). However, it is unlikely that factory farmed pigs in Canada are the source of VRSA isolates in the USA. All seven reported VRSA isolates belong to ST5 whilst ST398 is the predominant ST reported among pigs but, ST398 has been shown to cause infection in humans (Tenover, 2008; van Belkum *et al.*, 2008). Further studies on the prevalence of MRSA among factory-farmed animals across North America and Canada are required.

Overuse and misuse of antimicrobials have contributed to increased bacterial resistance and rising drug costs. In recent years, new antimicrobial agents such as daptomycin, linezolid, quinupristin-dalfopristin and tigecycline that are effective in treating MRSA infection have been introduced but the development of new antimicrobial agents has slowed partly because of the cost which is estimated to be between $400 and $800 million per approved agent (Rice, 2006; Payne & Tomasz, 2004; Thomson *et al.*, 2004; Spellberg *et al.*, 2004). New glycopeptide antibiotics under study with superior pharmacodynamic properties compared to vancomycin include dalbavancin, telavancin and oritavancin (Lentino *et al.*, 2008). Thus now more then ever, control of both the appropriate and inappropriate use of antimicrobial agents is critical to successfully optimise and extend the effectiveness of currently available antimicrobial agents.

Reducing the overall rates of MRSA infection in healthcare facilities is the most effective infection control measure. Implementing infection control guidelines successfully requires sufficient personnel and resources (Tenover, 2006). Strategies to promote the judicious use of antibiotics have been previously described (Fishman, 2006). Education, hospital formulary restriction, prior approval programmes and computer-based programmes have been described

as key factors involved in promoting the prudent use of antibiotics (Fishman, 2006). Antimicrobial streamlining, whereby broad-spectrum empirical therapy is modified (following laboratory testing) to therapy with a narrow regimen has also been described as an effective means of improving antimicrobial usage. In addition, antibiotic cycling whereby antimicrobial therapy is switched between two agents to prevent resistance occurring to a single agent has been another effective intervention for the improved use of antimicrobials (Fishman, 2006). Compliance with issued guidelines for effective infection control is imperative to successfully reduce the rates of MRSA in health-care environments.

In 2001, the Strategy for the Control of Antimicrobial Resistance in Ireland (SARI) was launched to combat the problem of the emergence and development of antimicrobial resistance (Anon., 2001). In 2005, new guidelines to control the spread of MRSA in hospitals and in the community in Ireland and guidelines for hand hygiene in health-care settings were issued by SARI (Anon., 2005c; Anon., 2005a). Included among guidelines were (i) recommendations for the appropriate use of glycopeptide therapy to treat MRSA infection, (ii) guidelines to control the spread of GISA and GRSA strains and (iii) recommendations for hospital hygiene audits and in particular audits of compliance with hand-hygiene guidelines (Anon., 2005a). Interestingly, during Q4, 2007, the proportion of MRSA among bloodstream *S. aureus* isolates was 28.8% compared to 40% during Q3, 2007 (Anon., 2007). This decline resulted in the annual proportion of MRSA among bloodstream *S. aureus* isolates decreasing from 42% (which had been the yearly average since 2002) to 38%. It remains to be seen whether these reduced rates are the beginning of a decline in the prevalence of MRSA in Ireland. A decrease in the incidence and prevalence of MRSA has been reported in Europe (Monnet & Kristinsson, 2008).

Whilst at present, the challenges associated with reduced susceptibility to glycopeptides among *S. aureus* isolates are great, they are not insurmountable. Early recognition of these isolates is imperative as the lack of development of new antimicrobial agents to treat serious MRSA infections is a potential threat to public health. Strict observance and compliance with infection control measures is required to prevent the emergence of VRSA. Further studies to elucidate the complex mechanisms involved in the process of reduced susceptibility to glycopeptides among *S. aureus* isolates will provide invaluable insights into antimicrobial resistance among *S. aureus* isolates.

Appendices

Appendix I. Molecular Biology Reagents

AI.1 Water

All water used was purified through a water treatment process to provide ultra pure (UP) water with low levels of impurities (Maxima Ultra Pure, ELGA LabWater, Veolia Water Systems, Celbridge, Co. Kildare, Ireland). Water was deionised, purified by reverse osmosis (RO) and treated with UV light to attain an 18MΩ quality.

AI.2 Stock Solutions

0.5 M EDTA (ethylenediamine tetraacetic acid) disodium salt, pH 7.5 (Sigma E-7889)

5X Tris-Borate-EDTA (TBE) (Sigma T-3913) was prepared according to manufacturer's instructions using UP water.

Ethidium Bromide (10 µg/ml) (Sigma E-4391) was prepared by dissolving one 10-mg tablet in 1 ml RO water. A working concentration (0.5 µg/ml) was prepared by diluting stock solution using RO water.

Stock solutions of the following were prepared as described by Sambrook & Russell (Sambrook & Russell, 2001):

1 M Tris, pH 7.6

1 M Tris, pH 8.0

5 M NaCl.

AI.2.1 Buffers

Tris-EDTA (TE) buffer pH 7.5

10 mM Tris, pH 7.6, 1 mM EDTA, pH 7.5

To prepare TE buffer (pH 7.5), 10 ml of 1 M Tris, pH 7.6 and 2 ml of 0.5 M EDTA were diluted with UP water in a 1 litre (L) volumetric flask.

Tris-NaCl (TN) buffer

10 mM Tris, pH 8.0, 10 mM NaCl

TN buffer was prepared by diluting 10 ml of 1 M Tris, pH 8.0 and 2 ml of 5 M NaCl with UP water in a 1L volumetric flask.

0.5X / 1X TBE

5X TBE was diluted 1/10 and 1/5 in 1L volumetric flasks with RO water to prepare working solutions of 0.5X and 1X TBE, respectively.

AI.2.2 Other Reagents

50 mM EDTA

0.5 M EDTA was diluted 1/20 in sterile UP water to prepare a 50 mM EDTA working solution.

Lysostaphin (1 mg) (Sigma L 7386)

The contents of one vial were dissolved in 1 ml of sterile UP water to give a working concentration of 1 mg/ml.

Lysozyme (1 g) (Sigma L 7651)

To prepare a working concentration of 100 mg/ml, the contents of one vial were dissolved in 10 ml sterile UP water, aliquoted in 120-µl volumes and stored at -20°C.

Lytic Enzyme Mix

Sufficient lytic enzyme mix (120 µl per isolate) containing 50 µl lysostaphin (1 mg/ml), 6 µl lysozyme (100 mg/ml) and 64 µl of Cell Lysis Solution (Promega #A793A) was prepared for the total number of isolates being tested, held on ice and aliquoted in 120-µl volumes when required.

Magnesium Chloride ($MgCl_2$) (1.0 M) (Sigma M-1028)

To prepare a working concentration of 50 mM $MgCl_2$, stock solution of 1.0 M $MgCl_2$ was diluted 1/20 using sterile UP water and stored at 4°C until required.

Achromopeptidase (ACP) (100,000 U) (Sigma A-3547)

The contents of one vial were reconstituted in 10 ml sterile TE buffer (10U/µl), aliquoted in 450-µl volumes and stored at -20°C until required.

Blue/Orange Loading Dye, 6X (Promega #G190A)

Isopropanol (Propan-2-ol, BDH 437433T)

Stored at RT in a flammable liquid storage cabinet.

70% Ethanol (Ethanol absolut, BDH 437433T)

Prepared by adding 30 ml of UP water to 70 ml of absolute ethanol. Stored at RT in a flammable liquid storage cabinet.

Appendix II. Molecular Biology Methods

AII.1 DNA Extraction Methods

AII.1.1 Chromosomal DNA Extraction for PFGE

Chromosomal DNA was extracted from bacterial cells *in situ* in agarose plugs. Bacterial cells were suspended in low melting temperature agarose with a lysing agent (ACP), incubated and following lysis, were washed and prepared for digestion with *Sma*I as described below.

AII.1.1.1 DNA Extraction Method

A heavy suspension of colonies from an overnight culture on CBA was prepared in 3 ml cold sterile TE buffer and transferred to a microcentrifuge tube on ice. The preparations were centrifuged at 14,000 rpm for 60 s at 4°C (Eppendorf 5417R, Eppendorf – Netheler – Hinz GmbH, Hamburg, Germany) and held on ice while supernatants were removed. Pellets were resuspended in 200-µl volumes of cold TE buffer and diluted to a density equivalent to a 3.0 McFarland turbidity standard in cold TE buffer. Cells were centrifuged as described above and pellets were resuspended in 200 µl cold TE buffer and held on ice until required. Low melting temperature agarose 2% (w/v) SeaPlaque Agarose (SeaPlaque 50101: BioWhittaker UK Ltd., Wokingham, UK) in sterile TN buffer at 50°C was dispensed in 200-µl volumes into microcentrifuge tubes and held at 50°C in a heating block (Thermomixer comfort 5355; Eppendorf – Netheler – Hinz GmbH, Hamburg, Germany). Twenty microlitres of ACP (10U/µl) solution were added and mixed well immediately prior to adding 200 µl of the cell suspension. The cell/agarose suspension was mixed well and cast into a plug mold (Sample Mold, Bio-Rad #170-3622) kept on ice, taking care not to introduce air bubbles. Plugs were allowed to solidify on ice for approximately 10 min before being transferred to sterile Universal containers containing 1 ml TN buffer and incubated at 50°C (in a shaking waterbath

at 120 rpm) for 60 min. Following lysis, the TN buffer was decanted and the plugs were washed three times in 10 ml volumes of TE buffer for at least 15 min at 50°C with shaking at 120 rpm. After the final wash, a 2 mm slice of each plug and the remainder of each plug were each transferred to separate microcentrifuge tubes containing 1.5 ml fresh TE buffer and stored at 4°C.

AII.1.2 Genomic DNA Extraction for Amplification using PCR

DNA extraction for amplification studies was performed using the Wizard™ Genomic DNA Purification Kit protocol for isolation of genomic DNA from Gram-positive bacteria (Promega #A1120, Promega Corporation, Madison W1, USA). Reagents not supplied with the kit {50 mM EDTA, lysostaphin (1 mg/ml), lysozyme (100 mg/ml), isopropanol and 70% ethanol} were prepared as outlined in AI.2.2.

AII.1.2.1 Wizard™ Genomic DNA Purification Kit Protocol

One millilitre of overnight cultures in TSB was added to microcentrifuge tubes held on ice and centrifuged at 14,000 rpm for 2 min at 4°C. Supernatants were removed, cells were resuspended in 480-µl volumes of 50 mM EDTA and held on ice. Lytic enzyme mix (120 µl per isolate) prepared as described in A1.2.2 was added to each cell suspension held on ice. Preparations were incubated at 37°C for 30–60 min in a waterbath (JB1 Grants Instruments) and centrifuged for 2 min at 14,000 rpm at 4°C. Following removal of supernatants, 600 µl of Nuclei Lysis Solution (Promega #A7941) was added to each preparation and gently pipetted to resuspend the cells. Preparations were incubated for 5 min at 80°C in a waterbath to lyse the cells and cooled to RT. RNase Solution (Promega #A797A) (3 µl) was added to each cell lysate, tubes were inverted 2–5 times and incubated at 37°C for 15–60 min. Preparations were

placed on ice, cooled to RT and 200 µl of Protein Precipitation Solution (Promega #A795A) was added. Lysates were vortexed at high speed for 20 s and incubated on ice for 5 min. Following centrifugation at 14,000 rpm for 3 min at 4°C, supernatants (containing the DNA) were transferred to new microcentrifuge tubes containing 600 µl of isopropanol at RT. Tubes were inverted gently several times until thread-like strands of DNA formed a visible mass. Samples were centrifuged for 2 min at 14,000 rpm at 4°C to pellet the DNA. Supernatants were carefully decanted and tubes were drained on clean absorbent paper prior to the addition of 600 µl of 70% ethanol (at RT). Tubes were inverted gently several times to wash the DNA pellet, centrifuged at 14,000 rpm for 2 min at 4°C and the ethanol was carefully aspirated. Tubes were drained on clean absorbent paper and pellets were allowed to air-dry for 10–15 min. DNA Rehydration Solution (Promega #A796A) (100 µl) was added to each tube and the DNA was held at 4°C overnight. Following rehydration, the DNA was aliquoted in 25-µl volumes and stored at 4°C until required.

AII.2 Amplification of DNA by Multiplex PCR

All PCR reaction preparation was performed in a PCR laminar flow cabinet (LaminAir Holten) which was switched on at least 15 min prior to use and cleaned with spirit. An aliquot of nuclease-free water (NFH_20) and an aliquot of 50 mM $MgCl_2$ were removed from a dedicated PCR reagent preparation refrigerator and placed on ice. PCR Master Mix {50 U/ml *Taq* DNA polymerase, 400 µM each of dATP, dGTP, dCTP, dTTP, and 3 mM $MgCl_2$ (Promega M 7502)} and an aliquot of each primer were removed from storage at -20°C and placed on ice. When thawed, the PCR Master Mix and primers were centrifuged for approximately 10 s in a microcentrifuge (Eppendorf MiniSpin 5452 000.018, Eppendorf, Hamburg, Germany) and replaced on ice. PCR tubes (Molecular Bioproducts RNase/DNase-

free, 0.2 ml #3412Y, 0.5 ml #3430Y) were prepared and labelled as required in the laminar flow cabinet.

AII.2.1 agr Typing

Reaction volumes were 25 µl containing 12.5 µl of PCR Master Mix, 0.075 µl of each primer (total=0.375 µl), 2 µl of 50 mM $MgCl_2$, 9.125 µl NFH_20 and 1 µl of genomic DNA. Reaction mixtures were transferred to a separate rack and transported to the PCR Product Detection Laboratory where amplification was carried out in a Hybaid Px2 Research Thermal Cycler using the following temperature programme: one cycle of 5 min at 94°C; 26 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C; and a final cycle of 72°C for 10 min.

AII.2.2 van Assay

One microlitre of purified genomic DNA was added to a total volume of 99 µl containing 50 µl PCR Master Mix, 0.4 µl of each primer pair (4.8 µl) except for *vanE* (2 µl of *vanE* primers were used) and 40.2 µl NFH_20. Reaction mixtures were transferred to the PCR Product Detection Laboratory with minimum delay. Amplification was performed on a Hybaid Px2 Research Thermal Cycler with the following thermal cycling profile: one cycle of 3 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C; and a final cycle of 72°C for 7 min.

AII.3 Preparation of Agarose Gels

AII.3.1 PFGE Running Gel Agarose

A 1% (w/v) agarose gel {Pulsed Field Certified Agarose, (Bio-Rad 162-0137)} in 0.5X TBE was prepared using a 21 cm x 14 cm tray with a casting stand (Bio-Rad 170-3704) and a 30-well comb (Bio-Rad 170-3628).

AII.3.2 Agarose Gel for PCR Product Detection

A 2% (w/v) agarose gel (Molecular Biology Certified Agarose, Bio-Rad 161-3100) in 1X TBE was prepared using a casting stand with either a large (10 cm x 15 cm) or small (7 cm x 10 cm) gel tray (Sub-cell tray 170-4416 or Sub-cell tray 170-4435, respectively). For large gels, a 30-well comb (Bio-Rad 170-4449) was used whilst a 15-well comb (Bio-Rad 170-4446) was used for smaller gels.

AII.4 Agarose Gel Electrophoresis

AII.4.1 PFGE

Following digestion of chromosomal DNA with *Sma*I, the comb was removed from the gel; plug slices were trimmed and loaded into the wells starting at the fourth well. Outermost lanes on the gel were not used to avoid distortion. Molecular size marker (Lambda Ladder PFG Marker, New England Biolabs, N0340S; New England Biolabs Ltd., Hitchin, UK) was loaded into the central and two outermost wells. The wells were sealed with 1% sealing agarose (PFGE running gel agarose) prepared as described in AII.3.1 and left to solidify for approximately 30 min.

Electrophoresis was performed in a CHEF DRIII PFGE apparatus (Bio-Rad 170-3913) using 0.5X TBE running buffer, held at 14°C by a chiller (Model 1000 Mini-Chiller, Bio-Rad 170-3702) and recirculated through the chiller and the electrophoresis tank with a pump. The gel was allowed to equilibrate to 14°C for 15 min and electrophoresis was performed with a 120° angle and ramped linearly with switch times of 6.8–63.8 s over 23 h.

AII.4.2 PCR Product Detection

Electrophoresis of amplified DNA was performed in designated electrophoresis tanks {Wide Mini GT System 170-4469 (large) and Subcell Mini GT 170-4486 (small)} using 1X TBE running buffer. Gel loading buffer (Blue/Orange Loading Dye, Promega #G190A) was added to all PCR products and to a 50 bp DNA Step Ladder (Promega #G4521) at a ratio of 1:5. A mixture of 4 µl sterile UP water, 1 µl of blue/orange loading dye and 1 µl of PCR product (or 1 µl of 50 bp DNA ladder) was prepared and loaded directly into the wells of the gel. The power supply (PowerPac 300 System, 220/240, Bio-Rad 165-4352) was set at the appropriate conditions for electrophoresis and switched on. Electrophoresis conditions for separation of amplified DNA fragments of *agr* sequences and *van* resistance genes were 90 V for 90 min and 70 V for 180 min, respectively.

AII.5 Staining and Photography

After electrophoresis, gels were stained with ethidium bromide (0.5 mg/L) (Sigma E-4391) for 30 min, transferred to a destaining container, rinsed with RO water and left to destain in fresh RO water for at least one hour.

Gels were transferred to a benchtop transilluminator (UV Benchtop Transilluminator; Model M-26, Ultra-Violet Products, Cambridge, UK) and photographed under UV light with a digital camera (Olympus Camedia C-3020 zoom). The image was adjusted accordingly using Doc-It Acquisition software (Ultra-Violet Products) and printed using a digital graphic printer (SONY UP-D895) on high gloss black and white thermal paper (SONY UPP-110HG).

PFGE gel images were saved as TIFF (tagged image file format) files for importation to GelCompar (Version 4.1, Applied Maths, Sint-Martens-Latem, Belgium) software analysis programme.

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Publications

List of Publications

1. **Fitzgibbon M. M., Rossney A. S. & O' Connell, B.** Investigation of glycopeptide resistance in MRSA in Ireland between 1998 and 2003. SARI/AMRAP Joint Implementation Meeting; Dundalk, Ireland. November 2004.

2. **Fitzgibbon M. M., Rossney A. S. & O' Connell, B.** Characterisation of 73 hGISA isolates recovered in Ireland between 1998 and 2003. *International Symposium on Staphylococci and Staphylococcal Infections*; Maastricht, The Netherlands. September 2006.

3. **Fitzgibbon M. M., Rossney A. S. & O' Connell, B.** Teicoplanin improves detection of hetero-glycopeptide-intermediate resistance in MRSA. SARI/AMRAP Joint Implementation Meeting; Dublin, Ireland. November 2006.

4. **Rossney A. S., Fitzgibbon M. M. & O' Connell, B.** Glycopeptide–intermediate *Staphylococcus aureus* (GISA) in Ireland: First Report. (www.ndsc.ie/hpsc/EPI-Insight/Volume72006/File,2067,en.PDF). November 2006.

5. **Fitzgibbon M. M., Rossney A. S. & O' Connell, B.** Investigation of reduced susceptibility to glycopeptides among methicillin-resistant *Staphylococcus aureus* isolates from patients in Ireland and evaluation of agar screening methods for detection of heterogeneously glycopeptide-intermediate *S. aureus*. *J. Clin. Microbiol.* 2007, **45**, 3263-3269.

Investigation of glycopeptide resistance in MRSA in Ireland between 1998 and 2003

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Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious nosocomial problem and therapeutic options may be limited to the glycopeptides vancomycin and teicoplanin. Three types of reduced susceptibility to glycopeptides have been described: 1) vancomycin-resistant *S. aureus* (VRSA) where the vancomycin mimimum inhibitory concentration (MIC) is ≥32 mg/L; 2) glycopeptide-intermediate *S. aureus* (GISA) where the vancomycin MIC is 8 mg/L; 3) heteroglycopeptide-intermediate *S. aureus* (hGISA) where the majority of the bacterial population has a vancomycin MIC of ≤4 mg/L but small minority of cells (perhaps as few as 10^{-6}) have MICs of 8 mg/L[1,2]. There have been three reported cases of VRSA worldwide[2]. GISA have been reported from many countries but are still rare[1]. hGISA are more common but their clinical relevance is debated. hGISA may be a pre-GISA state which could lead to treatment failure over time[1,3].

Routine disk diffusion and MIC methods fail to detect GISA and hGISA. A screening method – the E-test macro-method – which uses a heavy inoculum (equivalent to a 2 McFarland turbidity standard) on brain heart infusion agar (BHI) with vancomycin and teicoplanin E-test strips and 48 hours incubation has been advocated[4]. This method has a reported sensitivity and specificity of 96% and 97% but is expensive for widespread screening[4]. Various preliminary agar screening methods have been proposed: BHI containing 6 mg/L vancomycin (BHIV6) detects GISA only; other methods include Mueller-Hinton (MH) agar containing teicoplanin at either 5 or 8 mg/L (MHT5, MHT8) or BHI with teicoplanin at 6mg/L[5,6,7]. Final confirmation of GISA and hGISA require population analysis profiling (PAP)[1]. A modified PAP method in which the ratio of the area under the curve (AUC) of the test isolate relative to the AUC of the prototype hGISA (*S. aureus* ATCC 700698, Mu3) has been described[4].

The present study investigated 1177 MRSA isolates from collections of MRSA from 1998 to 2003 comprising 2569 isolates from 2417 patients to 1) determine the prevalence of VRSA, GISA and hGISA, 2) evaluate screening methods including a new formulation of BHI containing teicoplanin at 5 mg/L (BHIT5) and 3) investigate epidemiological types of GISA and hGISA in these MRSA populations.

Materials and Methods

The MRSA collections, numbers of isolates in original studies, numbers screened in the present study, screening methods, numbers investigated by broth microdilution (using NCCLS methodology) and by PAP-AUC analysis are summarised in Table 1. With Collections 1a and 1b, only isolates that grew on BHI containing 4 mg/L vancomycin in previous studies were included. Isolates in Collections 1 and 4 were recovered from all body sites, those from Collections 2 and 3 were from blood cultures only. PAP-AUC ratios were determined for any isolate with E-test macro-method values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone. PAP-AUC ratio interpretative criteria were: glycopeptide-susceptible *S. aureus* (GSSA), <0.9; hGISA ≥0.9 and <1.3; GISA ≥1.3.

A panel of isolates consisting of all isolates confirmed as hGISA and a selection of isolates confirmed as glycopeptide-susceptible *S. aureus* (GSSA) by PAP-AUC analysis and a matched number of GSSA isolates (confirmed by E-test macro-method) was used to evaluate MHT5 and BHIT5. Inocula were prepared in saline to densities equivalent to 0.5 and 2 McFarland turbidity standards and from stationary phase broth cultures.

All isolates confirmed as hGISA were typed by macrorestriction fragment length polymorphism analysis using *Sma*I digestion followed by pulsed field gel electrophoresis (PFGE). Isolates had previously been typed by antibiogram-resistogram (AR) typing (using a panel of 23 antimicrobials).

Table 1. Numbers of isolates investigated by screening methods, MIC determination and PAP-AUC analysis. Figures in parentheses indicate numbers of patients in original studies.

MRSA Collection			No. of Isolates		Screening Methods			MIC Methods		
No.	Year	Hospitals	Original Study	Present Study	BHIV6	MHT5	E-testM*	E-Test**	Broth Micro-dilution	PAP
1a	1998	H1	188† (169)	84	84	84	84	7	84	7
1b	1999	All (N/S Study)[8]	714† (682)	489	489	489	489	75	489	75
2	1999-'02	All (EARSS)	1150†† (1091)	87	87	87	(87)‡	10	87	10
3	2003	All (EARSS)	430 (416)	430	430	…	430	4	…	4
4	2000-'03	NMRSARL Referrals‡‡	87 (59)	87	87	…	(87)‡	9	…	9
Total	**1998-'03**		**2569 (2417)**	**1177**	**1177**	**660**	**1177**	**105**	**660**	**105**

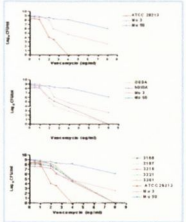
…, not done; *E-TestM, E-test macro-method; **E-Test performed on isolates with E-Test macro-method values ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone; †, isolates screened on BHI with 4 mg/L vancomycin during previous studies; ††, isolates screened by E-Test macro-method; ‡, tests performed previously in NMRSARL; ‡‡, isolates referred to NMRSARL for investigation of glycopeptide susceptibility. All, all Irish hospitals; EARSS, blood culture isolates from the Irish European Antimicrobial Resistance Surveillance System participants; H1, one Dublin hospital; N/S, North/South Study of MRSA in Ireland 1999[8]; PAP, PAP-AUC ratio determination.

Results

No VRSA or GISA were detected. PAP-AUC analysis confirmed 61 hGISA (overall prevalence 2.5%; 61/2417). Prevalence in each study period is shown in Table 2. During the North/South Study 1999, prevalence in the North and South was 14% and 5%, respectively. PAPs of representative isolates are shown in the figure.

No hGISA grew on BHIV6. Fifteen hGISA failed to grow on MHT5 when the inoculum was delivered by 10 µl loop. Repeat screening on MHT5 with the inoculum delivered by pipette yielded growth of all 15 isolates. Table 3 summarises the evaluation of screening on MHT5 and BHIT5. BHIT5 with an inoculum density equivalent to a 0.5 McFarland turbidity standard performed best (with sensitivities of 100% and 98% and specificities of 38% and 72%, for the detection of hGISA and positive E-Test macro-method results, respectively).

Epidemiologcal typing showed that AR types AR13 and AR14 were exhibited by 62% and 16% of hGISA, respectively. Analysis of PFGE results await completion but data to date suggest that in 1999 at least, certain patterns were associated with hGISA in individual hospitals.



Population analysis profiles of control *S. aureus* isolates and a selection of test isolates.

Summary

- **No VRSA or GISA were detected**
- **Prevalence of hGISA was 2.5%**
- **Prevalence varied from 0.2% (among blood culture isolates) to 14% (among isolates from the North during the North/South Study, 1999)**
- **Inoculating screening media with a 10 µl loop yields false negative results**
- **BHIT5 (inoculum density equivalent to 0.5 McFarland standard) is the most effective screening medium to predict hGISA and E-Test macro-method-positive isolates**
- **Epidemiological typing suggests that particular AR types and PFGE patterns are associated with hGISA in individual hospitals**

Table 2. Prevalence of hGISA in Ireland (1998 to 2003).

		No. of Patients		hGISA	
No.	Year	Original Study	Present Study	PAP-AUC ratio >0.9	Prevalence
1a	1998	169	77	3	2%
1b*	1999*	682*	474*	48*	7%*
(North)*	1999*	(192)*	(135)*	(26)*	(14%)*
(South)*	1999*	(490)*	(339)*	(22)*	(5%)*
2	1999-'02	1091	87	2	0.2%
3	2003	416	416	1	0.2%
4	2000-'03	59†	59†	7†	12%†
Total	**1998-'03**	**2417**	**1113**	**61**	**2.5**

*, isolates investigated during the North/South Study, 1999; †, eight hGISA isolates received from an unrelated study were not included.

Table 3. Agar screening methods for hGISA.

Screening Medium	Sensitivity %	Specificity %	PPV %	NPV %
MHT5 (0.5)*	71 (51)	86 (94)	78 (82)	81 (65)
MHT5 (2.0)*	100 (95)	41 (68)	54 (62)	100 (100)
MHT5 (Stat)*	100 (99)	9 (17)	44 (59)	100 (100)
BHIT5 (0.5)*	100 (98)	38 (72)	53 (60)	100 (100)
BHIT5 (2.0)*	100 (100)	18 (36)	46 (59)	100 (100)
BHIT5 (Stat)*	100 (100)	6 (11)	43 (59)	100 (100)

*, 0.5, 2.0 indicate McFarland turbidity standard equivalence of bacterial inoculum density used; Stat, stationary phase broth subculture inoculum. PPV, positive predictive value; NPV, negative predictive value. Figures in parentheses indicate values for detecting isolates positive by the E-Test macro-method.

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Acknowledgements

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Characterisation of 73 hGISA Isolates Recovered in Ireland between 1998 and 2003

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Introduction

Reports of meticillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to glycopeptides are becoming more numerous in recent years and laboratory confirmation is difficult.[1] During an investigation of 2909 MRSA isolates from 2728 patients in Ireland between 1998 and 2003, 73 isolates (2.7%) were confirmed as hetero-glycopeptide-intermediate *S. aureus* (hGISA).[2] No vancomycin-resistant *S. aureus* (VRSA) or GISA were found. It is reported that hGISA do not carry vancomycin resistance determinants similar to those found in enterococci[1] but that they carry accessory gene regulator (*agr*) I or II, are defective in *agr* function (as indicated by lack of delta haemolysin production) and have increased ability to bind to artificial surfaces such as polystyrene.[3] This study investigated these characteristics among the 73 hGISA isolates together with epidemiological typing to determine whether there were any traits specific to hGISA that could be used as possible markers to facilitate recognition of potential hGISA.

Materials and Methods

- Confirmation of hGISA Phenotype

The preliminary study used agar screening and the E-test macromethod as screening methods. Isolates with E-test macromethod values ≥8mg/L for both vancomycin and teicoplanin or ≥12mg/L for teicoplanin alone were confirmed as hGISA by vancomycin PAP-AUC ratio determination. PAP-AUC ratio interpretive criteria were: glycopeptide-susceptible *S. aureus* (GSSA), <0.9; hGISA, 0.9-1.29; GISA, ≥1.3.[1]

- Epidemiological Typing

hGISA isolates were typed by antibiogram-resistogram (AR) typing by determining susceptibility to a panel of 23 antimicrobials, by biotyping (pigment production and hydrolysis of urea and Tween 80) and by DNA macrorestriction digestion using *Sma*I followed by pulsed field gel electrophoresis (PFGE). PFGE patterns were assigned 5-digit PFGE type (PFT) numbers which were abbreviated to 2-digit PFT groups (PFG). AR and PFGE typing results were combined to give an AR-PFG type.[4] AR-PFG types of the hGISA isolates were compared with AR-PFGs of MRSA from blood cultures recovered in Irish hospitals between 1999 and 2003 (n = 1580).[4]

- *agr* Type, Delta-haemolysin and Adherence Studies

The 73 hGISA isolates were matched with glycopeptide-susceptible (GS) MRSA isolates exhibiting similar AR-PFG types (n=70). Both sets of isolates were investigated for *agr* type, delta haemolysin production and their ability to bind to polystyrene. *Agr* typing was performed by DNA amplification studies to distinguish between *agr* groups I, II, III and IV.[5,6] Delta-haemolysin expression was determined using sheep red blood cells.[3] Adherence ability was assessed using a polystyrene adherence assay in which bacteria were grown in microtitre trays and, following staining, absorbance at 540 nm measured the isolate's ability to adhere.[3]

- *van* gene detection

Presence of enterococcal glycopeptide resistance genes (*vanA, vanB, vanC, vanD, vanE, vanG*) among hGISA was investigated by a multiplex PCR assay.[7]

Results

- Confirmation of hGISA Phenotype

PAP's of representative hGISA isolates are shown in Figure 1. Also shown are the PAPs of *S. aureus* Mu3 and Mu50 the prototype hGISA and GISA strains, and a GSSA control strain (*S. aureus* ATCC 29213).

- Epidemiological Typing

The most frequently occurring AR-PFG types among hGISA were AR-PFG 13-00 and 14-00 accounting for 59% and 14% of isolates, respectively (Figure 2). In contrast, the predominant AR-PFG among GS-MRSA was AR-PFG 06-01 being exhibited by 57% of isolates; only 14% and 12% of isolates exhibited AR-PFG 13-00 and 14-00, respectively. Distribution of AR-PFG types among the remaining hGISA was: New02-00, 6.9%; 44-99, 5.5%; 43-00, 2.7%; 07-02, 2.7%, 06-01, 1.4% and single isolates exhibiting other AR-PFGs 6.9%, respectively (Figure 2). Previous studies have shown that AR-PFG types 13-00, 14-00, New02-00, 44-99 and 43-00 belong to clonal complex (CC) 8.[4,8]

- *agr* Type, Delta-haemolysin and Adherence Studies

The 73 hGISA and 70 GS-MRSA isolates gave similar results when tested for *agr* type, delta haemolysin production and ability to bind to polystyrene (Table 2).

- *van* Gene Detection

No *van* genes were detected.

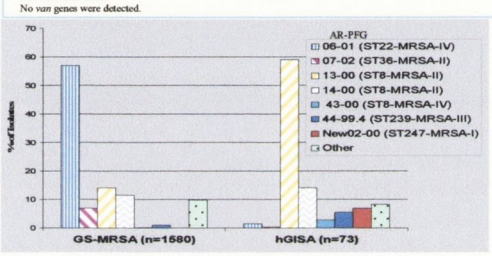

Figure 2. Antibiogram-resistogram type-pulsed field group (AR-PFG) types of glycopeptide susceptible (GS)-MRSA (1999–2003) & hGISA (1998–2003)

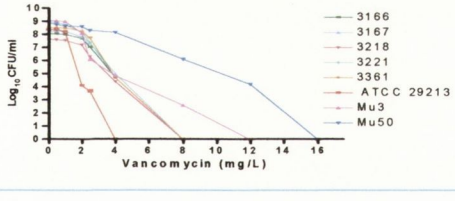

Figure 1. Population analysis profile of five hGISA isolates and control strains {*S. aureus* ATCC 29213, *S. aureus* Mu3 (hGISA) and *S. aureus* Mu50 (GISA)}

Table 2. Comparison of *agr* type, δ-haemolysin production and adherence ability of hGISA and GS-MRSA

	n=	*agr* type I	II	III	δ-Haemolysin	Adherence
hGISA	**73**	**96%**	**2%**	**2%**	**64%**	**90%**
13-00	43	100%	0	0	53%	100%
14-00	10	100%	0	0	90%	100%
07-02	2	0	0	100%	50%	50%
43-00	2	100%	0	0	50%	100%
44-99	4	100%	0	0	100%	100%
New02-00	5	100%	0	0	100%	20%
Other	7	71%	29%	0	57%	71%
GS-MRSA	**70**	**93%**	**3.0%**	**4.0%**	**71%**	**91%**
13-00	44	98%	2%	0	52%	100%
14-00	10	100%	0	0	90%	100%
07-02	2	0	0	100%	0	100%
43-00	2	100%	0	0	50%	100%
44-99	5	100%	0	0	100%	100%
New02-00	2	100%	0	0	100%	0
Other	5	60%	20%	20%	60%	80%

Conclusions

- **AR-PFG type distribution differed between hGISA and GS-MRSA isolates**
- **Most hGISA (89%, 65/73) belong to clonal complex 8**
- **Most hGISA (96%, 70/73) exhibited *agr* type I**
- **Comparison of *agr* type, delta haemolysin and adherence ability yielded similar results for both hGISA and GS-MRSA isolates**
- **No hGISA carried *van* resistance genes**
- **Further studies are needed to identify potential markers for hGISA**

Discussion

Howe *et al.* reported that hGISA has emerged in the five major epidemic MRSA lineages (CC5, CC8, CC22, CC30 and CC45).[9] A previous study from Ireland has shown that AR-PFG types 13-00 and 14-00 belong to CC8 (ST8-MRSA-II).[8] Other AR-PFG types represented in CC8 among hGISA from Ireland include New02-00 (ST247-MRSA-I); 44-99 (ST239-MRSA-III) and 43-00 (ST8-MRSA-IV). Among the 73 hGISA investigated, only eight isolates {07-02 (ST36-MRSA-II) (n = 2); 07-03 (ST5-MRSA-II) (n = 1); 06-01 (ST22-MRSA-IV) (n = 1); ST-MRSA typing results unavailable (n = 4)} were not known to belong to CC8. Although AR-PFG type 13-00 (ST8-MRSA-II) predominated among the hGISA isolates, 14% (223/1580) of GS-MRSA exhibited this AR-PFG. Thus AR-PFG typing cannot be used as a surrogate marker to help recognise potential hGISA. Unlike the predominant strain among GS-MRSA – AR-PFG 06-01 (ST22-MRSA-IV) which tends to be resistant to ≤5 antimicrobials in the AR typing panel, 13-00 (ST8-MRSA-IV) is resistant to up to 15 antimicrobials. Its predominance among hGISA may reflect the fact that this strain has been present in the Irish MRSA population since the early 1990s, whereas AR-PFG 06-01 (ST22-MRSA-IV) did not begin to predominate until 2000.[4,9]

When the 73 hGISA isolates were compared with 70 GS-MRSA exhibiting similar AR-PFG types, the predominant *agr* type in both groups was *agr* I, only 36% hGISA and 29% of GS-MRSA were delta haemolysin negative (i.e. were deficient in *agr* function) and there was little difference in adherence ability between both groups. Hence these characteristics cannot be used to distinguish between hGISA and GS-MRSA isolates in the Irish MRSA population. Lack of *van* determinants among the hGISA isolates confirmed that there was no covert glycopeptide resistance among these isolates.

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Teicoplanin Improves Detection of Hetero-Glycopeptide-Intermediate Resistance in MRSA

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Introduction

The glycopeptide antibiotics vancomycin and teicoplanin play an important role in the treatment of serious staphylococcal disease. Three types of glycopeptide resistance have been described in *Staphylococcus aureus*: vancomycin-resistant *S. aureus* (VRSA) with vancomycin minimum inhibitory concentrations (MIC) of ≥16 mg/L; glycopeptide-intermediate *S. aureus* (GISA) with vancomycin MICs of 4–8 mg/L and/or teicoplanin MICs of 16 mg/L, and hetero-glycopeptide-intermediate *S. aureus* (hGISA) where the majority population has a susceptible MIC but a minority population exhibit MICs in the intermediate category. A hGISA isolate may be defined as a hetero-vancomycin-intermediate *S. aureus* (hVISA) and/or a hetero-teicoplanin-intermediate *S. aureus* (hTISA). These definitions utilise the Clinical and Laboratory Standards Institute (CLSI) glycopeptide breakpoints that were revised in 2006[1] but previous CLSI {formerly National Committee for Clinical Laboratory Standards (NCCLS)}and current British and European breakpoints define GISA as isolates with vancomycin MICs of 8 mg/L.[2,3,4]

Reports of resistance and/or reduced susceptibility of meticillin-resistant *Staphylococcus aureus* (MRSA) to glycopeptides are increasing. To date, six vancomycin-resistant MRSA (VRSA) isolates have been documented in the U.S.A.[5] Reports of GISA remain relatively rare but reports of hGISA vary reflecting difficulties with definitions, diagnostic laboratory detection methods and reference laboratory methods. In a previous study in Ireland, 3,189 MRSA isolates were screened for glycopeptide resistance by agar screening and the E-test™ macromethod; isolates with macromethod values of 8 mg/L for both vancomycin and teicoplanin or 12 mg/L for teicoplanin alone (n = 139) were investigated by vancomycin population analysis profile-area under the curve (PAP-AUC) ratio determination (vPAP-AUC). No VRSA or GISA were detected but 2.4% of isolates (73/2999) were confirmed as hGISA.[6] The present study investigated the role of teicoplanin population analysis profiling (tPAP) for the recognition of hGISA and the effect of reducing the teicoplanin E-test™ macromethod cut-off value to 8 mg/L.

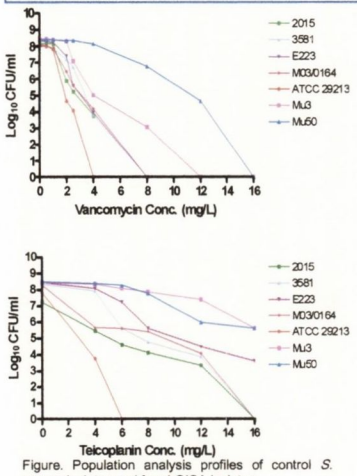

Figure. Population analysis profiles of control *S. aureus* isolates and four hGISA isolates.

Summary

- **Using teicoplanin for PAP and E-test™ macromethod cut-off values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone increased the numbers of hGISA detected by 30% (22/73)**
- **Using teicoplanin for PAP and E-test™ macromethod cut-off values of ≥8 mg/L for teicoplanin alone increased the numbers of hGISA detected by 143% (105/73)**
- **Overall prevalence of hGISA was 6% using both vPAP-AUC and tPAP methods**
- **Early detection of hGISA isolates is important due to the ever increasing threat of emerging glycopeptide resistance among *S. aureus* isolates.**

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Materials and Methods

Population analysis profiling using teicoplanin was performed on all 139 isolates yielding E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone. A further 120 isolates with E-test™ macromethod values of 8 mg/L for teicoplanin were tested by both vPAP-AUC ratio determination and by tPAP. An additional 30 isolates with teicoplanin E-test™ macromethod values of 6 mg/L were also tested by both vPAP-AUC ratio determination and by tPAP.

Overnight tryptic soy broth subcultures and dilutions ($10^{-5}$and 10^{-6}) prepared in saline were inoculated onto Brain Heart Infusion agar plates containing 0.5, 1.0, 2.0, 2.5, 4.0, 8.0, 12.0 and 16.0 mg/L vancomycin and 4.0, 6.0, 8.0, 12.0, and 16.0 mg/L teicoplanin using a spiral plater. After 48 h incubation at 35°C, colonies were counted and the log of the CFU/ml was plotted against both vancomycin and teicoplanin concentrations using GraphPad Prism software (GraphPad Software™, Inc., San Diego, California, USA) . The vancomycin graph was used to calculate the AUC of each isolate and a ratio of the AUC of the test strain to the AUC of *S. aureus* Mu3 was calculated. vPAP-AUC ratio criteria was: GSSA, <0.9; hGISA, 0.9–1.29; GISA, ≥1.3. hTISA isolates were defined as isolates which had resistant subpopulations which grew at concentrations above the susceptible breakpoint (>8 mg/L teicoplanin). *S. aureus* ATCC 700699 (Mu50) the prototype GISA strain, *S. aureus* ATCC 700698 (Mu3) the prototype hGISA strain and *S. aureus* ATCC 29213 which is glycopeptide-susceptible, were used as control strains.

Results

Using the E-test™ macromethod recommended cut-off value of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone 73 isolates were confirmed as hGISA by vPAP-AUC ratio determination, 95 hGISA's were confirmed by tPAP and 108 isolates were confirmed as hGISA by both methods (Table). When the cut-off value for the teicoplanin E-test™ macromethod was lowered to 8 mg/L, an additional 120 isolates required PAP. An additional 70 hGISA's were confirmed of which 17 were confirmed by vPAP-AUC and 70 by tPAP. Lowering the cut-off value to 6 mg/L did not yield any further hGISA. The Figure shows PAP using both vancomycin and teicoplanin of control strains *S. aureus* ATCC 700699 (Mu50), *S. aureus* ATCC700698 (Mu3) and *S. aureus* ATCC 29213 and four hGISA isolates.

Using a teicoplanin E-test™ macromethod cut-off value of 8 mg/L and tPAP, the number of hGISA detected increased by 143% (105/73) and the overall prevalence of hGISA was 6% (178/2999). Further investigation of the characterisation of hTISA isolates is underway.

Numbers of hGISA isolates detected using vancomycin and teicoplanin population analysis profiles

E-test™ macromethod cut -off values (mg/L) Vanc	Teic	Isolates (n =)	vPAP-AUC[a]	tPAP[b]	vPAP-AUC[a] or tPAP[b]	Breakdown of hGISA
8	8	139	73	95	108	60 hVISA and hTISA 35 hTISA / 13 hVISA
…	12					
…	8	120	17	70	70	17 hVISA and hTISA 53 hTISA
…	6	30	0	0	0	
	Total		**90**	**165**	**178**	77 hVISA and hTISA 88 hTISA / 13 hVISA

[a], Population analysis profile-area under the curve ratio determination using vancomycin; [b], Population analysis profiling using teicoplanin

Acknowledgements

We are grateful to the National SARI Committee for funding this work. We thank the participants and organisers of the North / South Study of MRSA in Ireland, 1999 and the EARSS project. We would also like to thank Sanofi-Aventis for the gift of teicoplanin.

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Teicoplanin Improves Detection of Hetero-Glycopeptide-Intermediate Resistance in MRSA

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Introduction

The glycopeptide antibiotics vancomycin and teicoplanin play an important role in the treatment of serious staphylococcal disease. Three types of glycopeptide resistance have been described in *Staphylococcus aureus*: vancomycin-resistant *S. aureus* (VRSA) with vancomycin minimum inhibitory concentrations (MIC) of ≥16 mg/L; glycopeptide-intermediate *S. aureus* (GISA) with vancomycin MICs of 4–8 mg/L and/or teicoplanin MICs of 16 mg/L, and hetero-glycopeptide-intermediate *S. aureus* (hGISA) where the majority population has a susceptible MIC but a minority population exhibit MICs in the intermediate category. A hGISA isolate may be defined as a hetero-vancomycin-intermediate *S. aureus* (hVISA) and/or a hetero-teicoplanin-intermediate *S. aureus* (hTISA). These definitions utilise the Clinical and Laboratory Standards Institute (CLSI) glycopeptide breakpoints that were revised in 2006[1] but previous CLSI {formerly National Committee for Clinical Laboratory Standards (NCCLS)}and current British and European breakpoints define GISA as isolates with vancomycin MICs of 8 mg/L.[2,3,4]

Reports of resistance and/or reduced susceptibility of meticillin-resistant *Staphylococcus aureus* (MRSA) to glycopeptides are increasing. To date, six vancomycin-resistant MRSA (VRSA) isolates have been documented in the U.S.A.[5] Reports of GISA remain relatively rare but reports of hGISA vary reflecting difficulties with definitions, diagnostic laboratory detection methods and reference laboratory methods. In a previous study in Ireland, 3,189 MRSA isolates were screened for glycopeptide resistance by agar screening and the E-test™ macromethod; isolates with macromethod values of 8 mg/L for both vancomycin and teicoplanin or 12 mg/L for teicoplanin alone (n = 139) were investigated by vancomycin population analysis profile-area under the curve (PAP-AUC) ratio determination (vPAP-AUC). No VRSA or GISA were detected but 2.4% of isolates (73/2999) were confirmed as hGISA.[6] The present study investigated the role of teicoplanin population analysis profiling (tPAP) for the recognition of hGISA and the effect of reducing the teicoplanin E-test™ macromethod cut-off value to 8 mg/L.

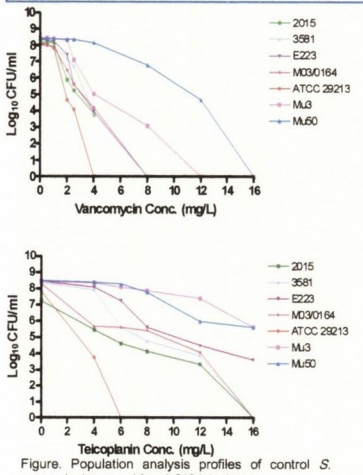

Figure. Population analysis profiles of control *S. aureus* isolates and four hGISA isolates.

Summary

- **Using teicoplanin for PAP and E-test™ macromethod cut-off values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone increased the numbers of hGISA detected by 30% (22/73)**
- **Using teicoplanin for PAP and E-test™ macromethod cut-off values of ≥8 mg/L for teicoplanin alone increased the numbers of hGISA detected by 143% (105/73)**
- **Overall prevalence of hGISA was 6% using both vPAP-AUC and tPAP methods**
- **Early detection of hGISA isolates is important due to the ever increasing threat of emerging glycopeptide resistance among *S. aureus* isolates.**

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Materials and Methods

Population analysis profiling using teicoplanin was performed on all 139 isolates yielding E-test™ macromethod values of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone. A further 120 isolates with E-test™ macromethod values of 8 mg/L for teicoplanin were tested by both vPAP-AUC ratio determination and by tPAP. An additional 30 isolates with teicoplanin E-test™ macromethod values of 6 mg/L were also tested by both vPAP-AUC ratio determination and by tPAP.

Overnight tryptic soy broth subcultures and dilutions ($10^{-3}$and 10^{-6}) prepared in saline were inoculated onto Brain Heart Infusion agar plates containing 0.5, 1.0, 2.0, 2.5, 4.0, 8.0, 12.0 and 16.0 mg/L vancomycin and 4.0, 6.0, 8.0, 12.0, and 16.0 mg/L teicoplanin using a spiral plater. After 48 h incubation at 35°C, colonies were counted and the log of the CFU/ml was plotted against both vancomycin and teicoplanin concentrations using GraphPad Prism software (GraphPad Software™, Inc., San Diego, California, USA) . The vancomycin graph was used to calculate the AUC of each isolate and a ratio of the AUC of the test strain to the AUC of *S. aureus* Mu3 was calculated. vPAP-AUC ratio criteria was: GSSA, <0.9; hGISA, 0.9–1.29; GISA, ≥1.3. hTISA isolates were defined as isolates which had resistant subpopulations which grew at concentrations above the susceptible breakpoint (>8 mg/L teicoplanin). *S. aureus* ATCC 700699 (Mu50) the prototype GISA strain, *S. aureus* ATCC 700698 (Mu3) the prototype hGISA strain and *S. aureus* ATCC 29213 which is glycopeptide-susceptible, were used as control strains.

Results

Using the E-test™ macromethod recommended cut-off value of ≥8 mg/L for both vancomycin and teicoplanin or ≥12 mg/L for teicoplanin alone 73 isolates were confirmed as hGISA by vPAP-AUC ratio determination, 95 hGISA's were confirmed by tPAP and 108 isolates were confirmed as hGISA by both methods (Table). When the cut-off value for the teicoplanin E-test™ macromethod was lowered to 8 mg/L, an additional 120 isolates required PAP. An additional 70 hGISA's were confirmed of which 17 were confirmed by vPAP-AUC and 70 by tPAP. Lowering the cut-off value to 6 mg/L did not yield any further hGISA. The Figure shows PAP using both vancomycin and teicoplanin of control strains *S. aureus* ATCC 700699 (Mu50), *S. aureus* ATCC700698 (Mu3) and *S. aureus* ATCC 29213 and four hGISA isolates.

Using a teicoplanin E-test™ macromethod cut-off value of 8 mg/L and tPAP, the number of hGISA detected increased by 143% (105/73) and the overall prevalence of hGISA was 6% (178/2999). Further investigation of the characterisation of hTISA isolates is underway.

Numbers of hGISA isolates detected using vancomycin and teicoplanin population analysis profiles

E-test™ macromethod cut -off values (mg/L) Vanc	Teic	Isolates (n =)	vPAP-AUC[a]	tPAP[b]	vPAP-AUC[a] or tPAP[b]	Breakdown of hGISA
8	8	139	73	95	108	60 hVISA and hTISA
…	12					35 hTISA / 13 hVISA
…	8	120	17	70	70	17 hVISA and hTISA
						53 hTISA
…	6	30	0	0	0	
Total			**90**	**165**	**178**	77 hVISA and hTISA
						88 hTISA / 13 hVISA

[a], Population analysis profile-area under the curve ratio determination using vancomycin; [b], Population analysis profiling using teicoplanin

Acknowledgements

We are grateful to the National SARI Committee for funding this work. We thank the participants and organisers of the North / South Study of MRSA in Ireland, 1999 and the EARSS project. We would also like to thank Sanofi-Aventis for the gift of teicoplanin.

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Glycopeptide-intermediate *Staphylococcus aureus* (GISA) in Ireland: First Report

The glycopeptide antibiotics vancomycin and teicoplanin play a major role in the treatment of serious staphylococcal disease. Reports of resistance and/or reduced susceptibility of meticillin-resistant *Staphylococcus aureus* (MRSA) to glycopeptides are increasing.[1] To date, six vancomycin-resistant MRSA (VRSA) isolates have been documented in the U.S.A.[2] Three types of glycopeptide resistance have been described in *S. aureus*: full vancomycin resistance {minimum inhibitory concentration (MIC) ≥16 mg/L}, glycopeptide-intermediate *S. aureus* (GISA) (vancomycin MIC 4–8 mg/L and/or teicoplanin MIC 16 mg/L) and hetero-glycopeptide-intermediate *S. aureus* (hGISA) where the majority population has a susceptible MIC but a minority population exhibit MICs in the intermediate category.[2] These definitions utilise the Clinical and Laboratory Standards Institute (CLSI) glycopeptide breakpoints that were revised in 2006[3] but previous CLSI (formerly National Committee for Clinical Laboratory Standards (NCCLS) and current British and European breakpoints define GISA as isolates with vancomycin MICs of 8 mg/L.[4,5,6]

In Ireland, glycopeptide resistance in MRSA has been monitored since 1999 in the National MRSA Reference Laboratory (NMRSARL). NMRSARL investigates MRSA isolates recovered from blood from patients in Irish hospitals that participate in the European Antimicrobial Resistance Surveillance System (EARSS), by agar screening on brain heart infusion (BHI) agar containing 6 mg/L vancomycin (BHIV6) and by the E-test macro-method with both vancomycin and teicoplanin.[7,8] Isolates showing E-test macro-method values of 8 mg/L for both vancomycin and teicoplanin or 12 mg/L for teicoplanin alone are investigated by vancomycin population analysis profile-area under the curve (PAP-AUC) ratio determination. Criteria for interpreting PAP-AUC ratios are: ≤0.89, glycopeptide susceptible *S. aureus* (GSSA); 0.9–1.29, hGISA; ≥1.3, GISA.[8]

No VRSA or GISA were detected among 2,866 MRSA isolates received in NMRSARL between January 1999 and July 2006 but nine isolates exhibited the hGISA phenotype. In August 2006, two isolates from patients in two different hospitals yielded the results shown in table 1.

Table 1. Microbiological data on two MRSA isolates with reduced glycopeptide susceptibility

Patient	BHIV6[a]	E-test[b] (mg/L)	E-test MIC[c] (mg/L)	Broth MIC[c,d] (mg/L)	PAP-AUC Ratio
1	26 colonies	12[c] (16)[e]	4.0	4.0	1.48 (GISA)
2	5 colonies	12[d] (6)[e]	4.0	4.0	1.25 (hGISA)

[a]number of colonies growing on brain heart infusion containing 6 mg/L vancomycin; [b]E-test macro-method; [c]vancomycin; [d]broth microdilution MIC; [e]teicoplanin.

According to CLSI breakpoints (from 2006) both isolates are GISA.[3] PAP-AUC analysis confirms that the isolate from Patient 1 is GISA but suggests that the isolate from Patient 2 is hGISA. Both isolates fail to meet British and European definitions of GISA. Both isolates were sent to the Bristol Centre for Antimicrobial Research and Evaluation (BCARE) and to the Centers for Disease Control (CDC, Atlanta, Georgia, USA) for confirmation. CDC reported that both isolates were GISA on the basis of MIC testing using CLSI 2006 breakpoints but BCARE could not confirm the isolate from Patient 2 as GISA because its PAP-AUC ratio fell below the criterion for GISA.

Clinical details of the two patients are summarised below. Patient 1 was a 67 year-old male who had undergone aortic valve replacement in one hospital (H1) where MRSA was recovered from blood. He was later admitted to another hospital (H2) where MRSA was again recovered and was subsequently transferred to a third hospital (H3) with endocarditis and an aortic root abscess. In H3, the patient's first blood culture isolate was susceptible to vancomycin and resistant to rifampicin. Treatment with vancomycin and gentamicin was initiated but following two weeks treatment with vancomycin, GISA was recovered from blood. Patient 2 was a 58 year-old female on renal dialysis in an unrelated hospital (H4). She had been treated with vancomycin for two two-week periods prior to detection of GISA. Intensive screening of staff and patient contacts in both hospitals yielded no secondary cases.

GISA isolates have been associated with failure of glycopeptide therapy for serious MRSA infection, may arise from GSSA during failed glycopeptide treatment, and are often associated with prosthetic materials.[1] Early reports suggested that GISA isolates were the progeny of one pandemic clone but more recent work has demonstrated the phenotype in all five major MRSA lineages. The emergence of GISA in Ireland emphasises the need for good antibiotic stewardship, the provision of sufficient resources for patient isolation, effective infection control, and adequate laboratory back-up. Failure on any of these fronts increases the risk of the emergence of MRSA with full glycopeptide resistance. To minimise the risk of this very real possibility, major efforts are needed to control the present epidemic of MRSA in Irish hospitals.

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Investigation of Reduced Susceptibility to Glycopeptides among Methicillin-Resistant *Staphylococcus aureus* Isolates from Patients in Ireland and Evaluation of Agar Screening Methods for Detection of Heterogeneously Glycopeptide-Intermediate *S. aureus*[▽]

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Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (n = 3,189) from 2,990 patients were investigated by agar screening and by the Etest macromethod for reduced susceptibility to glycopeptide. No vancomycin-resistant *S. aureus* or glycopeptide-intermediate *S. aureus* (GISA) isolates were detected, but 178 isolates were confirmed as hetero-GISA (hGISA) by vancomycin population analysis profile (vPAP)-area under the curve (AUC) ratio determination and/or teicoplanin PAP (tPAP) methods. Of 139 isolates detected using the recommended Etest macromethod cutoff values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone, 73 were confirmed as hGISA by vPAP-AUC, 95 were confirmed as hGISA by tPAP, and 108 were confirmed as hGISA by both methods. An Etest macromethod cutoff value of 8 mg/liter for teicoplanin alone detected a further 70 hGISA (17 were confirmed by vPAP-AUC and 70 were confirmed by tPAP). Agar screening utilizing brain heart infusion (BHI) agar containing 6 mg of vancomycin/liter (BHIV6) and Mueller-Hinton (MH) agar containing 8 mg of teicoplanin/liter (MHT8) failed to detect hGISA. MH agar containing 5 mg of teicoplanin/liter (MHT5) and BHI containing 5 mg of teicoplanin/liter (BHIT5) were evaluated using 10-μl volumes of three inoculum concentrations (with densities equivalent to 0.5 and 2.0 McFarland turbidity standards and stationary-phase BHI broth subcultures [$MHT5_{0.5}$, $MHT5_{2.0}$, $MHT5_S$, $BHIT5_{0.5}$, $BHIT5_{2.0}$, and $BHIT5_S$]). The sensitivity of all methods except $MHT5_{0.5}$ and $MHT5_{2.0}$ was 100%. The specificity ranged from 4 to 82%. $BHIT5_{0.5}$ yielded the best performance, with a specificity of 84% for detecting isolates with teicoplanin Etest macromethod values of ≥8 mg/liter. Screening on $BHIT5_{0.5}$ is useful where screen-positive isolates are investigated with the Etest macromethod and confirmed by vPAP-AUC and tPAP. The prevalence of hGISA among patients with blood culture isolates recovered in Irish hospitals between 1999 and 2003 was 2.6%, whereas the prevalence among patients with isolates from all specimen sites collected during a 2-week survey in 1999 was 12%. The prevalence in one hospital decreased from 5.3% in 2003 to 1.5% in 2004.

Clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) with reduced susceptibility to glycopeptide were first described in Japan in 1997 (15). To date, three types of reduced susceptibility to glycopeptides have been described in *S. aureus*: vancomycin-resistant *S. aureus* (VRSA), glycopeptide-intermediate *S. aureus* (GISA), and hetero-GISA (hGISA). VRSA isolates exhibit vancomycin MICs of ≥16 mg/liter (5, 9, 13). According to the 2006 revision of the Clinical and Laboratory Standards Institute (CLSI) guidelines, isolates are considered GISA if they have vancomycin MICs of 4 to 8 mg/liter and/or teicoplanin MICs of 16 mg/liter, but current British and European breakpoint criteria define GISA as isolates with vancomycin MICs of 8 mg/liter (5, 9, 13). With isolates described as hGISA, the majority of bacteria exhibit susceptible MICs, but a minority population (perhaps as few as 10^{-6} cells) exhibit MICs in the intermediate category (15). The terms GISA or hGISA have been proposed to describe isolates with reduced susceptibility to vancomycin (vancomycin-intermediate *S. aureus* [VISA] or hetero-VISA [hVISA]) or to teicoplanin (teicoplanin-intermediate *S. aureus* [TISA] or hetero-TISA [hTISA]) or to both vancomycin and teicoplanin (6, 24).

Reports of VRSA, GISA, and hGISA among MRSA isolates are increasing (16). Between 2002 and 2005, six clinical VRSA isolates (MIC of ≥32 mg/liter) were reported from the United States, where resistance has been shown to be mediated by *vanA*, which encodes glycopeptide resistance in vancomycin-resistant enterococci (7). More recently, two VRSA isolates with vancomycin MICs of 32 to 64 mg/liter have been reported from India, but *vanA* genes were not detected (25). There has also been a report of VRSA from Jordan, where isolates were investigated by agar dilution and Etest MIC determination only (4). GISA isolates have been reported from many countries worldwide but are still relatively rare (6, 27). Isolates of hGISA occur more frequently with reported prevalence rates ranging from 0 to 74%; this variation reflects difficulties with definitions, screening methods, confirmatory techniques, and interpretative criteria used in different studies (6, 17, 27). Attempts to assess the clinical significance of GISA and especially hGISA are complicated by these problems, but infection with

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TABLE 1. Numbers of isolates investigated by screening methods and by MIC determination

MRSA collection no., name, yr (no. of isolates)[a]	No. of isolates tested (no. positive) by screening method[b]:						No. of isolates tested for MIC as determined by:	
	BHIV4	BHIV6	MHT8	$MHT5_S$	$BHIT5_{0.5}$	Etest macromethod	Etest	CLSI[c]
1, H1 Q4, 1998 (188)	188 (84)	84 (0)	–	84 (33)	–	84 (7)	7	84
2, N/S Study, 1999 (714)	714 (489)	489 (0)	–	489 (178)	–	489 (74)	74	489
3, EARSS, 1999–2003 (1,580)	–[e]	1580 (0)	–	87[d] (30)	–	1,580 (15)	169	87[d]
4, NMRSARL, 2000–2003 (97)	–	97 (0)	–	–	–	97 (19)	19	–
5, H1 Q2, 2003 (330)	–	330 (0)	330 (0)	330 (37)	330 (73)	73 (18)	18	–
6, H1 Q4, 2004 (280)	–	280 (0)	–	–	280 (25)	25 (6)	6	–
Total (3,189)	902 (573)	2,860 (0)	330 (0)	990 (278)	610 (98)	2,348 (139)	293	660

[a] Abbreviations: N/S Study, North/South Study of MRSA in Ireland 1999.
[b] The numbers in parentheses indicate the numbers of strains determined to be positive by each screening method. For screening methods BHIV6, MHT8, $MHT5_S$, and $BHIT5_{0.5}$, growth of ≥1 colony indicated a positive result.
[c] CLSI broth microdilution.
[d] Isolates recovered between 1999 and 2002 exhibiting Etest macromethod values of >4 mg/liter for vancomycin and/or teicoplanin.
[e] –, Not done.

GISA and hGISA has been associated with glycopeptide therapy failure (17).

Guidelines for the detection of VRSA and GISA have been published, but finding suitable guidance for the detection of hGISA is problematic (6, 17). Standard disk diffusion, broth microdilution MIC, and standard Etest MIC determinations all fail to detect hGISA, probably because the initial inoculum density used with these tests is too low to detect the resistant subpopulation (6, 26). The population heterogeneity of hGISA isolates is demonstrated when isolates are investigated by population analysis (15). A modified population analysis profile (PAP) method where the area under the curve (AUC) is calculated for each test isolate and compared to the AUC of the prototype hGISA isolate Mu3 (*S. aureus* ATCC 700698) has been described (29). This PAP-AUC ratio method has been reported to be a reliable and reproducible method of detecting hGISA but is time-consuming and unsuitable for use in clinical laboratories (27). Walsh et al. have described a modification of the Etest system—the Etest macromethod—wherein a heavy inoculum (200 μl of bacterial suspensions with densities equivalent to a 2.0 McFarland turbidity standard [6×10^8 CFU/ml]; nutritious medium, i.e., brain heart infusion [BHI] agar) and prolonged incubation (48 h) have been shown to reliably detect isolates exhibiting reduced susceptibility to glycopeptides with sensitivity and specificity values of 96 and 97%, respectively, compared to the PAP-AUC method (26). However, widespread use of Etest strips for diagnostic laboratories is expensive, and a simple, reliable, cost-effective screening method for the detection of hGISA is needed for use in clinical laboratories.

Several agar screening methods that differ in medium composition, inoculum density, choice of glycopeptide (vancomycin or teicoplanin), and/or antibiotic concentration have been proposed (6, 10, 14, 15, 19). Hiramatsu et al. screened with BHI agar containing 4 mg of vancomycin/liter (BHIV4) and an inoculum density of 10^6 CFU/ml and used population analysis for confirmatory testing (15). The sensitivity and specificity of screening on BHIV4 were shown to be 71 and 88%, respectively, compared to PAP-AUC ratio determination (26). In the same study, screening with Mueller-Hinton (MH) agar containing 5 mg of vancomycin/liter (MHV5) and BHI containing 6 mg of vancomycin/liter (BHIV6) failed to detect hGISA. Other studies suggested screening with BHI agar containing 6 mg of teicoplanin/liter (BHIT6) or MH agar containing 5 mg of teicoplanin/liter (MHT5) (10, 14). The European Antimicrobial Resistance Surveillance System (EARSS) recommends screening on MHT5 with a stationary-phase broth culture (using 10-μl volumes) and incubation for 48 h (12).

In Ireland, MRSA is a serious nosocomial problem, and the rates of methicillin resistance in *S. aureus* isolates recovered from blood are among the highest in Europe (20). A study in 1999 reported that there were no VRSA or GISA among 714 MRSA isolates investigated, and although 5% of the isolates yielded screening results suggestive of hGISA, the hGISA phenotype was not confirmed by PAP (22). In the present study, 3,189 MRSA isolates from 2,990 patients recovered between 1998 and 2004 were investigated to determine the prevalence of VRSA, GISA, and hGISA among MRSA isolates in Ireland and to evaluate agar screening methods for the detection of hGISA.

MATERIALS AND METHODS

Bacterial isolates. MRSA isolates (n = 3,189 isolates from 2,990 patients) from six collections of MRSA recovered between 1998 and 2004 were investigated. Isolates were collected from the following sources: (i) all MRSA isolates recovered in one 936-bed tertiary-referral adult university hospital (H1) during quarter 4 (Q4), 1998 (n = 188) (21); (ii) all MRSA isolates recovered during a 2-week study period in 1999 from all hospitals that participated in the North/South Study of MRSA in Ireland (n = 714) (22); and (iii) MRSA isolates from blood cultures (one isolate per patient per quarter) from Irish laboratories that participated in the EARSS project between 1999 and 2003 (n = 1,580). In Ireland, participation in EARSS provides almost 100% population coverage, and all MRSA isolates reported are sent to the National MRSA Reference Laboratory (NMRSARL) for confirmation and further investigation (20). Collection 4 consisted of all MRSA isolates submitted to the NMRSARL between 2000 and 2003 for investigation of reduced susceptibility to glycopeptides (n = 97). Collection 5 comprised all MRSA isolates recovered from H1 during Q2, 2003 (n = 330) (20), while collection 6 consisted of all MRSA isolates recovered from H1 during a prospective study in Q4, 2004 (n = 280). In general, one isolate per patient was included unless routine susceptibility tests in the diagnostic laboratory suggested the patient was carrying more than one strain of MRSA. Isolate details are summarized in Table 1.

Isolates were identified as MRSA as described previously and stored at −70°C

on cryoprotective beads (Protect Bacterial Preservers, Technical Service Consultants, Ltd., Hayward, United Kingdom) (11). All investigations were undertaken by using overnight subcultures on Columbia agar (LabM, Lab 1; International Diagnostics Group, plc, Bury, Lancashire, United Kingdom) containing 7% horse blood.

Preliminary agar screening. Isolates from collections 1 (n = 188) and 2 (n = 714) were screened on BHIV4 using a heavy inoculum (200-μl volumes of bacterial suspensions with densities equivalent to a 2.0 McFarland turbidity standard [6×10^8 CFU/ml]) and incubation for 48 h at 35°C as described previously (11, 21). All isolates that grew on BHIV4 from collections 1 and 2 (n = 84 and n = 489, respectively) were included in the present study.

All isolates (n = 2,860), the 573 isolates from collections 1 and 2 and the 2,287 isolates from collections 3 to 6 were screened with BHI agar (CM0375; Oxoid, Ltd., Basingstoke, England) containing 6 mg of vancomycin (vancomycin hydrochloride [V2002]; Sigma-Aldrich, Ltd., Tallaght, Ireland)/liter (BHIV6) as previously described (24). In a pilot study, all 330 isolates in collection 5 were screened on MH agar (catalog no. 225250; Becton-Dickinson and Company [BD], Columbia, MD) containing teicoplanin (teicoplanin sodium salt; Sanofi-Aventis, Paris, France) at a concentration of 8 mg/liter (MHT8) using an inoculum of 10^6 CFU/ml (19).

All isolates that grew on BHIV4 from collections 1 and 2 (n = 84 and n = 489, respectively), all isolates from collection 3 recovered between 1999 and 2002 yielding Etest macromethod values of >4 mg/liter for vancomycin and/or teicoplanin (n = 87), and all isolates in collection 5 (n = 330) were screened on MH agar containing 5 mg of teicoplanin/liter using 10-μl volumes of stationary-phase BHI broth (CM0225; Oxoid) subcultures ($MHT5_S$) delivered by using 10-μl loops (12). In all, 990 isolates were screened on $MHT5_S$. Plates were incubated at 35°C, and growth was observed after 24 and 48 h. Growth of ≥1 colony after 48 h of incubation indicated a positive result.

The following control strains were used: *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* Mu3 ATCC 700698, *S. aureus* Mu50 ATCC 700699, *Enterococcus faecalis* ATCC 29212, and *E. faecalis* ATCC 51299.

Etest macromethod. Etest macromethod values were determined according to the manufacturer's instructions using vancomycin and teicoplanin Etest strips (AB Biodisk, Ltd., Solna, Sweden) (2). Briefly, 200-μl volumes of bacterial suspensions prepared in saline to a density equivalent to 2.0 McFarland turbidity standard (6×10^8 CFU/ml) were pipetted onto each BHI agar plate, and the inoculum was spread evenly with a swab. Plates were dried at room temperature, Etest strips were applied, and plates were incubated at 35°C for 48 h. The interpretative criteria for reduced susceptibility were values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone as recommended by the manufacturer (2, 26). All isolates from collections 1 and 2 that grew on BHIV4, all isolates in collections 3 and 4, and any isolate from collections 5 and 6 that grew on any screening media were investigated by the Etest macromethod. Details of the isolates investigated are summarized in Table 1.

MIC determination. Etest MICs for vancomycin and teicoplanin were performed according to the manufacturer's instructions on all isolates exhibiting Etest macromethod values ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone (n = 139). Etest MICs were also determined for any isolate from collection 3 that yielded Etest macromethod values >4 mg/liter for vancomycin and/or teicoplanin (n = 169). Broth microdilution MICs for vancomycin and teicoplanin were performed on all isolates requiring further investigation from collections 1 and 2 (n = 84 and 489, respectively) and on all collection 3 isolates recovered between 1999 and 2002 yielding Etest macromethod values of >4 mg/liter for vancomycin and/or teicoplanin (n = 87) (8). The quality control strains used with MIC determinations were *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212. Details of the isolates investigated are summarized in Table 1.

Population analysis profiling. Colonies from cultures grown overnight on Columbia agar containing 7% horse blood were inoculated into tryptic soy broth (211825; BD). After incubation for 24 h, dilutions of 10^{-3} (10^5 CFU/ml) and 10^{-6} (10^2 CFU/ml) were prepared in saline. Volumes (50 μl) were inoculated onto BHI agar plates containing 0, 0.5, 1.0, 2.0, 2.5, 4.0, 8.0, 12.0, or 16.0 mg of vancomycin/liter and 0, 4.0, 6.0, 8.0, 12.0, or 16.0 mg of teicoplanin/liter, respectively, using a spiral plater (Don Whitley Scientific, Ltd., Shipley, United Kingdom) (18, 29). After 48 h of incubation at 35°C, the colonies were counted, and the log of the CFU/ml was plotted against the antibiotic concentration by using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) to obtain vancomycin PAPs (vPAP) and teicoplanin PAPs (tPAP). The vPAP graph was used to calculate the AUC of each isolate, and the ratio of the AUC of the test isolate to the AUC of *S. aureus* Mu3 (ATCC 700698) was calculated (29). The vPAP-AUC ratio criteria were as follows: glycopeptide-susceptible *S. aureus* (GSSA), <0.9; hGISA, 0.9 to 1.29; and GISA, ≥1.3. The criterion to define hTISA isolates using tPAP was any isolate with a resistant subpopulation that grew at concentrations above the susceptible breakpoint (>8 mg of teicoplanin/liter) (18). PAPs were performed on all isolates with Etest macromethod values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone (n = 139). In addition, PAPs were also established for any isolate yielding a teicoplanin macromethod value of 8 mg/liter (n = 119). A pilot study of isolates yielding Etest macromethod values of 6 mg/liter (n = 36) also had PAPs performed. Control isolates *S. aureus* Mu3 (ATCC 700698) (prototype hGISA strain), *S. aureus* Mu50 (ATCC 700699) (prototype GISA strain), and *S. aureus* ATCC 29213 (GSSA) were included with each batch of isolates tested.

Evaluation of agar screening methods. Six agar screen methods were investigated using 110 hGISA and 68 GSSA isolates. The hGISA isolates were detected by initial screening with the Etest macromethod using both vancomycin and teicoplanin and were confirmed by PAP methods using both vancomycin and teicoplanin. The 68 GSSA isolates comprised 24 isolates that yielded values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone by Etest macromethod screening and were negative by vPAP and tPAP methods and 44 isolates that yielded negative screening results with the Etest macromethod. The latter GSSA isolates included 10 with Etest macromethod values of ≥4 mg/liter and ≤8 mg/liter for both vancomycin and teicoplanin and 34 isolates with Etest macromethod values of ≤2 mg/liter for both vancomycin and teicoplanin. During preliminary agar screening, it had been noted that 51 isolates shown subsequently to be hGISA by vPAP and/or tPAP methods failed to grow on $MHT5_S$ when the inoculum was delivered by a 10-μl loop. Therefore, when evaluating the six agar screen methods, the inoculum was delivered by pipette.

The media evaluated were MHT5 and BHI agar containing 5 mg of teicoplanin/liter (BHIT5). Each medium was investigated by using 10-μl volumes from three inoculum preparations consisting of bacterial suspensions prepared in saline to densities equivalent to 0.5 and 2.0 McFarland turbidity standards, respectively (i.e., $MHT5_{0.5}$, $BHIT5_{0.5}$, $MHT5_{2.0}$, and $BHIT5_{2.0}$) and from stationary-phase subcultures grown in BHI broth (10^8 CFU/ml) ($MHT5_S$ and $BHIT5_S$). Twelve isolates were inoculated onto each plate, and screening tests were performed in duplicate. Plates were incubated at 35°C, and growth was observed after 24 and 48 h. Growth of ≥1 colony after 48 h of incubation indicated a positive result. After the evaluation, the medium with the best overall performance ($BHIT5_{0.5}$) was used to screen isolates from collections 5 (n = 330) and 6 (n = 280). Any isolate that yielded growth on $BHIT5_{0.5}$ was investigated further by the Etest macromethod as described above. The sensitivity, specificity, and positive and negative predictive values were calculated for all media evaluated.

RESULTS

No VRSA or GISA isolates were detected among the 3,189 isolates investigated, but 5.6% of isolates (178 of 3,189) from 5.8% of patients (172 of 2,990) exhibited the hGISA phenotype (Table 2). The criterion used to define hGISA was any isolate recovered by any screening method with a vPAP-AUC ratio of 0.9 to 1.29 and/or a tPAP with a resistant subpopulation growing at concentrations >8 mg of teicoplanin/liter (18, 29).

Preliminary agar screening. The agar screening methods used are summarized in Table 1. No isolates grew on BHIV6 or MHT8 agar screen plates. Of 990 isolates screened on $MHT5_S$, 278 yielded growth. These isolates comprised 81 hGISA isolates, but an additional 51 hGISA isolates failed to grow on this medium.

Etest macromethod. The numbers of isolates yielding positive screening results by the Etest macromethod are shown in Tables 1 and 2. In total, 2,348 isolates were screened by the Etest macromethod and 139 yielded values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone (Tables 1 and 2). When a cutoff value of 8 mg/liter for teicoplanin alone was accepted as the criterion for a positive macromethod result, an additional 119 isolates required further investigation by PAP methods (Table 2).

TABLE 2. Numbers of isolates yielding positive screening results as determined by the Etest macromethod and numbers of hGISA isolates detected by the vPAP and/or tPAP methods

MRSA collection no., name, yr (no. of isolates/no. of patients)[a]	No. of isolates yielding positive screening results by the Etest macromethod			hGISA[c]	
	V and T ≥ 8 or T ≥ 12 (mg/liter)[b]	T = 8 (mg/liter)[c]	T ≥ 8 (mg/liter)[d]	No. of isolates (%)	No. of patients (%)
1, H1 Q4, 1998 (188/169)	7	8	15	10 (5.3)	10 (5.9)
2, N/S study, 1999 (714/682)	74	62	136	81 (11)	81 (12)
3, EARSS, 1999–2003 (1,580/1,507)	15	31	46	39 (2.5)	39 (2.6)
4, NMRSARL, 2000–2003 (97/59)	19	11	30	28* (29)	22* (37)
5, H1 Q2, 2003 (330/303)	18	7	25	16 (4.9)	16 (5.3)
6, H1 Q4, 2004 (280/270)	6	0	6	4 (1.4)	4 (1.5)
Total (3,189/2,990)	139	119	258	178 (5.6)	172 (5.8)

[a] See Table 1, footnote *a*.
[b] Cutoff values of ≥8 mg/liter for both vancomycin (V) and teicoplanin (T) or ≥12 mg/liter for teicoplanin alone.
[c] Cutoff value of 8 mg/liter for teicoplanin (T) alone.
[d] Cutoff value of ≥8 mg/liter for teicoplanin (T).
[e] *, hGISA isolates comprised of 27 hGISA isolates from 22 patients and 1 isolate from an environmental source. Single isolates were recovered from 19 patients; two isolates were recovered from 1 patient, and three isolates were recovered from 2 patients.

MIC determination. Etest MIC results for both vancomycin and teicoplanin showed that all 293 isolates investigated had MICs ≤2 mg/liter for vancomycin and ≤8 mg/liter for teicoplanin. Similarly, all 660 isolates selected for testing by using the broth microdilution method yielded vancomycin and teicoplanin MICs of ≤2 mg/liter and ≤8 mg/liter, respectively. Hence, there were no VRSA or GISA strains among these MRSA isolates.

Population analysis profiling. Of 139 isolates yielding Etest macromethod values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone, 73 were confirmed by vPAP-AUC ratio to be hGISA (Table 3). When tPAP was used for confirmation of hGISA, an additional 35 isolates were recognized as hGISA (shown in Table 3). When the Etest macromethod cutoff value was decreased to 8 mg/liter for teicoplanin alone, a further 119 isolates required PAP and 70 additional hGISA were confirmed. Lowering the cutoff value to 6 mg/liter for teicoplanin did not yield any further hGISA. In total, 178 hGISA were detected comprising 13 hVISA, 88 hTISA, and 77 hVISA and hTISA isolates (shown in Tables 2 and 3). vPAP-AUC and/or tPAP analysis showed that 69% (178 of 258) of isolates with a macromethod value of ≥8 mg/liter for teicoplanin were hGISA.

TABLE 3. Numbers of hGISA isolates detected using vancomycin and/or teicoplanin population analysis profiling and breakdown into hVISA, hTISA, and hVISA and hTISA

Group	No. of isolates investigated by PAP	No. of hGISA isolates detected					
		vPAP-AUC	tPAP	Total hGISA	hVISA[c]	hTISA[d]	hVISA and hTISA
1[a]	139	73	95	108	13	35	60
2[b]	119	17	70	70	0	53	17
Total	258	90	165	178	13	88	77

[a] Group 1 includes isolates exhibiting Etest macromethod values of ≥8 mg/liter for vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone.
[b] Group 2 includes additional isolates requiring PAP when the teicoplanin Etest macromethod cutoff value was decreased to 8 mg/liter.
[c] hVISA is defined as isolates with vPAP-AUC ratios ranging from 0.9 to 1.29.
[d] hTISA is defined as isolates with resistant subpopulations capable of growth at >8 mg of teicoplanin/liter.

Evaluation of screening methods. The sensitivity, specificity, and positive and negative predictive values of $MHT5_{0.5}$, $MHT5_{2.0}$, $MHT5_S$, $BHIT5_{0.5}$, $BHIT5_{2.0}$, and $BHIT5_S$ for the detection of hGISA are shown in Table 4. The sensitivity of all methods except $MHT5_{0.5}$ was ≥98% (the sensitivity of $MHT5_{0.5}$ was 66%), but the specificities ranged from 4 to 82%. During preliminary agar screening with $MHT5_S$, when the inoculum was delivered by using a 10-μl loop, the sensitivity and specificity of $MHT5_S$ were 61 and 77%, respectively. When the inoculum was delivered by pipette, sensitivity improved to 100%, but the specificity decreased to 6%.

When the specificity of each of the six agar screen methods to detect isolates with Etest macromethod values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone was considered, the methods with the best performance were $BHIT5_{0.5}$ and $MHT5_{2.0}$, with sensitivities of 98 and 95% and specificities of 70 and 60%, respectively. In the course of the present study, the use of tPAP analysis and a criterion for a positive Etest macromethod result of 8 mg/liter for teicoplanin alone allowed detection of an additional 70 hGISA isolates. Using this criterion, the sensitivity and specificity of $BHIT5_{0.5}$ to detect isolates with Etest macromethod values of ≥8 mg/liter for teicoplanin were 99 and 84%, resepctively. The two isolates with positive Etest macromethod values that failed to grow on $BHIT5_{0.5}$ were shown by vPAP-AUC and tPAP analysis to be GSSA.

$BHIT5_{0.5}$ was used to screen all isolates in collections 5 and 6. Seventy-three isolates from collection 5 and 25 isolates from collection 6 were screen positive (Table 1). Of these, 25 isolates from collection 5 and 6 isolates from collection 6 yielded positive macromethod values, whereas 16 isolates from collection 5 and 4 from collection 6 were confirmed as hGISA (Table 2).

Prevalence of VRSA, GISA, and hGISA. No VRSA or GISA isolates were detected. Although the overall proportion of hGISA isolates was 5.6% (178 of 3,189), the proportions varied among different groups of isolates (Table 2). Among patients

TABLE 4. Evaluation of six agar screening methods to detect hGISA and isolates yielding positive Etest macromethod values

Method	Determined value (%)[a]											
	hGISA				Etest macromethod (approach 1)[b]				Etest macromethod (approach 2)[c]			
	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV
$MHT5_{0.5}$	66	82	89	61	59	85	95	47	58	91	95	42
$MHT5_{2.0}$	98	53	77	95	95	60	85	84	96	73	91	84
$MHT5_S$	100	6	63	100	99	6	71	75	99	7	76	75
$BHIT5_{0.5}$	100	57	79	100	98	70	88	95	99	84	95	95
$BHIT5_{2.0}$	100	18	66	100	100	23	75	100	100	27	80	100
$BHIT5_S$	100	4	63	100	100	6	71	100	100	7	77	100

[a] Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.
[b] Values for detecting isolates with Etest macromethod values of ≥8 mg/liter for vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone.
[c] Values for detecting isolates with Etest macromethod values of ≥8 mg/liter for teicoplanin alone.

with blood culture isolates investigated under the EARSS project, the prevalence was 2.6% (39 of 1,507), but among patients investigated during the North/South Study of MRSA in Ireland, 1999 prevalence was 12% (81 of 682). In H1, between Q2 2003 and Q4 2004, the proportion fell from 5.3% (16 of 303) to 1.5% (4 of 270).

DISCUSSION

Since its emergence in 1997, the clinical significance of hGISA has been debated and, although it has been suggested that hGISA may be a laboratory-induced phenomenon, hGISA isolates have been associated with treatment failure and have been shown to be precursors of GISA (3, 15, 23, 28). Accurate assessment of the clinical significance of hGISA requires reliable screening and confirmatory methods (17). Studies have reported that the original method described by Hiramatsu et al. (10^6 CFU/ml inoculated onto BHIV4) did not reliably detect hGISA isolates (29). In preparation for the present study, the preliminary selection of isolates from collections 1 and 2 using screening with BHIV4 utilized an inoculum concentration of 10^8 CFU/ml to ensure no hGISA isolates went undetected (11, 21).

In the clinical laboratory, it is likely that many hGISA isolates are unrecognized because the recommended screening methods present problems for diagnostic laboratories. The Etest macromethod is expensive if it is to be performed on all *S. aureus* isolates, and confirmatory testing with population analysis is labor-intensive, time-consuming, and unsuitable for routine use. In the present study, when the efficacy of various preliminary agar screen methods was assessed, the method of inoculum delivery was found to be a crucial factor. EARSS guidelines for detection of reduced susceptibility to glycopeptides suggest preliminary screening with MHT5 using a stationary-phase broth subculture inoculum and investigating any growth with the Etest macromethod (12). In the present study, the value of MHT5 as a preliminary screening method varied depending on whether the inoculum was delivered by a standard 10-μl loop when the sensitivity and specificity were 61 and 77%, respectively, or by pipette, where the sensitivity increased to 100% but the specificity fell to 6%. This poor specificity makes the method unsuitable for routine diagnostic use. Of the other agar screening media evaluated, $BHIT5_{0.5}$ using an inoculum of 10^6 CFU/ml proved to be the most useful agar screen for detecting hGISA, with a sensitivity of 100% and a specificity of 57%. The specificity increased to 84% when the method was used as a screening technique to detect isolates with Etest teicoplanin macromethod values of ≥8 mg/liter. $BHIT5_{0.5}$ also has the practical advantage for the diagnostic laboratory that the inoculum density (equivalent to a 0.5 McFarland turbidity standard) is the same as that used for routine CLSI disk diffusion susceptibility testing.

Subsequent to the present study, further evaluation of $BHIT5_{0.5}$ was undertaken at the NMRSARL to investigate sensitivity and specificity when the criterion for a positive result was growth of >1 colony. Among 492 blood culture MRSA isolates screened with both the Etest macromethod and $BHIT5_{0.5}$ agar screening between June 2006 and May 2007, sensitivity and specificity values for the detection of (i) hGISA, (ii) isolates exhibiting Etest macromethod values of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone, and (iii) isolates exhibiting teicoplanin Etest macromethod values of ≥8 mg/liter were 100 and 84% for all three categories when the criterion for a positive result was the growth of ≥1 colony. When the criterion was changed to >1 colony, the sensitivity and specificity values were 100 and 92% for categories i and ii and 95 and 94% for category iii, respectively. Population analysis profiling showed that the single isolate that exhibited a teicoplanin Etest macromethod value of 8 mg/liter and yielded one colony on $BHIT5_{0.5}$ was GSSA.

Recently, Wootton et al. compared three screening methods for detecting GISA and hGISA isolates and found that $MHT5_{2.0}$ and the Etest macromethod (using an 100-μl inoculum volume) yielded sensitivity and specificity values of 86 and 76% and 82 and 89%, respectively (30). In an earlier study in which the inoculum volume was 200 μl, the sensitivity and specificity of the Etest macromethod was reported to be 96 and 97% when the interpretative criteria for a positive screening result were ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone (26). In the present study, 200-μl inoculum volumes were used as recommended by the manufacturer's technical guide for the detection of reduced susceptibility to glycopeptides, but there is a discrepancy between this recommendation and the recommendation to use a 100-μl volume inoculum in the manufacturer's Etest application sheet for staphylococci (1, 2). When the interpretative criterion of 8 mg/liter for teicoplanin alone for a positive Etest macromethod was used, the numbers of hGISA detected increased by 23% (17 of 73) when the vPAP-AUC ratio deter-

mination was used as the confirmatory method. The sensitivity and specificity of screening with $BHIT5_{0.5}$ to detect isolates with this positive Etest macromethod value were 99 and 84%, whereas screening on $MHT5_{2.0}$ yielded sensitivity and specificity values of 96 and 73%, respectively. Thus, a practical approach to detecting hGISA isolates in clinical laboratories is to screen on $BHIT5_{0.5}$ and to investigate positive results by the Etest macromethod, with final confirmation using PAP.

In the present study, when the Etest manufacturer's criteria of ≥8 mg/liter for both vancomycin and teicoplanin or ≥12 mg/liter for teicoplanin alone and vPAP-AUC only was used, the proportion of hGISA isolates detected was 2.3% (73 of 3,189). However, decreasing the teicoplanin Etest macromethod cutoff value to 8 mg/liter and including tPAP to confirm the hGISA phenotype increased the number of hGISA detected by 144% (105 of 73), bringing the overall proportion of hGISA to 5.6% (178 of 3,189). Rates varied from 1.5 to 37% depending on the patient population studied. A prevalence rate of 2.6% was found among blood culture isolates submitted to NMRSARL under the EARSS project where the protocol required data on the first isolate per patient per quarter. Since the hGISA phenotype tends to be associated with prolonged exposure to glycopeptide, a lower prevalence among this population might be expected (27). However, the lowest prevalence was observed in H1 during the prospective study in 2004 when isolates from all specimen sites were investigated. During that study, additional isolates were sought from patients on prolonged glycopeptide therapy, but only one further hGISA isolates was detected.

In summary, the present study has shown that $BHIT5_{0.5}$ is a useful method of screening for hGISA where screen-positive isolates are investigated by the Etest macromethod and confirmed by PAP. The currently recommended Etest macromethod cutoff criteria need to be reevaluated. tPAP is needed to ensure that the phenotype of all presumptive hGISA isolates is confirmed. A simpler hGISA confirmatory method suitable for use in clinical laboratories is required. The prevalence of hGISA among MRSA isolates recovered from blood culture in Ireland is 2.6%.

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