Methicillin-resistant *Staphylococcus aureus*: a novel approach to molecular detection and a US countywide study of strain diversity and distribution among healthcare facilities

Lyndsey Olivia Hudson

Imperial College London

Department of Infectious Disease Epidemiology

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I declare that all work presented in this thesis is my own, unless otherwise stated.

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global public health problem and is a major cause of morbidity and mortality worldwide, imposing serious economic costs on patients and hospitals. Prior to the mid-1990s, MRSA was largely a healthcare-associated pathogen, causing infection predominantly in people with frequent or recent contact with healthcare facilities (HA-MRSA). Since then, community-associated MRSA (CA-MRSA), which often causes infection among healthy children and young adults with no exposure to the healthcare setting, has become increasingly prevalent. Worryingly, there is evidence that CA-MRSA is penetrating the healthcare MRSA reservoir, and even replacing traditional HA-MRSA strains. This highlights the need to keep abreast of the changing epidemiology of MRSA in order to implement effective infection control strategies. To investigate the composition of the healthcare MRSA reservoir and ascertain the extent to which CA-MRSA has penetrated this reservoir, a countywide, population-based cohort study of MRSA in hospital inpatients and nursing home residents was conducted in Orange County (OC), California, covering a total of 46 facilities. CA-MRSA was found to be fully mixed with HA-MRSA in the hospital setting. The predominant CA-MRSA clone in the US, USA300, was the most commonly isolated MRSA clone in OC hospitals. In OC nursing homes, HA-MRSA (specifically a variant of USA100 that is also very common in OC hospitals but has not been reported elsewhere) predominates, but USA300 made up just over a quarter of the isolates and was the second most frequently isolated clone. Both OC hospitals and nursing homes were dominated by the same three strains: USA300, USA100 and a variant of USA100. Not only are community-based infection control strategies needed to stem the influx of community associated strains, in particular USA300, into the hospital setting, but also strategies tailored to the complex problem of MRSA transmission and infection

in nursing homes, to minimise the impact of the unique nursing home MRSA reservoir on overall regional MRSA burden. A key component of effective infection control strategies is prompt isolation of MRSA carriers, facilitated by rapid diagnostics. PCR-based methods of MRSA detection offer a much faster alternative to traditional culture techniques, but are expensive and often complex to operate. A novel nucleic acid amplification technique developed by my industrial sponsor, TwistDx Ltd, called recombinase polymerase amplification (RPA), has been incorporated into a probe based detection system called TwistAmp MRSA, and offers a simple and cheap alternative to current commercial PCR-based assays, amplifying MRSA to detectable levels within 20 minutes. I tested the assay with diverse collections of MRSA and discovered that 4% of isolates from a UK MRSA collection could not be detected by the assay. I subsequently developed RPA primers for their detection. Nonetheless, TwistAmp MRSA was able to detect most MRSA strains, and was comparable to current commercial assays in this respect. Despite a very high analytical sensitivity of approximately 20 CFU/swab, the clinical sensitivity of TwistAmp MRSA was lower than expected with respect to the current market leader, Xpert MRSA. I investigated lysis and filtration methods to improve the assay's clinical sensitivity, but found that such methods did not currently warrant inclusion in the TwistAmp MRSA protocol. While TwistAmp MRSA performance is in line with current assays, and is a faster, cheaper and simpler assay, a problem faced by all molecular methods of MRSA detection is the constant emergence of undetectable MRSA strains, necessitating continual assay evaluation and improvement where possible.

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LIST OF ABBREVIATIONS

$1-D$	Simpson's index of diversity
5′ or 3′	5-prime (upstream) or 3-prime (downstream) of DNA
°C	Degrees centigrade
χ^2	Chi-square
μg	Microgram
μl	Microlitre
μm	Micrometre
μM	Micromolar
ABCs	Active Bacterial Core surveillance
ACME	Argenine catabolic mobile element
AIDS	Acquired immunodeficiency syndrome
ATP/ADP/ AMP	Adenosine tri-/di-/mono-phosphate
β	Beta
BLAST	Basic local alignment search tool
bp	base pair
BURP	Based upon repeat pattern
BURST	Based upon related sequence type
CA-MRSA	Community-associated MRSA
CC	clonal complex
ccr	Cassette chromosome recombinase
CDC	US Centers for Disease Control and Prevention
CFU	Colony forming unit
CI	Confidence Interval
cm	Centimetre
CMFT	Central Manchester University Hospitals NHS Foundation Trust
CNS	Coagulase negative staphylococci
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DLV	Double locus variant
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP)

dTTP	Deoxythymidine triphosphate
EARSnet	European Antimicrobial Resistance Surveillance network
EDSI	Excision, duplication, substitution and indels (insertion/deletions)
EDTA	Ethylenediaminetetraacetic acid
EMRSA	Epidemic MRSA
EQA	External quality assessment
FAM	Carboxyfluorescin
FDA	US Food and Drug Administration
GISA	Glycopeptide-intermediate *Staphylococcus aureus*
HA-MRSA	Healthcare-associated MRSA
HEX	Hexachlorofluorescein
ICU	Intensive care unit
IgG	Immunoglobulin G
IQR	Interquartile range
IS	Insertion sequence
IWG-SCC	International Working Group on the Classification of SCC Elements
kb	kilobase
KC	Kansas City, US
L	Litre
LA-MRSA	Livestock-associated MRSA
LOD	Limit of detection
LTAC	Long-term acute care
MEGA	Molecular evolutionary genetics analysis
mer	Length of an oligonucleotide e.g. a 35-mer is 35bp in length
mg	Milligram
Mg	Magnesium
Mg	Magnesium acetate
$MgCl_2$	Magnesium chloride
ml	Millilitre
mM	Millimolar
MLST	Multi-locus sequence typing
MR-CNS	Methicillin-resistant coagulase negative staphylococci
MREP	*mec* right extremity polymorphism
MREJ	*mec* right extremity junction
MRSA	Methicillin-resistant *Staphylococcus aureus*

MS-CNS	Methicillin-sensitive *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesive matrix molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
mV	MilliVolt
NF	No filter/unfiltered
ng	Nanogram
NGS	Next generation sequencing
$(NH_4)_2SO_4$	Ammonium sulphate
NPV	Negative predictive value
NT	Non-typeable
NTC	No template control
OC	Orange County
ORF	Open reading frame
p	P-value (probability)
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFGE	Pulsed field gel elctrophoresis
Pol I	Polymerase I
PPV	Positive predictive value
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
PVL	Panton-Valentine leukocidin
QCMD	Quality Control for Molecular Diagnostics
r	Correlation coefficient
ROX	Carboxy-x-rhodamine
RPA	Recombinase polymerase amplification
s	Second
S. aureus	*Staphylococcus aureus*
SCC	Staphylococcal cassette chromosome
SCC*mec*	Staphylococcal cassette chromosome *mec*
SDW	Sterile distilled water
SE	Standard error
SLV	Single locus variant
SNP	Single nucleotide polymorphism

spa	Staphylococcal protein A
spa-CC	*spa*-clonal complex
SSB	Single-stranded DNA binding protein
SSTI	Skin and sof tissue infection
ST	Sequence type
SWP	Southwest Pacific
TAMRA	Carboxytetramethylrhodamine
TAT	Turnaround time
TBE	Tris-Borate-EDTA
Tris-Cl/HCL	Tris-chloride/hydrochloride
TSB	Tryptic soy broth
TSST	Toxic shock syndrome toxin
u	Unified atomic mass unit
UCI	University of California, Irvine
V	Volt
VISA	Vancomycin-resistant *Staphylococcus aureus*
VRSA	Vancomycin-intermediate *Staphylococcus aureus*
w/v	weight/volume

CHAPTER 1: INTRODUCTION

1.1 STAPHYLOCOCCUS AUREUS CARRIAGE AND DISEASE

Staphylococcus aureus is the most important pathogenic species of the *Staphylococcus* genus, which contains more than 30 species. In contrast to most other Staphylococci, *S. aureus* has pathogenic potential even in the absence of clear host conditions that predispose them to infection, such as immunodeficiency. *S. aureus* is a non-motile, non-spore-forming, gram-positive, catalase-positive and primarily coagulase-positive facultative anaerobe. Occuring as cluster-forming cocci, and forming white-grey to golden-yellow colonies, *S. aureus* bacteria are often haemolytic on blood agar and most ferment mannitol.

S. aureus is one of the most important human pathogens, occurring worldwide, and responsible for healthcare-, community- and livestock-associated colonisation and infection. It is an opportunistic pathogen that colonises the human skin and mucosa, the primary reservoir being the anterior nares, and is present in 30% to 50% of healthy adults, about 20% of which are persistently colonised [Lowy 1998, Wertheim et al. 2005]. Extra-nasal sites include the groin, pharynx, axillae, skin, perineum and vagina [Wertheim et al. 2005]. Those colonised with *S. aureus* are at increased risk of subsequent infection and disease, ranging from mild skin and soft tissue infections (SSTIs) such as folliculitis and furunculosis to life-threatening, invasive infections such as pneumonia, deep abscesses and sepsis [Lowy 1998, Wertheim et al. 2005]. *S. aureus* also colonises several different animal species, where it can cause disease such as bovine mastitis [Annemuller, Lammler and Zschock 1999].

The diverse range of *S. aureus* disease has been attributed to its ability to

produce an array of virulence factors [Begun et al. 2005] (Figure 1.1) which include MSCRAMMs (microbial surface components recognising adhesive matrix molecules) such as protein A, clumping factor A and fibronectin binding protein A, and exoproteins such as pyrogenic toxin superantigens, exfoliative toxins, and leukocidin [Dinges, Orwin and Schlievert 2000]. Infections are initiated when a breach of the skin or mucosal barrier allows *S. aureus* to infiltrate adjoining tissues or the bloodstream. Whether the infection spreads or not depends on a complex interplay between *S. aureus* virulence determinants and host defence mechanisms [Lowy 1998]. Risk factors for infection therefore include: colonisation, immunodeficiency (e.g. AIDS), underlying medical conditions (e.g. type 1 diabetes), surgical wounds, intravenous drug use, and invasive medical devices (e.g. catheters). *S. aureus* is usually transmitted by direct skin-to-skin contact or by sharing contaminated items or using contaminated surfaces [Boyce et al. 1997, Shiomori et al. 2002, Miller and Diep 2008, Snyder et al. 2008, Caron and Mousa 2010, Uhlemann et al. 2011].

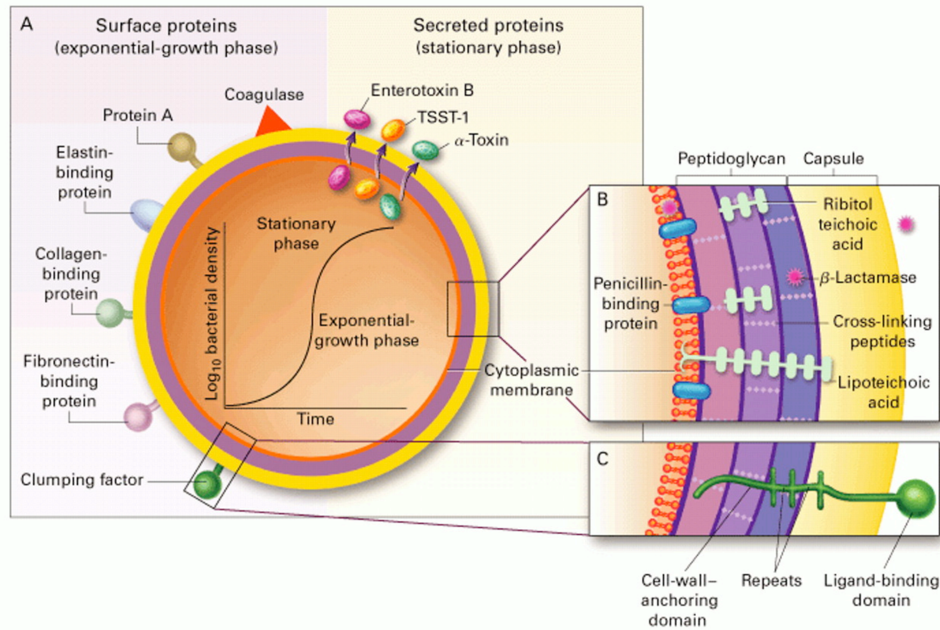


Figure 1.1 *Staphylococcus aureus* structure: surface and secreted proteins/virulence factors. A: surface protein synthesis is usually dependent on the growth phase and secreted protein synthesis on the stationary phase. B: cross section of the cell envelope. C: many of the surface proteins have a structural organization similar to that of clumping factor, including repeated segments of amino acids. TSST-1=toxic shock syndrome toxin 1 (a pyrogenic toxin superantigen). Figure taken from [Lowy 1998].

1.2 ANTIBIOTIC RESISTANCE: EMERGENCE AND MECHANISMS

The first clinical isolate of methicillin-resistant *S. aureus* (MRSA) was reported in 1961 in the UK. Now often called the 'archaic' clone, it carries staphylococcal cassette chromosome *mec* type I (SCC*mec* I). SCC*mec* is a mobile genetic element that carries *mecA*, the gene conferring methicillin resistance in *S. aureus* (see section 1.2.1). The archaic MRSA clone was reported just 1 year after the introduction of methicillin, a β-lactam antibiotic developed to counter the increasing prevalence of penicillin resistance in gram-positive bacteria [Jevons 1961]. Since then, *S. aureus* has developed or acquired resistance mechanisms to almost all

antibiotics that have been introduced over the past decades, including β-lactams, aminoglycosides, quinolones and glycopepetides [Lowy 2003]. After the first reports of MRSA in the 1960s, it gradually disseminated around Europe [Crisostomo et al. 2001], and began causing serious hospital infections worldwide in the 1970s [Hiramatsu et al. 2001]. By the 1980s, the archaic clone had largely disappeared from European hospitals, and descendents of this clone (e.g. the Iberian clone) as well as new lineages of MRSA had emerged (Table 1.1), causing significant clinical and epidemiological problems in hospitals [Oliveira, Tomasz and de Lencastre 2002]. In 1982, the New York/Japan Clone (SCC*mec* II) was discovered and also spread worldwide, followed by the discovery in 1985 of the 85/2082 MRSA strain in New Zealand (SCC*mec* III). These and new MRSA strains disseminated around the world during the 1990s, contributing to the worldwide healthcare-associated MRSA (HA-MRSA) pandemic in hospitals and other healthcare facilities such as nursing homes, that continues today (Table 1.1). From the 1990s, MRSA harbouring a new SCC*mec* element, type IV, had emerged, and the WIS MRSA strain (SCC*mec* V) was described in Australia [Udo, Pearman and Grubb 1993, Ma et al. 2002, Vandenesch et al. 2003, Ito et al. 2004].

Table 1.1 Common healthcare-associated MRSA strains. Taken from Chambers and Deleo [2009].

Clonal complex	Sequence type	Common name(s)	Comment and SCC*mec* allotype
CC5	ST5	USA100, NewYork or Japan clone	The most common health care-associated MRSA strain in the United States; SCC*mec*II
	ST5	EMRSA-3	SCC*mec*I
	ST5	USA800 or paediatric clone	Prevalent in Argentina, Colombia and the United States; SCC*mec*IV
	ST5	HDE288 or paediatric clone (in Portugal)	SCC*mec*VI
CC8	ST250	Archaic	The first MRSA clone to be identified, includes the COL strain; SCC*mec*I
	ST247	Iberian clone or EMRSA-5	A descendant of COL-type strains; SCC*mec*I
	ST239	Brazilian or Hungarian clone	SCC*mec*III
	ST239	EMRSA- 1	An Eastern Australian epidemic clone of the 1980s; SCC*mec*III
	ST239	AUS-2 and AUS-3	Common Australian multidrug-resistant clones of the early 2000s; SCC*mec*III
	ST8	Irish-1	Common hospital-acquired isolate in the 1990s in Europe and the United States; SCC*mec*II
	ST8	USA500, EMRSA-2 or EMRSA-6	SCC*mec*IV
CC22	ST22	EMRSA-15	An international clone that is prominent in Europe and Australia; SCC*mec*IV
CC30	ST36	USA200 or EMRSA-16	The single most abundant cause of MRSA infections in UK hospitals and the second most common cause of MRSA infections in US hospitals in 2003; SCC*mec*II
CC45	ST45	USA600	SCC*mec*II
	ST45	Berlin clone	SCC*mec*IV

CC, clonal complex; MRSA, methicillin-resistant *Staphylococcus aureus*; SCC*mec*, staphylococcal chromosome cassette *mec*; ST, sequence type.

The emergence of new strains harbouring SCC*mec* types IV and V coincided with the emergence of community-associated MRSA (CA-MRSA), which were susceptible to most antibiotics other than β-lactams, and caused infection in otherwise healthy children and young adults with no risk factors for MRSA [Udo, Pearman and Grubb 1993, Herold et al. 1998, CDC 1999, Coombs et al. 2004, O'Brien et al. 2004]. Although CA-MRSA tend to be associated with skin and soft tissue infections (SSTIs), they are also highly virulent, causing severe, invasive infection, often leading to death. CA-MRSA have since been reported in virtually every geographic region of the world and in various populations, such as indigenous

peoples, competitive athletes, prison inmates, men who have sex with men, military recruits and personnel, children in day care centres, contacts of patients with CA-MRSA infection, and adult emergency room patients [Adcock et al. 1998, Shahin et al. 1999, Groom et al. 2001, CDC 2003c, CDC 2003b, CDC 2003a, Baggett et al. 2004, CDC 2004b, Zinderman et al. 2004, Aiello et al. 2006, Johansson, Gustafsson and Ringberg 2007, Tristan et al. 2007a, Diep et al. 2008a, Wallin, Hern and Frazee 2008]. CA-MRSA strains are also being increasingly reported as a cause of hospital-onset and healthcare-associated infections [O'Brien et al. 1999, Saiman et al. 2003, Bratu et al. 2005, Klevens et al. 2006, Seybold et al. 2006, Liu et al. 2008, Patel et al. 2008, Park et al. 2009b].

In the last decade, MRSA strains harbouring three new SCC*mec* elements were reported, in Portugal (a healthcare-associated paediatric clone; SCC*mec* VI) [Oliveira, Milheirico and de Lencastre 2006], Sweden (a community-associated strain; SCC*mec* VII) [Berglund et al. 2008] and Canada (a healthcare-associated strain; SCC*mec* VIII). Most recently, a further three SCC*mec* elements have been described, SCC*mec* types IX, X and XI, which are associated with livestock-associated MRSA (LA-MRSA) [Garcia-Alvarez et al. 2011, Li et al. 2011]. Livestock are an increasingly recognised reservoir for MRSA, with LA-MRSA carriage and infection reported in both farm animals and human beings [de Neeling et al. 2007, van Loo et al. 2007, Mulders et al. 2010, Van Cleef et al. 2010, van Cleef et al. 2010, Garcia-Alvarez et al. 2011].

The continual discovery of novel SCC*mec* elements represents an ongoing evolution of antibiotic resistance in *S. aureus* (although novel SCC*mec* acquisition is not necessarily driven by antibiotic resistance). Since β-lactams (such as methicillin) have been the first-line antibiotics for treatment of *S. aureus* infections, such

evolution may likely impact on therapeutic options [Monecke et al. 2011]. *S. aureus* has quickly acquired resistance to all antibiotics introduced for clinical use, and many MRSA isolates are multiply antibiotic-resistant. Alternative antibiotics to β-lactams for treatment of MRSA infections include daptomycin, linezolid, vancomycin and rifampicin, but they are expensive or have problems with tissue penetration and toxicity [Monecke et al. 2011]. Until recently, all MRSA were considered susceptible to glycopeptide antibiotics such as vancomycin - considered the antibiotic of last resort for MRSA infections - and investigational drugs [Enright et al. 2002, Lowy 2003]. However, due to intensive selective pressure as a result of increased glycopeptide use, MRSA isolates increasingly resistant to vancomycin have been reported worldwide (vancomycin intermediate/resistant *S. aureus*; VISA/VRSA) [Hiramatsu et al. 1997a, Hiramatsu et al. 1997b, Howe et al. 1998, Ploy et al. 1998, Sieradzki et al. 1999, Smith et al. 1999, Ferraz et al. 2000, Kim et al. 2000, Wong et al. 2000, Boyle-Vavra, Carey and Daum 2001, Hageman et al. 2001, Oliveira et al. 2001, CDC 2002a, CDC 2002b, Weigel et al. 2003, CDC 2004a, Howe et al. 2004], leading to treatment failures and poor outcomes [Fridkin et al. 2003, Moore, Perdreau-Remington and Chambers 2003, Charles et al. 2004, Howden et al. 2004]. MRSA resistant to linezolid, daptomycin and rifampicin have also been reported [Schmitz et al. 2000, Mangili et al. 2005, Long et al. 2006, Marty et al. 2006, Skiest 2006, Murthy et al. 2008, Kehrenberg et al. 2009, Shore et al. 2010, Tan et al. 2011], posing a great problem for antimicrobial therapy.

1.2.1 Mechanism of methicillin resistance

MRSA produces a modified penicillin-binding protein, PBP2A, which has a low affinity for β-lactam antibiotics [Hartman and Tomasz 1984, Reynolds and

Brown 1985, Utsui and Yokota 1985], conferring resistance to all β-lactams (including penicillins, cephalosporins (except ceftobiprole [Stein, Goetz and Ganea 2009]), carbapenems, and monobactams), the most commonly used antibiotics to treat *S. aureus* infections. β-lactams bind to PBPs in the cell wall, inhibiting peptidoglycan synthesis in susceptible microbes, but PBP2A retains effective transpeptidase activity in the presence of β-lactams, unlike the PBPs native to *S. aureus*, allowing cell wall synthesis to continue. The transpeptidase domain of PBP2A functions cooperatively with the transglycosylase domain of the native staphylococcal PBP2 to achieve cell wall synthesis in the presence of β-lactams [Pinho, de Lencastre and Tomasz 2001]. PBP2A is encoded by the *mecA* gene, carried on the mobile genetic element SCC*mec*. MRSA arises when methicillin-susceptible *S. aureus* (MSSA) acquires SCC*mec*. Evidence suggests this acquisition comes from coagulase negative staphylococci (CNS) [Archer et al. 1996, Kobayashi et al. 1999, Wielders et al. 2001, Robinson and Enright 2003, Wisplinghoff et al. 2003, Qi et al. 2005, Grundmann et al. 2006], and has occurred several times into different *S. aureus* lineages [Musser and Kapur 1992, Crisostomo et al. 2001, Fitzgerald et al. 2001, Oliveira, Tomasz and de Lencastre 2001, Enright et al. 2002, Gomes, Westh and de Lencastre 2006] i.e. the multi-clone theory.

The SCC*mec* element contains the *mec* gene complex (the *mecA* gene and its regulators, *mecI*, encoding a repressor protein, and *mecR1*, encoding a signal transducer protein, both of which are sometimes truncated) and the *ccr* (cassette chromosome recombinase) gene complex, which encodes site-specific recombinases (*ccrA*, *ccrB* and *ccrC*) responsible for the mobility of SCC*mec* [Ito et al. 2004]. There are curently 11 different SCC*mec* elements (21-53kb in size) formed by different combinations of the *mec* and *ccr* gene complexes (Tables 1.2 and 1.3, Figure 1.2).

The recombinases catalyse the insertion/excision of SCC*mec* into/from the *S. aureus* genome at a specific site (the bacterial chromosome attachment site for SCC*mec* DNA, attBscc) at the 3′ end of an open reading frame (ORF) of unknown function, *orfX*, located near the origin of replication of *S. aureus*. The various SCC*mec* types can be further classified into subtypes based upon variations in the so-called J regions (or 'joining regions'), J1 (the region between *ccr* and the chromosomal region flanking SCC*mec*), J2 (the region between *mec* and *ccr*), and J3 (the region between *orfX* and *mec*), which constitute nonessential components of the cassette [IWG-SCC 2009]. The presence of specific DNA sequences in these J regions are used to define SCC*mec* subtypes, including mobile genetic elements such as insertion sequences (ISs), plasmids or transposons, most of which encode antibiotic resistance (e.g. to aminoglycosides or macrolides), resistance to heavy metals (e.g. Cd and Hg), or other determinants, and characteristic genes, pseudogenes or non-coding regions in the J regions [Oliveira, Wu and de Lencastre 2000, Ito et al. 2001, IWG-SCC 2009]. Mobile genetic elements encoding antibiotic resistance are mainly integrated into the J2 or J3 regions, while subtype-specific ORFs are used to distinguish the several different J1 regions in SCC*mec* types II and IV [Chongtrakool et al. 2006]. Horizontal transfer of DNA from other strains or species plays an important part in antibiotic resistance in *S. aureus*, despite *S. aureus* evolution being regarded as predominantly clonal [Enright et al. 2000, Grundmann et al. 2002, Feil et al. 2003, Murchan et al. 2003, Melles et al. 2004]. For example, the recent emergence of VRSA is due to the acquisition by conjugative transposition of *vanA*-containing elements from vancomycin-resistant enterococci [CDC 2002b, CDC 2002a, Chang et al. 2003].

Table 1.2 Currently identified *ccr* and *mec* gene complexes in *S. aureus*. Adapted from IWG-SCC [2009].

***ccr* complex**	***ccr* genes**
1	*ccrA1* and *ccrB1*
2	*ccrA2* and *ccrB2*
3	*ccrA3* and *ccrB3*
4	*ccrA4* and *ccrB4*
5	*ccrC*
6	*ccrA5* and ccr*B3*
7	*ccrA1* and *ccrB6*
8	*ccrA1* and *ccrB3*
***mec* complex**	
A	IS*431*-*mecA*-*mecR1*-*mecI*[a]
B	IS*431*-*mecA*-Δ*mecR1*-IS*1272*
C1	IS*431*-*mecA*-Δ*mecR1*-IS*431*
C2	IS*431*-*mecA*-Δ*mecR1*-IS*431*[b]
D	IS*431*-*mecA*-Δ*mecR1*
E	blaZ-*mec*A_{LGA251}-*mec*$R1_{LGA251}$-*mec*I_{LGA251}

[a] IS = insertion sequence.

[b] In the C2 *mec* gene complex the orientation of IS*431* upstream of *mecA* is reversed.

Table 1.3 SCC*mec* types defined by the combination of *mec* and *ccr* gene complexes. Adapted from IWG-SCC [2009].

SCC*mec* type	*ccr*/*mec* gene complex combination	Size (kb)	Representative strain(s)
I	1B	34	NCTC10442, COL
II	2A	53	N315, Mu50, Mu3, MRSA252, JH1, JH9
III	3A	35	85/2082, ANS46
IV	2B	21-24	CA05, MW2m 8/6-3P, 81/108, 2314, cm11, JCSC4469, M03-68, EMRSA-15, JCSC6668, JCSC6670
V	5C2	28	WIS, TSGH17, PM1
VI	4B	21	HDE288
VII	5C1	33	JCSC6082
VIII	4A	32	C10682, BK20781
IX	1C2	44	JCSC6943
X	7C1	51	JCSC6945
XI	8E	29	LGA251

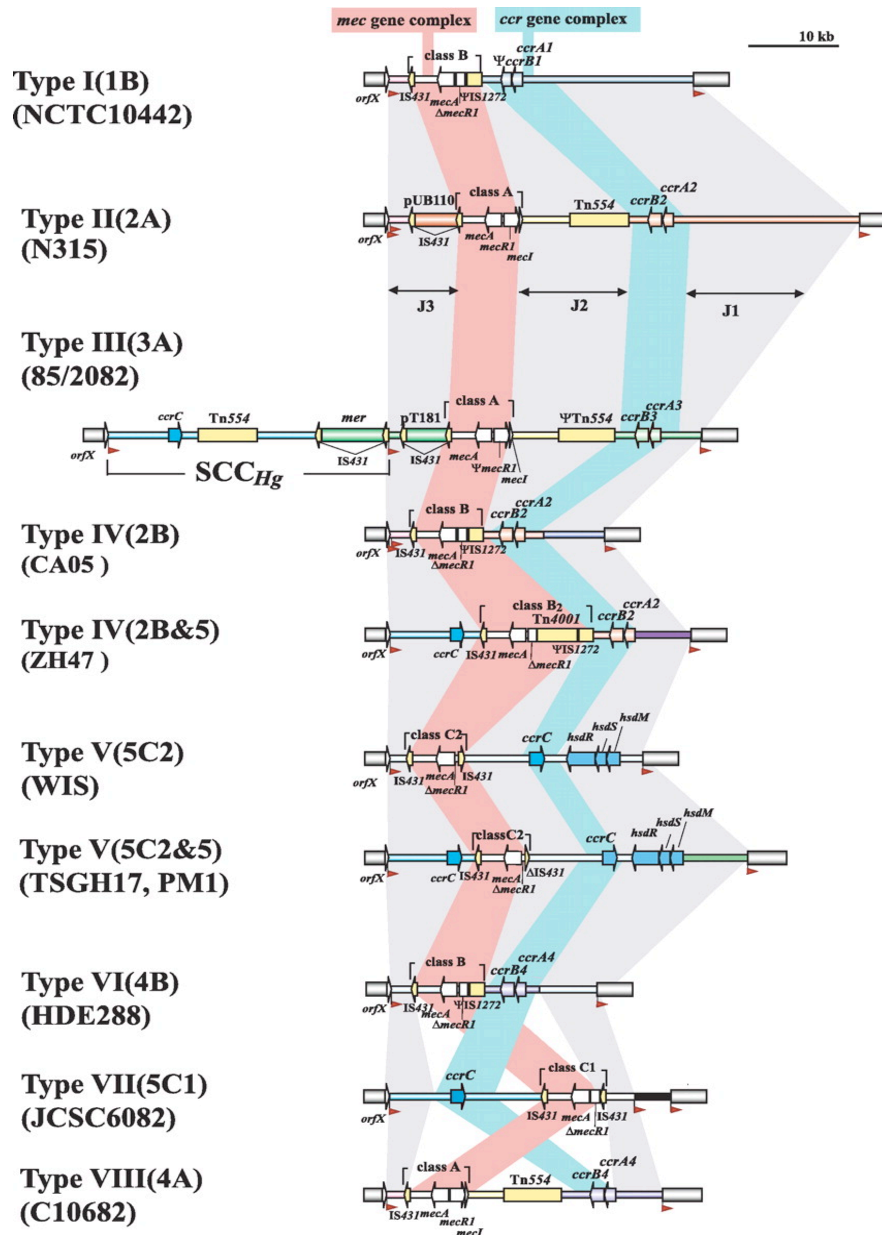


Figure 1.2 Structural comparison of SCC*mec* types I-VIII. Figure taken from IWG-SCC [2009]. Structures of recently described types IX-XI not shown (see Garcia-Alvarez et al. 2011 and Li et al. 2011).

1.3 MOLECULAR EPIDEMIOLOGY OF *S. AUREUS*

Clones of *S. aureus* are relatively stable, and the acquisition of SCC*mec* elements through horizontal DNA transfer is a relatively rare event. As a result of studies using molecular typing methods such as multi-locus sequence typing (MLST,

see section 1.3.2), it is thought that a limited number of genetically distinct epidemic clones circulate and disseminate worldwide (Table 1.1), with SCC*mec* and other mobile genetic elements conferring enhanced virulence and antibiotic resistance, maintained in the predominantly clonal genomic background [Oliveira, Tomasz and de Lencastre 2002, Enright 2003, Robinson and Enright 2003]. However, a more recent study, using a highly discriminatory single nucleotide polymorphism (SNP)-discovery method, provided evidence that the population of MRSA comprising the so-called EMRSA-3, New York/Japan and Paediatric clones (multi-locus sequence type ST5) is geographically structured, and that MRSA could have emerged very frequently in different parts of the world through independent imports of the methicillin resistance determinant into their genomes [Deurenberg and Stobberingh 2008, Nubel et al. 2008]. Studies on the population structure of a different lineage, ST239, have suggested dissemination rather than repeated emergence is the cause of its global prevalence, although phylogeographic structure was also found as in the ST5 group [Harris et al. 2010, Smyth et al. 2010, Gray et al. 2011]. A study of the emerging ST225 clone also suggests long-distance dissemination as opposed to repeated importation of SCC*mec* [Nubel et al. 2010]. More studies employing SNP-discovery and genomic comparison methods are required to elucidate the relative contributions of dissemination and local emergence to global MRSA population structure.

In the absence of frequent inter-strain recombination, *S. aureus* clones mainly diversify through the accumulation of single nucleotide polymorphisms (SNPs), thus making it possible to distinguish clones and clonal lineages using genetic markers [Feil et al. 2003, Grundmann et al. 2010]. Molecular typing methods use these genetic markers to not only track the transmission and spread of clones, but answer questions

regarding their evolution and epidemiology, in order to develop effective strategies for controlling the spread of MRSA. The most commonly used molecular typing methods are pulsed field gel electrophoresis (PFGE), MLST, *spa* typing and SCC*mec* typing. All allow the typing of unrelated strains but do so with different accuracy, discriminatory power, and reproducibility [Melles et al. 2007]. Recent advances in whole genome sequencing have shown that almost all isolates of a single strain differ to some extent in nucleotide sequence, allowing the detailed spread and microevolution of *S. aureus* strains to be studied.

1.3.1 Pulsed field gel electrophoresis (PFGE)

PFGE is the most commonly used and one of the most discriminatory typing methods for studying local MRSA epidemiology such as outbreaks and nosocomial transmission [Cookson et al. 2007]. PFGE is based on digestion of chromosomal DNA with restriction enzyme *Sma*I followed by agarose gel electrophoresis. The resulting banding patterns are analysed using software such as Bionumerics (Applied Maths), compared to banding patterns of reference strains, and PFGE types defined based on a similarity coefficient. Due to the nature of the method, efforts to standardize PFGE at an international level have not been successful in terms of reproducibility, speed and analysis costs. A common nomenclature is needed, which has been achieved only at a national level [Deurenberg and Stobberingh 2008].

1.3.2 Multi locus sequence typing (MLST)

MLST has become established as an important tool for unambiguously defining strains and for studying MRSA clonal evolution, although whole genome sequencing will inevitably supersede it. MLST is based on the sequence analysis of seven *S.*

aureus housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*) [Enright et al. 2000]. For each locus, different sequences are assigned as different alleles, and each isolate is assigned a sequence type (ST) based on its allelic profile (the set of alleles at all seven loci) (www.mlst.net). *S. aureus* STs are grouped within clonal complexes (CCs), which are groups of STs where every member of the group has a 6/7 allelic match to at least one other ST in the group. The putative ancestor or founder of each CC is the ST with the largest number of single-locus variants (SLVs), and sub-founders are SLVs or double locus variants (DLVs) of a predicted founder that has become prevalent in a population and diversified to produce its own SLVs and DLVs [Enright and Spratt 1999, Enright et al. 2000, Enright et al. 2002, Spratt et al. 2004]. CCs are defined using the based upon related sequence types (BURST) algorithm (www.eburst.mlst.net) and are named by the ST number of the predicted founder [Spratt et al. 2004].

Although highly discriminatory, MLST may lack the necessary power to discriminate between epidemiologically unrelated strains [Cooper and Feil 2004], and is laborious and time-consuming. Nonetheless, MLST offers a major advantage over pulsed field gel electrophoresis (PFGE) as a reference method due to the unambiguous nature of the procedure allowing excellent reproducibility.

A common nomenclature for MRSA is the combination of ST and SCC*mec* type. For example the New York/Japan clone is ST5-MRSA-II and EMRSA-15 is ST22-MRSA-IV.

1.3.3 spa typing

The *spa* locus of *S. aureus* encodes staphylococcal protein A, a species-specific protein known for its immunoglobulin G (IgG) binding capacity. *spa* typing

targets the highly polymorphic region X of the *spa* gene. This region consists of a variable number of mainly 24-bp repeats, the variation being largely due to deletions and duplications of the different repeats [Shopsin et al. 1999]. *spa* typing is simple compared to MLST as it requires the sequencing of just one locus, and its discriminatory power lies between that of PFGE and MLST [Malachowa et al. 2005]. Unlike MLST, both molecular evolution and hospital outbreaks can be studied with *spa* typing, and comparability and a common nomenclature is possible thanks to dedicated software [Harmsen et al. 2003, Deurenberg et al. 2007]. Because it is a single-locus typing method, it is less expensive, less laborious and less time consuming than MLST. The *spa* typing database, spaserver.ridom.de (Ridom GmbH and SeqNet), synchronises public *spa* typing data and currently comprises over 10,000 *spa* types that consist of different combinations of over 500 *spa* repeats from over 200,000 *S. aureus* isolates typed in 90 countries worldwide.

Cluster analysis of *spa* typing data groups *spa* types into *spa*-clonal complexes (*spa*-CCs) using the based upon repeat pattern (BURP) algorithm in the StaphType software (Ridom GmbH). *spa* typing has a higher discriminatory power than MLST and so a single MLST ST can be resolved into several *spa* types, typically within the same *spa*-CC. Good concordance has been reported between MLST and *spa* typing but anomalies can occur with *spa* typing/BURP [Cookson et al. 2007, Mellmann et al. 2008, Strommenger et al. 2008]. For example, the same or related *spa* types or *spa* repeat patterns can occur in different clonal lineages, maybe due to recombination events involving the *spa* gene, or recombination within the *spa* locus [Robinson and Enright 2004, Strommenger et al. 2008]. It has been suggested that the discriminatory power of *spa* typing/BURP can be improved by combining it

with an additional genetic marker, e.g. SCC*mec* for MRSA [Strommenger et al. 2008].

1.3.4 SCCmec typing

SCC*mec* typing exploits differences in the various SCC*mec* elements of MRSA. SCC*mec* typing methods are able to detect various ranges of SCC*mec* types and subtypes, including I-IV [Okuma et al. 2002, Oliveira and de Lencastre 2002, Francois et al. 2004, Motoshima et al. 2010], subtypes of IV [Milheirico, Oliveira and de Lencastre 2007b], I-V [Ito et al. 2001, Zhang et al. 2005, Boye et al. 2007, Kondo et al. 2007, Valvatne et al. 2009, Ghaznavi-Rad et al. 2010b], I-VI [Milheirico, Oliveira and de Lencastre 2007a, Cai et al. 2009] and even I-VI and VIII [Chen et al. 2009]. These methods are based on the *mec* complex and *ccr* genes, or the *mecA* gene and other loci on SCC*mec*, mainly using multiplex PCR. However, each method determines different structural properties of SCC*mec*, with some methods giving different results for the same MRSA isolate [Shore et al. 2005, Kim et al. 2007]. There is no single universal method available for the classification of this mobile element, but the most commonly used methods are those developed by Milheirico, Oliveira and de Lencastre [2007a] and Ito et al. [Ito et al. 2001, Okuma et al. 2002] that target several loci. Simpler methods have been developed by Boye et al. [2007] and Zhang et al. [2005] but they only target a single locus for most SCC*mec* types, and thus have less discriminatory power.

1.3.5 Advances in S. aureus molecular epidemiology

The field of *S. aureus* molecular epidemiology has really advanced in the last few years, with the advent of new technologies such as whole genome sequencing and

microarrays, which also have great utility in the field of *S. aureus* diagnsotics [van Belkum et al. 2009, Lindsay 2010]. The increasing availability of whole genome data has aided the development of multi-strain DNA microarrays for whole genome comparisons [van Belkum et al. 2009, Lindsay 2010], and next generation sequencing (NGS) technologies such as the Illumina Genome Analyzer make it feasible to rapidly generate whole genome data for large population samples of bacteria [Harris et al. 2010]. NGS technologies bridge the gap between sequence-based approaches such as MLST, which lack the ability to distinguish between closely related isolates, and full-genome sequencing, which is impractical for large population samples [Harris et al. 2010]. NGS technologies can provide the sequence of the core genome and, using sequence read assemblies, the non-core gene content of each isolate, providing the ability to show the fine-scale evolutionary changes that have occurred among isolates of a single ST or strain. A recent study highlighted the value of NGS technologies in elucidating the epidemiology and microevolution of ST239-MRSA-III, and demonstrated their potential to track transmission within healthcare facilities, improving contact tracing in endemic and outbreak settings [Harris et al. 2010]. The consistency between high-resolution SNP data and *spa* typing was high in the ST239 study, but another study noted inconsistencies for the ST5 lineage [Nubel et al. 2008]. Microarray and whole genome sequencing technologies are currently too expensive to be adopted as routine laboratory methods for studying epidemiology and evolution of *S. aureus*, and thus sequenced-based typing methods such as MLST and *spa* typing remain common practice. Undoubtedly, NGS will become routine for molecular epidemiology once new platforms allow genome sequences to be obtained simply, cheaply and rapidly. Spatial and temporal dynamics of the ST22 lineage have recently been determined using NGS methods to elucidate its pandemic spread [Holden et al.,

submitted for publication], and high-resolution SNP data from over 1,000 isolates of the USA300 clone (ST8-MRSA-IV) are currently being generated to investigate its transmission and spread across healthcare facilities in a US county (see Part 2 final discussion).

1.4 THE CHANGING EPIDEMIOLOGY OF MRSA

As highlighted in section 1.2, the emergence of novel SCC*mec* elements has generally coincided with the emergence of epidemiologically novel MRSA strains. Over the last few decades, MRSA has largely been a nosocomial pathogen, causing infection in people with frequent or recent contact with healthcare facilities. The epidemic MRSA clones that currently pose a major public health problem in healthcare facilities worldwide, termed HA-MRSA, are listed in Table 1.1. However, MRSA isolated from young, otherwise healthy patients with no identifiable risk factors (including recent hospitalisation, surgery, residency in a long-term care facility, dialysis or invasive medical devices), termed CA-MRSA, have become increasingly prevalent since the 1990s and are now seen worldwide. The major CA-MRSA clones currently circulating are listed in Table 1.4.

SCC*mec* types I, II and III are typically associated with HA-MRSA and are not frequent among the healthy, younger population, while the smaller SCC*mec* types IV and V are commonly associated with CA-MRSA that not only infect hospitalised patients but also healthy contact persons, and spread easily in the community [Kazakova et al. 2005, Hota et al. 2007, Larsen et al. 2007, Huang et al. 2008, Tong et al. 2008]. These latter SCC*mec* allotypes are more readily transmissible between staphylococci than the larger elements and may provide a lower fitness cost to the pathogen [Grundmann et al. 2006]. This could lead to competitive exclusion of HA-

MRSA by CA-MRSA if a reservoir of the latter was established in hospitals [D'Agata et al. 2009], for which there is increasing evidence [Moran et al. 2006, Seybold et al. 2006, Huang et al. 2007b, Patel et al. 2008, Popovich, Weinstein and Hota 2008, Song et al. 2011]. The larger HA-MRSA SCC*mec* allotypes correlate with a slower growth rate, and strains with these elements may be at a selective disadvantage in the absence of antibiotics, i.e. in community settings [Ender et al. 2004, Lee et al. 2007]. Successful HA-MRSA clones harbour the SCC*mec* IV element however. For example, one of the most common healthcare-associated clones in the UK is ST22-MRSA-IV, and the Paediatric clone (ST5) harbours SCC*mec* IV [Holmes et al. 2005]. Furthermore, SCC*mec* types I, II and III have been observed in CA-MRSA isolates [Chung et al. 2004, Wannet et al. 2005].

CA-MRSA are considered more virulent than HA-MRSA due to the presence of various virulence factors [Chambers 2001, Davis et al. 2007, Otto 2010], which has clear implications in terms of morbidity and mortality in the healthcare setting. While frequently associated with chronic or recurrent SSTIs, CA-MRSA can also cause septic arthritis, bacteraemia, toxic shock syndrome, necrotising fasciitis and necrotising pneumonia [Mongkolrattanothai et al. 2003, Francis et al. 2005, Gonzalez et al. 2005a, Gonzalez et al. 2005b, Miller et al. 2005, Bocchini et al. 2006, King et al. 2006, Moran et al. 2006, Davis et al. 2007, Tristan et al. 2007b, Lo and Wang 2011, Shilo and Quach 2011]. Panton-Valentine leukocidin (PVL) is a virulence factor that can cause tissue necrosis and destruction of leukocytes by forming pores in the cellular membrane [Bassetti, Nicco and Mikulska 2009], and is directly associated with staphylococcal necrotising pneumonia [Gillet et al. 2002, Labandeira-Rey et al. 2007], but its association with other CA-MRSA invasive disease is debatable [Lina et al. 1999, Voyich et al. 2006, Ellington et al. 2007]. Despite the predominant CA-

MRSA clone in the US being PVL-positive (USA300) [Okuma et al. 2002, Vandenesch et al. 2003] several CA-MRSA lineages are PVL-negative, so PVL cannot be considered a marker for CA-MRSA [Nimmo et al. 2006, Rossney et al. 2007b, Otter and French 2008, Zhang et al. 2008]. Another factor that may contribute to the virulence of CA-MRSA is the argenine catabolic mobile element (ACME), which has also been shown to contribute to the growth and survival of USA300, the clone in which it seems to be exclusively observed [Diep et al. 2006b, Goering et al. 2007, Diep et al. 2008b, Ellington et al. 2008]. The pore-forming toxin α-haemolysin has also been shown essential for USA300 and USA400 to cause lethal pneumonia in a mouse model of the disease, and increasing severity of the disease has been shown *in vitro* to correlate with increasing amounts of the toxin produced by these strains [Bubeck Wardenburg et al. 2007, Burlak et al. 2007, Montgomery et al. 2008].

CA-MRSA is particularly well established in the US, with USA300 the predominant cause of MRSA infection in North America [Gonzalez et al. 2006, Moran et al. 2006, Klevens et al. 2007]. In contrast, USA300 is uncommon in Europe despite being reported in most countries. However, USA300 appears to be increasing in prevalence there [Larsen et al. 2007, Witte et al. 2007a], and CA-MRSA is partly responsible for the increase in MRSA prevalence in northern European countries that have a traditionally low prevalence of HA-MRSA [Bartels et al. 2007, Stam-Bolink et al. 2007, Fang et al. 2008, Larsen et al. 2008]. European CA-MRSA are more clonally diverse and vary geographically, but the European clone (ST80-MRSA-IV) is widespread on this continent (Table 1.4) [Otter and French 2010]. In stark contrast to the US that is dominated by a single clone, there is considerable CA-MRSA diversity in Australia, with over 100 clones described there [Chua et al. 2011]. Nonetheless, CA-MRSA prevalence in other parts of the world still remains much lower than in the

US [Otter and French 2010, Otto 2010, Johnson 2011]. The USA300 clone, so predominant in North America, is already disseminating globally, which could lead to a rapid worldwide increase in CA-MRSA. Other than Europe, it is also present in Latin America and the Caribbean, the Middle East and the Western Pacific [Nimmo 2012].

It has been shown that CA-MRSA lineages are distinct from those of HA-MRSA, and CA-MRSA are associated with several specific *S. aureus* lineages [Groom et al. 2001, Naimi et al. 2001, Tristan et al. 2007a, David and Daum 2010]. In addition, the larger clonal diversity of CA-MRSA compared to HA-MRSA suggests that more MSSA lineages have the ability to become CA-MRSA [Enright et al. 2002, Okuma et al. 2002, Feng et al. 2008, Francois et al. 2008]. It is unclear whether CA-MRSA was originally MSSA that acquired SCC*mec*, or CA-MRSA originated from HA-MRSA, but more evidence suggests that it descended from virulent strains of MSSA via horizontal phage transfer and integration of SCC*mec* from CNS [Okuma et al. 2002, Aires de Sousa and de Lencastre 2003, Bradley 2005, Robinson et al. 2005, Ma et al. 2006, Boyle-Vavra and Daum 2007, Monecke et al. 2007c, Wallin, Hern and Frazee 2008].

In contrast to HA-MRSA, CA-MRSA are susceptible to most non β-lactam antibiotics, have a faster growth rate and express methicillin resistance at lower levels and heterogeneously [Laurent et al. 2001, Okuma et al. 2002], but multidrug-resistant CA-MRSA have started to emerge [Boyle-Vavra et al. 2005, Ramdani-Bouguessa et al. 2006, Diep et al. 2008a], posing a serious public health concern because of their associated virulence and their ability to cause outbreaks in otherwise healthy individuals, as well as their rapid spread in countries worldwide [Monecke et al. 2011]. Moreover, the lack of clear definitions for HA-MRSA and CA-MRSA due to

increasingly blurred molecular and epidemiological distinctions between the two groups, make it difficult to develop effective infection control strategies in healthcare and community settings. One study in the US highlighted this blurred line by obtaining MRSA isolates from patients and classifying them as either HA-MRSA or CA-MRSA based on both epidemiological and molecular definitions, which were performed separately by blinded investigators. Sixty percent of strains classified as HA-MRSA based on epidemiological definitions were identified as CA-MRSA based on molecular definitions, and CA-MRSA was found to cause healthcare-associated bloodstream infection just as likely as it causes community-associated infection [Gonzalez et al. 2006].

The common epidemiological definition for CA-MRSA is that used by the Active Bacterial Core surveillance (ABCs) program of the US Centers for Disease Control and Prevention (CDC): any MRSA infection diagnosed in an outpatient or within 48 hours of hospitalisation if the patient lacks healthcare-associated MRSA risk factors (haemodialysis, surgery, residence in a long-term care facility, recent hospitalisation, or invasive medical devices) [Morrison, Hageman and Klevens 2006, Klevens et al. 2007]. All other MRSA are considered HA-MRSA. A simpler temporal definition of CA-MRSA is often used, without considering the presence of MRSA risk factors [David and Daum 2010], while the use of a strictly molecular definition is becoming increasingly problematic, for example because HA-MRSA also possess the traditionally community-associated SCC*mec* IV element [Miller and Kaplan 2009]. One such strain, ST22-MRSA-IV has been reported in the community in Ireland [Mollaghan et al. 2010]. In East Asia, CA-MRSA with SCC*mec* IV (ST59, ST30 and ST72) have spread from the community into the hospital, while healthcare-associated strains ST239-MRSA-III and ST5-MRSA-II have been found in the community [Song

et al. 2011]. Nevertheless, in this thesis, HA-MRSA and CA-MRSA are defined on the basis of their clone types, as determined by molecular typing methods (MLST, SCC*mec* typing and *spa* typing).

Table 1.4 Common community-associated MRSA strains. Adapted from Deurenberg and Stobberingh [2008] and Chambers and Deleo [2009].

Clonal complex	**Sequence type**	**Common name(s)**	**Comment and SCC*mec* allotype**
CC80	ST80	European	The predominant CA-MRSA in Europe, SCC*mec* IV
CC30	ST30	Southwest Pacific (SWP), USA1100	Most frequent clone in Eastern Australia, SCC*mec* IV
CC1	ST1	USA400, WA MRSA-1	Earliest CA-MRSA clone in US, important PVL-negative CA-MRSA clone in Australia, SCC*mec* IV
CC8	ST8	USA300	The predominant CA-MRSA in the US having supplanted USA400, isolated infrequently in Europe, can also cause healthcare-associated infections, SCC*mec* IV
CC59	ST59	USA1000	Main CA-MRSA in Taiwan, SCC*mec* IV/V

The first isolation of MRSA from animals was in mastitic cows in the 1970s [Devriese, Van Damme and Fameree 1972, Devriese and Hommez 1975], and MRSA has since been reported in several domestic species including dogs, cats, horses, sheep, chickens and pigs [Leonard and Markey 2008]. The MRSA strains associated with companion animals typically belonged to human nosocomial lineages, leading to the assumption that transmission was occurring from humans to animals - a

'humanosis' [Morgan 2008]. Since 2003, MRSA strains carrying SCC*mec* types IV and V have been reported among livestock, termed LA-MRSA [Nemati et al. 2008], predominantly belonging to the clonal lineage CC398. CC398 first emerged as a coloniser among farmed pigs, and later in other livestock such as calves and poultry. It also colonises humans in contact with these livestock, where it infrequently causes infection, ranging from SSTIs to severe, invasive infections such as endocarditis, necrotising fasciitis and pneumonia [Ekkelenkamp et al. 2006, van Rijen, Van Keulen and Kluytmans 2008, Pan et al. 2009, Catry et al. 2010, Hartmeyer et al. 2010, Mammina et al. 2010, Schijffelen et al. 2010, Soavi et al. 2010], demonstrating the zoonotic potential and virulence of this lineage [Voss et al. 2005, van Loo et al. 2007, Witte et al. 2007b, Khanna et al. 2008, Smith et al. 2008, van Belkum et al. 2008, Cuny et al. 2009, Mulders et al. 2010, van Cleef et al. 2010]. CC398 is now reported in different countries around the world, with livestock representing another reservoir for MRSA colonisation and infection in humans [Armand-Lefevre, Ruimy and Andremont 2005, Witte et al. 2007b, Lewis et al. 2008, Smith et al. 2008, Bhat et al. 2009, Denis et al. 2009b, Krziwanek, Metz-Gercek and Mittermayer 2009, Loeffler et al. 2009, Pan et al. 2009, Mulders et al. 2010, Potel et al. 2010, Soavi et al. 2010, van Cleef et al. 2010, Vanderhaeghen et al. 2010, Haenni et al. 2011]. It has been shown however that CC398 MRSA are poor persistent human colonisers in the absence of animal contact [Graveland et al. 2011].

Studies of *S. aureus* population genetics have demonstrated the existence of host-specific clonal lineages, with the majority of LA-MRSA belonging to a small number of animal-associated clones (Table 1.5) [Sung, Lloyd and Lindsay 2008, McCarthy et al. 2011, Fitzgerald 2012]. Most ruminant lineages are host-specific, for example ST97, ST705, ST126, ST151 and ST133 [Cuny et al. 2011, Fitzgerald 2012].

However, ST398 and human lineages such as ST5, ST239 and ST1 have been associated with bovine mastitis [Cuny et al. 2011], and CC130, which was recently found to harbour the novel divergent *mecA*$_{LGA251}$ gene carried by SCC*mec* XI, appears to have no host restrictions, infecting both bovine and human populations [Cuny et al. 2011, Garcia-Alvarez et al. 2011, Shore et al. 2011]. Clones such as ST398 appear to have the ability to colonise and infect multiple host species. The novel *mecA*$_{LGA251}$ found in CC130 clones was also identified in CC705 and ST425, from bovine and human populations in the UK, Denmark, Ireland and Germany, highlighting the possibility of additional circulating novel *mecA* alleles that could be acquired by *S. aureus* to create new MRSA strains [Cuny et al. 2011, Garcia-Alvarez et al. 2011, Shore et al. 2011, Fitzgerald 2012]. In addition, *mecA*$_{LGA251}$ cannot be detected by molecular diagnostic tests for MRSA, leading to false negative results, and the ramifications of this should be considered by diagnostic protocols [Garcia-Alvarez et al. 2011].

Not only do livestock serve as a potential source of zoonotic *S. aureus* infection, but also humans represent an important source of new pathogenic strains affecting economically important livestock [Lowder et al. 2009, Guinane et al. 2010, Sakwinska et al. 2011, Fitzgerald 2012], with a recent study providing strong evidence that LA-MRSA CC398 originated in humans as MSSA [Price et al. 2012]. While the potential impact of the community MRSA reservoir on current infection control strategies is clear, the impact of the livestock MRSA reservoir on public health is less so. Current evidence suggests LA-MRSA have not spread significantly into healthcare settings in Europe, but CC398 spread seems to be dependent on the region and the intensity of pig farming [Stefani et al. 2012]. A better understanding of the origin, evolution and epidemiology of both CA-MRSA and LA-MRSA is required

to ensure that future MRSA control strategies are effective in the face of these increasingly important MRSA reservoirs.

Table 1.5 Common livestock-associated MRSA strains. Adapted from Fitzgerald [2012].

Clonal complex/ Sequence type	**Host species**
ST1	Human, cow, horse, chicken
CC5	Human, chicken, turkey
ST8	Human, horse, cow
ST9	Pig, chicken
CC97	Cow, human
ST121	Human, rabbit
CC126	Cow
CC130	Cow, sheep, human
CC133	Sheep, goat, cow
CC705	Cow
CC385	Chicken, wild birds
ST398	Pig, human, cow, chicken, horse
ST425	Cow, human
ST1464	Sheep

1.5 MRSA PREVALENCE AND BURDEN

MRSA is the most important cause of antibiotic-resistant healthcare-associated infections worldwide, and the most commonly identified antibiotic-resistant pathogen in many parts of the world, including Europe, the Americas, North Africa, the Middle East, and East Asia [Grundmann et al. 2006]. MRSA carriage based on Dutch and US

prevalence figures is conservatively estimated at 0.1-2.65% of the expected 2 billion *S. aureus* carriers globally [Grundmann et al. 2006]. MRSA prevalence varies greatly between countries, as well as within (Figures 1.3 and 1.4). It is hard to make international comparisons of MRSA prevalence however, due to the differences in the way prevalence rates are obtained between studies, and the scarcity of routine surveillance systems for MRSA. In general, the US, South America and East Asia (including South Korea, Japan and Taiwan) appear to have the highest MRSA rates, (>50%), with intermediate rates in Canada, Latin America, Australia, southern and central Europe, and parts of Africa and the Middle East (mostly between 25 and 50%) [Bouchillon et al. 2004, Grundmann et al. 2006, Perovic et al. 2006, Laxminarayan and Malani 2007, Reinert et al. 2007, Mejia, Zurita and Guzman-Blanco 2010, Song et al. 2011]. Scandinavia and the Netherlands have the lowest MRSA rates, at <5%, while rates in resource-poor countries, including most of Asia and Africa, are largely unknown, due to a dearth of prevalence studies in these regions [Nickerson et al. 2009], although MRSA rates appear to be intermediate [Kesah et al. 2003, Ramdani-Bouguessa et al. 2006, Song et al. 2011]. High MRSA rates tend to be seen in countries with high rates of antibiotic use (current or historical) and poor infection control strategies, whereas the low MRSA rates seen for example in the Netherlands are attributable to national 'search and destroy' policies to limit MRSA spread. Such policies are expensive to implement, but in countries with low endemic MRSA incidence, the benefits outweigh the costs [Laxminarayan and Malani 2007, Simoens, Ophals and Schuermans 2009, van Rijen and Kluytmans 2009].

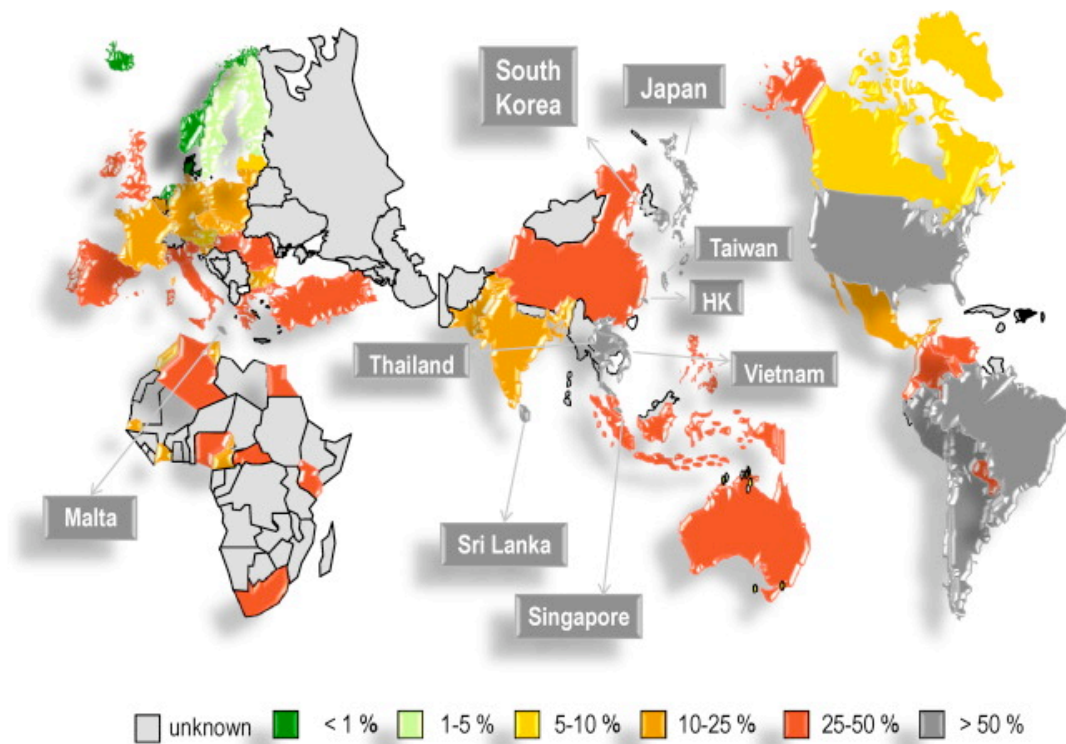


Figure 1.3 Global prevalence of healthcare-acquired MRSA. HK = Hong Kong. Taken from Stefani et al. [2012].

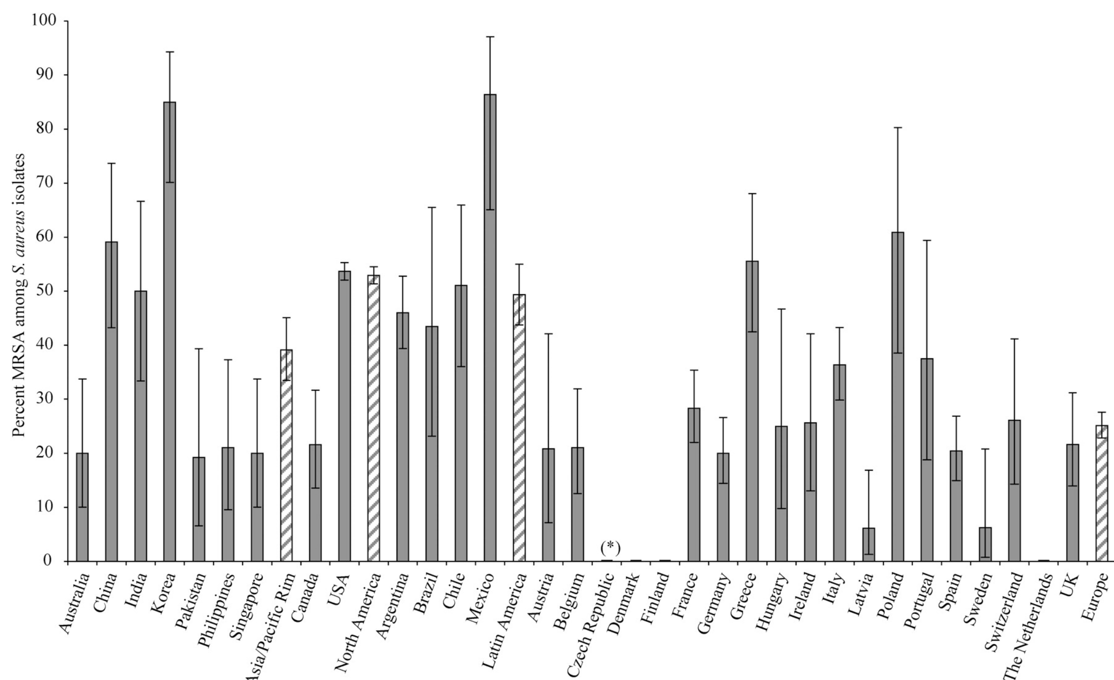


Figure 1.4 Frequency of MRSA among *S. aureus* isolates (collected from blood, respiratory tract, urine, skin, wound, body fluids and other defined sources between January 2004 and August 2006), by country. Only countries submitting at least 10 isolates are shown (with 95% confidence intervals). *Indicates data not shown as country submitted <10 *S. aureus* isolates. Taken from Reinert et al. [2007].

The European Antimicrobial Resistance Surveillance Network (EARSnet) was set up in 1999 using sentinel hospital laboratories across the continent to survey cases of bacteraemia caused by several bacterial species including *S. aureus*. Although bacteraemia is less common than other types of infection such as SSTIs, surveillance systems tend to focus on bloodstream infections as they are clinically significant and are likely to be investigated microbiologically [Johnson 2011]. Through EARSnet, in 2009, nine European countries reported less than 10% invasive MRSA isolates, nine countries reported 10-25%, another nine reported 25-50%, and one country reported over 50% invasive MRSA isolates (Figure 1.3) [ECDC 2011]. Eight countries reported a decreasing trend for MRSA while just one country reported an increase [ECDC 2011]. In the UK, the percentage of invasive MRSA isolates was between 25 and 50% in 2009, but a significantly sustained decrease in this percentage was observed between 2006 and 2009 [ECDC 2011]. This decrease likely reflects the government action in England to make reporting of MRSA bacteraemia mandatory for all hospitals, and setting hospitals the target of halving their MRSA rates [Liebowitz 2009, Pearson, Chronias and Murray 2009]. Although proportions of MRSA seem to be stabilising, and even decreasing in some European countries, the percentage of MRSA is still more than 25% in 10 of 28 reporting countries, and thus MRSA control remains a public health priority in Europe [ECDC 2011].

In the US, the ABCs population-based sentinel surveillance program was used to evaluate the incidence of healthcare-associated MRSA infections between 2005 and 2008 [Kallen et al. 2010]. Over the four-year period, rates of hospital onset and healthcare-associated, community onset invasive MRSA infections decreased among US hospital inpatients [Kallen et al. 2010], supporting a previous study on bloodstream infections in intensive care units (ICUs) [Burton et al. 2009], and

mirroring the pattern observed in some European countries like the UK. Despite this decrease, MRSA prevalence remains high in the US [Grundmann et al. 2006], likely due to the rapid increase in rates of CA-MRSA infection in the last decade and its emergence as a major cause of healthcare-associated infection [Carleton et al. 2004, King et al. 2006, Moran et al. 2006, Patel et al. 2008].

MRSA is a large and increasing global burden on healthcare resources, and is associated with increased morbidity and a higher risk of mortality [Abramson and Sexton 1999, Cosgrove et al. 2003, Engemann et al. 2003, Chu et al. 2005, Cosgrove et al. 2005, Gould 2005, Klein, Smith and Laxminarayan 2007, Shurland et al. 2007]. The poorer therapeutic outcome of MRSA infections compared to MSSA infections can be attributed to the underlying medical problems of the often sicker, older patients that are infected with MRSA, plus the use of more toxic or ineffective antibiotics [Cosgrove et al. 2003, Lowy 2003, Simoens, Ophals and Schuermans 2009]. The additional burden imposed by resistance not only aggravates the clinical outcome but also adds to the overall caseload of patients with invasive *S. aureus* infections [de Kraker et al. 2011]. Increased risk of treatment failure, implementation of isolation measures, antimicrobial treatment and extended hospital stay all add to the financial burden on healthcare facilities [Shorr 2007]. Additional financial costs can be accrued through the containment of outbreaks and changes in antibiotic prescribing habits [Grundmann et al. 2006]. The average excess costs per MRSA-positive patient have been estimated to range from €5,700 to €10,000 [Monecke et al. 2011]. MRSA infections also impact on sufferers psychologically (e.g. due to isolation) and financially, through loss of productivity and long-term disability, in turn impacting on societal costs [Tarzi et al. 2001, Grundmann et al. 2006].

1.6 PREVENTATIVE MEASURES

Although HA-MRSA prevalence is stabilising and even decreasing in some regions, notably Europe, the need for effective MRSA control strategies remains, as HA-MRSA prevalence is still high and increasing in many countries around the world. Several hurdles to effective control exist however, including the increasing prevalence of CA-MRSA, which appears to be emerging as a potent combination of transmissibility, virulence and resistance, the ever-increasing healthcare MRSA reservoir, to which CA-MRSA is increasingly contributing, the likely impossibility of eradicating endemic MRSA, and the significant costs and disruption to patient care associated with active surveillance and control [Marshall et al. 2004].

There are four main approaches to MRSA control: reduction in antibiotic use through education and restriction, transmission prevention through hand hygiene, identification of carriers through screening and isolation, and elimination of reservoirs through decontamination [Harbarth 2006]. There is much debate regarding the most cost-effective strategy for MRSA control, but it is clear that strategies developed with HA-MRSA pathogenesis in mind may not work for prevention of CA-MRSA infection, attenuating our ability to control overall MRSA burden [Miller and Diep 2008]. The increasing non-β-lactam resistance in CA-MRSA clones, particularly USA300, highlights the need for accurate therapeutic decisions, guided by active screening and surveillance [Chua et al. 2011].

The limited choice of therapeutic options available, and the declining investment by pharmaceutical companies in antimicrobial research and development [Talbot et al. 2006] (Figure 1.5), has shifted the spotlight onto infection prevention and control measures such as hand hygiene, protective clothing and equipment, and accommodating patients in isolation rooms or wards [Monecke et al. 2011]. As well

as a better understanding of *S. aureus* pathogenesis and improved non-antimicrobial approaches, an important aspect of effective MRSA control is the rapid screening for early identification of MRSA carriers, using accurate and rapid MRSA diagnostics.

The stringent search and destroy policies employed in Scandinavian countries and the Netherlands, where MRSA prevalence is low, involve a combination of measures, such as isolation of identified MRSA carriers, patient decolonisation, admission screening of high-risk patients with pre-emptive isolation, screening of all contact patients, healthcare workers and index cases, temporarily sending healthcare workers home, and closing wards to prevent outbreaks. A study investigating the individual contribution of each of these measures found that admission screening combined with pre-emptive isolation could be the most beneficial, even in high MRSA prevalence settings [Bootsma, Diekmann and Bonten 2006b]. Rapid MRSA detection is crucial in such a strategy, to limit transmission risk and isolation costs, as well as the impact on patient care [Wassenberg et al. 2010]. Conventional microbiological cultures have a turnaround time (TAT) of at least 48 hours, and longer if a broth enrichment technique is used. Novel and faster diagnostic tests for MRSA screening have been introduced in recent years that reduce the TAT to a diagnostic result, and these are discussed in section 1.7.

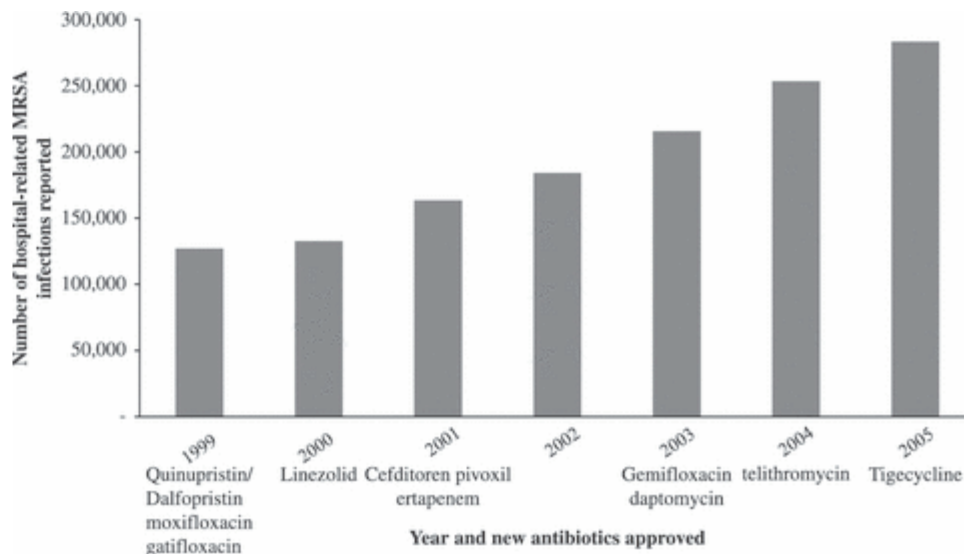


Figure 1.5 Number of HA-MRSA infections reported annually in the US between 1999 and 2005. The new antibiotics approved for each of these years are also shown. Taken from Stein, Goetz and Ganea [2009].

1.7 MRSA DIAGNOSIS

Several organisations have recommended that patients be screened upon admission to hospitals where the prevalence of infection is high, and that persons identified as being colonised be placed on contact isolation [Muto et al. 2003, Gastmeier et al. 2004, Carroll 2008]. This has focused attention on rapid and accurate detection methods for *S. aureus*, particularly MRSA.

Approaches to rapid detection of MRSA include culture methods and molecular tests, but many of the available tests differ markedly in their specificity, sensitivity, ease of use and cost. The advantages and disadvantages of culture and molecular methods are shown in Table 1.6. Despite commercialisation of some molecular methods, the most common method for MRSA detection in routine laboratories is culturing on selective and chromogenic agar from nasal swab specimens, or nasal specimens combined with those from the throat, groin or rectum

to improve bacterial recovery [Eveillard et al. 2006]. These rapid culture methods differ from traditional culture methods in that they are selective and differential for MRSA. Such media inhibits the growth of other organisms through the use of antibiotics in the agar, and the presence of chromogenic substrate, which is hydrolysed by an MRSA-specific enzyme, creates colonies of a distinct colour. Using this type of agar allows identification of MRSA from primary isolation plates within 24 to 48 hours, obviating the need for further subcultures and additional biochemical confirmatory tests [Malhotra-Kumar et al. 2008].

Table 1.6 Advantages and disadvantages of culture and molecular methods. Taken from Marlowe and Bankowski [2011].

Method	**Sensitivity**	**Specificity (%)**	**Time to results (hours)**	**Costs**	**User skill level required**
Culture	Low, but improved with chromogenic agar and broth enrichment	100	18-48	Low	Moderate
Molecular	High	<100	<24	High	Moderate to high

Selective media containing oxacillin have been found to produce unsatisfactory clinical sensitivity and specificity, and are sensitive to incubation temperature and inoculum density [Cherkaoui et al. 2007]. Thus, cefoxitin- or cephamycin-containing media is now recommended [CLSI 2005]. Several chromogenic media exist on the market, but CHROMagar MRSA (Beckton Dickinson and CHROMagar Microbiology) is currently the most popular for MRSA detection [Lindsay 2008]. When grown on CHROMagar MRSA agar plates, MRSA

will grow in the presence of cefoxitin (6mg/L) and produce mauve colonies as a result of hydrolysis of the chromogenic substrate. Additional selective agents are incorporated for the suppression of gram negative organisms, yeast and some other gram positive cocci, but other bacteria may grow and produce differently coloured colonies, e.g. bacteria that utilise chromogenic substrates in the medium produce blue or blue/green colonies and those that don't utilise chromogenic substrates will produce white or colourless colonies [BD Diagnostics 2010]. Other cefoxitin- or cephomycin-containing media include chromID MRSA (bioMérieux), MRSA Select (Bio-Rad) and Chromogenic MRSA/Denim Blue agar (Oxoid). All produce uniformly high specificities after 24 hours, but sensitivities tend to vary both between media and studies [Cherkaoui et al. 2007, Malhotra-Kumar et al. 2008, Luteijn et al. 2011], although they are higher than non-chromogenic media (93-99%) [Malhotra-Kumar et al. 2010a]. Sensitivities can be improved by 48 hour incubation, but then specificities are affected, requiring confirmatory MRSA tests [Malhotra-Kumar et al. 2008]. The variable performances highlight the need for a gold standard media for MRSA screening [Cherkaoui et al. 2007].

Molecular techniques for direct detection of MRSA have become increasingly commonplace [Marlowe and Bankowski 2011]. MRSA may be hetero-resistant to β-lactam antibiotics due to repression of *mecA* by *mecI* and consequently the gold standard method for molecular detection of MRSA is PCR detection of *mecA* in *S. aureus*. However, coagulase-negative staphylococci (CNS), such as *S. epidermidis* and *S. haemolyticus*, can be positive for *mecA* and are frequently carried in the human population. Commercial PCR kits for detection of *mecA* have been available for several years but these are not widely used due to the problem of false-positives caused by *mecA*-carrying CNS isolates. In CNS, SCC*mec* elements can be identical

to those in *S. aureus*, but the location of the integration site (*attBscc*) differs. In the *S. aureus* chromosome, *attBscc* is located near the *S. aureus* origin of replication, in an open reading frame of unknown function, *orfX*, which is highly conserved among clinical strains of *S. aureus*. Multiplex PCR approaches have been developed in recent years to take advantage of this fact, in an attempt to overcome the problem of false positives. Specifically, the approach involves detection of a single amplicon, which includes the right junction of the SCC*mec* element and a part of the adjacent *S. aureus*-specific *orfX* gene. This amplified region is termed the *mec* right extremity junction (MREJ) (section 3.1.1).

Commercial assays like GenoType MRSA Direct (Hain Lifescience), BD GeneOhm MRSA (BD Diagnostics) and Xpert MRSA (Cepheid Diagnostics) have successfully utilised the approach, with the latter two being US Food and Drug Administration (FDA)-approved for detection of MRSA from nasal surveillance samples. Colonisation of the nose has been shown to be a risk factor for subsequent infection [Carroll 2008].

GenoType MRSA Direct targets SCC*mec* types I to V in a multiplex PCR using biotinylated primers followed by a reverse hybridisation step [Malhotra-Kumar et al. 2008]. Direct detection of MRSA from diverse body sties (nose, throat, groin, axilla, wound, and other sites) gives a sensitivity and specificity ranging from 68% to 95% and 96% to 99%, respectively [Harbarth et al. 2011]. There is a newer version of this assay, the GenoQuick MRSA dipstick assay, which excludes the reverse hybridisation step, reducing the total assay time from 4 hours to 2 hours 20 minutes [Carroll 2008, Malhotra-Kumar et al. 2008]. This assay has been shown to have very high sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) (100%, 99.4%, 96% and 100% respectively) [Eigner et al. 2007].

BD GeneOhm MRSA is a multiplex qualitative real-time PCR assay that can be semi-automated using Cepheid's SmartCycler instrument and has been extensively evaluated [Huletsky et al. 2004, Warren et al. 2004, Bishop et al. 2006, Desjardins et al. 2006, Drews et al. 2006, Oberdorfer et al. 2006, Francois et al. 2007, Paule et al. 2007, Rossney et al. 2007a, van Hal et al. 2007, Zhang et al. 2007, Bartels et al. 2009, Kelley et al. 2009, Kimura et al. 2009, Park et al. 2009a, Hombach et al. 2010, Lucke et al. 2010, Peterson et al. 2010, Snyder, Munier and Johnson 2010, Hassan and Shorman 2011, Patel et al. 2011]. The assay contains primers targeting the right junction sequences of SCC*mec* types I to VI and VIII [Boyle-Vavra and Daum 2010], combined with a consensus primer and three molecular beacons specific for *orfX* [Malhotra-Kumar et al. 2008]. Since the assay is able to simultaneously link identification with resistance detection, it can differentiate MRSA from MSSA and *mecA*-positive CNS in clinical samples. Clinical performance characteristics are compared with those of the Xpert MRSA assay in Table 1.7. In general, the sensitivity and NPVs are equivalent or slightly better than culture, so the assay can be used to decide which patients should be put into or removed from isolation [Malhotra-Kumar et al. 2008]. However, the PPV appears poor (Table 1.7) so the impact of isolating non-colonised patients should be assessed before using this assay [Carroll 2008]. Studies show that BD GeneOhm MRSA, Genotype MRSA Direct and Xpert MRSA assays fail to detect nontypeable SCC*mec* elements or certain variants of known SCC*mec* types [Francois et al. 2007, Rossney et al. 2007a, Bartels et al. 2009, Boyle-Vavra and Daum 2010, Laurent et al. 2010], suggesting that these assays should be evaluated against the local MRSA diversity, and in areas where problematic SCC*mec* elements are prevalent, perhaps an alternative assay or culture-based method should be employed.

A new version of the BD GeneOhm MRSA assay, BD GeneOhm ACP MRSA, incorporates a novel lysis method for specimen preparation, reducing the preparation steps and time needed to perform them, thus facilitating a high-throughput, automated procedure for MRSA detection. Performance characteristics of the ACP version were comparable to the original assay, providing good sensitivity [Patel et al. 2011].

Table 1.7 Performance characteristics of the two FDA-approved molecular assays that target SCC*mec* sequences for the direct detection of nasal colonisation by MRSA. Adapted from Malhotra-Kumar et al. [2008] and Carroll [2008].

Assay	**Internal Control**	**Clinical characteristics (ranges)***	**Analytical sensitivity (CFU/swab)**	**Assay time**	**Comments**	**References**
BD GeneOhm MRSA	Yes	Sensitivity 81-100% Specificity 78-99% PPV 56-99% NPV 94-100%	25-325	2-4 hrs	Amplifies some *mecA* negative *S. aureus*; doesn't amplify some SCC*mec* variants; false positive rate as high as 5%.	[Huletsky et al. 2004, Warren et al. 2004, Bishop et al. 2006, Desjardins et al. 2006, Drews et al. 2006, Oberdorfer et al. 2006, Francois et al. 2007, Paule et al. 2007, Rossney et al. 2007a, van Hal et al. 2007, Zhang et al. 2007, Kelley et al. 2009, Kimura et al. 2009, Park et al. 2009a, Hombach et al. 2010, Lucke et al. 2010, Malhotra-Kumar et al. 2010b, Peterson et al. 2010, Snyder, Munier and Johnson 2010, Hassan and Shorman 2011, Luteijn et al. 2011, Patel et al. 2011, BD Diagnostics 2012]
Xpert MRSA	Yes	Sensitivity 69-100% Specificity 90-99% PPV 78-90% NPV 96-100%	58-80	75 mins	Has similar issues to BD GeneOhm MRSA.	[Mehta et al. 2007, Rossney et al. 2008, Kelley et al. 2009, Wolk et al. 2009, Brenwald, Baker and Oppenheim 2010, Creamer et al. 2010, Hombach et al. 2010, Laurent et al. 2010, Malhotra-Kumar et al. 2010b]

*Note that performance characteristics are influenced by study design, site MRSA prevalence, gold standards used, sample sites (e.g. nares, groin, axilla) and enrichment protocols, and should be borne in mind when comparing study results. PPV = positive predictive value, NPV = negative predictive value.

Xpert MRSA is a real-time PCR assay that works on a fully automated platform, and is the most sophisticated system available for MRSA detection. It is fully automated with minimal front-end processing, the level of expertise required to operate it are minimal, and it is a random access instrument allowing flexible testing of samples [Carroll 2008, Malhotra-Kumar et al. 2008, Rossney et al. 2008]. Xpert MRSA is able to detect SCC*mec* types I, II, III, IVa, V and VI. A comparison of Xpert MRSA and BD GeneOhm MRSA showed similar sensitivities and specificities (98.5% and 97.1%, and 90.4% and 89.2%, respectively) for MRSA detection from nasal samples [Mehta et al. 2007] (Table 1.7), and Wolk et al. found no statistical difference in performance between the two assays [Wolk et al. 2009]. The Xpert MRSA assay is able to detect both MSSA and MRSA and is thought to reliably detect MRSA in mixed cultures as the relative quantities of gene products is measured, thus making it a semi-quantitative assay.

These molecular assays show consistently high NPVs, making them ideal tools for the rapid isolation of MRSA carriers, and in turn dramatically reducing isolation time [Hassan and Shorman 2011]. The potential value of using such assays in point of care settings such as hospital wards has also been demonstrated by a major reduction in TAT (by more than ten hours) compared to their use in laboratories [Brenwald, Baker and Oppenheim 2010].

A study evaluating the savings made if Xpert MRSA, at a cost of €50 per test, was used for MRSA detection in patients and healthcare workers, found that at least €925 per exposed healthcare worker and €550 per exposed patient that were MRSA negative, would be saved [Andersen et al. 2010]. Another study found that decision-making based on molecular tests added between €154 (BD GeneOhm MRSA) and €194 (Xpert MRSA) per patient to overall costs, while chromogenic tests saved €31,

leading the authors to conclude that rapid diagnostic tests safely reduce the number of unnecessary isolation days, but only screening based on chromogenic testing, can be considered cost saving [Wassenberg et al. 2010]. This is in contrast to a Swiss study in a single healthcare facility that found replacement of culture-based testing with PCR-based methods more than halved the number of pre-emptive isolation days and was thus considered cost-effective [Uckay et al. 2008].

It is clear that molecular tests allow for prompt MRSA detection, but they are expensive, and so the effectiveness of their use must be carefully evaluated for each setting. In populations with low MRSA endemicity, broad use of molecular tests may not be cost-effective [Harbarth et al. 2011, Wassenberg et al. 2011], but in settings of low MRSA prevalence, rapid isolation of MRSA is crucial for effective MRSA control [Diederen 2010]. Rapid screening with chromogenic media is preferred due to the expense of molecular tests, but the latter could be cost-effective for high-risk healthcare units and critically ill patients [Harbarth et al. 2011]. While it is suggested in some populations that transmission rates do not differ between patients screened with culture-based methods and those screened with molecular-based methods, the real value of the latter lies in their ability to free up bed space more readily in the hospital [Marlowe and Bankowski 2011].

1.6 THESIS OBJECTIVES

My thesis draws on two themes identified in the introduction: the need for a simpler, cheaper and quicker MRSA diagnostic test that enables widespread use of a molecular test for MRSA screening (addressed in Part 1); and the need for a better understanding of healthcare MRSA reservoirs due to the ever-changing epidemiology of MRSA, specifically investigating the extent to which HA-MRSA and CA-MRSA

strains have merged in these reservoirs (Part 2 of the thesis). These themes are introduced more fully in their respective parts.

CHAPTER 2: METHODS

Note: This chapter outlines methods used throughout my thesis. Methods specific to a chapter are described in that chapter.

2.1 BACTERIAL CULTURE

All bacterial isolates were stored in 15% glycerol (TSB-glycerol) at −80°C. Cells were harvested on Oxoid blood agar base No.2 and incubated at 37°C overnight. Single colonies from the overnight growth were picked and subcultured, from which DNA extractions were performed.

2.2 DNA EXTRACTION

DNA was extracted from subcultured growth using a DNeasy Blood & Tissue Kit (Qiagen), according to guidelines for the purification of total DNA from gram-positive bacteria. Briefly, this involved the standard purification procedure but with a pretreatment step of incubation with enzymatic lysis buffer to lyse cell walls (20mM Tris-Cl pH 8.0, 2mM sodium EDTA, 1.2% Triton X-100, lysostaphin to 2mg/ml and immediately before use, lysozyme to 20mg/ml). DNA samples were eluted in 200µl of AE buffer (10 mM Tris-Cl and 0.5 mM EDTA, pH 9.0) and stored at −20°C.

2.3 PCR AMPLIFICATION

Unless otherwise stated, all PCR amplification was performed in a total volume of 29µl, containing 25µl of 1.1x ReddyMix PCR Master Mix (Thermo Scientific; 1.25u Thermoprime Plus DNA Polymerase, 75mM Tris-HCl (pH8.8 at 25°C), 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2mM each of dATP, dCTP, dGTP and dTTP, plus precipitant and red dye for electrophoresis), 1µl

each of 100µM forward and reverse primer, and 2µl of cleaned DNA template. 5µl of each PCR product was checked by electrophoresis on a 1% agarose gel containing 5µl of SafeView nucleic acid stain (NBS Biologicals) at 15V/cm for 40 min in 1x TBE buffer. The remaining volume of each PCR product was then purified by polyethylene glycol (PEG) precipitation plus an ethanol wash and suspended in 12µl of sterile distilled water (SDW).

2.4 SEQUENCING OF PCR PRODUCTS

All purified PCR products were diluted to 2.5-5ng/µl using SDW prior to sequencing reactions. Products were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). A total reaction volume of 10µl contained 2µl of purified PCR product, 4µl of 1µM forward or reverse primer, 0.5µl of BigDye Terminator, 1.75µl of BigDye Terminator 5x Sequencing Buffer, and 1.75µl of SDW). Thermal cycling conditions for sequencing reactions included 25 cycles of denaturing, annealing and extension (10s at 96°C, 5s at 50°C and 2 min at 60°C) and a ramp of 0.1°C/s to 4°C. Reaction cleanup was performed by ethanol precipitation. When ready to sequence, cleaned reaction products were re-suspended in 10µl of Hi-Di Formamide (Applied Biosystems) and sequences determined using the ABI 3730xl DNA Analyser. All sequence data were assembled, trimmed, edited and aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 4.1 [Tamura et al. 2007], except data for the *spa* repeat region, which were analysed using software described in section 2.6.

2.5 MLST

Primer sequences used for PCR amplification of the seven housekeeping genes are listed in Table 2.1. Thermal cycling conditions consisted of an initial denaturation of 3 min at 95°C, 35 cycles of denaturation, annealing and extension (30s at 95°C, 30s at 60°C and 1 min at 72°C), and a final extension of 10 min at 72°C. Following sequencing of the seven loci and building of consensus sequences using MEGA version 4.1 [Tamura et al. 2007], consensus sequences were queried using the MLST *S. aureus* database (http://saureus.mlst.net/) to determine sequence types (STs). Relationships between STs were visualised using the eBURST (Based Upon Related Sequence Types) algorithm [Feil et al. 2004, Spratt et al. 2004] (http://saureus.mlst.net/eburst/).

2.6 *spa* TYPING

Primer sequences used for PCR amplification of the *spa* repeat region are listed in Table 2.1. Thermal cycling conditions consisted of an initial denaturation of 5 min at 80°C, 35 cycles of denaturation, annealing and extension (45s at 94°C, 45s at 60°C and 90s at 72°C), and a final extension of 10 min at 72°C. Following sequencing of the *spa* repeat region, *spa* types were determined using Ridom StaphType v1.5-2.2 (Ridom GmbH, Würzburg, Germany) [Harmsen et al. 2003]. To assess *spa* type diversity and relatedness, cluster analysis of *spa* types was performed using the Based Upon Repeat Pattern (BURP) algorithm, a built-in feature of the StaphType software [Mellmann et al. 2007]. The BURP algorithm is a heuristic variant of the Excision, Duplication, Substitution and Indels (EDSI) algorithm [Sammeth and Stoye 2006], and is the first automated and objective tool to infer clonal relatedeness from *spa* repeat regions [Mellmann et al. 2007].

Table 2.1 PCR Primers used for *spa* typing and MLST.

Primer	5′-3′ sequence	Amplicon Size (bp)	Reference
spa_1113F spa_1514R	TAAAGACGATCCTTCGGTGAGC CAGCAGTAGTGCCGTTTGCTT	Variable	[Ridom GmbH 2006]
arC_fwd arcC_rev	TTGATTCACCAGCGCGTATTGTC AGGTATCTGCTTCAATCAGCG	456	[Enright et al. 2000]
aroE_fwd aroE_rev	ATCGGAAATCCTATTTCACATTC GGTGTTGTATTAATAACGATATC	456	[Enright et al. 2000]
glpF_fwd glpF_rev	CTAGGAACTGCAATCTTAATCC TGGTAAAATCGCATGTCCAATTC	465	[Enright et al. 2000]
gmk_fwd gmk_rev	ATCGTTTTATCGGGACCATC TCATTAACTACAACGTAATCGTA	429	[Enright et al. 2000]
pta_fwd pta_rev	GTTAAAATCGTATTACCTGAAGG GACCCTTTTGTTGAAAAGCTTAA	474	[Enright et al. 2000]
tpi_fwd tpi_rev	TCGTTCATTCTGAACGTCGTGAA TTTGCACCTTCTAACAATTGTAC	402	[Enright et al. 2000]
yqiL_fwd yqiL_rev	CAGCATACAGGACACCTATTGGC CGTTGAGGAATCGATACTGGAAC	516	[Enright et al. 2000]

2.7 SCC*mec* TYPING

SCC*mec* typing was carried out according to either the method of Boye et al. [2007] or Milheirico, Oliveira and de Lencastre [2007a] using the primers listed in Tables 2.2 and 2.3. Multiplex PCR products were viewed on agarose gels (percentages method-specific) containing 5µl of SafeView nucleic acid stain (NBS Biologicals), run in 1x TBE buffer and at the rate recommended by each method. SCC*mec* types were determined on the basis of the amplification pattern obtained (Figure 2.1). Isolates with no visible bands, or with an amplification pattern not in agreement with one of the predicted patterns, were classified as non-typeable (NT).

Table 2.2 Primers used in the multiplex SCC*mec* PCR assay of Boye et al. [2007] for SCC*mec* types I-V.

Name	Primer sequence (5′→3′)	Amplicon size (bp)	Target	Primer specificity (SCC*mec* type)
β α	ATTGCCTTCATAATAGCCYTCT[a] TAAAGGCATCAATGCACAAACACT[a]	937	*ccrA2-B*	II and IV
ccrCF ccrCR	CGTCTATTACAAGATGTTAAGGATAAT[b] CCTTTATAGACTGGATTATTCAAAATAT[b]	518	*ccrC*	III and V
1272F1 1272R1	GCCACTCATAACATATGGAA[c] CATCCGAGTGAAACCCAAA[c]	415	*IS1272*	I and IV
5RmecA 5R431	TATACCAAACCCGACAACTAC[c] CGGCTACAGTGATAACATCC[c]	359	*mecA–IS431*	V

[a][Ito et al. 2001]; [b][Ito et al. 2004]; [c][Boye et al. 2007].

Table 2.3 Primers used in the multiplex SCC*mec* PCR assay of Milheirico, Oliveira and de Lencastre [2007a] for SCC*mec* types I-VI.

Name	Primer sequence (5′→3′)	Amplicon size (bp)	Target	Primer specificity (SCC*mec* type)
CIF2 F2 CIF2 R2	TTCGAGTTGCTGATGAAGAAGG[a] ATTTACCACAAGGACTACCAGC[a]	495	downstream of *pls,* J1 region	I
ccrC F2 ccrC R2	GTACTCGTTACAATGTTTGG[b] ATAATGGCTTCATGCTTACC[b]	449	*ccrC*	V
RIF5 F10 RIF5 R13	TTCTTAAGTACACGCTGAATCG[a] ATGGAGATGAATTACAAGGG[a]	414	J3 region	III
SCCmec V J1 F SCCmec V J1 R	TTCTCCATTCTTGTTCATCC[b] AGAGACTACTGACTTAAGTGG[b]	377	J1 region	V
dcs F2 dcs R1	CATCCTATGATAGCTTGGTC[a] CTAAATCATAGCCATGACCG[a]	342	*dcs* region	I, II, IV and VI
ccrB2 F2 ccrB2 R2	AGTTTCTCAGAATTCGAACG[b] CCGATATAGAAWGGGTTAGC[b]	311	*ccrB2*	II and IV
kdp F1 kdp R1	AATCATCTGCCATTGGTGATGC[a] CGAATGAAGTGAAAGAAAGTG[a]	284	*kdp* operon	II
SCCmec III J1 F SCCmec III J1 R	CATTTGTGAAACACAGTACG[b] GTTATTGAGACTCCTAAAGC[b]	243	J1 region	III
mecI P2 mecI P3	ATCAAGACTTGCATTCAGGC[a] GCGGTTTCAATTCACTTGTC[a]	209	*mecI*	II and III
mecA P4 mecA P7	TCCAGATTACAACTTCACCAGG[a] CCACTTCATATCTTGTAACG[a]	162	*mecA*	Internal positive control

[a]Oliveira and de Lencastre [2002]; [b]Milheirico, Oliveira and de Lencastre [2007a].

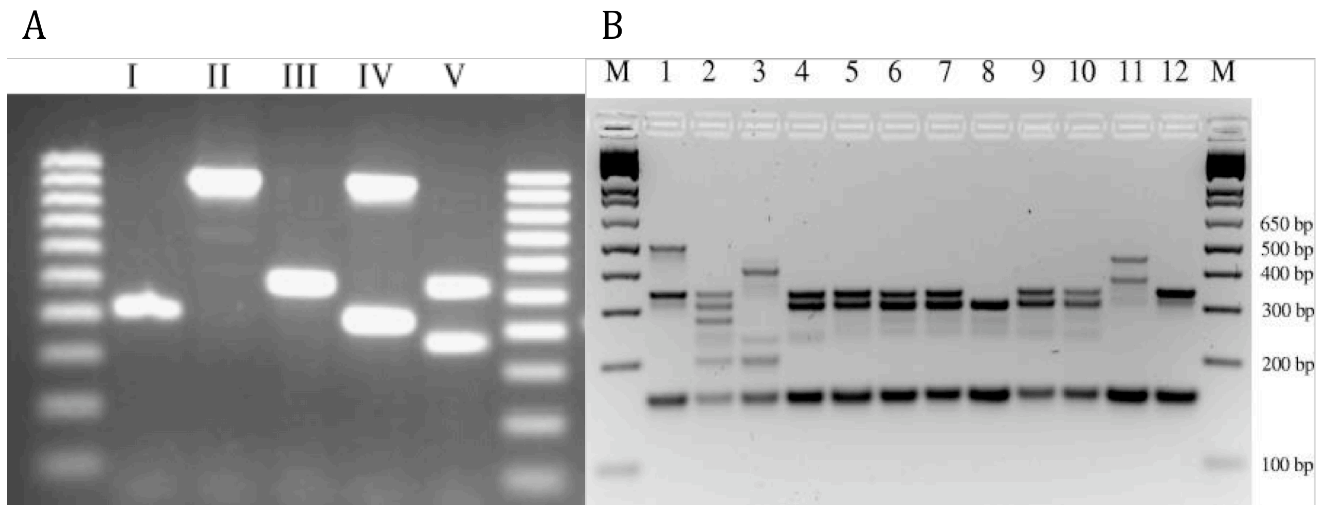


Figure 2.1 Amplification patterns obtained with the SCC*mec* multiplex PCR strategies of **A** Boye et al. [2007] (examples of SCC*mec* types I-V with 100bp DNA ladders) and **B** Milheirico, Oliveira and de Lencastre [2007a] (SCC*mec* types (prototype strains) as follows: lane 1, I (COL); lane 2, II (N315); lane 3, III (ANS46), lane 4, IVa (MW2); lane 5, IVb (8/6-3P); lane 6, IVc (Q2314); lane 7, IVd (JCSC4469); lane 8, IVE (AR43/3330.1); lane 9, IVg (M03-68); lane 10, IVh (HAR22); lane 11, V (WIS); lane 12, VI (HDE288); and M, 1kb DNA ladder). Gel images taken directly from Boye et al. [2007] and Milheirico, Oliveira and de Lencastre [2007a].

PART 1 INTRODUCTION: RPA-BASED DETECTION OF MRSA

The major advantage of rapid MRSA detection is the time saved over traditional culture methods. Although advances have been made with culture-based detection methods, particularly those based on chromogenic media, which have decreased the time to detection, molecular tests can provide results in a few hours; BD GeneOhm MRSA takes 2-4 hours, and Xpert MRSA under 70 minutes. However, both systems require expensive specialist platforms, the costs per test are high (BD GeneOhm MRSA excluding/including platform, personnel and additional costs (swabs, gloves and consumables), around €30/€56; Xpert MRSA around €43/€70) and the operator skill requirement for the assays is also relatively high, preventing their widespread routine use [Malhotra-Kumar et al. 2008, Wassenberg et al. 2010]. The training required and the size and expense of these platforms means they are typically employed only in central laboratories, significantly increasing the TAT for a diagnostic MRSA result. Nonetheless, PCR-based molecular detection methods allow a clinically relevant turnaround time for a diagnostic result, with comparable clinical sensitivity and specificity to culture-based methods [Luteijn et al. 2011]. There is still a need for a standalone rapid assay that can detect all MRSA strains, and a faster and cheaper diagnostic test that can be deployed at the point of care. This chapter describes and evaluates one such assay that is based on a novel method of nucleic acid amplification.

Recombinase polymerase amplification (RPA), developed by my industrial sponsor TwistDx, is a novel alternative to PCR for the amplification and detection of nucleic acids. It couples isothermal recombinase-driven primer targeting of template material with strand-displacement DNA synthesis (Figure I.1) [Piepenburg et al. 2006]. The key to RPA is the dynamic reaction environment that balances

recombinase-primer complex formation with disassembly (Figure I.2). UvsX recombinase of bacteriophage T4 binds cooperatively to primers in the presence of ATP. The resulting ATP-bound nucleoprotein complex (Figure I.2A) actively hydrolyses ATP and the consequent depletion of ATP substrate and accumulation of ADP/AMP products leads to replacement of UvsX with gp32 (Figure I.2B), the single stranded DNA binding protein (SSB) of T4 necessary for RPA. gp32 protects single-stranded DNA from nuclease digestion, removes secondary structure and promotes assembly of recombinase-primer complexes [Liu, Qian and Morrical 2006]. However, at high gp32 and salt concentrations, recombinase-primer complex formation is inhibited by the high affinity of gp32-primer interactions [Ando and Morrical 1998]. gp32 thus competes with UvsX for binding sites on the primer. Assembly of UvsX-primer complexes relies on the mediator function of the T4 UvsY protein, a recombinase loading factor that weakens gp32-primer interactions and strengthens UvsX-primer interactions, thus helping UvsX to displace gp32 and shift the equilibrium in favour of recombinase loading (Figure I.2C) [Jiang, Salinas and Kodadek 1997, Sweezy and Morrical 1999, Bleuit et al. 2004, Liu, Bond and Morrical 2006]. The presence of a crowding agent such as polyethylene glycol (PEG) further establishes reaction conditions that support RPA, by strengthening the interactions between the proteins and DNA. As well as a role in presynaptic filament formation, gp32 facilitates DNA strand exchange by binding to the displaced strand generated during UvsX-catalysed D-loop formation, as shown in Figure I.1 [Liu, Qian and Morrical 2006].

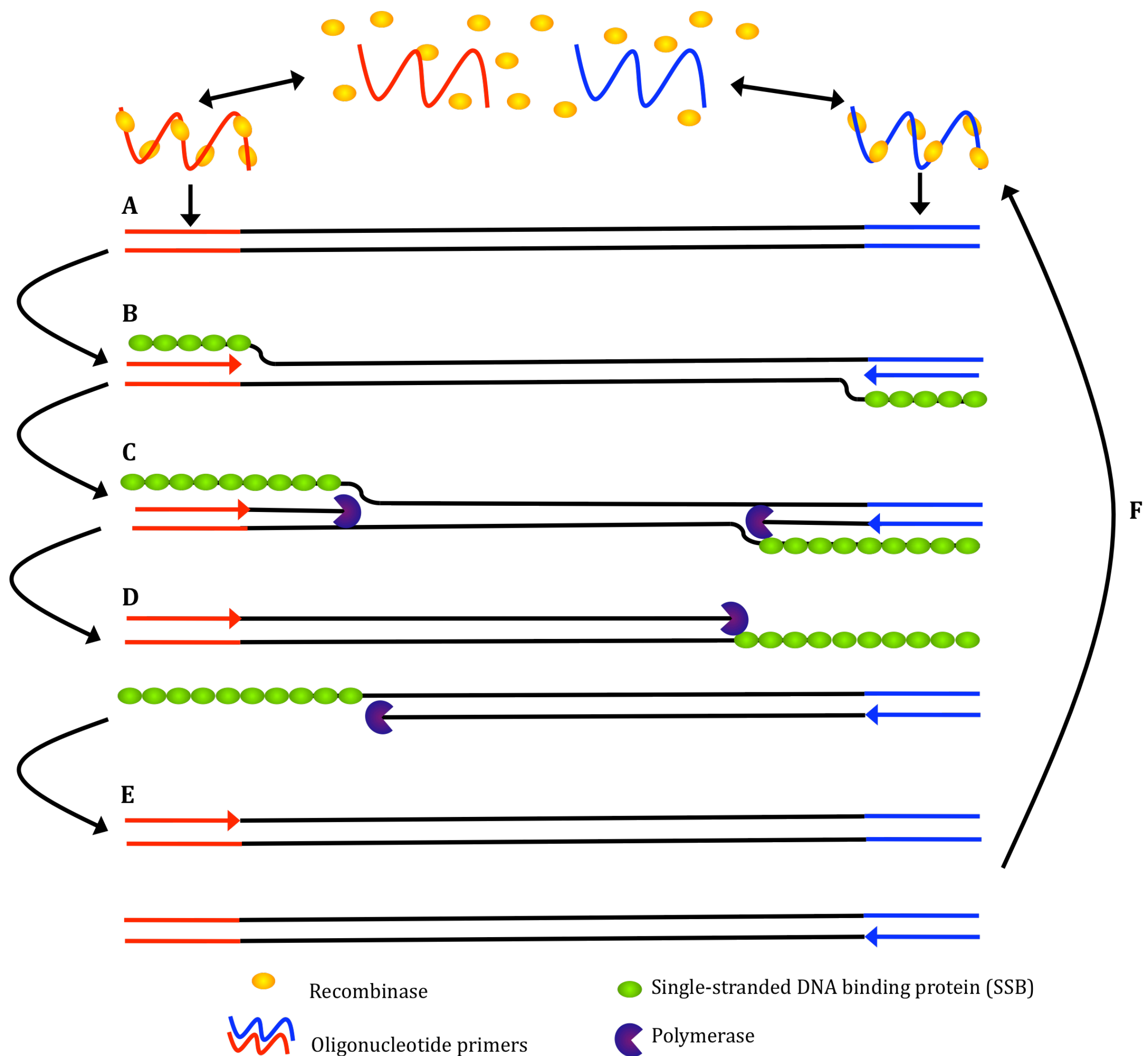


Figure I.1 Schematic of recombinase polymerase amplification (RPA). **A** Recombinase-oligonucleotide primer complexes form and target homologous DNA; **B** D-loops form due to strand displacement. The displaced strand is bound by SSB (gp32), which prevent reannealing of the double-stranded DNA; **C** Recombinase disassembly enables primer extension by the polymerase, initiating DNA synthesis; **D** Parental strands separate and synthesis continues; **E** Two duplexes form; **F** The process is cyclic, achieving exponential DNA amplification.

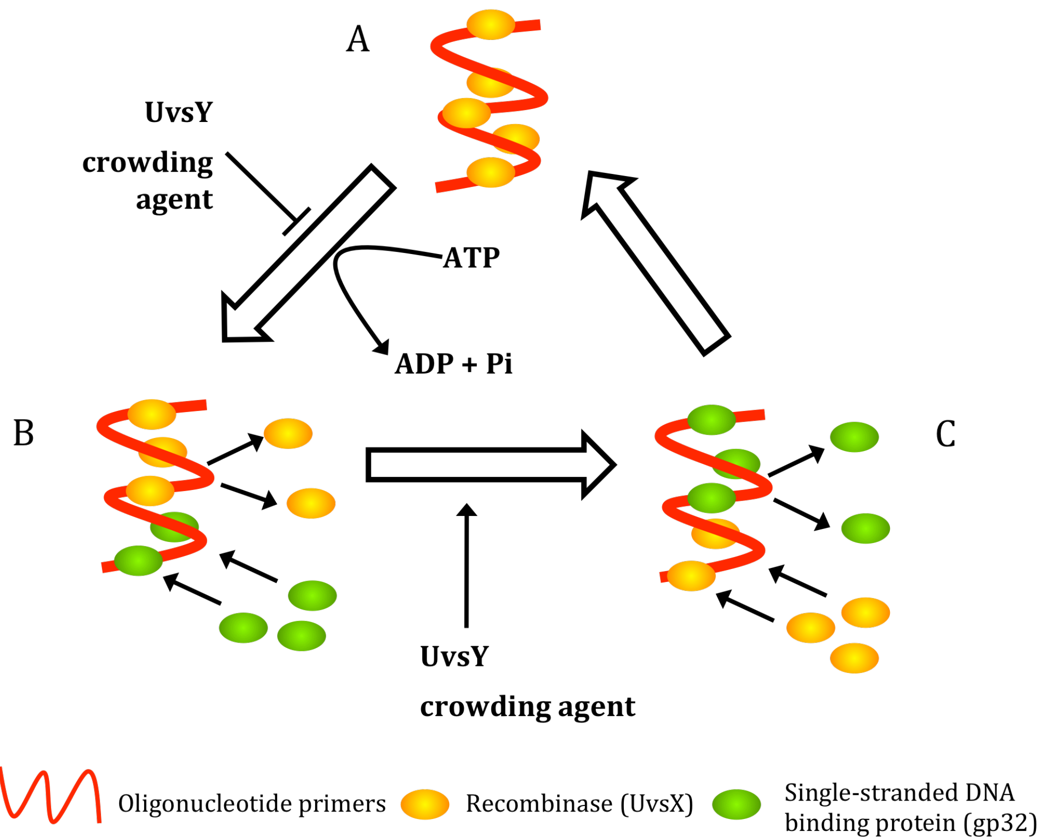


Figure I.2 The dynamic reaction environment of RPA. **A** ATP-binding results in a UvsX-primer complex; **B** replacement of UvsX with gp32 due to ATP hydrolysis; **C** UvsY and crowding agent support UvsX loading by weakening gp32-primer interactions.

In addition to primers, proteins (UvsX, UvsY and gp32) and a crowding agent, RPA reactions require salts, mainly acetate ions, to provide the appropriate ionic environment for RPA, dNTPs, Tris as a buffering component, and the cofactor magnesium to stabilise and support the reaction. For the polymerase, a Pol I large fragment from a mesophile is used, which has better characteristics for RPA than Klenow and other commercial relatives.

RPA has been shown to be sensitive to fewer than ten target copies of genomic DNA [Piepenburg et al. 2006] and exponentially amplifies the target to detectable

levels within 15 minutes, providing diagnostic results within 20 minutes. This is owing to a) the isothermal nature of the technology, which operates at an optimum of 37-42°C, obviating the need for denaturation and re-annealing steps as used in PCR, or for a specialist platform; and b) the robustness of RPA to crude complex samples with no pre-treatment required, allowing simple and rapid sample preparation.

An assay utilising the RPA technology has the potential to make a significant contribution to microbiological testing and molecular diagnostics by providing a portable, rapid and widely accessible nucleic acid-based test that can be used in point of care and field settings, from which current nucleic acid-based tests are almost entirely absent. As such, RPA has been incorporated into a probe-based detection system with the probe containing an abasic site mimic (a tetrahydrofuran residue or dSpacer) flanked by nucleotides modified with a fluorophore and quencher (Figure I.3). A 3′ block (e.g. a C3-spacer) prevents the probe from acting as an amplification primer, which could lead to non-specific amplification and signal generation from primer-dimers. On binding of the probe to complementary DNA, the double-strand-specific nuclease, Exonuclease III, cuts the probe at the abasic site, separating the fluorophore from the quencher. The probe remnant is then elongated by the polymerase, thus acting as an amplification primer. Since the probe's target sequence is located within the amplicon, cutting of the probe is indicative of the amplification event itself and can be used to monitor the progress of the reaction.

TaqMan probes are incompatible with RPA due to the use of a strand-displacing polymerase, which would displace the probe from the target but not cut the probe, thus generating no fluorescence. Likewise, molecular beacon probes are incompatible with RPA as the presence of SSB and recombinase would linearise the probe's hairpin structure, causing it to constantly fluoresce.

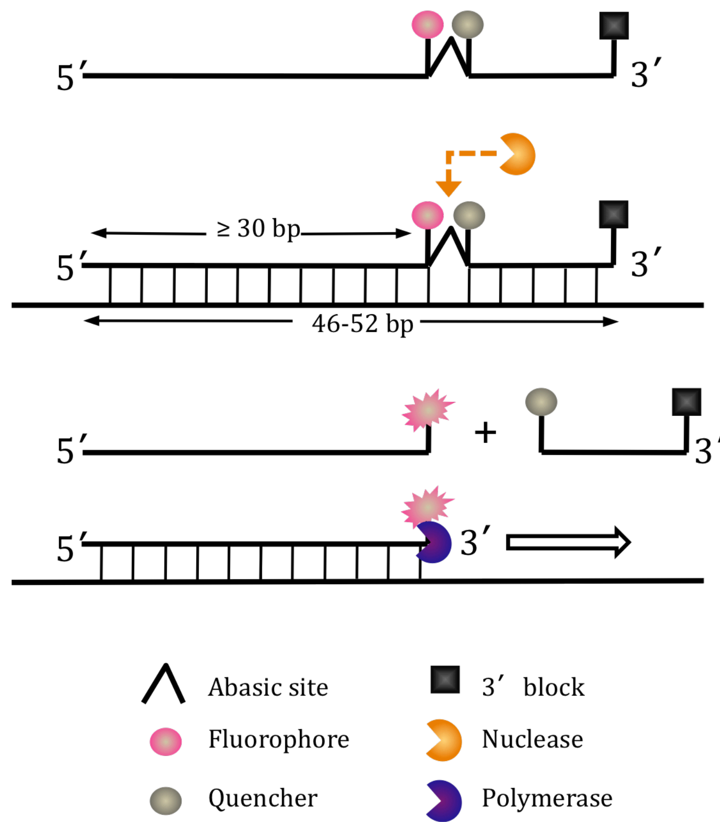


Figure I.3 RPA as a probe-based detection system.

Part 1 of my thesis focuses on the RPA-based detection system developed by TwistDx for the identification of MRSA in clinical samples, called TwistAmp MRSA. The first chapter evaluates its performance and characterises MRSA isolates that fail to be detected by the assay, in order to ascertain why they were not detected and how they can be detected in future. The second chapter explores potential reasons for its lower clinical sensitivity in comparison to the market leader in molecular diagnostics for MRSA, Cepheid's Xpert MRSA assay, and investigates possible methods for improving this sensitivity. Clinical sensitivity is the ability of a test to correctly identify MRSA, given as the percentage of persons with MRSA that are identified as having MRSA by the test. Analytical sensitivity is the smallest amount of MRSA (in CFU) that can be reliably detected by the test.

CHAPTER 3: TWISTAMP MRSA

3.1 INTRODUCTION

Having recognised the public health implications of MRSA worldwide and thus the need for a faster, simpler and cheaper diagnostic test compared to current commercial assays, TwistDx have developed an RPA-based diagnostic assay for MRSA, called TwistAmp MRSA. Proprietary primers amplify the different sequences between the SCC*mec* element and *orfX* (the *mec* right extremity junction; MREJ) found in MRSA strains, an approach similar to that used in the BD GeneOhm MRSA and Xpert MRSA assays. Proprietary FAM- and TAMRA-labelled TwistAmp exo probes then bind to the MREJ amplicons and internal control respectively and generate a fluorescence signal as a real-time readout.

3.1.1 The mec Right Extremity Junction (MREJ)

The MREJ is approximately 1kb in length and comprises the right extremity of SCC*mec*, the SCC*mec* integration site attBscc and the 3′ end of the *orfX* gene of the *S. aureus* chromosome (Figure 3.1) [Huletsky et al. 2004, Cuny and Witte 2005]. The first assay that utilised the MREJ as a MRSA-specific target was developed in 2000, using PCR primers that targeted the MREJs of SCC*mec* types I-III [Hiramatsu et al. 2000]. This led to the development of *mec* right extremity polymorphism (MREP) typing for SCC*mec* DNA, on the basis that SCC*mec* types I-III each have a different MREJ (MREJ types i-iii) [Hiramatsu, Kondo and Ito 1996, Ito et al. 2001]. After the discovery of SCC*mec* IV [Ma et al. 2002], it was found that the MREP typing method could not discriminate it from SCC*mec* II and therefore both were associated with MREJ ii [Huletsky and Rossbach 2002].

Whole-genome sequencing of MRSA strains N315 and Mu50 revealed that SCC*mec* elements are in fact located downstream of *orfX*, and thus the *mec* right extremity should actually be called the *mec* left extremity [Chongtrakool et al. 2006]. Consequently, MREP typing was renamed MLEP (*mec* left extremity polymorphism) typing due to the position of the SCC*mec* element in relation to *orfX* [Chongtrakool et al. 2006]. However, I shall use the original terminology (MREJ) for clarity as the literature largely refers to the *mec* right extremity.

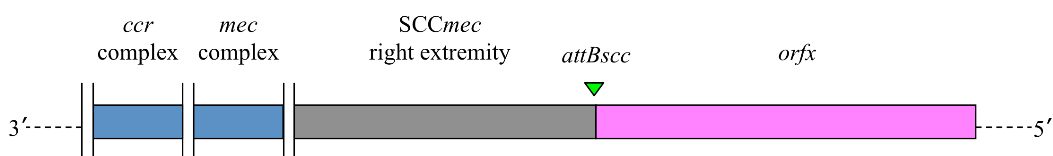


Figure 3.1 The *mec* right extremity junction (MREJ). Relative positions of the *ccr* and *mec* complexes of SCC*mec* are also shown. *attBscc* is the SCC*mec* integration site.

In 2002 the MREP typing method was used to test a variety of MRSA, MSSA and CNS strains. Approximately 50% (20/39) of the MRSA strains tested were not amplified [Huletsky and Rossbach 2002]. With the aim of developing more universal primers and probes for global MRSA detection, a new set of MRSA-specific primers was developed using the MREJ sequences of SCC*mec* types I-III plus type IV subtypes IVa, IVb and IVc [Huletsky and Rossbach 2002, Huletsky et al. 2004]. These sequences comprised MREJ types i-iii plus the newly discovered types iv, v and vii [Huletsky and Rossbach 2002], and their corresponding primers and probes were combined to create a multiplex PCR assay for MRSA [Huletsky et al. 2004]. MREJ sequences for types vi, viii, ix and x were also described but were not included

in this multiplex assay as they were infrequent among the strains characterised [Huletsky and Rossbach 2002].

In 2007 a further 10 MREJ types (xi-xx) were described, bringing the total number of known MREJ types to 20 [Huletsky and Rossbach 2002, Huletsky and Giroux 2007]. As a result, detection of more MRSA strains was now possible, allowing improvement of current assays for MRSA detection. However, due to the nature of multiplexing, where assay performance decreases as the extent of multiplexing increases, and taking into account the rarity of certain MREJ types and the predominance of others, it is not practical to detect all of these 20 MREJ types with a multiplex assay - there must be a compromise between the coverage of as many SCC*mec* variants as possible and as little loss of analytical sensitivity as possible.

The TwistAmp MRSA assay developed by my industrial sponsor, like current commercial assays, covers MREJ types i-v and vii (Figure 3.2) as the optimal set of MREJ types for the RPA-based system. These six MREJ types account for >98% of worldwide strains as tested by BD Diagnostics [2012].

In order for TwistDx to design primers and probes for the various MREJ types, sequences were obtained from either public databases or patents [Hiramatsu et al. 2000, Huletsky and Rossbach 2002, Huletsky and Giroux 2007]. Sequences are not publicly available for MREJ types vi and viii-x. Sequence data for MREJ xii and isolates possessing this MREJ type are available but corresponding RPA primers were not included in the multiplex assay since they proved counterproductive to incorporate (reactions failed, likely due to unfavourable changes in the kinetics of the reaction or primer interactions that have an inhibitory effect on the reaction) [Forrest 2009]. Isolates with the remaining MREJ types (xi and xiii-xx) have yet to be discovered by TwistDx, except for an isolate with MREJ type xiii, which I discovered

during my research. Primers for these types therefore cannot be, or haven't yet been, tested for possible incorporation into the multiplex assay. Nonetheless, an RPA-based assay targeting only MREJ types i-v and vii currently provides the optimal combination of primers, probes and reagents for rapid and sufficiently ubiquitous MRSA detection.

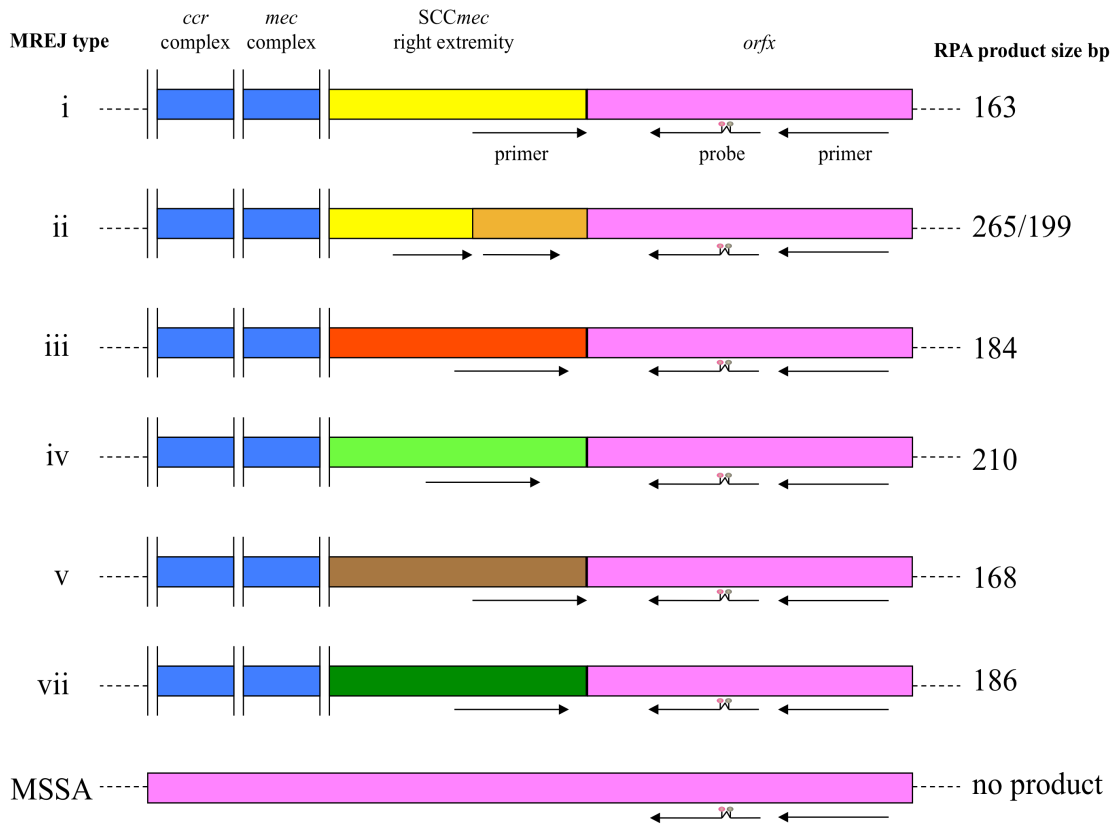


Figure 3.2 Schematic showing the relative positions of proprietary primers and probe for the different MREJ types covered by TwistAmp MRSA. The same *orfX* primer covers all MREJ types; the SCC*mec* right extremity primer varies for each MREJ type. A 102-bp insertion (orange region) differentiates MREJ ii from MREJ i. MREJ ii isolates will test positive for both MREJ i and ii due to the targets of the primers, but type ii primers are still included in the multiplex assay because of slow amplification of type ii template by type i primers alone. Figure adapted from Huletsky et al. [2004].

3.1.2 Chapter Objectives

3.1.2.1 TwistAmp MRSA performance

The aim of TwistAmp MRSA is to not only provide a more accurate and rapid alternative to traditional culturing methods for MRSA detection, but also to rival the current commercial PCR-based MRSA assays. A collection of Staphylococci from our laboratory, including MRSA, MSSA and CNS, was used to interrogate the assay in order to assess its performance (sensitivity, specificity, PPV and NPV). Two collections of MRSA isolates obtained from standard screening procedures at hospitals in the UK and US (hereafter called the UK and KC collections respectively; see section 3.2) were used to further determine the performance of the assay.

3.1.2.2 MREJ typing of MRSA isolates

TwistAmp MRSA uses a multiplex format with proprietary primers and probes to detect MRSA with MREJ types i-v and vii. Any MRSA with one of these MREJ types will produce a fluorescence signal, which provides the user with a positive identification of MRSA. Unlike MREP typing however, the MREJ type of the isolate is not provided by the method. MREJ types can, however, be obtained using a series of singleplex RPA-based assays with the primers and probes for the individual MREJ types, an approach called MREJ typing.

The MREJ has not been studied a great deal in an epidemiological sense. Since the prevalence of SCC*mec* types at the local and global level are well documented, MREJ typing was performed on the UK and KC MRSA collections in order to assess the diversity and distribution of MREJ types among local populations of MRSA. Similarly, MREJ typing of our laboratory collection of MRSA was

performed to provide a snapshot of the diversity and distribution of MREJ types among geographically diverse strains of HA- and CA-MRSA.

The commercial assays BD GeneOhm MRSA, Xpert MRSA and Genotype MRSA Direct all target the MREJ, but like TwistAmp MRSA only produce a yes/no output; they do not report the specific MREJ type. In their product literature, these three commercial assays state the different SCC*mec* types that they are able to detect, and the associated limit of detection (LOD) for each type. The LODs are based on testing of an MRSA strain of each SCC*mec* type, but the product literature does not clearly specify how SCC*me*c type correlates with MREJ type. The BD GeneOhm MRSA package insert lists the LOD for each of six strains tested, representing MREJ types i-v and vii and SCC*mec* types I-IV, but also states the ability to detect SCC*mec* types V and VI without specifying the associated MREJ type [BD Diagnostics 2012]. The Xpert MRSA and Genotype MRSA Direct assays can detect SCC*mec* types I-V but the associated MREJ types are not stated [Cepheid Diagnostics 2009, Hain Lifescience GmbH 2012].

To my knowledge only two comparisons of MREJ typing and SCC*mec* typing have been made. Huletsky et al. [2004] made three key findings: 1) no correlation was found between MREJ and SCC*mec* type for the MRSA strains described in the study; 2) exceptions were found to the typical association between SCC*mec* types I-III and MREJ types i-iii; and 3) strains with new MREJ types did not carry a new SCC*mec* element but rather have structural variations at the SCC*mec* right extremity, since known SCC*mec* types were assigned for most of the MRSA strains with the MREJ types described in the study [Huletsky et al. 2004]. The other study found a strong correlation between SCC*mec* type and MREJ type, but there were discrepancies and non-typeable isolates [Chongtrakool et al. 2006]. For example, of 370 SCC*mec* III

isolates, 86.4% were MREJ iii but 10.6% were MREJ ii and 3% could not be assigned a MREJ type [Chongtrakool et al. 2006]. Since TwistAmp MRSA also targets the MREJ, the correlation between SCC*mec* type and MREJ type was explored further using our laboratory and the KC collections of MRSA, as well as prototypic strains for SCC*mec* types I-XI.

3.1.2.3 Characterisation of false negative isolates

The evaluation of the performance of TwistAmp MRSA in identifying MRSA isolates in the UK and KC collections, followed by MREJ typing of these collections, resulted in MRSA that were not detected by this assay (false negatives) and an MSSA that was identified as MRSA (false positive). In order to contribute to the development and improvement of TwistAmp MRSA, these false positive and false negative isolates were characterised using MLST, *spa* typing and SCC*mec* typing. In this way it was hoped that STs, *spa* types or SCC*mec* types could then be identified that might pose a problem in terms of MRSA detection using TwistAmp MRSA. In the case of false negative isolates, sequencing of the MREJ region was performed to try and determine why these isolates were not detected, and if their MREJ sequences could be incorporated into the assay through modification of the primers and/or probes for currently covered MREJ types, or whether new primers and probes needed to be developed for novel MREJ types.

In the latter case, primers and/or probes were designed for any novel MREJ types and full primer screens conducted for potential incorporation into and therefore improvement of the multiplex assay. If RPA primers are already developed for all MREJ types encountered, whether known or novel, then incorporating them into the diagnostic assay prospectively, where required, will prove more efficient than

discovering the assay does not detect a percentage of MRSA strains in a certain region and designing them retrospectively.

3.1.2.4 Scope of TwistAmp MRSA

TwistDx aim to develop TwistAmp MRSA such that it can be marketed widely, as opposed to an assay that after marketing in a certain region, requires development of primers and probes for false negative variants discovered there. To assess whether the current set of primers and probes in TwistAmp MRSA is sufficient to detect most MRSA strain types, the assay was tested with strains possessing different SCC*mec* elements. All isolates from the collections described above that were characterised by SCC*mec* typing were tested, as well as prototypic strains for SCC*mec* types I-XI.

Recent literature and personal communications suggest some MRSA strains can be problematic with current commercial diagnostic assays such as BD GeneOhm MRSA and Xpert MRSA [Thomas et al. 2008, Bartels et al. 2009, Snyder et al. 2009, Voss 2009, Malhotra-Kumar et al. 2010b]. Bartels et al. [2009] hypothesised that the same SCC*mec* type might have minor variations in different MRSA lineages and that this variation could be in the primer regions. While studies of BD GeneOhm MRSA performance suggest misidentifications are uncommon [Stamper et al. 2007, Grobner et al. 2009], other studies observe low assay sensitivities due to the prevailing SCC*mec* types [Thomas et al. 2008, Bartels et al. 2009]. TwistAmp MRSA was therefore tested with identical strains or ones similar to those that have been reported to be problematic, to see if it too was unable to detect them. Similarly, MRSA isolates of clonal lineage ST398, a major livestock-associated MRSA able to cause infections in humans, have been reported to be missed by current diagnostic MRSA assays

[Reischl et al. 2009, Voss 2009, Malhotra-Kumar et al. 2010b]. ST398 has been isolated mainly from livestock but also other animals including horses, chickens and pets [Voss et al. 2005, de Neeling et al. 2007, Monecke et al. 2007b, Cuny et al. 2008, Nemati et al. 2008, Loeffler et al. 2009, Nienhoff et al. 2009, Walther et al. 2009, Mulders et al. 2010]. It has also been isolated from humans with or without a history of contact with livestock [Aubry-Damon et al. 2004, Sergio et al. 2007, van Loo et al. 2007, Witte et al. 2007b, Khanna et al. 2008, Smith et al. 2008, Wulf et al. 2008, Yu et al. 2008, Cuny et al. 2009, Krziwanek, Metz-Gercek and Mittermayer 2009]. ST398 has been reported in both community and healthcare settings in several European countries [Nemati et al. 2008, Krziwanek, Metz-Gercek and Mittermayer 2009, Loeffler et al. 2009, Lozano et al. 2009, Pan et al. 2009, Battisti et al. 2010, Potel et al. 2010, Vanderhaeghen et al. 2010], the Americas [Khanna et al. 2008, Smith et al. 2008, Bhat et al. 2009], Australia [Monecke et al. 2011], Singapore [Sergio et al. 2007] and China [Yu et al. 2008]. Most worryingly, ST398 has shown the ability to cause severe infection in humans, such as bacteraemia and pneumonia, including necrotising pneumonia [Witte et al. 2007b, Nulens et al. 2008, van Belkum et al. 2008, van Rijen, Van Keulen and Kluytmans 2008, Hartmeyer et al. 2010, Mammina et al. 2010, Rasigade et al. 2010, Soavi et al. 2010]. TwistAmp MRSA was therefore tested with a reference ST398 isolate (SO385, provided by Angela Kearns, Staphylococcal Reference Unit, HPA, UK) to assess its ability to detect a strain from this important lineage.

3.2 METHODS

Note: I performed all methods unless otherwise stated.

3.2.1 The UK MRSA collection

MRSA isolates were obtained from the Central Manchester University Hospitals NHS Foundation Trust (CMFT) and Addenbrooke's Hospital (Cambridge University Hospitals NHS Foundation Trust) as part of collaborations with TwistDx. MRSA isolates were obtained from clinical samples collected via standard hospital screening procedures (nasal and groin swabs were combined and processed according to a gold standard broth enrichment culture technique). CMFT isolates were collected between December 2008 and June 2009 (n=580); Addenbrooke's isolates included a random selection of MRSA collected throughout 2008, plus all MRSA samples collected in December 2008 (n=550). The 1,130 MRSA isolates were sub-cultured by TwistDx and collaborators, and one colony from each sub-culture resuspended in sterile distilled water (SDW) in a 1.5ml cryovial or eppendorf tube and boiled in water for 20 minutes to kill the bacteria. Boiled bacteria were then diluted 1:1000 in SDW in new 1.5ml tubes. A separate collaboration between TwistDx and CMFT (July 2009 to November 2009) obtained a further 146 culture-positive MRSA isolates from nasal and groin screening swabs that were cultured via standard methods employed at CMFT and stored as glycerol stocks. A total of 1,276 MRSA isolates - 1,130 boiled isolates in SDW and 146 viable isolates in glycerol - were provided to me for MREJ typing.

3.2.2 Kansas City paediatric hospital MRSA collection

Fifty-two hospital screening samples (nasal swabs) positive for MRSA by culture were collected from a paediatric hospital in Kansas City, US, between May 2011 and August 2011, as part of a further collaboration with TwistDx. Samples were determined as MRSA using CHROMagar plates as part of the standard screening protocol employed at the hospital. Collaborators collected 35 isolates positive for MRSA by culture from outpatients visiting a dermatology clinic and 17 from hospital inpatients. These 52 MRSA isolates were provided to me as glycerol stocks. One MR-CNS and four MSSA isolates from clinic outpatients were also provided to me as negative controls. All 57 isolates were tested with TwistAmp MRSA and the MRSA isolates were also MREJ typed.

3.2.3 Laboratory collection of Staphylococci

Our laboratory collection of Staphylococci comprises 57 MRSA isolates, 59 MSSA isolates and three CNS isolates (one MR-CNS and two MS-CNS), totalling 119. This collection contains members of previously described EMRSA clones - including glycopeptide-intermediate *S. aureus* (GISA) isolates - and MSSA isolates from disease and carriage [Enright et al. 2002, Feil et al. 2003]. The isolates represent lineages of geographically diverse HA- and CA- MRSA (Appendix 1). The MSSA isolates represent 52 different STs and are mainly from the UK, but also include one isolate from the Netherlands (ST281), one from Canada (ST289), a putative ancestor to the Berlin clone from Germany (ST46), two isolates from Cuba (ST30 and ST94) and two MSSA isolates recovered in the early 1960s from Denmark (ST30 and ST250) [Crisostomo et al. 2001, Robinson and Enright 2003]. The CNS isolates

include one positive for the type III SCC*mec* element. These 119 isolates were used as a screening collection for TwistAmp MRSA, to assess the sensitivity, specificity, PPV and NPV of the assay. The 57 MRSA isolates were also used to further investigate MREJ types.

In summary I had available for study 1,276 MRSA isolates from the UK, 52 MRSA isolates from KC, US, and 57 MRSA isolates from our own laboratory collection.

3.2.4 TwistAmp MRSA protocol

TwistAmp MRSA reactions come freeze-dried in strips of 8x0.2ml tubes, so up to 7 samples plus a control reaction can be run at once. The freeze-dried reactions contain all the proteins needed for RPA and the proprietary primer/probe set used for MRSA detection. RPA resuspension buffer containing salts, crowding agent and Mg, as well as the test DNA, is added to the freeze-dried reactions to start RPA. The same standard protocol was used throughout my thesis for testing clinical isolates, culture or DNA, and is as follows:

1. One colony taken using a sterile inoculating loop from an agar plate **OR** one loop scrape of frozen isolate stock **OR** 1μl of DNA (at a concentration of approximately 50ng/μl unless otherwise stated), was added to 50μl of RPA resuspension buffer in a 1.5ml eppendorf tube. For a no template control (NTC), 1μl of SDW was added. The resuspension buffer containing template DNA was briefly spun down, vortexed and spun down again.

2. The 50μl of the resuspension buffer containing template DNA was added to a 0.2ml TwistAmp MRSA reaction tube, sealed firmly with a lid, and briefly vortexed and spun down.
3. The TwistAmp MRSA reaction tube was placed in a Twista machine (Figure 3.3A), a fluorometer that detects the TwistAmp exo FAM- and TAMRA-labelled probes. Reaction conditions were set up and monitored in real-time using Twista Studio software (Figure 3.3B) via a Twista-connected computer. The standard conditions for RPA reactions are 38 or 39°C for 20 minutes, with a 4 min reminder to shake (see below).
4. Because RPA is so rapid, operates at a constant temperature, and the reactions are so viscous (due to the RPA resuspension buffer), diffusion and convection do not adequately mix the amplicons throughout the reaction mixture. At 4 min, the reaction tube was therefore removed from the machine, briefly agitated by vortexing, and spun down before replacing in the machine, to disperse the amplicons throughout the reaction mixture so the reagents were not locally limiting.
5. The Twista machine was left to run for the remaining 16 minutes to generate the reaction curves (Figure 3.3B).

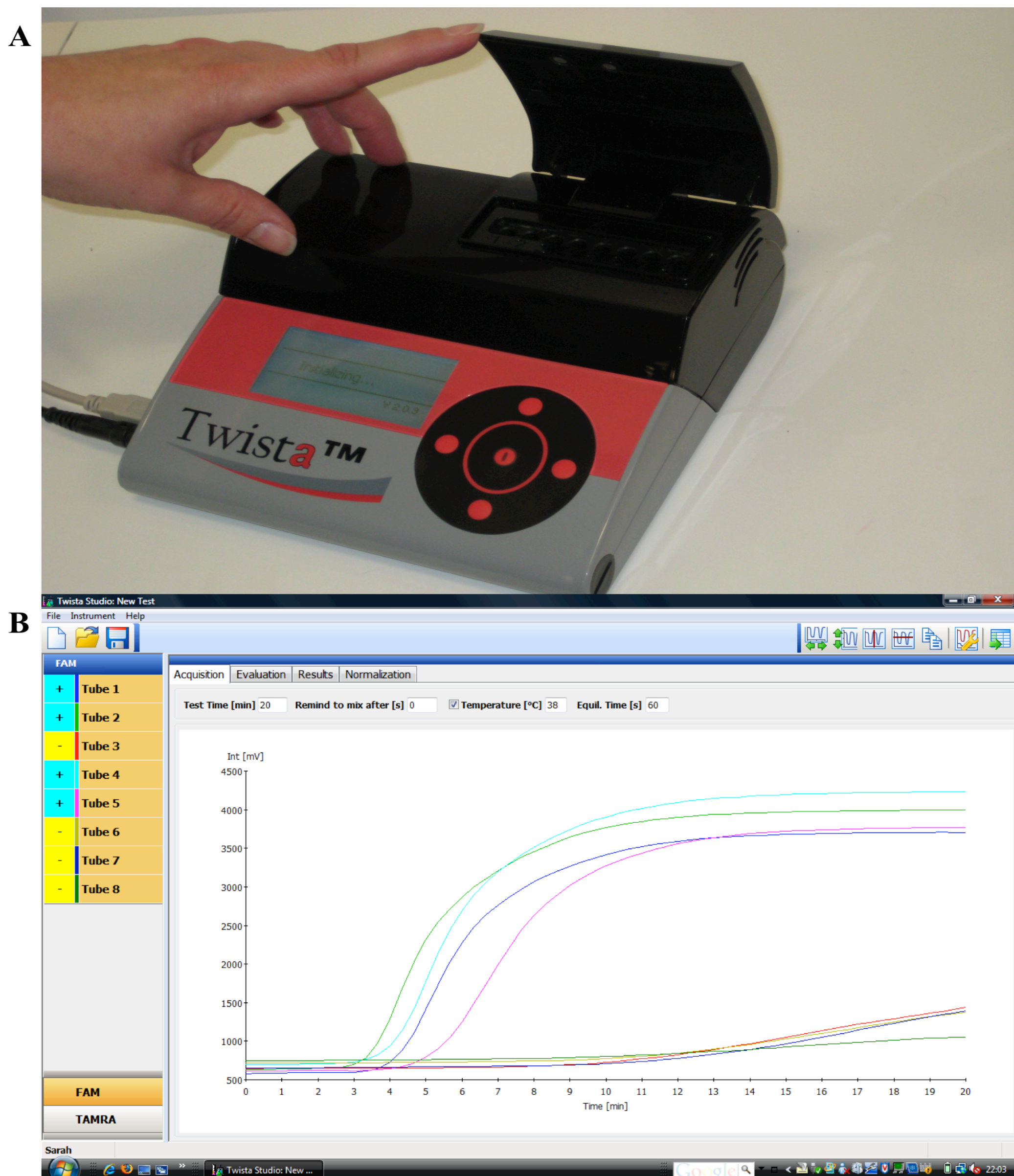


Figure 3.3 A Twista portable real-time fluorometer (photograph courtesy of TwistDx). **B** Typical Twista Studio output allowing fluorescence to be monitored in real-time. Tubes 1, 2, 4 and 5 are MRSA positive; tubes 3, 6 and 7 are MRSA negative. Tube 8 =NTC. Positive/negative results are declared on the left.

The TwistAmp MRSA protocol was used for screening all 119 isolates from our laboratory collection of Staphylococci. I also performed TwistAmp MRSA testing of the MRSA culture positive KC isolates direct from TSB-glycerol stocks diluted 1:1000 in SDW. Prototypic strains for SCC*mec* types I-XI were tested with TwistAmp MRSA from DNA samples.

3.2.5 MREJ typing protocol

The MREJ typing protocol was used to type all MRSA isolates from the UK, KC and laboratory collections. Prototypic strains for SCC*mec* types I-XI were also MREJ typed. The protocol used was as in 3.2.4, but using singleplex RPA reactions for the individual MREJ types. MREJ typing was performed in the following order to first distinguish type i isolates from type ii isolates and to process the isolates further in the most efficient manner (reactions for more common MREJ types performed first): ii, i, iii, vii, v, xii, iv. Isolates negative for these MREJ types were then tested with PCR reactions for types xi and xiii-xx (as RPA assays for these types had not been developed). Statistical analyses were performed using STATA (release 11, StataCorp). Fisher's exact test was used a) to compare the distributions of MREJ types among CMFT and Addenbrooke's isolates from the UK MRSA collection; and b) to investigate the correlation between SCC*mec* type and MREJ type among the 57 laboratory MRSA isolates.

3.2.6 Characterisation of isolates

I characterised the false negative isolates from the UK collection using MLST, SCC*mec* typing (Boye et al. [2007] method then Milheirico, Oliveira and de Lencastre

[2007a] method to retest unexpected banding patterns) and *spa* typing, all as described in Chapter 2, in order to ascertain their diversity and define clones based on the currently used nomenclature. All 57 isolates from the KC collection were characterised by Alere Technologies GmbH using their StaphyType DNA microarray, as previously described [Monecke and Ehricht 2005, Monecke et al. 2006, Monecke et al. 2007a, Monecke et al. 2008, Monecke, Slickers and Ehricht 2008, Monecke et al. 2011]. The array allows comprehensive genotyping of *S. aureus* isolates by simultaneous detection of a large number of genes and alleles thereof, including species markers, regulatory genes, and genes related to antibiotic resistance, virulence and pathogenicity. Markers for typing SCC*mec* were also included in the array, covering *mecA* and its regulatory genes as well as the different recombinase and accessory genes that make up the various SCC*mec* elements [Monecke et al. 2007a]. Alere Technlogies determined affiliation of isolates to clonal complexes or STs, as defined by MLST, by comparison of hybridisation profiles to a collection of reference strains previously characterised by MLST [Monecke and Ehricht 2005, Monecke et al. 2007a, Monecke et al. 2011]. Results of the microarray testing were sent to me for analysis and epidemiological interpretation.

3.2.7 Primer design and primer screening for novel MREJ types

False negative isolates from the Addenbrooke's MRSA collection were unavailable for whole genome sequencing as permission for their use could not be obtained. Collaborators at King Abdullah University of Science and Technology, Saudi Arabia, obtained the genome sequences of the false negative MRSA isolates from the CMFT collection, by assembly of 110-nucleotide reads from an Illumina Genome Analyzer IIx (Illumina, Inc.). The assemblies obtained from the 110-

nucleotide sequence reads were then sent to me by these collaborators, and for each sequenced isolate, the contig containing the MREJ was identified by BLAST using a segment of *orfX* (position 33692-34111 of GenBank entry BA000018). This contig was compared to all known MREJ sequences by ClustalW2 alignment. Positive control isolates of known MREJ types were also sequenced and assembled to confirm the ability of the procedure to correctly assemble MREJ sequences.

For novel MREJ sequences (18 successfully sequenced in total), I grouped together identical sequences and for each group (i.e. each novel MREJ sequence) chose 35bp-primers from within the SCC*mec* element a sufficient distance downstream from the TwistAmp MRSA *orfX* primer (~100bp), to amplify the SCC*mec*-*orfX* junction region. I then selected a further 14 primers, each time 5bp further downstream of *orfX* than the previous one. Thus, 15 candidate RPA primers for each novel MREJ were generated and tested.

I prepared template DNA for each novel MREJ using PCR primers flanking the RPA MREJ primers (primer sequences are commercially sensitive and cannot be shown), to generate amplicons of at least 500bp that span the TwistAmp MRSA target region within each novel MREJ. The *orfX* PCR primer was positioned approximately 200bp upstream of the *orfX* RPA primer. PCR reactions were performed using genomic DNA preparations of isolates with novel MREJ types, in a total volume of 50μl using DreamTaq DNA polymerase (Fermentas), according to the manufacturer's protocol. PCR products were viewed on a 1% agarose gel and extracted and purified using a QIAquick Gel Extraction Kit (Qiagen). DNA quantification of the purified products was performed using a TwistDx protocol (Appendix 2) and each product was serially diluted in Eppendorf LoBind tubes to ~50 copies/μl in T0.1E buffer (10 mM Tris–HCl, pH 8 and 0.1 mM EDTA, pH 8) supplemented with 1ng/μl of human DNA

(Promega). The use of LoBind tubes and inclusion of human DNA ensured minimal adsorption of target amplicons onto tube surfaces.

To conduct the primer screen, I used freeze-dried RPA reactions (50μl total volume) containing all the enzymes necessary for RPA but no primers or probe. These are called TwistAmp exo reactions, referring to the Exonuclease III used for probe cutting [TwistDx Ltd 2009a]. I then added the *orfX* and novel MREJ primers separately. The primer screening protocol was as follows:

1. For each novel MREJ, a mastermix for the required number of reactions was created:

	1x
• *orfX* primer (6μM)	4μl
• *orfX* probe (6μM)	1μl
• RPA resuspension buffer with no MgAc	29.5μl
• SDW	8μl
• novel MREJ template (~50 copies/μl)	1μl
	Total: 43.5μl

2. For each reaction, 43.5μl of the mastermix and 4μl of the appropriate candidate primer (6μM) were added to a 1.5ml eppendorf tube, vortexed and spun down.
3. 47.5μl of this solution was added to each freeze-dried TwistAmp exo reaction. TwistAmp exo reactions come in strips of eight reactions, so each candidate primer was tested in duplicate. Four candidate primers were therefore tested in one run.

4. 2.5µl of 280mM MgAc was added to the inside of the lid of each reaction tube, the lids on the reaction tubes secured, and the reaction tubes briefly spun, vortexed and spun again (50µl total reaction volume).
5. The TwistAmp protocol was then followed from step 3, as in section 3.2.4.

The best candidate primer from the first stage of the primer screen was selected and tested alongside a further eight primers, staggered by 1bp downstream (four primers) and 1bp upstream (four primers) from the initial best candidate primer. The best 35bp-candidate from this second stage was tested in duplicate with no template and 10^6 copies of MSSA to ensure specificity for MRSA detection, then modified in length to create another 8 primers 30-38bp long. The length was modified at the 3′ end of the primer sequence i.e. towards *orfX*. This final stage of the primer screen determined the optimal MREJ-specific RPA primer to use with the universal *orfX* RPA primer for detection of each novel MREJ type.

3.2.8 Detection of SCCmec variants and problematic strains

To determine the range of SCC*mec* types that TwistAmp MRSA was able to detect, prototype strains for SCC*mec* types I-XI (Table 3.1) were tested with the assay. A number of strains that have been reported not to be detected by other MREJ-based diagnostic assays were also examined, as follows. Bartels et al. [2009] found a common variant of SCC*mec* type IVa (ST8-MRSA-IVa/t024) in Copenhagen, Denmark, that is not detected by the BD GeneOhm MRSA assay. This variant is the most abundant MRSA clone in Copenhagen, affecting mainly people in nursing homes and causing small outbreaks in local hospitals [Bartels et al. 2009]. One isolate with a similar SCC*mec* and *spa* type (IVh and t024 respectively) to the Copenhagen

clone reported in the Bartels study was tested with TwistAmp MRSA, MREJ typed and its MREJ sequenced. Another study of Australian MRSA isolates revealed only half (12/24) were correctly identified by BD GeneOhm MRSA with 11 of the 12 false negatives corresponding to the predominant Australian nosocomial clone (ST239-MRSA-III) and one to the Southwest Pacific clone (ST30-MRSA-IV) [Thomas et al. 2008]. Isolates of these clones are included in the laboratory collection of Staphylococci and were thus tested with TwistAmp MRSA as part of the screening performed in this chapter. The reference strain SO385 (ST398-MRSA-V) was also tested and further characterised by *spa* and MREJ typing, and its MREJ sequenced.

Table 3.1 SCC*mec* prototype strains.

SCC*mec* type	**Strain name(s)[a]**
I	NCTC10442, COL
II	N315, BK2464
III	85/2082, ANS46
IVa	JCSC4744, MW2
V	WIS
VI	HDE288
VII	JCSC6082
VIII	C10682
IX	JCSC6943
X	JCSC6945
XI	LGA251

[a] Strains were kindly provided by T. Ito, H. de Lencastre, B. Soderquist, K. Zhang, A. Larsen/R. Skov and M. Holmes.

3.3 RESULTS

3.3.1 TwistAmp MRSA performance

3.3.1.1 UK MRSA collection

Of the 1,276 MRSA isolates from the UK collection, 1,232 were MREJ typed as i-v or vii (the types covered by TwistAmp MRSA) (Table 3.3), giving TwistAmp MRSA a sensitivity of 96.6%.

3.3.1.2 KC MRSA collection

Of the 52 isolates that were culture positive for MRSA from the KC collection, 50 gave a positive result when tested with TwistAmp MRSA. Despite a culture positive result for MRSA, the two remaining isolates were found to be MSSA and MR-CNS (*S. succinus*) when characterised by Alere Technologies using their StaphyType DNA microarray (see section 3.3.3.2). The five isolates included in the KC collection as negative controls (four MSSA isolates and one MR-CNS (*S. haemolyticus*) isolate) correctly gave a negative result with TwistAmp MRSA. Thus, TwistAmp MRSA correctly identified all 50 MRSA isolates, giving the assay a sensitivity of 100%.

Two KC isolates that were culture positive for MRSA produced weak TwistAmp MRSA reaction curves compared to all other isolates (Figure 3.4). One of these was an isolate that was later found to be MSSA by microarray analysis (performed by Alere Technologies; see section 3.3.3.2) and therefore gave a false positive result with TwistAmp MRSA, perhaps due to an SCC remnant or *mec*-less cassette. I sequenced the MREJ of these two isolates, both of which were MREJ i, using PCR primers flanking the RPA *orfX* and RPA MREJ i-specific primers.

Sequencing revealed no single nucleotide polymorphisms (SNPs) in the primer/probe binding regions, except one SNP in the *orfX* primer binding region of the MSSA isolate (the same SNP found in most 'odd-performing' MREJ ii isolates of the CMFT collection; see Figure 3.7 in section 3.3.2.1). This C/T SNP however was deemed to have little effect on reaction performance due to its central position within the primer, which was confirmed by testing PCR product (1000 copies/μl) of a MREJ with the same *orfX* SNP, and directly comparing it to wild type PCR product with no SNPs in the *orfX* primer binding region (Figure 3.5). See section 3.3.3.2 for the results of further characterisation of these two isolates.

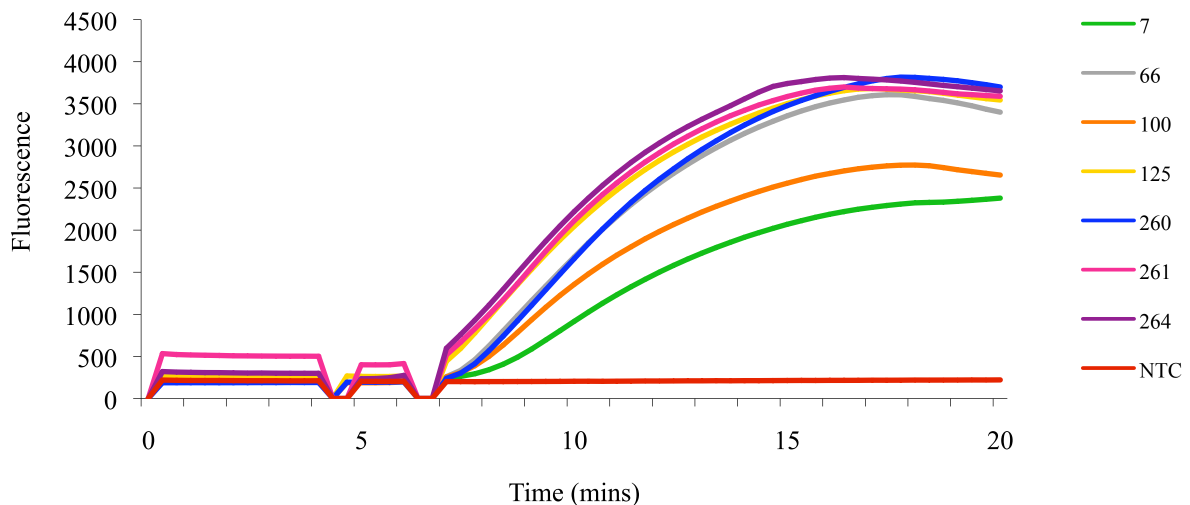


Figure 3.4 TwistAmp MRSA output showing weak reaction curves produced by two isolates from the KC MRSA collection (isolates 7 and 100; 7 was later found to be MSSA by microarray analysis). Legend numbers represent isolate numbers. NTC= no template control.

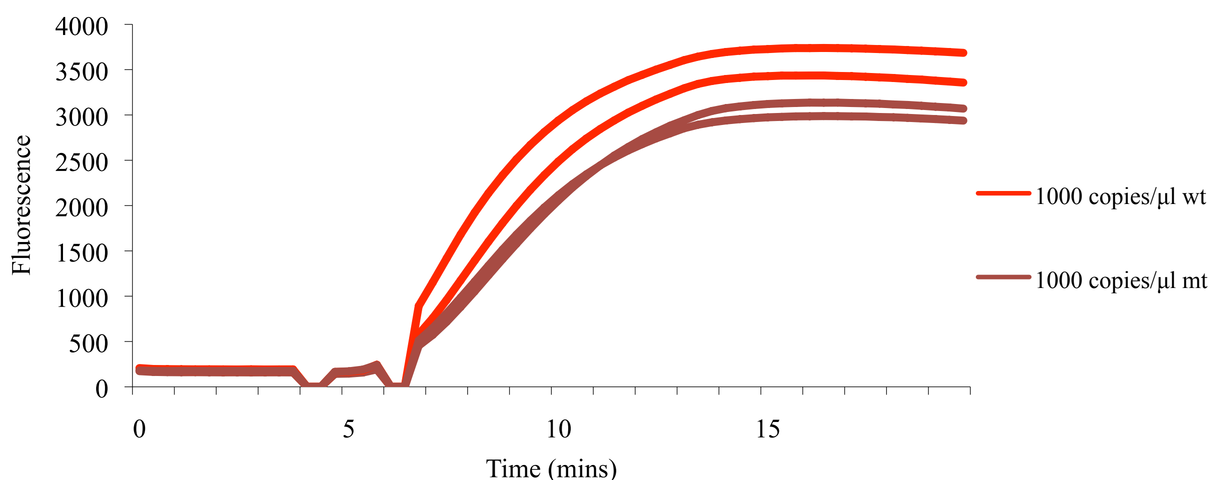


Figure 3.5 TwistAmp MRSA output showing the effect of the C/T SNP in the *orfX* primer binding region. wt = wild-type MREJ template; mt = mutant MREJ template.

3.3.1.3 Laboratory collection of Staphylococci

The results of screening our laboratory collection of Staphylococci (57 MRSA, 59 MSSA, 2 MS-CNS and 1 MR-CNS) with TwistAmp MRSA, directly from glycerol stocks, are shown in Table 3.2. The assay produced six false positives and one false negative and these isolates were retested, leading to slightly improved test results: one MRSA isolate that produced a negative result initially, was correctly identified upon retesting, and vice versa for one MSSA isolate, leaving five false positives but no false negatives. All false positive isolates were MSSA from the UK, representing five different STs (previously characterised as STs 1, 3, 8, 69 and 266 [Enright et al. 2002, Feil et al. 2003]).

Table 3.2 Test characteristics of TwistAmp MRSA for the laboratory Staphylococci collection (N=119). The first numbers/percentages in each box represent results of the initial screen, and the second numbers/percentages represent results of the repeat screen.

		Isolate		
		MRSA	MSSA or CNS	
Test outcome	TwistAmp MRSA positive	True positive *56* 57	False positive *6* 5	**PPV** *90.3%* 91.9%
	TwistAmp MRSA negative	False negative *1* 0	True negative *56* 57	**NPV** *98.2%* 100%
		Sensitivity *98.2%* 100%	**Specificity** *90.3%* 91.9%	

3.3.2 MREJ typing

3.3.2.1 UK MRSA collection

1,232 of the 1,276 MRSA isolates were typed as MREJ i-v or vii (Tables 3.3 and 3.4) using singleplex RPA reactions. A further two isolates were MREJ typed using PCR reactions for types xiii and xvi, but the latter was later found by whole genome sequencing to be incorrectly assigned and was re-assigned as a MREJ non-typeable (NT) isolate (see section 3.3.3.1). Forty-two isolates (3.3%; 28 from CMFT and 14 from Addenbrooke's) were negative for all MREJ types tested (i-v, vii and xi-xx). The predominant MREJ type was ii, making up 90.8% of all isolates. The distribution of MREJ types, including NT MREJs, was not significantly different between CMFT and Addenbrooke’s isolates (Table 3.4, p = 0.485).

25 CMFT isolates were assigned MREJ types (23 MREJ ii, one MREJ i and one MREJ iii) but produced weakly positive or lagging reaction curves (Figure 3.6). SNPs in the *orfX* primer binding region were observed among 19 of the 25 isolates, with one SNP in the MREJ i/ii isolates and a different SNP in the MREJ iii isolate. A further two SNPs were identified in the MREJ iii-specific primer binding region of the single odd-performing MREJ iii isolate (for *orfX* SNPs see Figure 3.7). Upon retesting the 25 isolates all produced strong reaction curves.

Table 3.3 MREJ typing results of the UK MRSA collection.

MREJ type	Frequency	%
i	21	1.6
ii	1159	90.8
iii	19	1.5
iv	2	0.2
v	3	0.2
vii	28	2.2
xiii	1	0.1
xvi/NT[a]	1	0.1
NT	42	3.3
Total	**1,276**	**100**

[a] NT = MREJ non-typeable. MREJ xvi isolate incorrectly assigned and later found to be NT (see section 3.3.3.1).

Table 3.4 MREJ typing results of the UK MRSA collection, by hospital.

MREJ type	Addenbrooke's Frequency (%)	CMFT Frequency (%)
i	8 (1.5)	13 (1.8)
ii	506 (92)	653 (89.9)
iii	6 (1.1)	13 (1.8)
iv	0	2 (0.3)
v	2 (0.4)	1 (0.1)
vii	14 (2.5)	14 (1.9)
xiii	0	1 (0.1)
xvi/NT[a]	0	1 (0.1)
NT	14 (2.5)	28 (3.9)
Total	**550**	**726**

[a] NT = MREJ non-typeable. MREJ xvi isolate incorrectly assigned and later found to be NT (see section 3.3.3.1).

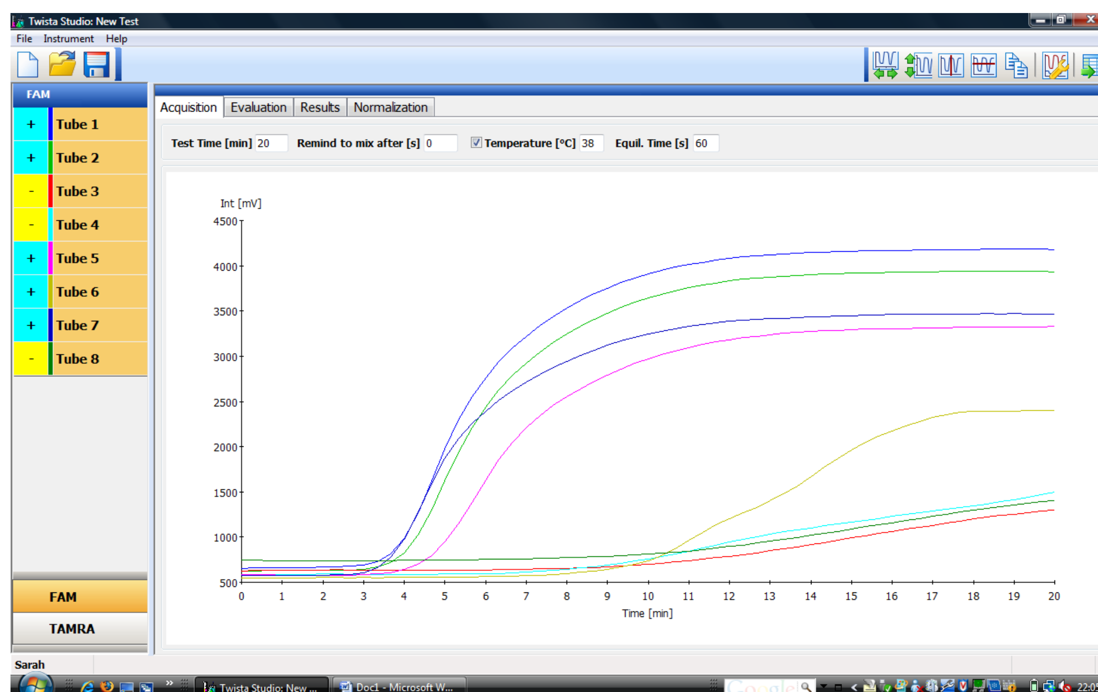


Figure 3.6 Twista Studio output for MREJ typing results showing a weakly positive reaction curve (Tube 6 - olive green line). Tube 8 (dark green line) = NTC.

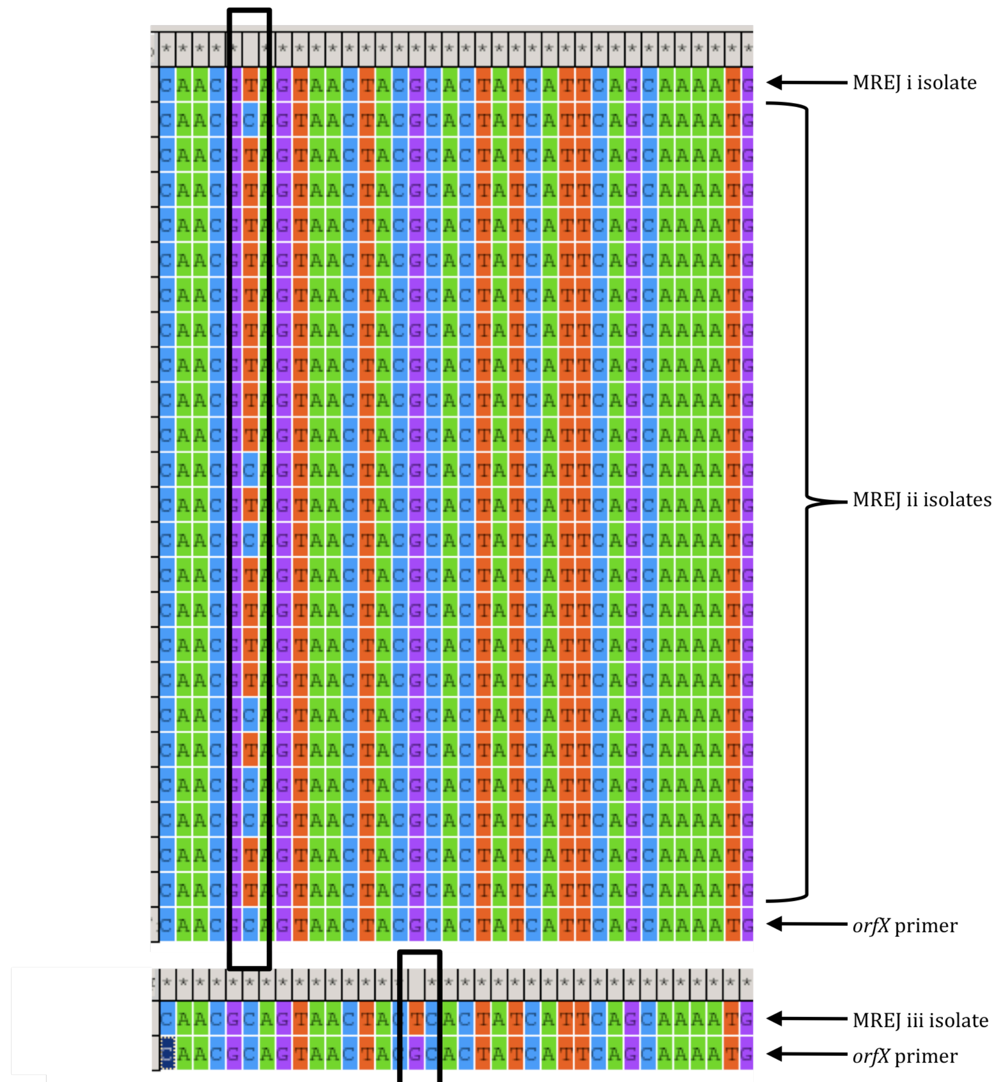


Figure 3.7 SNPs found in the *orfX* primer binding region of the 25 odd-performing CMFT isolates.

3.3.2.2 KC MRSA collection

Of the 50 isolates positive by TwistAmp MRSA, 49 were MRSA and one was MSSA (according to microarray analysis performed by Alere Technologies; section 3.3.3.2). 98% (48/49) of MRSA isolates were typed as MREJ ii with one isolate typed as MREJ i. The MSSA isolate was also typed as MREJ i.

3.3.2.3 Laboratory MRSA collection

MREJ typing of the 57 MRSA isolates in our laboratory collection of Staphylococci revealed the most common MREJ type was ii (80.7%;), with all isolates typed as either i, ii or iii (Table 3.5).

Table 3.5 MREJ typing results of the laboratory MRSA collection.

MREJ type	Frequency	%
i	7	12.3
ii	46	80.7
iii	4	7.0
Total	**57**	**100**

3.3.2.4 Correlation of MREJ type with SCCmec type

I compared the MREJ types determined for the 57 MRSA isolates from our laboratory collection of Staphylococci with the SCC*mec* types already established for these strains and confirmed the SCC*mec* types by multiplex PCR (Table 3.6). Only MREJ types i, ii and iii were detected among the isolates tested, using singleplex RPA reactions. A significant correlation was found between MREJ and SCC*mec* type ($p<0.001$), with 78.3% of MREJ ii isolates either SCC*mec* II or IV (91.7%). Most MREJ iii isolates (75%) were SCC*mec* III and most MREJ i isolates (71.4%) were SCC*mec* I. The single SCC*mec* V isolate was MREJ iii.

When confirming SCC*mec* types by multiplex PCR [Boye et al. 2007], one isolate gave an unusual amplification pattern (ST254-MRSA-IV/t009), producing

bands for both *ccrA2-B* and *ccrC*. Another isolate produced no bands (ST5-MRSA-III/t045). Retesting with the alternative multiplex PCR (Milheirico, Oliveira and de Lencastre 2007a) gave the expected amplification pattern for ST254-MRSA-IV but an unusual amplification pattern for ST5-MRSA-III (Figure 3.8). The ST254-MRSA-IV isolate likely carries SCC*mec* subtype IVk (type IV and *ccrC* (2B&5)), explaining the presence of *ccrA2-B* and *ccrC* bands using the Boye et al. [2007] method. ST254-MRSA-IV, otherwise known as EMRSA-10 or the Hannover clone, has already been reported to carry this SCC*mec* element [Chongtrakool et al. 2006, Monecke et al. 2011]. The lack of amplification pattern for the ST5-MRSA-III isolate using the Boye et al. [2007] method indicated the absence of *ccrC* in its SCC*mec* element. The Milheirico, Oliveira and de Lencastre [2007a] method produced an amplification pattern for this isolate consisting of bands for *mecA*, *mecI*, the SCC*mec* III-specific JI region and *dcs* (Figure 3.8). The *dcs* (downstream common sequence) is shared by SCC*mec* types I, II and IV, but previous studies have identified SCC*mec* III MRSA and MR-CNS isolates positive for *dcs* [Aires de Sousa and de Lencastre 2003, Qi et al. 2005, Budimir et al. 2006, Chongtrakool et al. 2006, Mombach Pinheiro Machado et al. 2007].

Table 3.6 Comparison of MREJ type and SCC*mec* type for the 57 MRSA strains from our laboratory collection.

SCC*mec* type	**Frequency**	**MREJ type (frequency)**		
		i	**ii**	**iii**
I	12	5	7	0
II	14	0	14	0
III	6	0	3	3
IV	24	2	22	0
V	1	0	0	1
Total	**57**	**7**	**46**	**4**

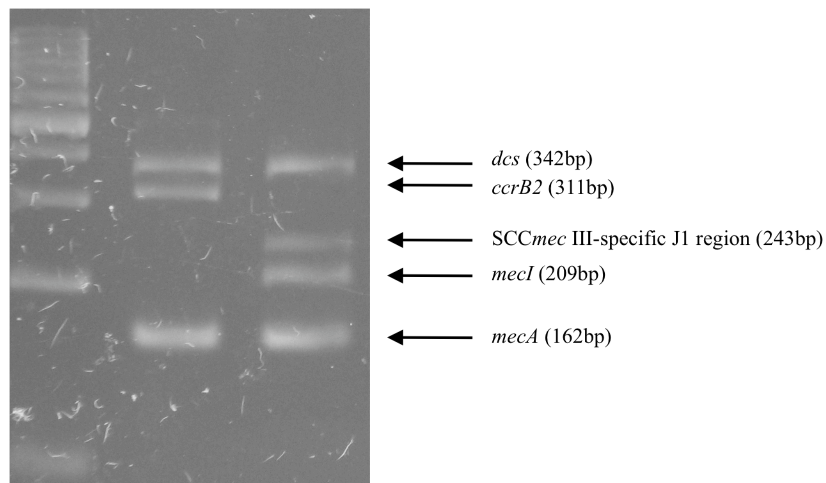


Figure 3.8 Amplification patterns for ST254-MRSA-IV (lane 2) and ST5-MRSA-III (lane 3) using the Milheirico, Oliveira and de Lencastre [2007a] multiplex PCR method. Lane 1, 100bp ladder.

Only two SCC*mec* types were found among the 49 KC MRSA isolates - II and IV. All SCC*mec* II isolates and most SCC*mec* IV isolates (97.4%) were MREJ ii (Table 3.7).

Table 3.7 Comparison of MREJ type and SCC*mec* type for 49 MRSA strains from the KC collection.

SCC*mec* type	**Frequency**	**MREJ type (frequency)**	
		i	**ii**
II	11	0	11
IV	38	1	37
Total	**49**	**1**	**48**

Among the 15 prototypic MRSA strains for the 11 currently known SCC*mec* types, MREJ types i, ii, iii and xii were identified (Table 3.8). Strains with SCC*mec* types II, IV and VI were MREJ ii, SCC*mec* types I, VII and VIII were MREJ i, and the SCC*mec* type III strains were MREJ iii. The prototypic SCC*mec* type V strain, WIS, was typed as MREJ xii. The recently described SCC*mec* types IX, X and XI were MREJ non-typeable for types i-v, vii and xi-xx, but SCC*mec* types IX and X were found to be novel MREJ types c and g, respectively, after BLAST alignment of their GenBank entries against sequences for novel MREJ types discovered in section 3.3.3.1.

Table 3.8 MREJ types of the 15 prototypic SCC*mec* strains.

SCC*mec* type (prototype(s))	**MREJ type**
I (NCTC10442, COL)	i
II (N315, BK2464)	ii
III (85/2082, ANS46)	iii
IVa (JCSC4744, MW2)	ii
V (WIS)	xii
VI (HDE288)	ii
VII (JCSC6082)	i
VIII (C10682)	i
IX (JCSC6943)	novel MREJ c[a]
X (JCSC6945)	novel MREJ g
XI (LGA251)	NT[b]

[a] see section 3.3.3.1 regarding novel MREJ types

[b] NT = MREJ non-typeable.

3.3.3 Characterisation of isolates

3.3.3.1 False negative MRSA isolates from TwistAmp MRSA screen of UK collection

Screening of the UK MRSA collection identified 42 isolates that were not recognised as MRSA by the TwistAmp MRSA assay, nor could they be MREJ-typed using singleplex RPA reactions for types i-v, vii and xii, and PCR reactions for types xi and xiii-xx. These false negative isolates were characterised by MLST, SCC*mec* and *spa* typing (Table 3.9). Eight STs in total were found, none of which were closely related according to eBURST, except for ST30, which is a SLV of ST36. Cluster analysis of the twenty *spa* types found revealed 4 *spa*-CCs, representing the four most

prevalent STs, ST22 (50% of isolates), ST36 (23.8%), ST149 (11.9%) and ST130 (4.8%) (Figure 3.9). Four isolates possessing novel *spa* types were found and submitted to the SpaServer database (http://spaserver2.ridom.de/index.shtml) via the StaphType software. These were designated types t5626 and t6419-21. t6419 also contains a novel repeat, designated r377.

Isolates with *spa* types t084, t657, t6419 and t1258 were classified as singletons because their repeat patterns differed by more than five repeats from those of all other *spa* types. The first three singleton *spa* types correspond to the single ST15, ST772 and ST59 isolates found among the false negative isolates. t1258 matched the first five repeats of t032 and would belong to *spa*-CC906 (ST22) if the BURP algorithm criteria were relaxed. *spa* type t5829 was excluded from BURP analysis because it was only 4 repeats in length and no reliable evolutionary history can be inferred from 'short' *spa* types [Mellmann et al. 2007]. SCC*mec* typing by the Boye et al. [2007] method described 45.2% of the false negative isolates as type IV, 23.8% as type II and one isolate each as type I and V. Eleven isolates (26.2%) were non-typeable (NT) for SCC*mec*.

ST22-MRSA-IV and ST36-MRSA-II, also known as EMRSA-15 and EMRSA-16 respectively, the two predominant nosocomial clones in the UK, were the two most common clones among the 42 false negative isolates (Table 3.9). The five ST149-MRSA-IV isolates, belonging to the major MLST clonal complex CC5 (ST149 is a SLV of ST5), have previously been reported only in Malta as an epidemic strain [Scicluna et al. 2010], and in a Libyan patient in Switzerland [Francois et al. 2008]. The single ST772-MRSA-V/t657 isolate is known as the Bengal Bay clone or WA MRSA-60, a multiply-resistant PVL-positive CA-MRSA that is becoming increasingly prevalent in India, where it has spread into hospitals [D'Souza, Rodrigues

and Mehta 2010]. It has also been identified in Malaysia, the UK, Italy, Australia, Germany, Hong Kong and Abu Dhabi [Neela et al. 2009, D'Souza, Rodrigues and Mehta 2010, Ellington et al. 2010, Monecke et al. 2011, Sanchini et al. 2011], and most recently in Ireland, where several ST772-MRSA-V/t657 isolates were reported [Brennan et al. 2012]. Many patients infected with ST772-MRSA-V outside of India had familial or travel links to India [Ellington et al. 2010, Brennan et al. 2012]. A single ST15-MRSA-I isolate was also found among the false negative isolates. MRSA from the CC15 lineage are extremely rare, with just one study reporting CC15-MRSA isolated in Italy in 1980 [Campanile et al. 2009, Monecke et al. 2011].

The two ST130-MRSA-NT isolates (*spa* types t843 and t1736) belong to the clonal lineage CC130 that has previously been reported in livestock (predominantly from bovine sources) and more recently in humans, in the UK, Denmark, Ireland and Germany [Cuny et al. 2011, Garcia-Alvarez et al. 2011, Shore et al. 2011]. These isolates were non-typeable using the SCC*mec* typing method of Boye et al. [2007], which was confirmed using the method of Milheirico, Oliveira and de Lencastre [2007a]. However, the most recently described SCC*mec* element - type XI - has been associated with ST130/t843 and ST130/t1736 strains [Cuny et al. 2011, Garcia-Alvarez et al. 2011, Shore et al. 2011].

Seven ST22-MRSA isolates (*spa* types t032 and t492) exhibited a novel amplification pattern with the Boye et al. [2007] typing method that combined the expected bands for SCC*mec* types III (*ccrC*) and IV (*ccrA2-B* and *IS1272*) (Figure 3.10). Re-typing using the Milheirico, Oliveira and de Lencastre [2007a] method showed they were SCC*mec* type IV (data not shown). A further two isolates, ST30/t017 and ST59/t6419 generated the single band expected for SCC*mec* type III (*ccrC*) plus one of the bands expected for SCC*mec* type IV (*ccrA2-B*) (Figure 3.10).

These 9 SCC*mec* NT isolates by the Boye et al. [2007] method could contain the most recently described SCC*me*c subtype, IVk (type IV and *ccrC* (2B&5)). This composite element, represented by MRSA strain ZH47, comprises a class B2 *mec* gene complex and a type 2 *ccr* gene complex, plus an SCC carrying *ccrC* located in the J3 region (between the right chromosomal junction and *mec* complex) [Heusser et al. 2007, IWG-SCC 2009]. If so, these isolates would be variants of ST22-MRSA-IV, ST30-MRSA-IV and ST59-MRSA-IV, respectively.

ST30-MRSA-IV is a CA-MRSA strain known as the Southwest Pacific clone or USA1100, and belongs to the major clonal complex CC30 of HA- and CA-MRSA, which includes ST36. The variant of ST59-MRSA-IV could be WA MRSA-15, the second most common CC59-MRSA strain in Australia [Coombs et al. 2010] that has a composite (SCC*mec* IV and V) or novel (SCC*mec* IV plus *ccrC*) SCC*mec* element (type IV (2B&5)) [Monecke et al. 2011]. Its *spa* type is t976, which differs from t6419 by two repeats. However, performing a BLAST alignment of the mobile element of MRSA strain ZH47 (accession number AM292304) against the MREJ amplicons of TwistAmp MRSA revealed this strain possesses MREJ iii and this strain would therefore be detected by the assay.

Table 3.9 Strain types of the 42 false negative isolates from the UK MRSA collection, according to MLST, SCC*mec* typing (using the method of Boye et al. [2007]), and *spa* typing.

Strain (ST and SCC*mec* type)	Frequency	*spa* types (frequency)
CMFT		
ST36-MRSA-II	10	t018 (10)
ST22-MRSA-IV	8	t906 (4), t032 (2), t6420 (1), t6421 (1)
ST149-MRSA-IV	5	t002 (1), t5181 (1), t5626 (1), t1062 (1), t5829 (1)
ST30-MRSA-NT[a]	1	t017
ST772-MRSA-V	1	t657
ST15-MRSA-I	1	t084
ST59-MRSA-NT	1	t6419
ST130-MRSA-NT	1	t843
Addenbrooke's		
ST22-MRSA-IV	6	t032 (2), t020 (2), t022 (1), t1258 (1)
ST22-MRSA-NT(IV)[b]	7	t032 (6), t492 (1)
ST130-MRSA-NT	1	t1736

[a] NT = non-typeable SCC*mec* element.

[b] NT by the method of Boye et al. [2007] but SCC*mec* type IV after re-typing by the method of Milheirico, Oliveira and de Lencastre [2007a].

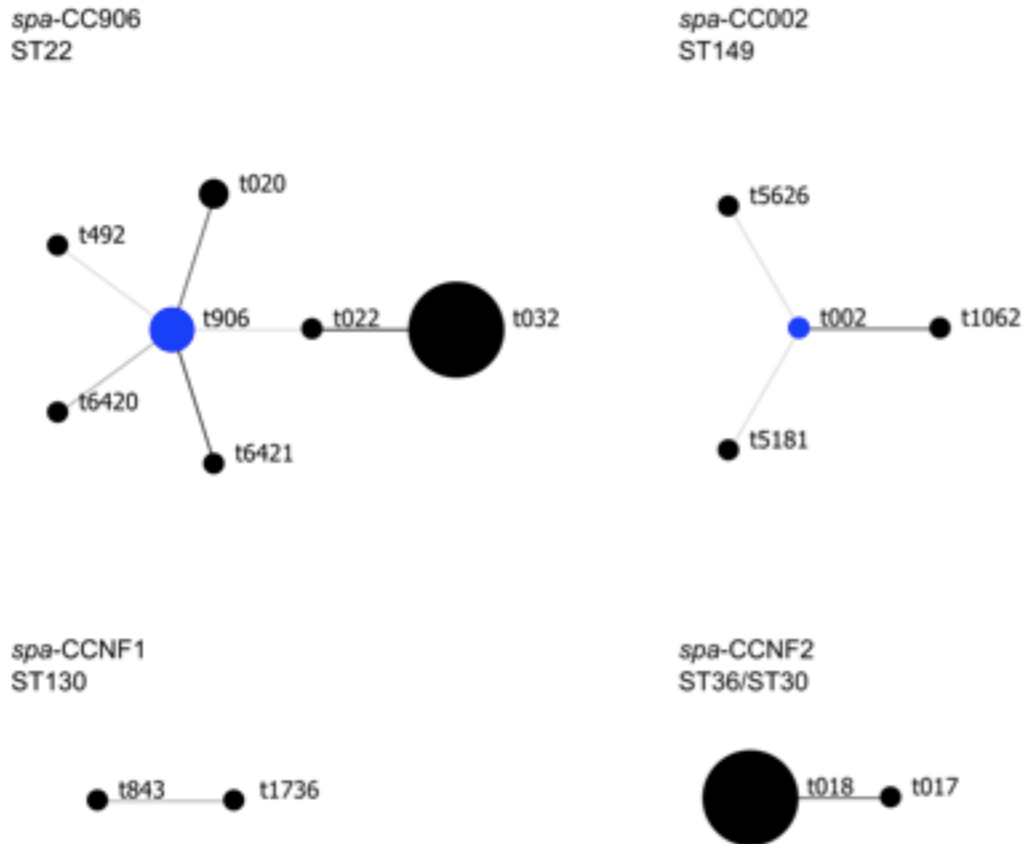


Figure 3.9 Relatedness of *spa* types among the 42 false negative isolates from the UK MRSA collection according to the BURP algorithm. Clusters of linked *spa* types correspond to *spa* clonal complexes (*spa*-CC). *spa* types are clustered into a *spa*-CC when their repeat patterns differ by no more than 5 repeats. BURP sums up 'costs' (a measure of relatedness based on the repeat pattern) to define a founder-score for each *spa* type in a *spa*-CC. The founder (blue node) is the *spa* type with the highest founder-score in its *spa*-CC. For example, *spa*-CC906 has founder t906. Each node represents a *spa* type. Node size represents the number of clustered strains that belong to that *spa* type. The shading of the branches represents the 'costs' (similarities in repeat patterns) between two *spa* types; the darker the branch, the lower the cost (more similar repeat patterns). The ST associated with each *spa*-CC is also shown. Singletons and excluded *spa* type not shown.

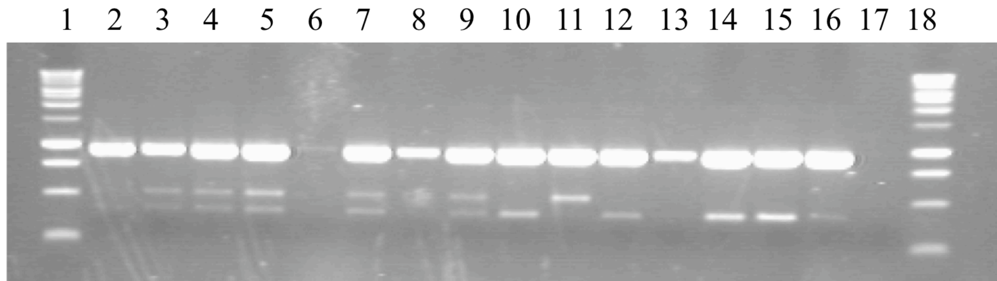


Figure 3.10 SCC*mec* typing using the Boye et al. [2007] method - PCR amplification patterns for 14 of the 42 false negative isolates from the UK MRSA collection. Lanes 1 and 18, 1kb DNA ladder (bottom four bands from top 1kb, 750bp, 500bp and 250bp); lane 16, positive control (MRSA-IV); lane 17, negative control; lanes 6, 8 and 13, SCC*mec* II; lanes 10, 12, 14 and 15, SCC*mec* IV; all other lanes, non-typeable.

Sequence analysis of the MREJs of 18 of the 28 false negative isolates from CMFT revealed six novel MREJ types, designated MREJ a, b, c, d, e and g. For the remaining 10 isolates, sequence data for seven are not yet available, two produced strange sequence assemblies and require further work, and one requires re-sequencing. I developed optimal RPA primers for the novel MREJ types a-d using the primer screening method outlined in section 3.2.7; these are listed in Table 3.10 but the sequences themselves are not available as they are commercially sensitive. Primer screens for MREJ types e and g have yet to be conducted. Example output from the primer screen is shown in Figure 3.11.

The novel MREJ types grouped as expected considering the strain types identified among the isolates, with the most common novel MREJ type being d (Table 3.11). CMFT isolates with known MREJ types included in the sequencing as controls (i-iv, vii, xii, xiii and xvi) gave the expected MREJ types when sequenced, except one. The isolate originally thought to be MREJ xvi turned out to be novel MREJ d

when sequenced. The positive PCR reactions for xvi could most likely be explained by non-specificity of the primers.

Table 3.10 Optimal MREJ-specific primers for novel MREJ types a to d. For example, primer d1+1 was located 1bp downstream from the initial best candidate primer, d1. The final stage of the primer screen determined that a length of 35bp was optimal for primer d1+1.

Novel MREJ type	Optimal MREJ-specific primer (all 35bp)
a	MREJ_a1+3
b	MREJ_b4+4
c	MREJ_c1+1
d	MREJ_d1+1

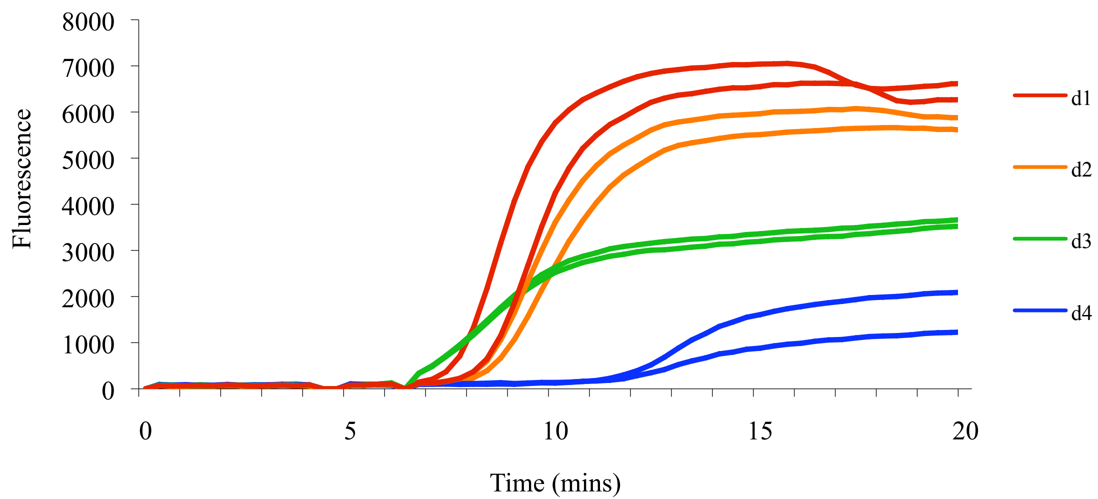


Figure 3.11 Example output for primer screen of novel MREJ type d. Primer candidates were tested in duplicate using novel MREJ PCR product (~50 copies/μl) as template. The original RPA primer designed for MREJ d (d1) is shown, together with three other candidate primers each 5bp further downstream of *orfX* than the previous one (i.e. d2, d3 and d4 were 5bp, 10bp and 15bp further downstream of *orfX* than d1, respectively). From this first stage of the primer screen, primer d1 was selected as the best candidate.

Table 3.11 Strain types of the 28 false negative CMFT isolates and associated novel MREJ types.

Strain	*spa* types (frequency)	Novel MREJ type (frequency)
Group 1		
ST36-MRSA-II	t018 (10)	d (8)
ST30-MRSA-NT	t017 (1)	g (1)
Group 2		
ST22-MRSA-IV	t906 (4), t032 (2), t6420 (1), t6421 (1)	c (4)
Group 3		
ST149-MRSA-IV	t002 (1), t5181 (1), t5626 (1), t1062 (1), t5829 (1)	e (3)
Singletons		
ST772-MRSA-V	t657 (1)	b (1)
ST15-MRSA-I	t084 (1)	sequence not available
ST59-MRSA-NT	t6419 (1)	a (1)
ST130-MRSA-NT	t843 (1)	sequence not available

3.3.3.2 Characterisation of the 49 KC MRSA isolates

ST8-MRSA-IV also known as USA300, the predominant CA-MRSA clone in the US, accounted for 65.3% of the 49 MRSA isolates tested with TwistAmp MRSA (Table 3.12). This included an ACME-negative variant (two isolates), which seems to be rare in the US [Diep, Sensabaugh and Perdreau-Remington 2007, Haenni et al. 2011], but has been reported in South America [Arias et al. 2008, Sola et al. 2012], Spain [Blanco et al. 2011] and most commonly, in Australia [Monecke et al. 2009]. One isolate of the ACME-negative variant produced a consistently weak reaction

curve with TwistAmp MRSA (see Figure 3.4) but when MREJ typed from DNA was strongly positive for MREJ i. The MREJ i false positive isolate mentioned in section 3.3.1.2, CC1-MSSA [PVL+], was also weakly positive with TwistAmp MRSA but strongly positive in singleplex RPA reactions for MREJ i. CC1 is a major worldwide MSSA lineage strongly associated with CA-MRSA, including USA400. All SCC*mec*-related microarray targets were negative for this isolate, removing the possibility of a *mec*-less cassette. However, the isolate could contain a remnant of an SCC element (*mec*-containing or otherwise) at the integration site (see section 3.4.1 for related discussion).

ST5-MRSA-II, including its SLV ST225-MRSA-II, made up 22.4% of the MRSA isolates tested. Also known as the NY/Japan clone, or USA100, this strain is the predominant HA-MRSA in the US. Four isolates belonging to the CC5-MRSA-IV, also known as the Paediatric clone, were found. This clone is prevalent in Argentina, Colombia and the US [Chambers and Deleo 2009].

Of the seven isolates that produced a negative result with TwistAmp MRSA, five were MSSA belonging to CC8, CC30, CC45, CC59 and CC152. CC8, CC30 and CC45 are major international MRSA lineages, and CC59 is a common CA-MRSA lineage. The CC8-MSSA isolate was PVL+ and is likely a USA300 that lost its SCC*mec* element [Brown et al. 2012]. The CC152-MSSA isolate was also PVL+. This clone is prevalent in West Africa [Ruimy et al. 2008, Okon et al. 2009, Breurec et al. 2011, Shittu et al. 2011], but the epidemic CC152-MRSA clone prevails elsewhere [Perez-Roth et al. 2010]. The two remaining isolates were MR-CNS, one of which, *Staphylococcus succinus*, was classified as MRSA by culture. There is only one report of *S. succinus* being isolated from human clinical material [Novakova et al. 2006].

Table 3.12 Strain types of the 57 isolates of the KC collection, as determined by Alere Technologies (using StaphyType DNA, as previously described [Monecke and Ehricht 2005, Monecke et al. 2006, Monecke et al. 2007a, Monecke et al. 2008, Monecke, Slickers and Ehricht 2008, Monecke et al. 2011]).

Strain	Further strain information	Frequency
True positive		**48**
ST8-MRSA-IV	PVL+/ACME+[1], USA300	31
ST5/ST225-MRSA-II	New York/Japan Clone, USA100	11
CC5-MRSA-IV	Paediatric clone	4
ST72-MRSA-IV	USA700/NRS386	1
ST8-MRSA-IV	PVL+/ACME-	1
Weak true positive		**1**
ST8-MRSA-IV	PVL+/ACME-	
False positive		**1**
CC1-MSSA	PVL+	
True negative		**7**
CC8-MSSA	PVL+	1
CC30-MSSA		1
CC45-MSSA		1
CC59-MSSA		1
CC152-MSSA	PVL+	1
MR-CNS		2 (one *S. haemolyticus*, one *S. succinus*)
Total		**57**

[1]PVL = panton valentine leukocidin; ACME = arginine catabolic mobile element.

3.3.4 Scope of TwistAmp MRSA

3.3.4.1 Testing a variant of a common MRSA clone in Copenhagen, Denmark

The isolate with similar ST, SCC*mec* and *spa* type (ST8, SCC*mec* IVh and t024 respectively) to the common variant of the Copenhagen clone (ST8-MRSA-IVa/t024) that was not detected by BD GeneOhm MRSA, was detected using TwistAmp MRSA and when tested with singleplex RPA reactions for MREJ types i-v, vii and xii it tested positive for MREJ ii (Figure 3.12). Since MREJ i and ii differ by a 102-bp insertion, an isolate with MREJ ii will test positive for both types. Sequencing of the MREJ amplicon of this isolate (data not shown) revealed three SNPs compared to the reference MREJ amplicon for type ii. One of these SNPs was in the probe binding region, but is accounted for in the probe design using a wobble base, so should not cause any detection problems. The other two SNPs were not present in the primer/probe binding regions so should not have any effect on RPA reaction performance. The preliminary data show that this variant is not problematic for TwistAmp MRSA, although the SCC*mec* type of the tested isolate was subtype IVh, whereas the problem variant was SCC*mec* subtype IVa. It is therefore likely that the tested isolate does not have the MREJ in question in the Bartels paper. Indeed, 23 MRSA isolates with SCC*mec* type IVh were tested by Bartels et al. [2009] and all were detected by BD GeneOhm MRSA. Two prototypic strains for SCC*mec* type IVa tested positive with TwistAmp MRSA and were MREJ typed as ii (section 3.3.2.4 above and 3.3.4.4 below) but had different STs and *spa* types to the variant of the Copenhagen clone (ST1/t128 and ST379/t375 versus ST8/t024). The PCR primers and/or probes used in BD GeneOhm MRSA may overlap SNPs in the MREJ of the Copenhagen clone, causing amplification and/or detection problems for their assay, or

the ST8-MRSA-IVa/t024 variant could possess an entirely novel MREJ not covered by the assay. Isolates of ST8-MRSA-IVa/t024 from the paper of Bartels et al. [2009] require testing with TwistAmp MRSA for proper comparison to BD GeneOhm MRSA.

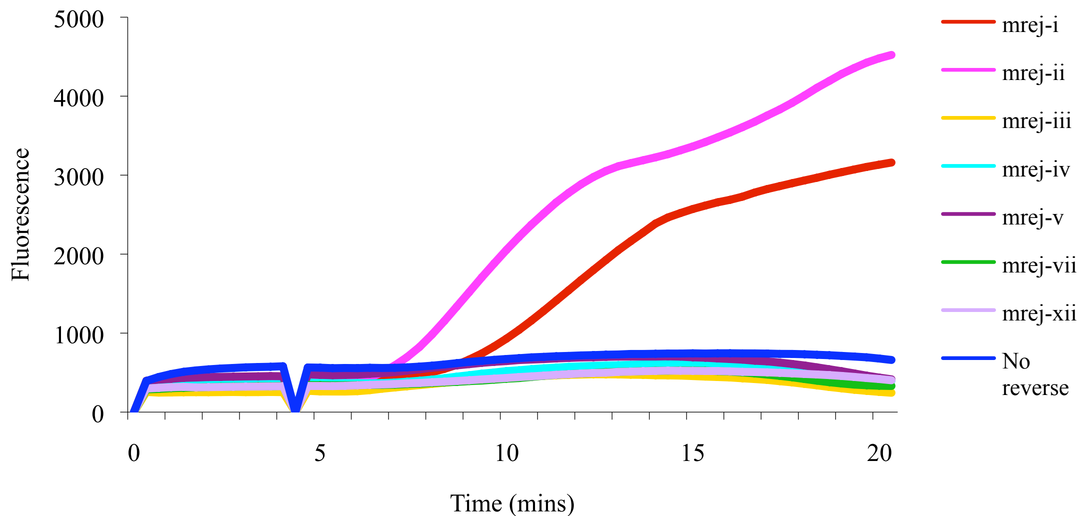


Figure 3.12 MREJ typing results for the SCC*mec* IVh/t024 isolate. No reverse = no MREJ-specific primer in reaction, to act as a negative control.

3.3.4.2 Testing common Australian MRSA clones not detected by BD GeneOhm MRSA

Two clones not detected by BD GeneOhm MRSA in an Australian study [Thomas et al. 2008], the predominant Australian nosocomial clone (ST239-MRSA-III) and the Southwest Pacific clone (ST30-MRSA-IV), were tested with TwistAmp MRSA. Four isolates from our laboratory collection of MRSA that corresponded to these two clones, were strongly detected by TwistAmp MRSA, with one isolate typed as MREJ iii (ST239-MRSA-III) and three isolates as MREJ ii. These clones therefore do not seem to cause detection problems with TwistAmp MRSA. Again, the primers

and/or probes used in BD GeneOhm MRSA may target more variable regions than those in TwistAmp MRSA.

3.3.4.3 Testing the livestock-associated MRSA clone ST398-MRSA-V

ST398 has previously been reported to cause detection problems with current commercial diagnostic assays [Reischl et al. 2009, Voss 2009, Malhotra-Kumar et al. 2010b]. Strain SO385 (ST398-MRSA-V) was therefore tested with TwistAmp MRSA, giving a weak positive result (data not shown). MREJ typing of this strain with RPA singeplex reactions for MREJ types i-v, vii and xii gave a weak positive result for MREJ type iii (Figure 3.13). Sequencing of the type iii amplicon revealed two SNPs in the probe binding region, compared to the typical MREJ iii sequence (sequences not shown as commercially sensitive). These two SNPs could well explain the weak positive result for the SO385 isolate compared to typical MREJ iii isolates, as well as the general ST398 detection problems seen with current commercial PCR diagnostic assays [Voss 2009]. The SO385 strain has since been whole genome-sequenced (GenBank accession number AM990992 [Schijffelen et al. 2010]). Aligning the SO385 MREJ iii amplicon I sequenced against the complete genome sequence of SO385 confirmed the presence of these two SNPs. Aligning the SO385 MREJ iii amplicon against other available ST398 sequences (all SCC*mec* V) revealed that the same two SNPs were consistent across all ST398-MRSA-V strains (GenBank accession numbers AB505629, FJ830606 (both SCC*mec* V (5C2)) and GQ902038 (SCC*mec* V (5C2&5)). It is likely that ST398 harbours more than one MREJ type since it is associated with more than one SCC*mec* type (mostly type V (shown to be MREJ iii) but also type IV; type III has also been reported but due to the typing method used may actually be type V [van Loo et al. 2007, Nemati et al. 2008, Jansen,

Box and Fluit 2009]). Recently, two new SCC*mec* types (IX and X) were reported in ST398 strains [Li et al. 2011], and as shown in section 3.3.2.4, were non-typeable by MREJ typing and thus not detected by TwistAmp MRSA. ST398 isolates with SCC*mec* type IV were unavailable for testing and no sequences were deposited in GenBank with this composition to allow comparison with the various TwistAmp MRSA amplicons.

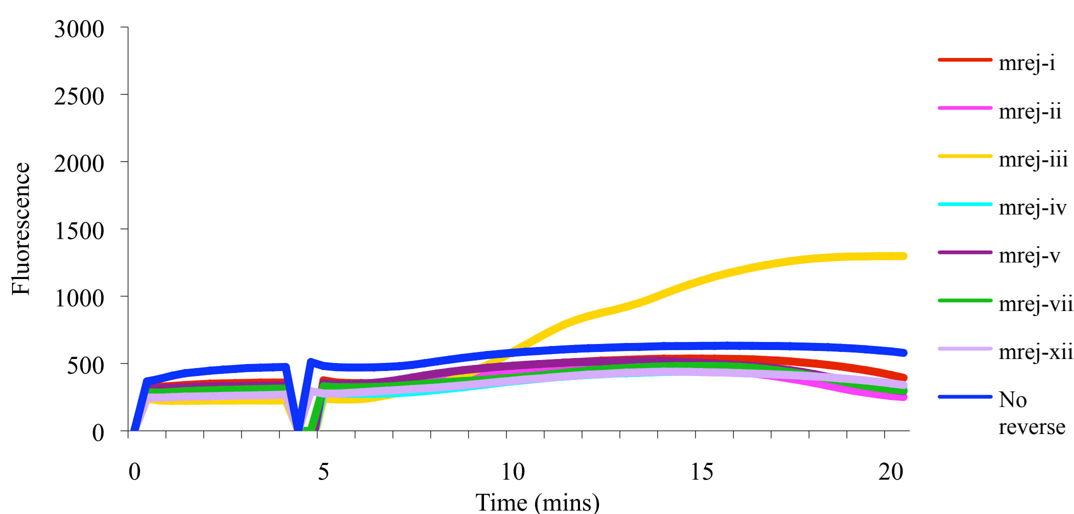


Figure 3.13 MREJ typing results for the ST398 isolate. No reverse = no MREJ-specific primer in reaction, to act as a negative control.

3.3.4.4 Testing prototype strains for SCCmec types I-XI with TwistAmp MRSA

To determine the range of SCC*mec* types that TwistAmp MRSA was able to detect, 15 prototypic strains for SCC*mec* types I-XI were tested with the assay. TwistAmp MRSA was able to detect 11, representing SCC*mec* types I-IV and VI-VIII (Figure 3.14A). The SCC*mec* type V prototype strain (WIS) was not detected by TwistAmp MRSA and was later MREJ typed by singleplex RPA reactions, as xii (Figure 3.14B), an MREJ type not covered by the assay. Performing a BLAST

alignment of all SCC*mec* type V entries in GenBank other than WIS (both types 5C2 and 5C2&5; accession numbers AB505629, AM990992, GQ902038, FJ830606, AB478780, AB512767, AB462393 and CP003166) against the MREJ sequences for all known MREJ types, revealed that they were all MREJ iii, rather than xii. As mentioned, all ST398-associated SCC*mec* type V sequences have two SNPs in the probe binding region compared to the typical MREJ iii sequence, which are likely to produce only weak positive results using TwistAmp MRSA, as shown by the ST398-MRSA-V reference strain SO385 above (Figure 3.13). The remaining four SCC*mec* type V sequences in GenBank possessed no SNPs in the primer or probe binding regions for MREJ iii, suggesting that TwistAmp MRSA would succesfully detect strains possessing this variant. Thus, it appears that some SCC*mec* type V strains will be detected by TwistAmp MRSA and others will not (or will give weak positive results), although the proportion that will be detected is at present unclear.

The recently described prototypic strains for SCC*mec* types IX-XI were not detected by TwistAmp MRSA and were MREJ non-typeable for types i-v, vii and xi-xx. However, a BLAST alignment of the sequences of SCC*mec* types IX, X and XI (GenBank accession numbers AB505628, AB505630 and FR823292, respectively) against the novel MREJ types a-e and g discovered in this chapter, revealed that the prototype strains for SCC*mec* types IX and X were novel MREJ types c and g, respectively. While the MREJ of the prototype strain for SCC*mec* X had no SNPs compared to the novel MREJ g sequence, SCC*mec* IX had one SNP in the *orfX* primer binding region compared to the novel MREJ c sequence (the same C/T SNP found in several CMFT MREJ ii isolates in section 3.3.2.1). SCC*mec* XI was not typeable by the novel MREJ types a-e and g.

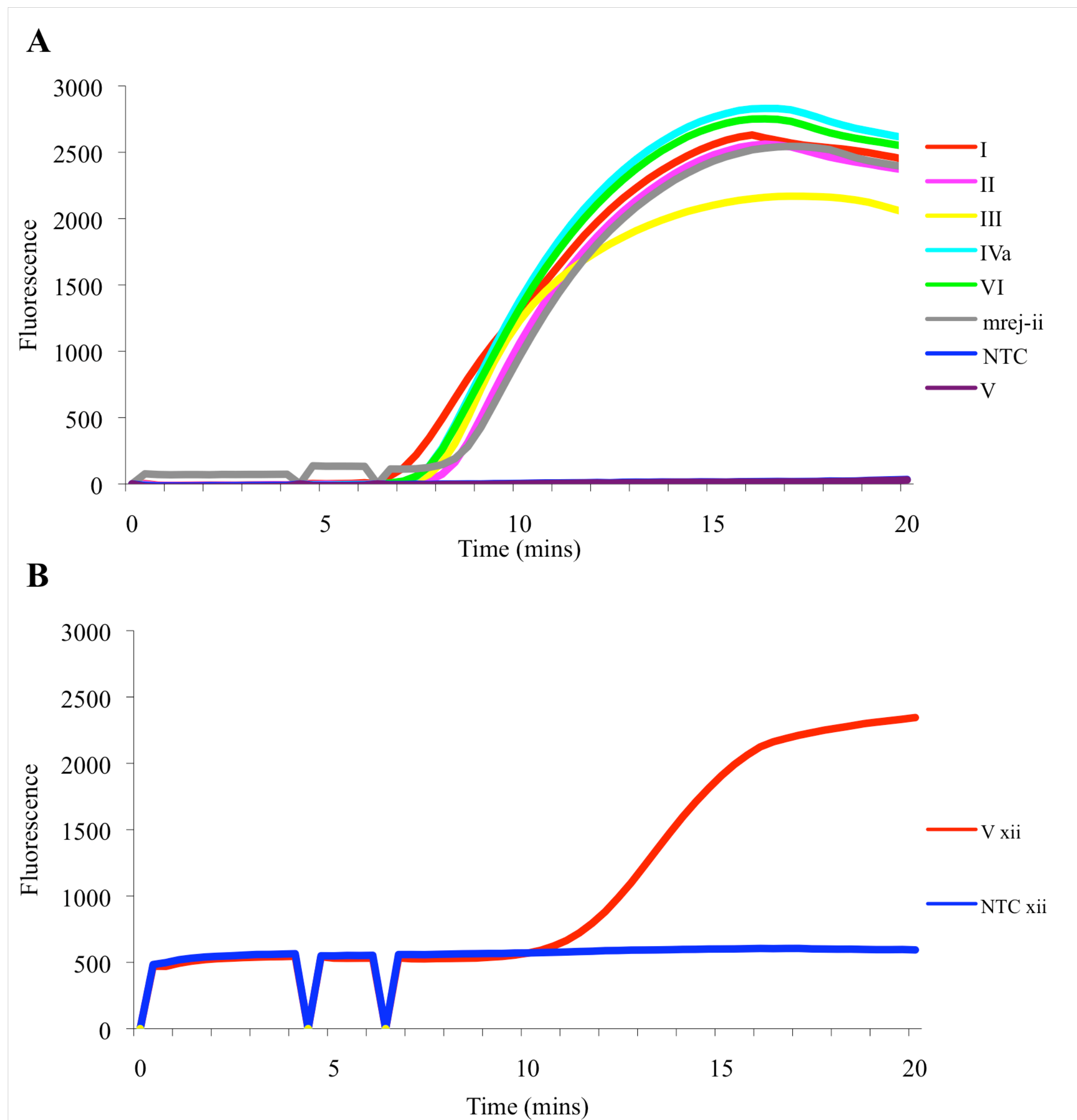


Figure 3.14 A TwistAmp MRSA output showing negative result for prototype strain WIS (SCC*mec* V). NTC = no template control; mrej-ii = MREJ type ii amplicon as a positive control (50 copies/µl). **B** Output from singleplex MREJ xii RPA reaction for prototype strain WIS (SCCmec V). NTC = no template control.

3.4 DISCUSSION

3.4.1 TwistAmp MRSA performance and scope

TwistAmp MRSA was shown to have a sensitivity, specificity, NPV and PPV of 98.2%, 90.3%, 98.2% and 90.3% respectively when tested with our laboratory collection of 119 geographically and clonally diverse MRSA and MSSA (and three CNS). Although not relevant to the use of the method in a clinical setting, all test characteristics were improved upon retesting those that gave false positive and false negative results (to 100%, 91.9%, 100% and 91.9% respectively). The one false negative isolate was correctly identified as MRSA upon retesting, suggesting a problem with the initial detection by TwistAmp MRSA (lack of assay sensitivity or perhaps poor protocol implementation). Five false positive isolates (all previously well-defined MSSA [Enright et al. 2002, Feil et al. 2003]) remained after retesting, indicating a lack of assay specificity, or possible contamination of the samples or reactions. The latter could occur as a result of MRSA-containing aerosols, for example created by pipetting, although negative controls should have detected this problem. Another possibility is MSSA containing *mec*-less cassettes or non-*mecA* SCC elements, which is discussed in greater detail below.

A limitation of using the laboratory collection of Staphylococci to test TwistAmp MRSA is that it does not include recently isolated or emerging strains, nor is it representative of a distinct region as it included MRSA isolates from 16 countries representing strains with an international distribution. Nonetheless, screening of the collection that includes diverse MRSA, has provided an initial insight into the performance of the assay, which is comparable to that of current commercial assays (see Table 1.7). A more up to date collection of MRSA, including diverse examples of

MR-CNS and more geographically diverse MSSA strains, will allow more comprehensive testing of TwistAmp MRSA.

Testing the MRSA collections from hospitals in the US and UK with TwistAmp MRSA (singleplex MREJ reactions for the UK collection) gave an assay sensitivity of 100% and 96.6% respectively. This showed that the assay delivered good coverage of the MRSA strains prevalent at each site. However, 25 isolates from the UK collection produced weakly positive or lagging reaction curves, but upon retesting were strongly positive, suggesting lack of assay sensitivity, or poor protocol implementation when first tested. The lower sensitivity observed for the UK collection of MRSA is explained by the presence of MREJ types not covered by TwistAmp MRSA, and a number of novel MREJ types. No such MREJ types were identified in the KC collection, although this may be an artefact of sample size - only 49 MRSA isolates in the latter compared to 1,276 from the UK collection. Testing more isolates from the site of the KC MRSA, or from a second US site, may reveal the presence of more MREJ types, including novel types, but it could in fact be that MREJ diversity, possibly as a consequence of the strain diversity or the presence of more conserved SCC*mec* elements among MRSA, is lower in the US compared to the UK (see section 3.4.2 for more on MREJ diversity and distribution). Indeed, a recent study of BD GeneOhm MRSA tested on a diverse range of MRSA genotypes and SCC*mec* types from the US and Taiwan, gave a very high assay sensitivity (99.7%) [Boyle-Vavra and Daum 2010]. The TwistAmp MRSA sensitivities determined for both the UK and KC MRSA collections may be overestimated because subculture of the isolates was used, not the swabs directly (which would contain mixed flora and potential reaction inhibitors).

Furthermore, two hospitals were involved in the UK collection compared to the single hospital in the KC collection (although MREJ typing revealed no significant difference between the two UK hospitals; see section 3.4.2 below). The nature of the hospitals could also play a role in the differing sensitivities seen. The US hospital served paediatric patients only. Differences in MRSA strain types have been observed between adult and paediatric patients [David et al. 2006b, Park et al. 2007, Hudson et al. 2012] (see also Chapter 5); for example, in the US a lower strain diversity and significantly more USA300 is associated with the latter group [Hudson et al. 2012] (see also discussion of KC isolate characterisation below and Chapter 5). An isolate collection from solely paediatric patients may affect the MRSA diversity observed and not represent the true diversity in the region. Simultaneously comparing TwistAmp MRSA with other diagnostic methods would allow more accurate assessment of assay sensitivity in a given region.

Although these preliminary test characteristics provide useful information about the general ability of TwistAmp MRSA to detect most MRSA, the performance of TwistAmp MRSA in a clinically relevant situation has been evaluated in two pre-clinical (feasibility) studies to obtain more clinically relevant performance data. TwistDx has used the assay to test clinical samples obtained as part of routine screening procedures at hospitals in the UK and US. Specifically, their aim was to assess whether the assay provided adequate coverage of the MRSA strains prevalent at a certain hospital/in a certain region and compare its performance to gold standards of MRSA detection.

In 2009 the UK study collected 5,433 nasal and groin swab samples from routine MRSA screening of patients admitted over a 12-week period at Manchester Royal Infirmary, CMFT [DoH 2011]. TwistAmp MRSA performed well when

compared to the gold standard method of broth enrichment and culture on chromogenic media. This was particularly the case for specificity and NPV, with clinical sensitivity at almost 75% (exact data confidential). However the PPV was only around 50%, which could be attributed to *mec*-less cassettes or non-*mecA* SCC elements, sampling error, or MRSA positive patients receiving systemic or topical MRSA therapy [DoH 2011]. The confidential US study was conducted in 2011 at a paediatric medical centre to simultaneously compare the clinical sensitivity and specificity of the assay to Xpert MRSA, using chromogenic agar and blood agar plate-based bacterial culture as the gold standard. TwistAmp MRSA performed similarly to Xpert MRSA, but showed a lower clinical sensitivity (data confidential).

Despite pre-clinical studies demonstrating the good performance of TwistAmp MRSA compared to current gold standard culture techniques and the market leader in molecular diagnostic assays, further assay development is required before the assay can be used clinically. There is potential to improve the assay further both by covering more MREJ types enabling detection of more MRSA strains and SCC*mec* variants, and by improving assay functionality (particularly since 25 isolates from the UK collection required retesting in order to produce strong positive results). The former was addressed in part in this chapter and is discussed below; the latter is addressed in Chapter 4.

In a screening situation where the goal is to detect MRSA colonised patients and to prevent nosocomial MRSA infections, an MRSA diagnostic assay with a high NPV is important [Bartels et al. 2009], especially in countries with a low MRSA prevalence (<1%), such as in northern Europe, where few MRSA carriers are expected [Tiemersma et al. 2004]. In these countries, hospitalised patients with MRSA are always isolated. If a patient is at risk of being colonised by MRSA, they

too are kept in isolation until a negative MRSA result is obtained. Thus, a false negative result would release patients with MRSA from isolation, which could lead to an MRSA outbreak [Bartels et al. 2009]. The proportion of invasive *S. aureus* isolates that are MRSA has been high in the UK (25-50% [ECDC 2011]), where patient isolation procedures are less stringent, but rates of MRSA bacteraemia are decreasing [HPA 2011]. It is important that for use in this country TwistAmp MRSA be adequate for screening the local diversity. Although a high NPV for TwistAmp MRSA has thus far been reported, this can be further improved by reducing the number of false negative results.

Several MRSA strains were identified among the 42 false negative isolates from the UK collection, including strains that, while occurring at a frequency of <0.5% in the MRSA population tested, harboured non-typeable SCC*mec* elements. The most salient finding however was that the majority of isolates not detected by TwistAmp MRSA (73.8%) belonged to the dominant UK clones EMRSA-15 and -16. It was therefore important to sequence the MREJs of all available false negative isolates (the 28 from CMFT) in order to determine why these strains or variants thereof were not detected, for subsequent coverage by the assay where possible. Six novel MREJ types were found among 18 of the 28 isolates where genome data were available. Genome data for the remaining ten isolates would likely reveal two more novel MREJ types based on the association of the current novel MREJ types with the strain types identified (Table 3.11). Given that sequence data for MREJ types vi and viii-x are not publicly available, up to four of the novel MREJ types discovered here could be one of these. It was not possible to modify the current set of primers and probe to account for the novel MREJ types due to great sequence variability, and so new RPA primers were designed and developed to detect them. Incorporation of these

into the current multiplex has not yet been attempted, but the most likely scenario would be incorporation of novel MREJ type d since it was the most common. Given that the current primer/probe set is optimal for the RPA-based system, and that previous attempts by TwistDx to include MREJ xii in the assay were counterproductive, the feasibility of adding a new primer pair to the set is questionable. Nevertheless, having optimised RPA primers that exist for all novel MREJ types identified will allow TwistDx to quickly and easily respond to future changes in the prevalences of these MREJ types, by developing as necessary separate primer/probe sets for MREJ types other than the 'core set', or by changing the 'core set' itself as a result of further research and development that facilitates easy incorporation of more MREJ types into TwistAmp MRSA. A recent study slightly adapted the PCR-based detection method of Huletsky et al. [2004] (the forerunner of BD GeneOhm MRSA) and added additional primers to allow detection of more MRSA types based on a literature search of strains and on the authors' discovery of 14 novel MREJ types that are not detected by commercially available assays, resulting in a megaplex PCR [van der Zee et al. 2011]. A total of 21 MREJ-specific primers were included in the megaplex, with no adverse effects on PCR reported [van der Zee et al. 2011]. PCR clearly works with many primers in a single reaction, but RPA is a new technology, and more research is needed into its multiplexing capabilities (see Part 1 summary).

In section 3.2.7 I used the whole genome sequence data provided by collaborators in Saudi Arabia to characterise the MREJs of 18 of the false negative CMFT isolates. My collaborators have subsequently used these genome data to assemble their SCC*mec* elements and compare them with the sequences of known SCC*mec* types using BLAST. Their results corroborate the findings of this chapter, in

terms of the non-typeable SCC*mec* elements I identified by SCC*mec* typing. Of the 18 isolates that were sequenced, most carried SCC*mec* IVk or variants thereof (Table 3.13). Four non-typeable (NT) SCC*mec* isolates were identified among the 42 UK false negatives, after re-typing the seven ST22 isolates as type IV using a second SCC*mec* typing method [Milheirico, Oliveira and de Lencastre 2007a]. Only three of these four were sequenced, with the remaining one from Addenbrooke's Hospital unavailable for sequencing (one ST130-MRSA-NT isolate). Of the three that were sequenced, the ST130-MRSA-NT isolate produced a novel sequence assembly that requires further analysis by collaborators, adding weight to the prediction that the two ST130 isolates identified could harbour the most recently described SCC*mec* element, type XI [Garcia-Alvarez et al. 2011, Shore et al. 2011]. ST59-MRSA-NT carried a IVk variant as predicted, and ST30-MRSA-NT carried an unusual composite SCC*mec* element primarily consisting of type IV.

Interestingly, the eight ST36 isolates and four ST22 isolates sequenced, which were SCC*mec* typed as II and IV respectively, all in fact carried IVk elements, or variants thereof, and in one case a composite element with some similarity to type IVk, but primarily to type I. This shows that SCC*mec* IVk, present in dominant UK clones, is clearly a problematic variant for TwistAmp MRSA, accounting for at least 82% of the false negatives identified in the UK collection and associated with four of the six novel MREJ types. This also highlights the problem of misidentifications experienced by PCR-based SCC*mec* typing methods, as discussed further in the Part 1 summary. Based on the SCC*mec* typing results of the Boye et al. [2007] method, the isolate representing the Hannover clone (ST254-MRSA-IV) in our laboratory collection of MRSA, and reported to carry SCC*mec* subtype IVk [Chongtrakool et al. 2006, Monecke et al. 2011], produced an identical, non-typeable amplification pattern

to the ST22-MRSA-NT false negative isolates of the UK collection, that were also thought to carry SCC*mec* subtype IVk. However, TwistAmp MRSA detected the isolate representing the Hannover clone. Perhaps, for some unknown reason, the ST254-MRSA-IVk clone, or German isolates containing SCC*mec* subtype IVk (since the isolate was obtained from Germany), have a detectable MREJ type (MREJ i) unlike the ST22-MRSA-IVk clone, or UK isolates containing this SCC*mec* subtype.

Finally, the MREJ xvi isolate included in the sequencing protocol as a control strain actually contained novel MREJ d upon sequencing, and consisted of a composite SCC*mec* element of types IVk and II. More detailed analysis of the SCC*mec* elements of these false negative isolates will be published at a later date.

Table 3.13 Strain types of the 28 false negative CMFT isolates, associated novel MREJ types (for 18 isolates) and SCC*mec* types as determined by whole genome sequencing (for 18 isolates).

Strain	*spa* types (frequency)	Novel MREJ type (frequency)	SCC*mec* type (frequency)	Similar currently described SCC*mec* (composites only)[a]
Group 1				
ST36-MRSA-II	t018 (10)	d (8)	IVk (5), IVk variant (1), composites (2),	Composite 1:**GU122149 (IVk), D86934 (II)** Composite 2: **CP000046 (I)**, AB435013 (II.5), BA000017 (II), GU122149 (IVk).
ST30-MRSA-NT	t017 (1)	g (1)	Composite	**HM030720 (IV)**, AB121219 (V), AP006716 (*S. haemolyticus*), AB425427 (III).
Group 2				
ST22-MRSA-IV	t906 (4), t032 (2), t6420 (1), t6421 (1)	c (4)	IVk (3), IVk variant (1)	
Group 3				
ST149-MRSA-IV	t002 (1), t5181 (1), t5626 (1), t1062 (1), t5829 (1)	e (3)	IVk (1), IVk variant (2)	
Singletons				
ST772-MRSA-V	t657 (1)	b (1)	V	
ST15-MRSA-I	t084 (1)	sequence not available		
ST59-MRSA-NT	t6419 (1)	a (1)	IVk variant	
ST130-MRSA-NT	t843 (1)	sequence not available		

[a] GenBank accession number (SCC*mec* type) for composite elements only. Primary SCC*mec* type in bold. All IVk isolates including variants similar to GenBank accession number GU122149.

All 49 MRSA isolates from the KC collection were detected by TwistAmp MRSA. Characterisation of these isolates by StaphyType DNA microarray analysis [Monecke and Ehricht 2005, Monecke et al. 2006, Monecke et al. 2007a, Monecke et al. 2008, Monecke, Slickers and Ehricht 2008, Monecke et al. 2011] showed 32 (65.3%) were USA300 and 11 (22.4%) were USA100, representing the predominant CA- and HA-MRSA clones in the US, respectively. This suggests that TwistAmp MRSA would perform well in the US given its ability to detect the major clones circulating there. One false positive isolate was detected however. This isolate was culture positive for MRSA, detected by TwistAmp MRSA and typed as MREJ i, yet when analysed by microarray was found to be CC1-MSSA and positive for the PVL locus. There are several possibilities. First, the isolate could have contained a mix of MRSA and MSSA, and when tested by microarray an MSSA colony was picked. Second, the isolate could have a novel *mecA* homologue not detected by mircoarray analysis, similar to that recently found in LA-MRSA, but combined with a detectable MREJ. Third, the isolate could be MSSA with a *mec*-less cassette or other SCC element. Given the culture positive result for MRSA this could only be possible if the first point was also true. Furthermore the negative microarray results for all SCC*mec*-related targets mean an intact SCC element is unlikely.

The isolate could have lost *mecA* while retaining PVL for fitness reasons (lack of antibiotic use in its environment) and because of evolutionary pressure [Ender et al. 2004, Lee et al. 2007, Brown et al. 2012], and there is evidence that some clinical MRSA isolates have lost all or part of the SCC*mec* element [Donnio et al. 2005, Wong et al. 2010]. Remnants of SCC*mec* in MSSA have been reported in several studies, but many refer to multi-resistant MSSA [Corkill et al. 2004, Huletsky et al. 2004, Donnio et al. 2005, Rupp et al. 2006, Donnio et al. 2007, Shore et al. 2008, Wong et al. 2010,

Lindqvist et al. 2011]. The false positive isolate was not positive for any antibiotic resistance determinants according to microarray analysis (except for penicillinase). Rupp et al. [Rupp et al. 2006] described an MSSA isolate containing only small fragments of the right extremity of SCC*mec* that tested positive with the GenoType MRSA Direct assay, speculating that the isolate arose from MRSA that experienced the deletion of large parts of its SCC*mec* element.

There has also been a report of an MRSA isolate rapidly losing SCC*mec* upon sub-culturing [Ciardo et al. 2010], which could have occurred in the false positive isolate found here, between the initial chromogenic culturing to determine MRSA and microarray analysis. Freezing-thawing of stored isolates has also been suggested as a possible cause of *mecA* loss [van Griethuysen et al. 2005]. If the false positive CC1-MSSA isolate did contain a *mec*-less element, then one would expect it to be genetically related to the predominant MRSA clones in the local area. No CC1-MRSA were identified among the KC isolates. Further investigation, ideally by whole genome sequencing, would reveal whether the isolate harbours remnants of a non-*mecA*-containing SCC element or indeed of an SCC*mec* element that is detected by TwistAmp MRSA. Since the isolate is CC1, it could harbour a remnant of SCC*mec* type IV present in USA400 (ST1) or a remnant of the mobile element SCC_{476} which is present in MSSA476 (ST1) [Holden et al. 2004]. One study reported 17 false positive isolates resembling USA400 and/or MSSA476 that contained an intact SCC integration site and a duplicate *dcs*. The binding region for one of the MREJ-specific primers in BD GeneOhm MRSA was identified in this duplicate *dcs*, which in the absence of SCC would generate a 176bp amplicon and thus a false positive result [Wong et al. 2010]. Again, further testing of the CC1-MSSA false positive isolate would reveal if it too possesses a duplicate *dcs*.

False positive isolates containing *mec*-less cassettes, non-*mecA*-containing SCC elements and SCC remnants that all possess a detectable MREJ are clearly a problem for current commercial assays [Huletsky et al. 2004, Huletsky et al. 2005, Desjardins et al. 2006, Oberdorfer et al. 2006, Rupp et al. 2006, Rossney et al. 2007a, Zhang et al. 2007, Farley et al. 2008, Shore et al. 2008, Snyder, Munier and Johnson 2010, Arbefeville et al. 2011, Blanc et al. 2011, Lindqvist et al. 2011, Stamper et al. 2011], and TwistAmp MRSA appears to be no exception. False positive results can lead to inappropriate patient care through unnecessary treatment (e.g. vancomycin therapy) and additional precautionary measures and costs. Further, current MREJ-based assays may not be suitable in regions with a high prevalence of multi-resistant or SCC remnant-containing MSSA [Lindqvist et al. 2011, Stamper et al. 2011]. For these reasons it is important that current and next generation tests minimise or even eradicate the limitation of false positives [Blanc et al. 2011].

To assess whether the current set of primers and probes in TwistAmp MRSA is sufficient to detect most MRSA strain types, the assay was tested with strains possessing different SCC*mec* elements as well as those possessing SCC*mec* variants that cause problems with current commerical assays. Of the 15 prototypic strains for known SCC*mec* types I-XI, TwistAmp MRSA was able to detect 11 strains, covering types I-IV and VI-VIII. WIS, the prototypic strain for SCC*mec* type V, was MREJ xii and therefore not covered by the assay. Rossney et al. [2007a] found BD GeneOhm MRSA also gave a false negative result for WIS, but was positive when tested from genomic DNA. Another study had to add an additional primer to the assay of Huletsky et al. [2004] in order to detect the MREJ of WIS [van der Zee et al. 2011]. A BLAST alignment of SCC*mec* type V sequences against TwistAmp MRSA amplicons showed all but the WIS type V element possessed MREJ type iii. A type V-containing isolate

from the laboratory collection of MRSA (ST59-MRSA-V) was also successfully detected by TwistAmp MRSA. With the exception of the element represented by WIS, these findings suggest that TwistAmp MRSA should be able to detect most type V elements, though it appears the variant in ST398-MRSA-V strains would produce weak results compared to others. Furthermore, the ST772-MRSA-V isolate from the CMFT collection could not be detected at all, due to the presence of the novel MREJ b, suggesting TwistAmp MRSA is unable to detect this emerging CA-MRSA clone. These findings warrant further investigation using more SCC*mec* type V isolates to directly test the assay.

SCC*mec* types IX-XI were not detected by TwistAmp MRSA due to the presence of novel MREJ types. Xpert MRSA also failed to detect SCC*me*c type XI (not yet tested with BD GeneOhm MRSA) [Shore et al. 2011]. This is unsurprising as SCC*mec* type XI not only contains a novel *mecA* homologue, *mecA*$_{LGA251}$, which is undetectable by PCR tests for *mecA*, but also does not have a J3 joining region [Garcia-Alvarez et al. 2011], the region of the SCC*mec* element that is targeted by MREJ-based molecular assays. Diagnostic protocols involving such assays, including TwistAmp MRSA, should consider the importance of not being able to detect this novel SCC*mec* type, particularly since LA-MRSA containing this element have been isolated from human blood and infected wound sites, demonstrating its lack of host-specificity and ability to cause clinical disease [Garcia-Alvarez et al. 2011]. Culturing and antimicrobial susceptibility testing were able to detect isolates containing this novel *mecA* homologue as MRSA [Garcia-Alvarez et al. 2011], perhaps providing further impetus for commercial assays to consider detection of its associated SCC*mec* element, although the potential to incorporate additional primers into current assays is

limited [Fluit 2011]. No commercial molecular assays have yet been tested with SCC*mec* types IX and X.

Testing TwistAmp MRSA with the laboratory collection of MRSA confirmed that it was able to detect a diverse collection of MRSA genotypes covering SCC*mec* types I-V. Similarly, TwistAmp MRSA detected all MRSA of the KC collection, which included types II and IV. Thus, TwistAmp MRSA is able to detect SCC*mec* types I-VIII, with the clear exception of WIS (SCC*mec* V/MREJ xii).

Xpert MRSA has been shown to detect SCC*mec* types I-VI [Rossney et al. 2008], and BD GeneOhm MRSA types I-VI and VIII [Boyle-Vavra and Daum 2010]. The coverage of TwistAmp MRSA is thus comparable to that of current commercial assays, however the various SCC*mec* subtypes, most notably those of type IV, have yet to be tested with the assay. While subtyping of SCC*mec* IV is mainly based on differences in the J1 region [IWG-SCC 2009] and MREJ-based assays use primers that amplify part of the J3 region, Bartels et al. [2009] showed that at least in type IVa, the J3 region exhibits some variability. Moreover, there are several subtype-specific targets present in the J3 region that may interfere with MREJ-based assay detection, for example the presence of an SCC carrying *ccrC* in subtype IVk (2B&5), which may be the cause of several false negative isolates identified in the UK collection of MRSA in this chapter. SCC*mec* IV subtypes are thus important to test with TwistAmp MRSA.

Despite the coverage of most known SCC*mec* types by current assays, many SCC*mec* variants, frequently of common SCC*mec* types, have been found that give false-negative results, often representing MRSA strains prevalent in the study location [Francois et al. 2007, Thomas et al. 2008, Bartels et al. 2009, Reischl et al. 2009, Sissonen et al. 2009, Snyder et al. 2009, Voss 2009, Laurent et al. 2010, Malhotra-Kumar et al. 2010b]. Furthermore, the results of this chapter and other studies suggest

composite SCC*mec* elements are more likely to test negative with MREJ-based detection assays [Boyle-Vavra and Daum 2010]. The implications of false negative isolates are clear, with the result that the assays cannot be relied upon in certain epidemiological situations, and must be combined with more MRSA-specific tests [Sissonen et al. 2009]. There are currently 20 known MREJ types (i-xx), the work presented in this chapter has identified at least six more (though up to four of these could be types vi and viii-x, for which sequence data are publicly unavailable), and the recent study of van der Zee et al. [2011] revealed several further MREJ types. These points highlight the importance of constantly developing and improving MREJ-based detection assays so they may accommodate the ever-changing diversity of SCC*mec* elements and thus maintain a high sensitivity, as well as continuous awareness of possible SCC*mec* variants giving false negative results [Laurent et al. 2010, Malhotra-Kumar et al. 2010b, van der Zee et al. 2011]. Ongoing evaluations of assays in regions where they are used are of great importance so that assays stay up to date with regional changes in MRSA epidemiology [Boyle-Vavra and Daum 2010]. Nonetheless, current assays including TwistAmp MRSA successfully cover the most common MREJ types and therefore SCC*mec* types, and currently provide the best compromise between coverage and multiplexing capacity [Reischl et al. 2009].

Preliminary testing of TwistAmp MRSA with strains or variants that prove problematic with current commerical assays, showed the former is potentially robust to polymorphisms that render some strains and variants thereof undetectable by other assays. However, much more work is required to fully substantiate the findings of these initial results. Specifically, testing more examples of ST398 will provide greater insight into the detectability of this lineage. Testing isolates representative of HA- and CA-MRSA for a specific region, for example, a collection representative of MRSA in

a given country, will allow the performance of TwistAmp MRSA to be assessed for that country before marketing it there, and perhaps enable the development of region-specific primer/probe sets. The laboratory collection of MRSA used to test TwistAmp only covers SCC*mec* types I-V, so a collection of MRSA chosen to represent a broad range of genetic backgrounds and harbouring variants of as many of the eleven currently described SCC*mec* types as possible, plus reported composite SCC*mec* elements undetectable by other assays, will more thoroughly test the performance of TwistAmp MRSA for today's use.

No MR-CNS were discovered that gave a false positive result with TwistAmp MRSA, but the sample size was very small (only three MR-CNS isolates were tested, all of which were assay-negative). It would therefore be of great interest to test more MR-CNS strains, as well as multi-resistant MSSA (MR-MSSA), to investigate potential cross-reactivity that would affect assay specificity. Cross-reactivity has been well described for MSSA and has also been reported for MR-CNS [Shore et al. 2008, Malhotra-Kumar et al. 2010b].

3.4.2 MREJ and SCCmec type

Few studies have explored the diversity and distribution of MREJ types and compared this to SCC*mec* type [Huletsky et al. 2004, Chongtrakool et al. 2006]. By far the most common MREJ type found among all collections of MRSA tested was MREJ ii, accounting for 80.7% to 98% of the isolates in each collection, followed by type vii (2.2% of the UK collection) or type i (1.6%, 2% and 12.3% of the UK, KC and laboratory collections respectively). In the UK collection, 96.6% of all isolates were covered by the MREJ types i-v and vii. Comparing the distribution of MREJ types between CMFT and Addenbrooke's hospital showed no significant difference (p =

0.485), suggesting that the MREJ diversity and distribution shown in the UK collection is representative of the MREJ types present in UK hospitals. Less diversity was seen among both the smaller KC (all MREJ ii except one MREJ i isolate) and laboratory collections (MREJs i-iii). This suggests that MREJ diversity differs between countries, perhaps reflecting the diversity of strain types, and also that examples of the major global HA- and CA-MRSA clones do not show great diversity in MREJ type. The laboratory collection represents MRSA isolates collected up to 2004; more modern examples of MRSA clones may reveal more MREJ types, since with the discovery of more SCC*mec* types and variants of known SCC*mec* types over time, more variation in the MREJ has been observed.

A strong correlation between SCC*mec* type and MREJ type was observed in this chapter. While variation exists, most SCC*mec* I-III strains are correspondingly MREJ i-iii. Most SCC*mec* IV strains are MREJ ii and most SCC*mec* V strains MREJ iii. This correlation was tested statistically and found to be highly significant among the UK MRSA collection (p<0.001). Chongtrakool et al. [2006] also found a strong correlation that matched the patterns seen here. While Huletsky et al. [2004] claimed no such correlation was found, performing a Fisher's exact test on their data reveals a significant correlation between SCC*mec* type and MREJ type (p<0.001), although slightly different patterns were found (for example MREJ iv isolates associated with SCC*mec* III and MREJ v isolates associated with SCC*mec* IV).

In conclusion, the work in this chapter is in agreement with two of the three key findings of Huletsky et al. [2004]. There *is* an association between SCC*mec* type and MREJ type, but exceptions to this typical association exist, and strains with new MREJ types do not necessarily carry a new SCC*mec* element but rather have structural variations at the SCC*mec* right extremity. It must therefore be borne in mind that when

reading reports of the SCC*mec* types detected by MREJ-based assays, this does not mean that all isolates of these SCC*mec* types will necessarily be successfully detected, or detected in the same way (for example detected strongly) by the same MREJ primers. MREJ and SCC*mec* typing much larger collections of MRSA would provide a much more detailed picture of the relationship between SCC*mec* elements and the sequences at their right extremity.

CHAPTER 4: IMPROVING ASSAY PERFORMANCE

4.1 INTRODUCTION

The TwistAmp MRSA diagnostic assay, the mechanisms of which are outlined in detail in the previous chapter, has been shown to work well when tested with diverse and representative collections of MRSA (Chapter 3). However, its clinical sensitivity still falls below that of the market leader, Cepheid's Xpert MRSA (TwistAmp MRSA confidential data; see Table 1.7 for Xpert MRSA performance). TwistDx collaborated with a hospital in Manchester, UK (Manchester Royal Infirmary, CMFT) to assess the performance of TwistAmp MRSA in comparison with microbiological culture, using nasal and groin swabs (Copan flocked swabs). A total of 5,433 patients were screened between July 2009 and November 2009. A lower than expected clinical sensitivity (nearly 75% [DoH 2011]) highlighted four possibilities: poor analytical limit of detection, prevalence of novel MREJ types, RPA inhibition and testing only a small fraction of the total sample (sub-sampling).

After the CMFT collaboration, TwistDx determined that greater sample dilution in RPA resuspension buffer, as well as changes to the buffer itself, could overcome most of the problems with inhibition (data not shown). These assay improvements were incorporated prior to a second collaboration with a paediatric hospital in Kansas City, MO. TwistAmp MRSA was compared to culture and Xpert MRSA using nasal swabs (custom Copan flocked swabs) collected from 250 dermatology clinic outpatients and 50 hospital inpatients between May 2011 and August 2011. Although specificities, PPVs and NPVs were similar between the two assays, sensitivity of TwistAmp MRSA was still lower than that of Xpert MRSA (confidential data), highlighting the need for further assay improvement.

Despite the discovery of several novel MREJ types (Chapter 3), and known MREJ types not covered by TwistAmp MRSA among the 726 CMFT MRSA isolates that I examined, the assay should have been able to detect 696 (96%) of CMFT MRSA. Among these 696, 25 (4%) isolates produced weak or lagging reaction curves, and required retesting to achieve a definitive diagnostic result. However, the sensitivity during the performance evaluation carried out by TwistDx in collaboration with CMFT was significantly lower than 96% (nearly 75% (p<0.001) [DoH 2011]).

In my study of KC MRSA, all isolates were successfully MREJ-typed as either i-v or vii, the types covered by TwistAmp MRSA, and therefore no novel MREJ types were detected among these isolates. Novel MREJ types, or known MREJ types not covered by the assay, are therefore unlikely to be a major reason for the observed performance gap seen during performance evaluations of TwistAmp MRSA by TwistDx and collaborators at CMFT and KC. This chapter investigates other potential reasons for the comparatively low TwistAmp MRSA clinical sensitivity observed as a result of TwistDx's work with their collaborators: poor analytical limit of detection, sub-sampling and RPA inhibition. While the latter was largely overcome by further sample dilution and changes to the RPA resuspension buffer by TwistDx (see above), some inhibition still remained and thus was investigated further.

4.1.2 Chapter Objectives

4.1.2.1 Limit of detection

The lower sensitivity observed for TwistAmp MRSA compared to Xpert MRSA could simply be due to a poorer limit of detection (LOD). TwistDx tested a sample of strain N315 (MREJ ii) from the Quality Control for Molecular Diagnostics (QCMD) 2008 MRSA external quality assessment (EQA) programme panel (Qnostics

Ltd; heat-inactivated cultured MRSA). The standard, lysis-free assay protocol was used, and TwistAmp MRSA was shown to reliably detect five colony forming units (CFU) within 20 minutes (Figure 4.1), demonstrating excellent analytical sensitivity. Because the QCMD sample consisted of inactivated MRSA cells, this chapter set out to confirm this low LOD using viable cells, which is clinically more realistic. Use of viable MRSA also allows accurate measurement of the CFU present in the samples that are tested. The reaction environment of RPA creates access to DNA within the MRSA cells, but it is not known how this occurs or to what extent, or whether greater access to the DNA can be achieved by addition of a lysis procedure. Thus, the aim was not only to determine TwistAmp's analytical sensitivity with viable MRSA, but also to assess whether a lysis step could increase this sensitivity by improving the LOD.

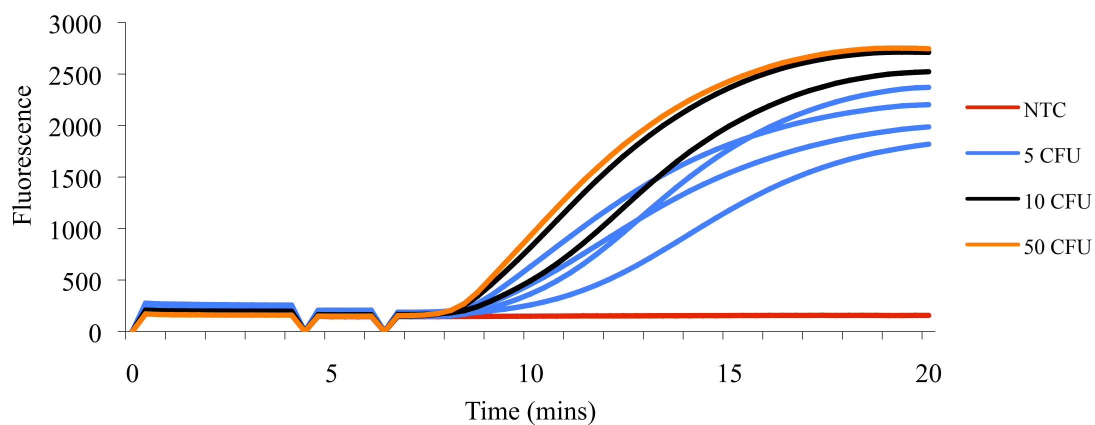


Figure 4.1 TwistAmp MRSA detection of strain N315 (MREJ ii; inactivated, cultured MRSA) at varying CFU per reaction. Troughs represent removal of the reaction tubes for vortexing and brief spinning at 4 minutes and 6 minutes. Figure courtesy of TwistDx Ltd.

4.1.2.2 Inhibition and sub-sampling

The reduced clinical sensitivity of TwistAmp MRSA is not due to molecular reasons such as an inability to detect certain MREJ types, or problems with the RPA chemistry. If the analytical LOD as determined in section 4.2.1 using viable MRSA is very low, and therefore does not affect clinical sensitivity, then sub-sampling and/or RPA inhibition may be the problem.

For the CMFT collaboration, duplicate swabs were taken from each patient: one for testing using the gold standard broth enrichment culture technique employed at the hospital, and one for testing with TwistAmp MRSA. The swabs for testing with TwistAmp MRSA were each eluted in 800μl of RPA resuspension buffer and 50μl of this solution was added to the reaction tube. For the KC collaboration, duplicate swabs were also taken from each patient. One swab was tested with Xpert MRSA and one swab was eluted in 1000μl of RPA resuspension buffer and 150μl of this solution tested with TwistAmp MRSA. The remaining solution was used to inoculate chromogenic and blood agar plates for detection of MRSA and MSSA, respectively.

A total reaction volume of 150μl was used in the KC trial after TwistDx found that greater sample dilution in RPA resuspension buffer solved most of the inhibition problems experienced during the CMFT trial, which used a total reaction volume of 50μl (see section 4.1). To compensate for the greater sample dilution, a greater reaction volume (greater volume of RPA resuspension buffer mixed with sample) was used. All TwistAmp MRSA reactions used in this chapter have a total volume of 150μl to replicate the reactions used in the KC trial. In Chapter 3, I used reactions with a smaller total volume of 50μl, since they require a third of the required RPA components, making them cheaper for TwistDx to produce.

The culturing procedure at CMFT involved breaking off the swab in broth, incubating for 24 hours at 37°C, and plating an amount of this broth on chromogenic media. The initial 24-hour broth culture allows any MRSA on a swab to grow and replicate such that when some of the broth is plated out it should contain MRSA (this also removes any inconsistencies caused by users, for example only one side of a swab being streaked directly onto a plate). With TwistAmp MRSA, the swab is placed in buffer and agitated for a few seconds, and the eluate tested. Thus, if the entire swab only holds small numbers of MRSA, e.g. 10 CFU, culturing may detect them, but TwistAmp MRSA may not, due to insufficient CFU (below the assay's LOD) being present in the fraction of eluate tested. The even distribution of cells in solution could be further affected by the cluster-forming nature of *S. aureus*, whose cells do not fully separate upon division. Cepheid's Xpert MRSA assay uses a larger swab (Copan Venturi Transystem double swab) and tests the entire eluate [Cepheid Diagnostics 2009] - procedures that are both likely to improve the possibility of detection compared to the TwistAmp MRSA protocol. However, in the KC collaboration, identical swabs were used for both TwistAmp and Xpert MRSA, and any difference would be due only to the fraction of eluate used in the test procedure.

Since the TwistAmp MRSA tested does not use any sample preparation, inhibitory substances or interfering organisms (e.g. MSSA and MR-CNS) in the sample may affect TwistAmp MRSA sensitivity. In contrast, Xpert MRSA includes DNA extraction and purification steps and thus tests cleaner samples. In order to ascertain the effects of potential inhibition and sub-sampling, experiments were performed to answer the following questions:

- Does the presence of MSSA or MR-CNS in the sample inhibit detection of MRSA thereby giving a false negative result? For example, the high sequence

similarity of closely related Staphylococcal species to MRSA could lead to the depletion of primer reserves required for MRSA-specific amplification.

- Do high levels of MSSA or CNS in samples with no MRSA present, cause false positive results, for example due to snap-back DNA synthesis as a result of the homology between *orfX* moieties in *S. aureus* and CNS, such as *S. haemolyticus* or S. *epidermidis* (GenBank accession numbers AY751823 and AY751825, respectively) [Francois et al. 2007]? MR-CNS, and MSSA and MS-CNS containing *mec*-less SCC cassettes such as SCC*cap1*, could also produce false-positive results due to the presence of MREJ sequences identical to those found in MRSA [Luong et al. 2002, Katayama et al. 2003, Mongkolrattanothai et al. 2004, Cuny and Witte 2005, Malhotra-Kumar et al. 2010b, Arbefeville et al. 2011].
- Does isolation of MRSA using filters reduce inhibition and thus improve detection? Which filters retain and therefore concentrate MRSA for subsequent testing, and which filters release MRSA?

4.2 METHODS AND RESULTS

Note: The troughs seen in all graphs at four minutes and six minutes represent removal of the reaction tubes for vortexing and brief spinning.

4.2.1 Limit of detection

To determine the analytical sensitivity of TwistAmp MRSA with viable, cultured MRSA, an isolate with the most common MREJ type (type ii), was cultured as in section 2.1. A colony from overnight growth on blood agar (Oxoid) was suspended in 1ml of RPA resuspension buffer and thoroughly mixed by vortexing, repeated

inversion, then orbital shaking for 30 minutes. Serial dilutions were performed, with thorough mixing between each dilution, giving final dilutions of 1:100, 1:10,000, 1:100,000, 1:1 million, 1:10 million and 1:100 million. 150µl of each dilution was tested in triplicate in 150µl TwistAmp MRSA reactions, following the standard, lysis-free protocol. In parallel, 100µl of each dilution was spread on blood agar plates in triplicate (Oxoid) and incubated at 37°C overnight. Colonies were counted using a Stuart colony counter (Bibby Scientific).

TwistAmp MRSA reliably detected viable MRSA in RPA resuspension buffer at a dilution of 1:1 million (all three replicates positive; Figure 4.2). This corresponded to a LOD of 2 CFU/100µl (Table 4.1). Weak detection of 1 CFU/100µl was also observed.

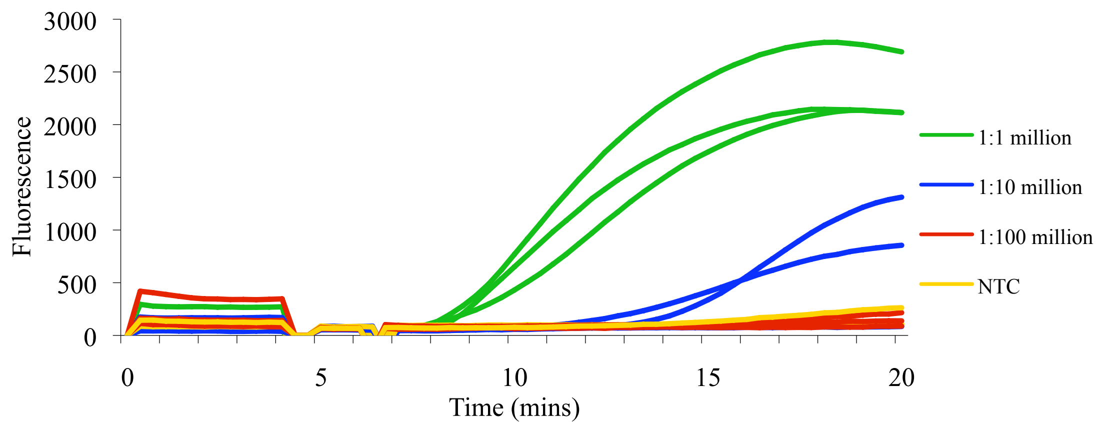


Figure 4.2 TwistAmp MRSA results of determining the LOD.

Table 4.1 Colony counts for serial dilutions of viable MRSA in RPA resuspension buffer. Dilution corresponding to the LOD (1:1 million) is shown, plus the dilution prior to this and dilutions down to <1 CFU/100μl.

	CFU/100μl			
Dilution	Replicate 1	Replicate 2	Replicate 3	**Average**
1:100,000	23	27	28	**26**
1:1 million	2	3	2	**2.3**
1:10 million	1	0	1	**0.7**
1:100 million	0	0	0	**0**

To determine whether a lysis step improved the analytical sensitivity of the assay, both MRSA from the QCMD 2008 MRSA EQA programme panel (inactivated, cultured N315/MREJ ii cells) and dilutions of the viable MRSA above were lysed and tested with TwistAmp MRSA. QCMD MRSA at a concentration of 25 CFU/μl was lysed and DNA purified in triplicate using Qiagen's DNeasy Blood & Tissue Kit (section 2.2). Samples were tested in duplicate. One μl of each sample was added to 150μl of RPA resuspension buffer, which was then added to a TwistAmp MRSA reaction tube (150μl total reaction volume). An unlysed sample of QCMD MRSA was diluted to 25 CFU/μl in the same buffer as the lysed samples (Qiagen's buffer AE: 10mM Tris-Cl; 0.5mM EDTA; pH 9.0) supplemented with 1ng/μl of human genomic DNA (Promega). No clear difference in MRSA detection was observed between lysed and unlysed QCMD MRSA samples (Figure 4.3).

Viable MRSA cells were also lysed and DNA purified using Qiagen's DNeasy Blood & Tissue Kit. First, the 1:10,000 and 1:100,000 dilutions of viable MRSA

created above were further diluted in enzymatic lysis buffer (see section 2.2 for lysis buffer composition) to achieve MRSA starting concentrations of 2 CFU/100µl (unlysed LOD; equivalent to 1:1 million dilution) and 1 CFU/100µl (equivalent to the 1:10 million dilution), respectively. These six samples were then lysed as per the protocol in section 2.2 and tested using 150µl TwistAmp MRSA reactions. Lysed MRSA samples improved TwistAmp MRSA detection, and thus the analytical sensitivity of the assay, or LOD, to 1 CFU/100µl (Figure 4.4).

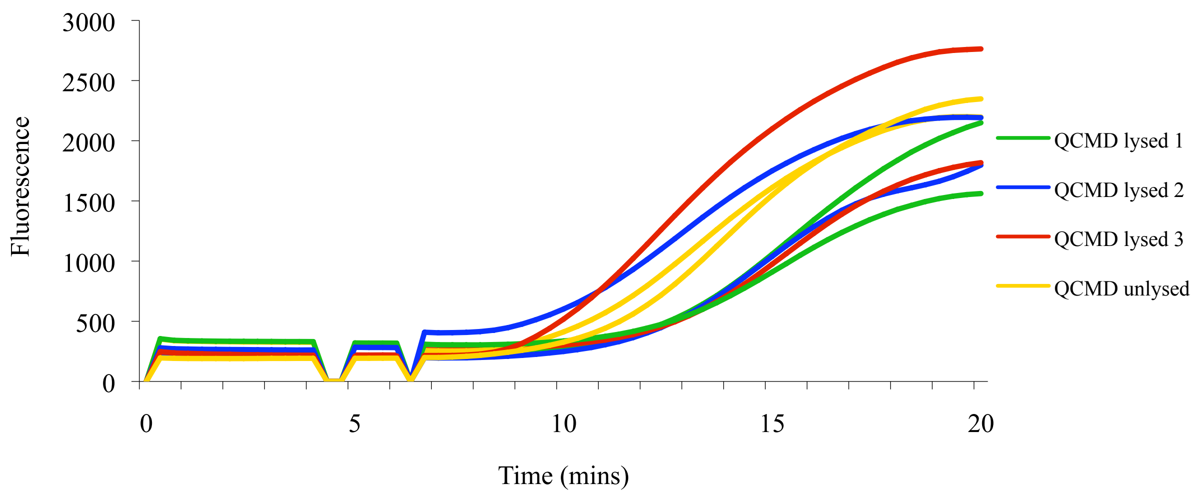


Figure 4.3 TwistAmp MRSA output for lysed and unlysed samples of QCMD MRSA (25 CFU/reaction).

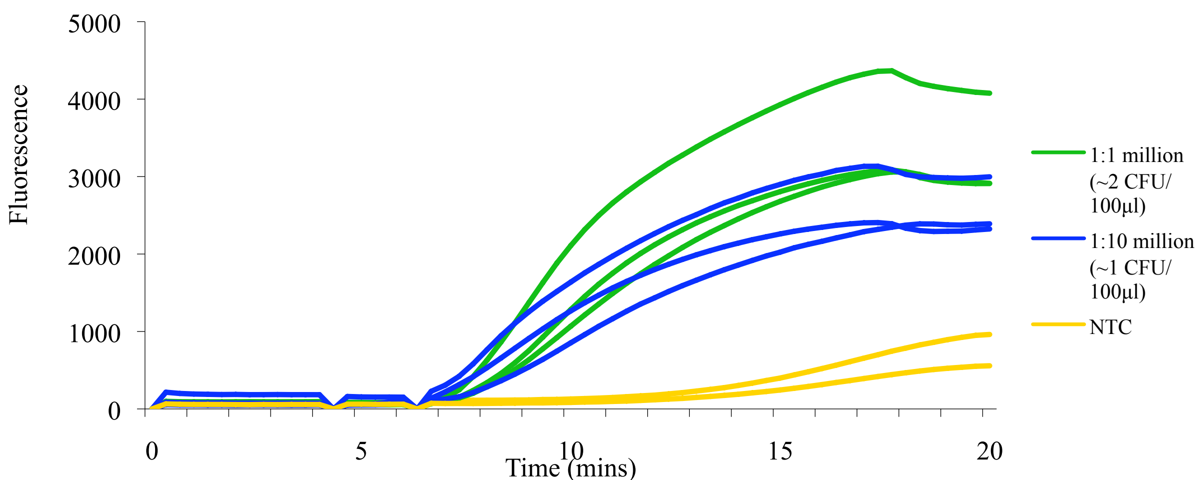


Figure 4.4 TwistAmp MRSA output for lysed samples of previously viable MRSA. NTC = no template control.

4.2.2 Inhibition and sub-sampling

To determine if TwistAmp MRSA is inhibited by the presence of large concentrations of MSSA or MR-CNS, leading to false negative results, MRSA, MSSA and MR-CNS samples from the QCMD 2008 MRSA EQA programme panel were used as template and tested with TwistAmp MRSA reactions as per the protocol in section 3.2.4. All samples were diluted to the necessary concentrations in *S. aureus* negative medium (Mueller-Hinton Broth) from the QCMD panel. MRSA template was added to each reaction at 50 CFU/reaction. MSSA and MR-CNS were tested separately, in duplicate, and at varying concentrations in combination with MRSA: 10^4, 10^5 and 10^6 CFU/reaction. To test for the possibility of false positive results in the presence of large concentrations of MSSA or MR-CNS but absence of MRSA, the same reactions were performed as above, but with no MRSA template (0 CFU/reaction).

Low concentrations of MRSA (50 CFU/reaction) in combination with high concentrations of MSSA or MR-CNS (10^4-10^6 CFU/reaction) in a sample did not inhibit TwistAmp MRSA reactions and cause false negative results (Figure 4.5). Shallower reaction curves with increasing concentrations of MR-CNS were observed, but did not affect definitive detection of MRSA (Figure 4.5B). High concentrations of MSSA or MR-CNS in a sample with no MRSA did not cause false positive results (Figure 4.6).

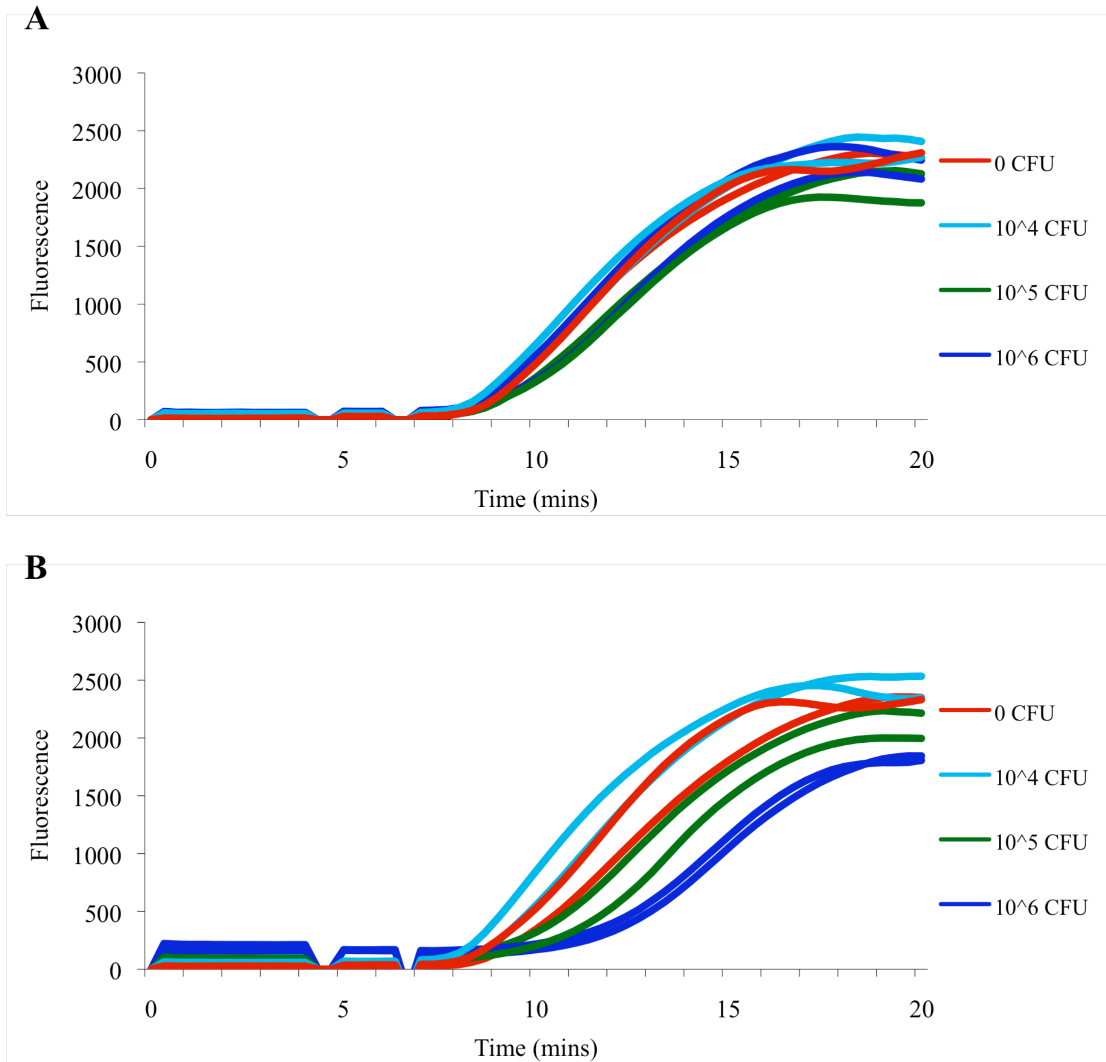


Figure 4.5 TwistAmp MRSA output for samples containing a constant concentration of MRSA (50 CFU/reaction) and increasing concentrations of **A** MSSA and **B** MR-CNS. Each assay was in duplicate.

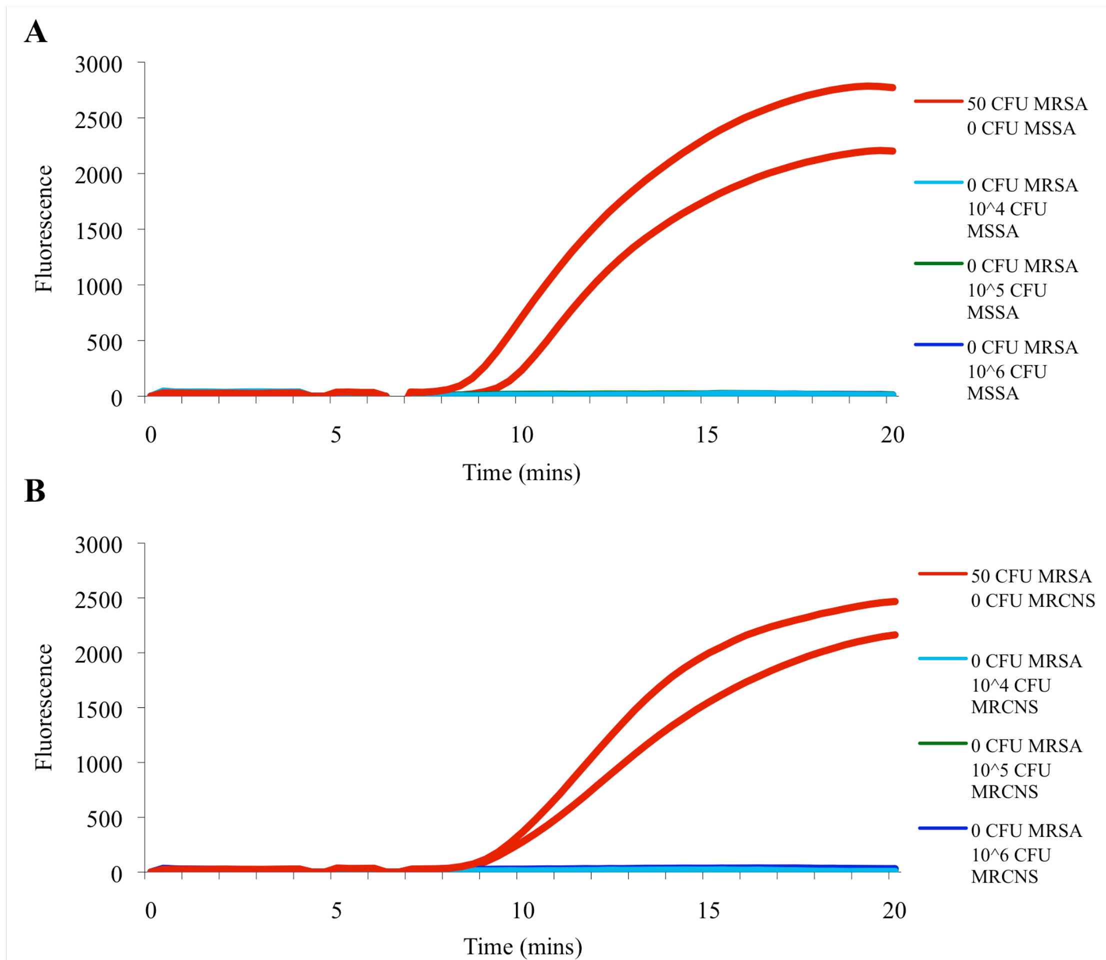


Figure 4.6 TwistAmp MRSA output for samples containing no MRSA and increasing concentrations of **A** MSSA and **B** MR-CNS. Positive control included MRSA at 50 CFU/reaction and no MSSA/MR-CNS. Each assay was in duplicate.

Several different filters manufactured by Millipore (Table 4.2) were tested with TwistAmp MRSA to assess whether MRSA detection is improved with filtration. These microporous membrane filters can be used for microfiltration of particles, including bacteria. *S. aureus* cocci are approximately 0.5-1μm in diameter, so any filters with a pore size larger than this should allow MRSA to pass through into the filtrate. Filters with pores smaller than 0.5μm should retain MRSA. Larger pore filters were tested for their porosity to MRSA with a view to their suitability as pre-filters that could remove larger particulate matter that might inhibit RPA, but not trap MRSA. This was tested by checking that filtration through these filters did not impact on TwistAmp MRSA assay performance. Smaller pore filters were tested for their ability to retain MRSA, evidenced by a negative reaction curve when the filtrate was added to TwistAmp MRSA reactions. MRSA from the QCMD 2008 MRSA EQA programme panel was used as template and mixed with RPA resuspension buffer at a concentration of 25 MRSA CFU/136μl, then filtered (Figure 4.7). The filtrate was then tested with 150μl TwistAmp MRSA reactions as per the protocol in section 3.2.4. Since MgAc immediately starts the RPA reaction, RPA resuspension buffer without MgAc was used for the filtering. Fourteen μl of 280mM MgAc was added separately to the reaction as a final step before placing reaction tubes in the Twista machine, to ensure simultaneous initiation of compared reactions.

To further test capture of MRSA, the filters themselves were included in TwistAmp MRSA reactions rather than the filtrate (Figure 4.8). Any filters that retain MRSA should produce a reaction curve; any filters that do not capture MRSA should produce no reaction curve.

Table 4.2 Millipore microporous 13mm membrane filters tested and their characteristics, sorted by decreasing pore size [Millipore Corporation 2012].

Filter code	Range	Material[a]	Wettability	Pore size (μm)	Protein binding capacity	TwistAmp MRSA filtrate result (+/-)
JCWP	Omnipore	PTFE	Hydrophilic	10	low	+
LCWP	Mitex	PTFE	Hydrophobic	10	low	+
TCTP	Isopore	Polycarbonate	Hydrophilic	10	low	+
SVLP	Durapore	PVDF	Hydrophilic	5	lowest Millipore offer (4 $\mu g/cm^2$)	+
DVPP	Durapore	PVDF	Hydrophilic	0.65	lowest (4 $\mu g/cm^2$)	+
FHLP	Fluoropore	PTFE	Hydrophobic	0.45	low	+
HPWP	Millipore Express	Polyethersulfone	Hydrophilic	0.45	low	+
HVHP	Durapore	PVDF	Hydrophobic	0.45	highest (150 $\mu g/cm^2$)	-
HVLP	Durapore	PVDF	Hydrophilic	0.45	lowest (4 $\mu g/cm^2$)	+
HTTP	Isopore	Polycarbonate	Hydrophilic	0.4	low	+
FGLP	Fluoropore	PTFE	Hydrophobic	0.22	low	+
GVHP	Durapore	PVDF	Hydrophobic	0.22	highest (150 $\mu g/cm^2$)	-
GVWP	Durapore	PVDF	Hydrophilic	0.22	lowest (4 $\mu g/cm^2$)	+
GTTP	Isopore	Polycarbonate	Hydrophilic	0.2	low	+

[a] PVDF = Polyvinylidene fluoride; PTFE = Polytetrafluoroethylene.

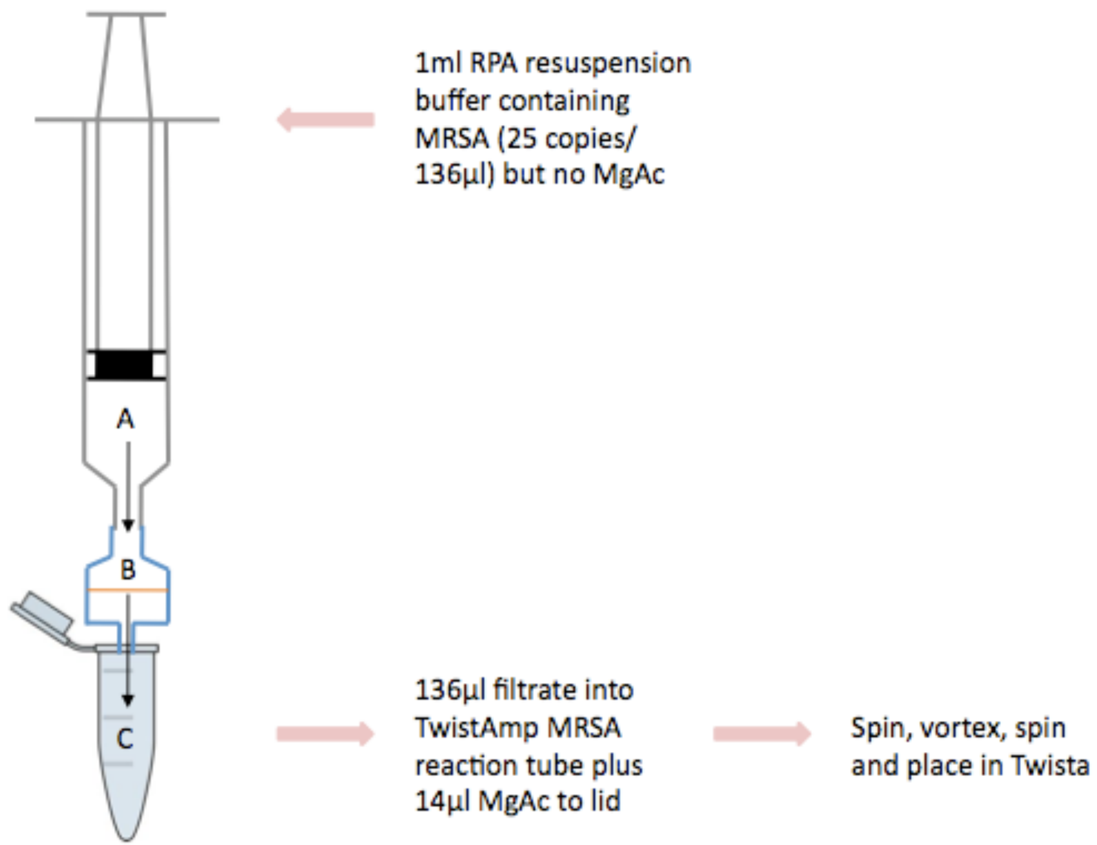


Figure 4.7 Schematic showing the buffer/template filtration process prior to filtrate testing with TwistAmp MRSA. 1ml of MgAc-negative buffer containing MRSA (25 CFU/136µl) was pushed through a Terumo 2ml syringe (**A**). The buffer then passed directly from the syringe through a Swinnex 13mm filter holder (blue; **B**) fitted with a 13mm membrane filter (orange line) and O-ring seal (all Merck Millipore). The filtrate was dispensed by the filter holder into a 1.5ml eppendorf tube (**C**) and 136µl of this filtrate plus 14µl of 280mM MgAc added to a TwistAmp MRSA reaction tube.

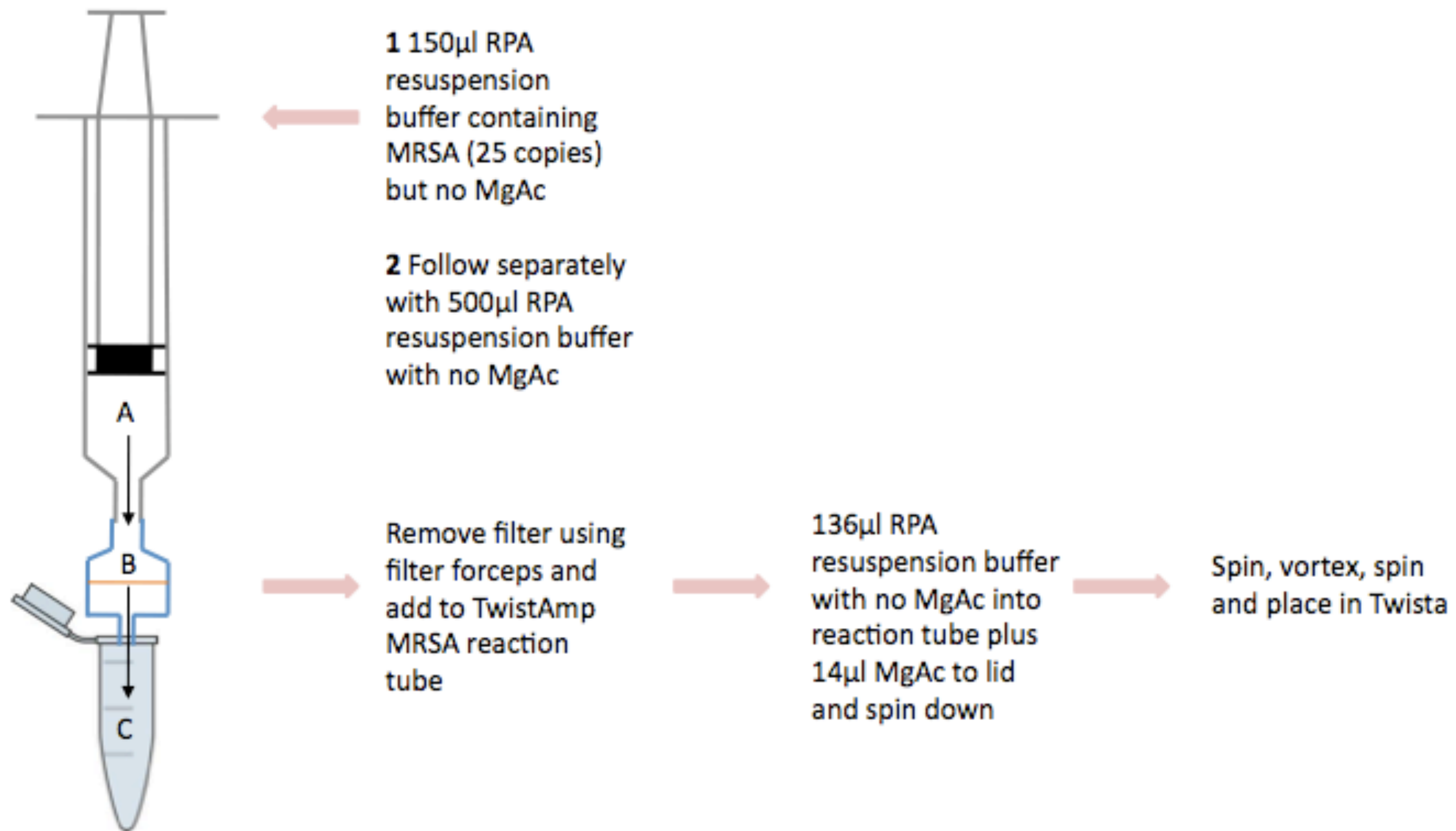


Figure 4.8 Schematic showing the buffer/template filtration process prior to filter testing with TwistAmp MRSA. 150µl of MgAc-negative buffer containing 25 CFU of MRSA was pushed through a Terumo 2ml syringe, followed by a further 500µl of MgAc-negative buffer (**A**). The buffer then passed directly from the syringe through a Swinnex 13mm filter holder (blue; **B**) fitted with a 13mm membrane filter (orange line) and O-ring seal (all Merck Millipore). The filtrate was dispensed by the filter holder into a 1.5ml eppendorf tube and disposed of (**C**). The filter was removed and placed in a 0.2ml TwistAmp MRSA reaction tube containing 136µl of MgAc-negative buffer. 14µl 280mM MgAc was then added to the tube to start the reaction.

Testing the filtrate of 14 different microporous membrane filters for their ability to capture MRSA and thus to separate MRSA from potential RPA inhibitors, revealed that filtrates from 12 filters gave a positive TwistAmp MRSA signal and filtrates from two filters a gave a negative signal (Table 4.2; Figure 4.9). No difference in signal strength was observed between the filtrate of each filter and the unfiltered control, with the exception of GVWP that produced a slightly stronger signal (Figure 4.9C). Reactions testing the filters themselves gave inconsistent results. Furthermore, the TAMRA-labelled internal control of these reactions often failed (Figure 4.10). For example, the first TwistAmp MRSA test of 0.4μm filters showed a positive result for HVHP (indicative of MRSA retention by the filter) and a negative result for the remaining four filters (FHLP, HPWP, HTTP and HVLP; Figure 4.10A left). However, the corresponding internal controls were weak for some of these filters (Figure 4.10A right). Repeating the experiment (Figure 4.10B) gave different results, for example HVHP was negative, but the internal controls improved. A final repeat experiment (Figure 4.10C) gave a strongly positive result for HTTP including the internal control, but the internal controls for the other filters were weak or negative.

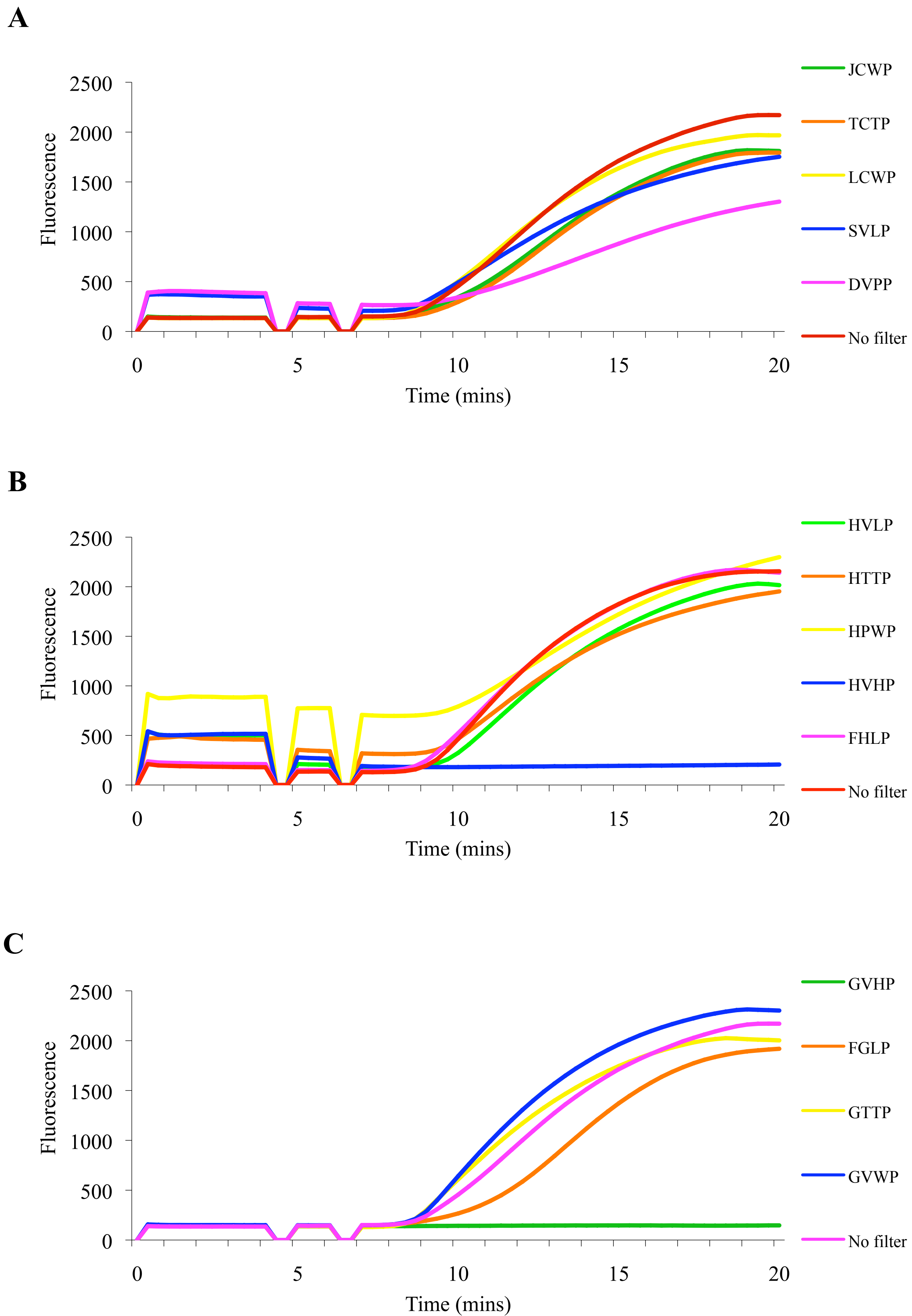


Figure 4.9 TwistAmp MRSA output for the filtrate of filters with pore sizes **A** >0.5μm; **B** 0.4μm; and **C** 0.2μm in diameter.

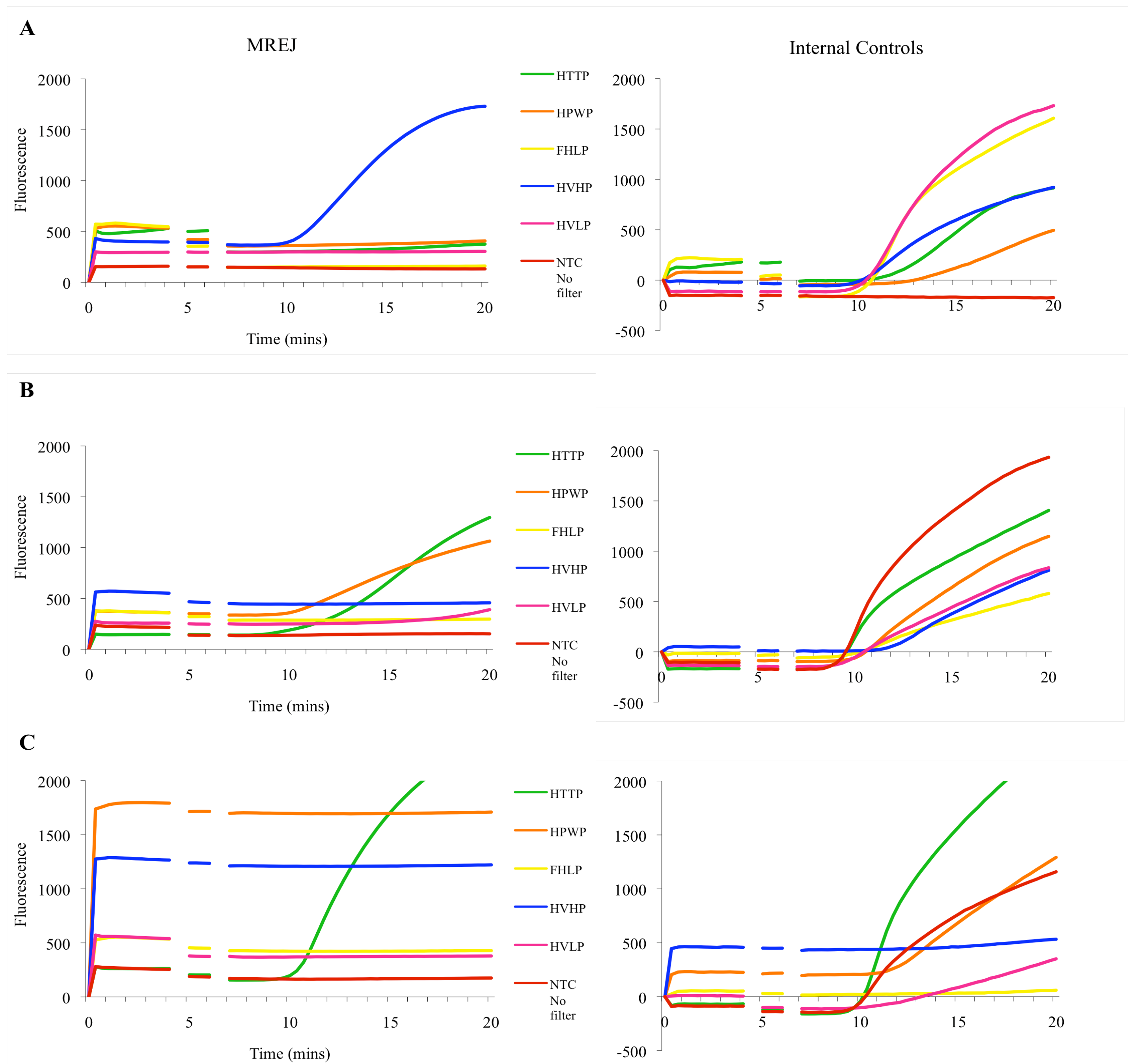
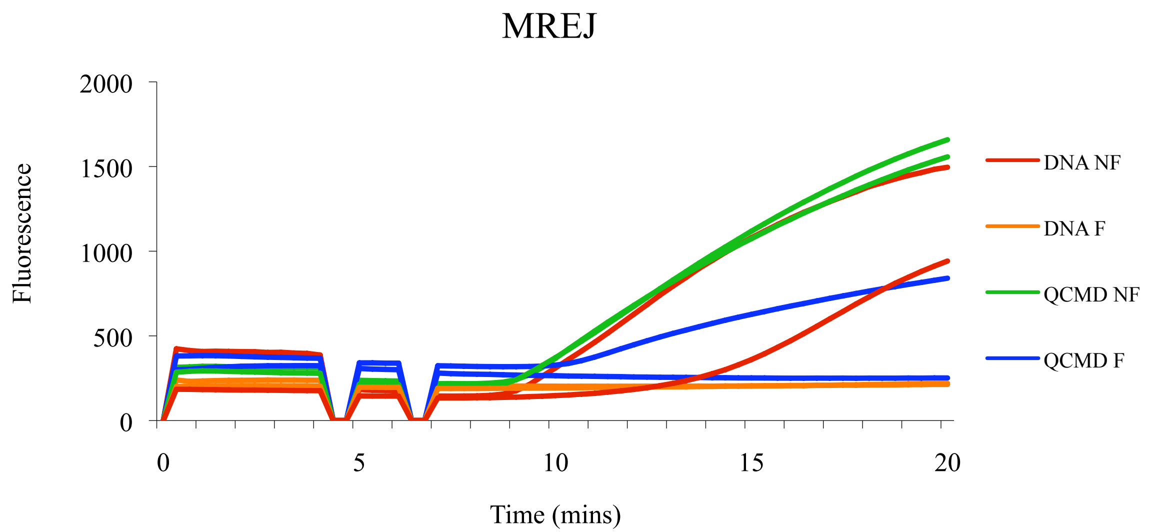


Figure 4.10 Example of TwistAmp MRSA output for reactions containing 0.4µm filters. Panels **A** to **C** show the output for three repeat experiments with the corresponding internal controls for each reaction shown on the right. NTC No filter = buffer containing no MRSA and no filter in reaction (negative control).

To investigate these problems further, three experiments were performed. First, the effect of filters on reaction performance and/or fluorescence readings was tested. Both MRSA from the QCMD 2008 MRSA EQA programme panel and DNA of known MREJ type (MREJ ii, and therefore known to give a positive result) were used as template in separate reactions (both at 25 CFU/150µl). Reactions were performed in duplicate, and each template tested with a filter (as in Figure 4.8) and without (as in Figure 4.7 but using no filter). In order to compare the results of including a filter in the reaction versus no filter, a filter that retains MRSA was needed. Thus, the 0.2µm filter GVHP was used as previous results suggested it retained MRSA.

Results showed that reactions with no filter performed better than the equivalent reactions containing the GVHP filter (Figure 4.11). The unexpectedly low duplicate for the MREJ ii DNA/no filter combination was likely due to an error in reaction setup. Internal controls were somewhat variable and also performed better without a filter in the reaction, with those of the QCMD MRSA template/GVHP filter combination producing very weak reaction curves (Figure 4.11).



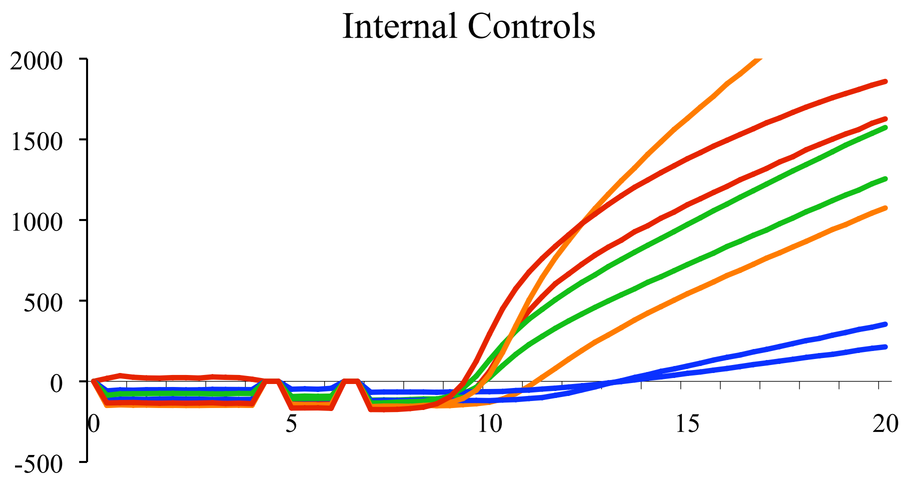


Figure 4.11 TwistAmp MRSA output, including internal controls, showing the effect of filters in the reaction tubes. Template (25 CFU/150µl): DNA = MREJ ii DNA; QCMD = MRSA from QCMD 2008 MRSA EQA programme panel. NF = no filter; F = GVHP filter.

Second, given the evidence of a potential negative effect of filters on reaction performance, the volume of RPA resuspension buffer retained by the filter was calculated. If a filter retains a large amount of buffer, adding the filter to a reaction may cause the optimal reaction volume to be exceeded, thus affecting reaction performance. The dry weight of three GVHP filters was measured and averaged (10.3μg). 200μl of RPA resuspension buffer containing no MgAc (to replicate the buffer used in the above experiments) was filtered by each GVHP filter and the used filters were weighed again and averaged (78.4μg). The difference in average weight of the GVHP filter before and after use was calculated and divided by the weight of 1μl of buffer (68.1μg/1.045μg). Results showed that 65.2μl of RPA resuspension buffer was retained by GVHP, taking the total reaction volume to over 200μl.

Third, to investigate whether the filters were affecting the ability of the Twista machine to accurately read fluorescence as a result of their opacity, 100μl of RPA resuspension buffer was added to each. In addition, each filter was submerged in 1ml buffer to test transparency. In both tests, filters were left for a total of two hours. Only the 0.4μm filter HTTP, and to a lesser extent HVLP became transparent, and did so within seconds of contact with the buffer (Figure 4.12). Their 0.2μm counterparts GTTP and GVWP also became transparent within seconds. All other filters remained opaque.

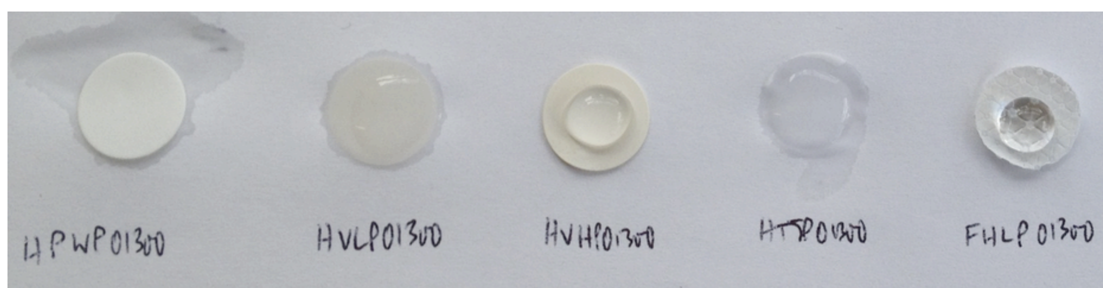


Figure 4.12 Photograph depicting the interaction between 100μl of RPA resuspension buffer and each 0.4μm microporous membrane filter.

Since no conclusive results could be obtained from testing the filters themselves with TwistAmp MRSA, the focus was turned to the filtrate experiments and the reasons why GVHP (0.2μm) and HVHP (0.4μm) showed evidence of MRSA retention. Filter retention would be expected since *S. aureus* cells are larger than 0.5μm, but given that all other 0.2μm and 0.4μm filters tested appeared to release MRSA cells, GVHP and HVHP were investigated. A component of the RPA resuspension buffer, polyethylene glycol (PEG), may somehow be retained by these filters, thus leading to a reaction failure. Buffer containing MRSA (25 CFU/110μl) but no PEG and no MgAc, was filtered through GVHP and HVHP. 20% w/v PEG (confidential molecular weight) and 280mM MgAc were then added to the reaction tubes prior to filtrate testing (Figure 4.13). The same reactions were performed with no filtration. Filters GVHP and HVHP did not appear to retain PEG, as shown by negative TwistAmp MRSA output for these filters, regardless of the presence of PEG in the buffer prior to filtration (Figure 4.14).

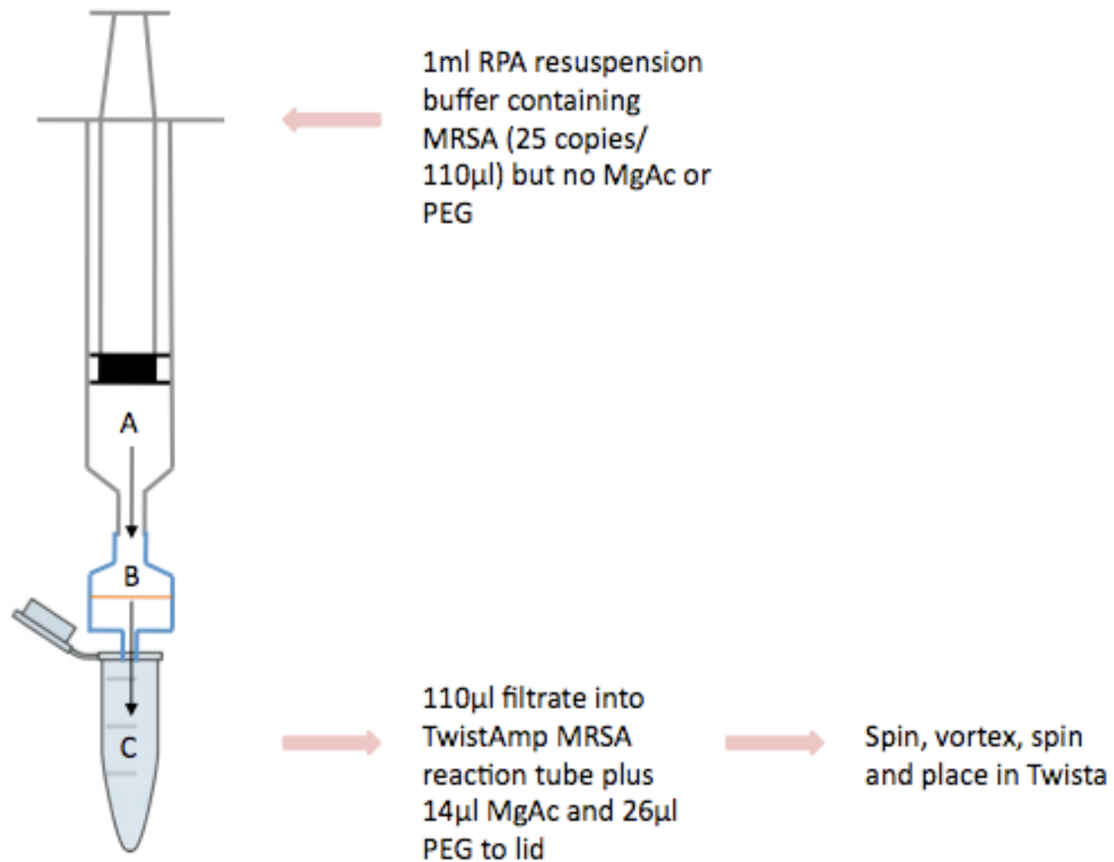


Figure 4.13 Schematic showing the filtration process prior to filtrate testing with TwistAmp MRSA, with buffer containing no polyethylene glycol (PEG). 1ml of buffer containing MRSA (25 CFU/110µl) but no MgAc or PEG was pushed through a Terumo 2ml syringe, followed by a further 500µl of buffer containing no MgAc or PEG (**A**). The buffer then passed directly from the syringe through a Swinnex 13mm filter holder (blue; **B**) fitted with a 13mm membrane filter (orange line) and O-ring seal (all Merck Millipore). The filtrate was dispensed by the filter holder into a 1.5ml eppendorf tube (**C**) and 110µl of this filtrate plus 14µl of 280mM MgAc and 26µl 20% w/v PEG (confidential molecular weight) added to a TwistAmp MRSA reaction tube.

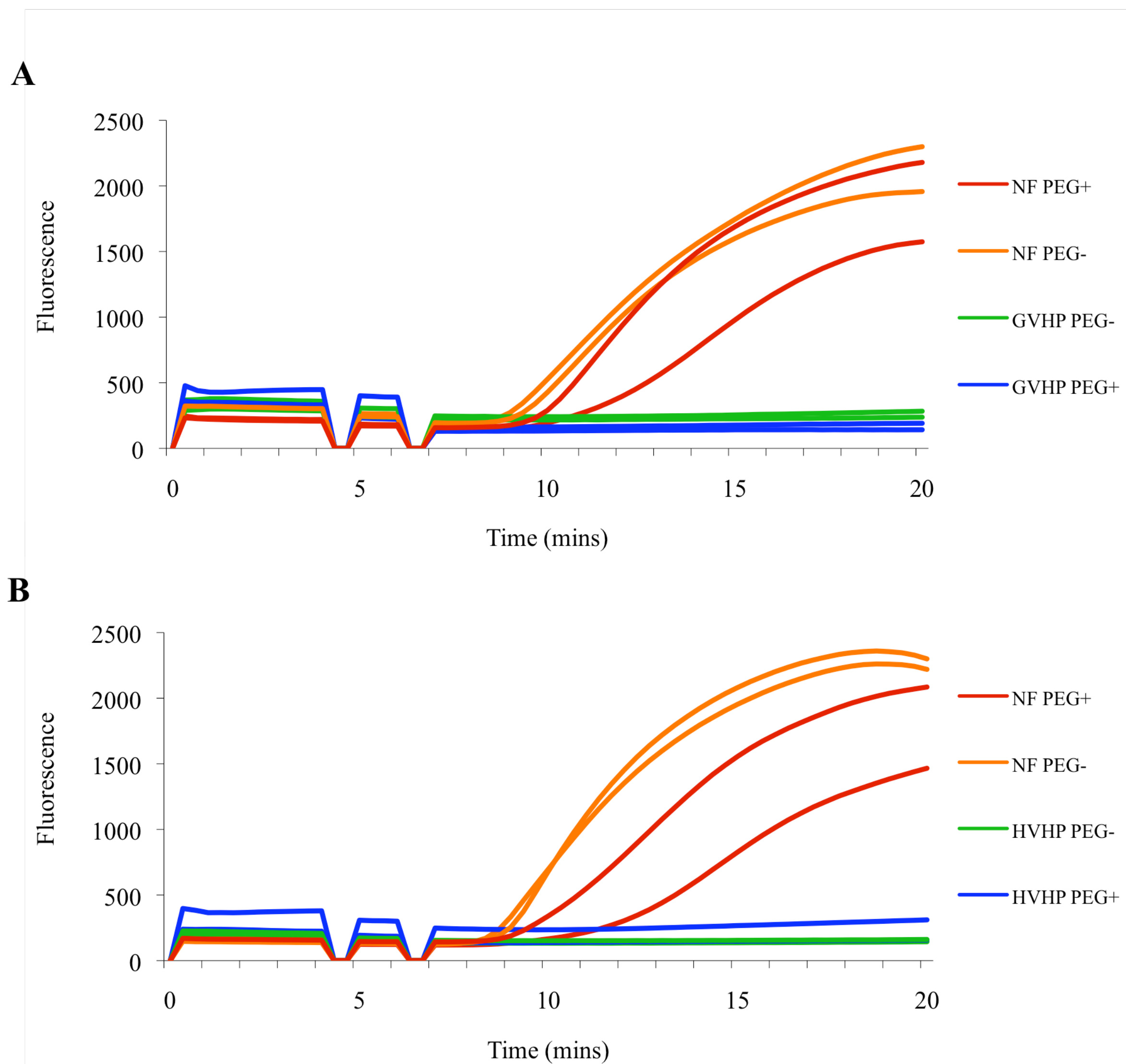


Figure 4.14 TwistAmp MRSA results of testing filter retention of polyethylene glycol (PEG). **A** shows the filtrate results of GVHP (0.2µm) and **B** those of HVHP (0.4µm). NF = unfiltered. PEG+ = buffer containing PEG prior to filtration (positive control; Figure 4.4). PEG- = PEG added to buffer after filtration. Template for all reactions was QCMD MRSA at 25 CFU/reaction.

The QCMD MRSA sample used as template throughout the filtration experiments consisted of heat-inactivated cultured cells. Heat has previously been shown to cause leakage and lysis of *S. aureus* cells [Allwood and Russell 1969], so it may be that free DNA was present, leading to RPA detection of MRSA in the filtrate. If this were the case, GVHP and HVHP retain DNA as well as *S. aureus* cells. In order to confirm that all other filters released cells and not just free DNA, filtration experiments were performed with live MRSA cells. Three filters with different characteristics were selected: HPWP (0.4μm), FGLP (0.2μm) and GVWP (0.2μm) (Table 4.2). MRSA of an MREJ type known to give a positive TwistAmp MRSA signal (MREJ ii) was cultured as in section 2.1. For each filter, a colony from overnight growth was suspended in 1.2ml of RPA resuspension buffer and thoroughly mixed by vortexing, repeated inversion, then orbital shaking for 30 minutes. Serial dilutions were performed from 1:100 up to the approximate TwistAmp MRSA LOD (1:1 million), with thorough mixing between each dilution. In parallel, and also for each filter, MRSA was suspended in sterile distilled water (SDW) and diluted up to 1:1 million. Three 100μl replicates of each 1:1 million and also 1:10,000 dilution (RPA resuspension buffer and SDW) were plated separately on blood agar plates (Oxoid) and incubated overnight at 37°C. Colonies were counted using a Stuart colony counter (Bibby Scientific). Filtration of the 1:1 million and 1:10,000 dilutions (buffer and SDW) was performed for each of the three filters. 600μl of each dilution was filtered, and 100μl replicates of each filtrate plated separately on blood agar plates and incubated overnight at 37°C. Comparing the filtrate of both the buffer and SDW dilutions by culture allowed the assessment of the effect of the buffer and/or filtration process on cell viability. 100μl of each buffer filtrate was tested with

TwistAmp MRSA (100µl reactions) and 100µl of each unfiltered buffer dilution was tested for direct comparison.

No difference was observed in the colony counts between buffer and SDW dilutions prior to filtration (Table 4.3), suggesting that RPA resuspension buffer does not lyse cells or cause cell leakage, and thus presumably creates no free DNA that may pass through the filters and cause a positive TwistAmp MRSA result. This was confirmed by testing the main component of RPA resuspension buffer besides SDW - PEG - for an effect during filtration (protocol Figure 4.13). No difference in TwistAmp MRSA output was observed between reactions with buffer that contained PEG prior to filtration, to reactions where PEG was added after filtration, using 0.2µm filters FGLP and GVWP (Figure 4.15).

No MRSA growth was observed from plating the filtrates. This was the case for both 1:10,000 and 1:1 million MRSA dilutions in SDW and resuspension buffer, and for all three filters tested (Table 4.3). TwistAmp MRSA output showed the buffer filtrates were MRSA positive at the 1:10,000 dilution but weaker than the unfiltered dilutions (Figure 4.16). Filter FGLP demonstrated the smallest difference in output before and after filtration, and at the 1:1 million dilution no difference was observed for this filter. Filtrates of HPWP and GVWP were negative at the 1:1 million dilution (Figure 4.16).

Table 4.3 Colony counts for 1:100,000 and 1:1m SDW and buffer dilutions of MRSA before and after filtration with microporous membrane filters HPWP, FGLP and GVWP.

	CFU/100µl RPA resuspension buffer		CFU/100µl Sterile distilled water	
	Before filtration	Filtrate	Before filtration	Filtrate
1:10,000 dilution				
FGLP	247	0	229	0
HPWP	155	0	122	0
GVWP	203	0	362	0
Average CFU/100µl	**202**	**0**	**238**	**0**
1:1 million dilution				
FGLP	3	0	7	0
HPWP	1	0	2	0
GVWP	9	0	3	0
Average CFU/100µl	**4**	**0**	**4**	**0**

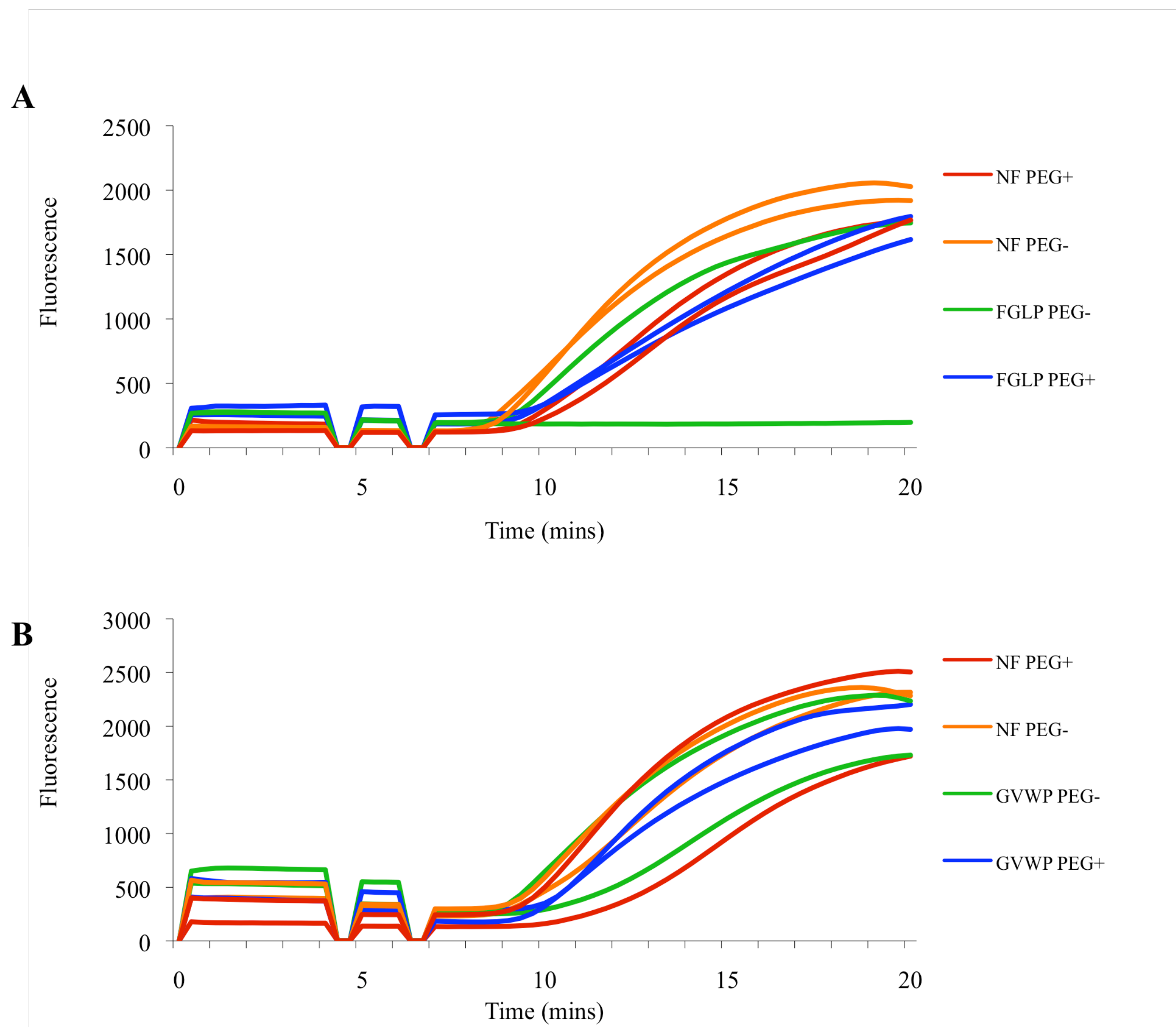


Figure 4.15 TwistAmp MRSA results of testing the effect of polyethylene glycol (PEG) in the resuspension buffer prior to filtration. **A** shows the filtrate results of FGLP (0.2µm) and **B** those of GVWP (0.2µm). NF = unfiltered. PEG+ = buffer containing PEG prior to filtration; PEG- = PEG added to buffer after filtration. Template for all reactions was QCMD MRSA at 25 CFU/reaction.

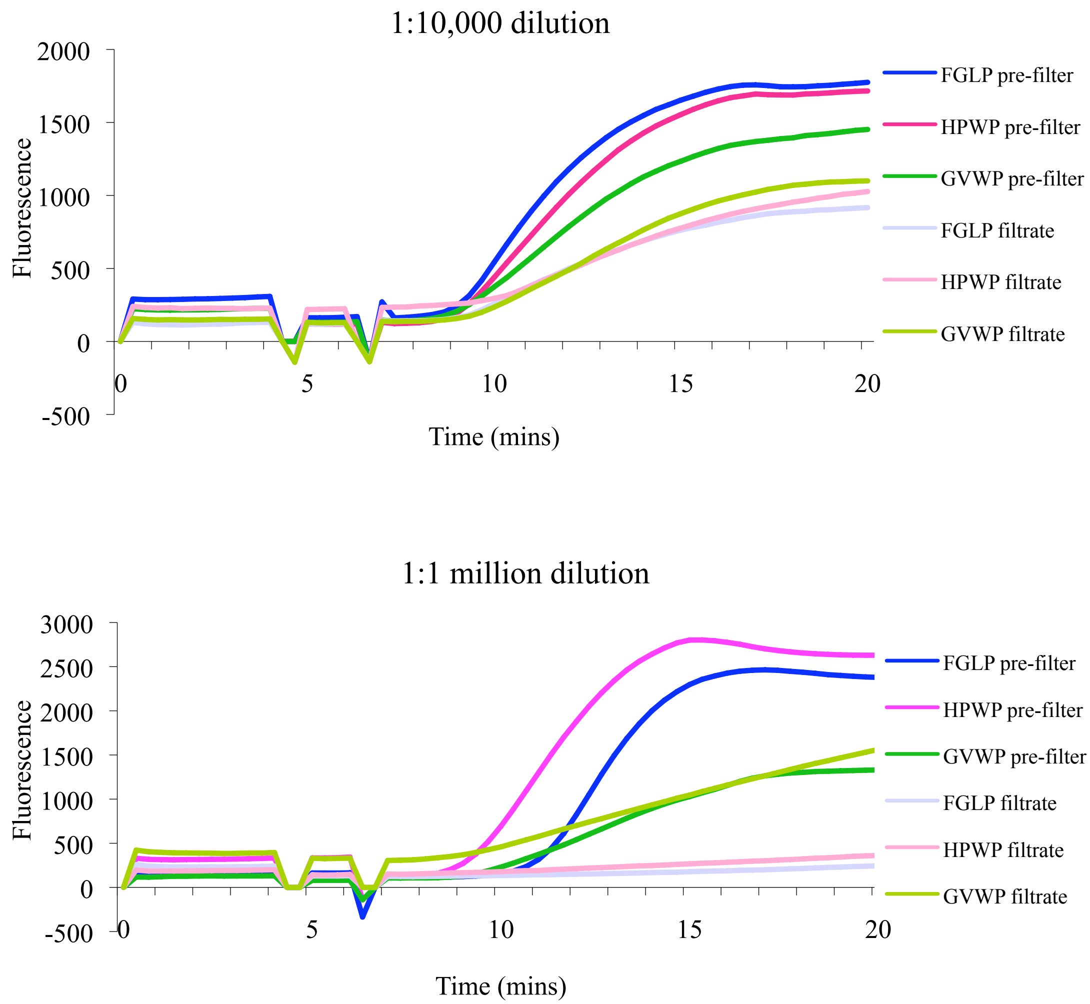


Figure 4.16 TwistAmp MRSA results of testing filtrates of MRSA diluted to 1:10,000 and 1:1 million in RPA resuspension buffer. Filters HPWP (0.4µm), FGLP (0.2µm) and GVWP (0.2µm) were used.

4.3 DISCUSSION

TwistAmp MRSA demonstrates a lower clinical sensitivity than the market leader in molecular MRSA diagnostics, Cepheid's Xpert MRSA. The purpose of this chapter was to investigate possible reasons for this (poor analytical LOD, RPA inhibition and sub-sampling) and assess whether methods such as lysis and/or filtration could improve the assay's analytical LOD and its ability to detect MRSA in clinical samples.

4.3.1 Limit of detection

The LOD of TwistAmp MRSA was determined as approximately 2 CFU/100μl using cultured MRSA. The assay sporadically and weakly detected 1 CFU/100μl. This clearly demonstrates the assay is very sensitive. Cepheid states an overall LOD of 80 CFU/swab for Xpert MRSA, and provides the LODs for individual SCC*mec* types. For Xpert MRSA, the LOD is 10 CFU/swab for SCC*mec* types I-III and V, 50 CFU/swab for type IV, and 100 CFU/swab for type IVa [Cepheid Diagnostics 2009]. The LOD of TwistAmp MRSA was determined using an MREJ type ii isolate, which accounted for about 90% of all MRSA tested in Chapter 3. SCC*mec* type II was exclusively associated with MREJ type ii in the MRSA strains typed in Chapter 3. Thus, results show TwistAmp MRSA has a slightly lower analytical sensitivity than Xpert MRSA (2 CFU/100μl, equivalent to 20 CFU/swab, versus 10 CFU/swab).

Xpert MRSA's LOD of 80 CFU/swab was determined with 95% confidence i.e. the lowest number of MRSA CFU per swab that can be reproducibly distinguished from negative samples with 95% confidence. A 95% confidence interval for the analytical LOD of TwistAmp MRSA is yet to be determined. Further work is also

needed to determine the LOD for each of the less prevalent MREJ types covered by TwistAmp MRSA, as well as for each of the prototypic strains for the different SCC*mec* elements detected by the assay (types I-IV and VI-VIII). Proprietary primers for MREJ types iv, v and vii performed less well than those for MREJs i-iii (data not shown), so the LOD is likely to be poorer for the former types. The SCC*mec* type V element represented by strain WIS (MREJ xii) was not detected by TwistAmp MRSA, and one other type V strain tested (ST398) produced only weak positive results for MREJ iii (Figure 3.16). All GenBank entries of type V elements with the exception of WIS are MREJ iii (see Chapter 3 section 3.3.4.4). TwistAmp MRSA would successfully detect half of these but the other half, all belonging to the livestock-associated lineage ST398, would likely produce weak positive results due to SNPs in the probe-binding region. Given this fact, it would be interesting to ascertain the SCC*mec* type V strain used in Cepheid's performance testing of Xpert MRSA. Because detection of SCC*mec* type V strains by TwistAmp MRSA is not reliable, determining a LOD for this element would only be informative if all variants were tested. Despite a correlation between MREJ type and SCC*mec* type existing, there are exceptions, so caution should be taken when interpreting SCC*mec* type-specific LODs.

A lysis and purification step was incorporated into the TwistAmp MRSA protocol in an attempt to further improve the assay's analytical sensitivity. No improvement was observed with inactivated N315/MREJ ii MRSA cells, however the limit of detection was improved to approximately 1 CFU/100μl after lysis of viable MREJ ii MRSA. This is equivalent to 10 CFU/swab, matching the LOD of Xpert MRSA for MREJ ii MRSA. The lack of difference observed between unlysed and lysed QCMD MRSA could be a result of free DNA already present in the heat-

inactivated sample. Heat has previously been shown to cause cell shrinkage and leakage of intracellular constituents from Staphylococcal cells [Allwood and Russell 1969, Berkman and Wyatt 1970]. Despite the improved LOD with lysis and purification of viable MRSA, high analytical sensitivity is still achieved with no lysis. It was therefore concluded that the addition of a lysis step would not improve the analytical sensitivity to an extent that would warrant such an addition, given the modifications that would have to be made to the current assay.

4.3.2 Inhibition and sub-sampling

Given the high analytical sensitivity of TwistAmp MRSA (2 CFU/100μl), the potential effects of RPA inhibition and sub-sampling on the assay's clinical sensitivity were investigated. Testing RPA with samples of MRSA mixed with high concentrations of MSSA or MR-CNS did not inhibit amplification of MRSA DNA and cause false negative results. A similar experiment performed for a rival assay, BD GeneOhm MRSA, showed that it too was not inhibited by MSSA or CNS [Huletsky et al. 2004]. While increasing concentrations of MR-CNS in MRSA samples did not produce false negative results, they did appear to weaken detection although not to an extent that would affect a definitive positive MRSA result. This is likely due to the depletion of MREJ-specific primer reserves in the reaction as they bind to the SCC*mec* element conferring methicillin resistance in the CNS, leaving less MREJ primer for MRSA DNA in the sample. The MR-CNS likely had the same MREJ type as the MRSA in the sample (MREJ ii), which explains the weaker reaction curves for MRSA as the concentration of MR-CNS increased.

Samples containing high concentrations of MSSA or MR-CNS but no MRSA did not produce false positive results, demonstrating good analytical specificity. More

comprehensive testing of MSSA, MS-CNS and MR-CNS, plus strains representing species phylogenetically related to *S. aureus* and members of the nasal commensal flora, is required for accurate determination of the analytical specificity of TwistAmp MRSA [Cepheid Diagnostics 2009]. Five MSSA isolates from our laboratory collection of Staphylococci, and one MSSA isolate from the KC collection gave false positive results with the assay (Chapter 3). The KC MSSA false positive appears to contain a remnant of an SCC element because it is MREJ positive (type i) but *mecA* negative. The QCMD 2008 MRSA EQA programme panel was used in this chapter, but the 2009 EQA panel contains two samples of MSSA with *mec*-less cassettes (SCC*mec* I and III). These samples would likely cause a similar effect to MR-CNS on MRSA detection, and would cause false positive results in the absence of MRSA.

mec-less SCC elements are known to cause false positive results with Xpert MRSA and BD GeneOhm MRSA [Francois et al. 2007, Arbefeville et al. 2011]. te Witt et al. [2010] found commercial real-time PCR tests incorrectly reported the two QCMD 2009 MSSA containing *mec*-less SCC elements as positive in 89% of datasets (40 of 45 datasets, including 13 tested with GeneOhm MRSA and 11 with Xpert MRSA). Xpert MRSA, GeneOhm MRSA and TwistAmp MRSA all target the MREJ and so false positive results are inevitable with MREJ-positive MSSA. If these assays were used in laboratories with high false positive rates or in regions with low MRSA prevalence, confirmation by culture or a second molecular test would be necessary [Kerremans et al. 2008, te Witt et al. 2010]. The only way to improve specificity with such samples would be to incorporate a *mecA* internal control into the assays. However, *mecA* is not unique to MRSA, so false positives could still arise due to co-colonisation by MSSA and MR-CNS. Overall, MREJ-negative MSSA and MR-CNS do not affect accurate MRSA detection by TwistAmp MRSA.

When directly using swabs, interfering substances such as blood or mucus present in clinical samples could cause RPA inhibition, but this has not yet been investigated. Cepheid evaluated these potentially interfering substances and concluded that they did not significantly inhibit PCR or cause false negative results with Xpert MRSA [Cepheid Diagnostics 2009], but this assay filters and lyses the sample, and the released DNA is eluted prior to PCR testing, thus separating reaction inhibitors such as blood or mucus from the target DNA. TwistAmp MRSA does not include any sample preparation.

RPA resuspension buffer containing MRSA was filtered using various microporous membrane filters to assess whether filtration improved detection of MRSA with TwistAmp MRSA. Filters and filtrates were tested with the assay for both retention and passage of MRSA. Of 14 filters, only two appeared to retain MRSA: GVHP (0.2μm) and HVHP (0.4μm). This was surprising given that *S. aureus* cells are at least 0.5μm in diameter - all 0.4μm and 0.2μm filters should retain MRSA. This highlighted the possibility of either free DNA present in the sample, or lysis/leakage of cells at some point during the filtration process, causing positive results despite the retention of cells by the filters. Since the MRSA used as template consisted of heat-inactivated cells, free DNA was likely present, since heat can cause cell leakage [Allwood and Russell 1969]. This was confirmed by filtering viable MRSA cells through selected 0.2μm and 0.4μm filters. The cultured filtrates grew no MRSA, yet TwistAmp MRSA testing of the same filtrates gave MRSA-positive results, albeit weak. It is likely therefore that all of the filters with pores less then 0.5μm in diameter retained MRSA cells. Alternatively, the filtration process could have affected cell viability in an unknown way. Millipore states that fluid viscosity and chemical interactions between filter membranes and particles in the solution could

affect bacterial retention [Millipore Corporation 2012]. However, RPA resuspension buffer, which is fairly viscous owing to PEG, was confirmed not to have an effect on filtration of MRSA cells.

MRSA retention by the 0.2μm and 0.4μm filters could not be confirmed with TwistAmp MRSA reactions containing the filters themselves. The idea was that adding filters to the reactions could test for the presence of MRSA cells on the filter membranes. These experiments could not produce consistent results, and internal controls failed. TwistAmp MRSA reaction tubes are 0.2ml in size, and so the 13mm filters were not only difficult to insert into these but likely interfered with the RPA reactions or measurements thereof due to their size and/or composition. Optical properties of the filters, such as autofluorescence or transmittance, could have affected fluorescence readings [Millipore Corporation 2012]. Of the filters tested for opacity when in contact with RPA resuspension buffer, only HTTP (0.4μm) and GTTP (0.2μm) filters were fully transparent. These filters are both part of Millipore's Isopore range, recommended for analyses in which the sample is viewed on the surface of the membrane, for example using optical or electron microscopy [Millipore Corporation 2012]. However, the optical compatibility of this filter type with the FAM and TAMRA fluorophores used in TwistAmp MRSA is unknown, so it's possible that despite their transparency to the naked eye, they still cause optical interference with this assay.

Filter GVHP was found to retain a significant volume of RPA resuspension buffer, which would have caused the optimal reaction volume to be exceeded, resulting in reaction failure. This could explain the negative results obtained when testing filters GVHP and HVHP (they were thought to retain MRSA so testing the filters were expected to give positive results). Indeed, both GVHP and HVHP filters

were hydrophobic. Retention of buffer as well as MRSA cells due to hydrophobicity renders these filters unsuitable for use with TwistAmp MRSA. The only filter characteristic GVHP and HVHP had in common that also differed from all other filters tested was their high protein binding capacity. It is not clear why this filter property would result in bacterial retention. One possibility is the surface proteins present on *S. aureus* cell walls binding to the filter.

Millipore describe a 0.2μm filter (GPWP) that is bacterially retentive and low protein-binding, meaning that MRSA would be retained by the filter, but the liquid sample in which it is suspended and any potential RPA inhibitors would pass through the filter [Millipore Corporation 2003]. This Millipore Express filter is hydrophilic and sterilising-grade, the latter feature meaning that it can reproducibly retain viable microorganisms. A 0.4μm Millipore Express filter was tested in this chapter - HPWP - but could not be accurately tested by the filtration experiments performed. Using viable MRSA cells however did provide evidence of its bacterial retention property. This was also the case for GVWP (0.22μm), a hydrophilic Durapore membrane with the lowest protein-binding capacity offered by Millipore. Thus, these filters would likely be the most appropriate for use with TwistAmp MRSA. Confirmation that these filters retain MRSA cells could be achieved by visualising the filters through a fluorescence microscope, but most bacterial retention studies measure the number of viable microorganisms present in the filtrate, rather than the number retained. One study used Millipore membrane filters spanning pore sizes of 0.4μm to 12μm to test their retention of *S. aureus*. Only 1μm filters or larger allowed passage of *S. aureus*, at both high (10^7 cells per ml) and low (10^2 cells per ml) cell concentrations [Bobbitt and Betts 1992]. If more time were available, the remaining 11 filters in this chapter would be tested with viable MRSA, and retention tested by measuring the CFU

present in solution before and after filtration (FGLP, GVWP and HPWP already tested here).

Despite evidence that 0.4μm and 0.2μm filters retain MRSA cells, no improvement in MRSA detection was observed as a result of filtration, suggesting that addition of a filtration step would not improve the clinical sensitivity of TwistAmp MRSA. Testing with clinical samples instead of cultured MRSA cells would allow more accurate evaluation of a filtration process for removal of potential RPA inhibitors, since it is clinical samples that most likely contain inhibitory substances, e.g. blood or mucus.

MRSA cells successfully retained on a membrane filter would not only be purified, but also concentrated. If a method for detecting the entire bacterial retention on a filter could be devised, the potential problem of sub-sampling would also be removed. Such a method could involve a double-filtration process whereby a swab is eluted in RPA resuspension buffer, and the resulting sample processed through a >0.5μm filter, allowing the passage of MRSA but retaining large sample constituents, followed by a 0.4μm filter that retains MRSA and allows passage of smaller sample constituents. The retained MRSA cells could then be eluted in more RPA resuspension buffer and the solution added to a TwistAmp MRSA reaction tube for testing. A similar process occurs in Cepheid's Xpert MRSA that uses a number of filters, ranging from a 5μm filter to a 0.2μm Durapore filter (Millipore) for cell capture, combined with glass beads and an ultrasonic horn for cell lysis to release DNA [Pourahmadi et al. 2000]. The released DNA is then eluted and the solution mixed with dried PCR reagents for testing [FDA 2007]. However, addition of a filtration step would complicate the TwistAmp MRSA assay and would have to provide a very substantial increase in performance to be considered.

4.3.3 Conclusions

Possible reasons for lower TwistAmp MRSA clinical sensitivity compared to that of Xpert MRSA were tested, but neither a poor analytical sensitivity, nor RPA interference due to the presence of MSSA or MR-CNS in the sample, were found to be significant causes. It is clear that filters retain MRSA, the main advantage of this being the concentration of MRSA cells for improved detection, but without testing clinical samples of MRSA that may contain inhibitory substances, it remains unclear to what extent clinical sensitivity is affected by reaction inhibition. It is also unclear what is causing positive TwistAmp MRSA results despite bacterial retention by filters. More comprehensive testing and development of the assay is needed to identify and minimise the causes of the lower than expected clinical sensitivity. Multiple manual steps are required in the current TwistAmp MRSA prorotcol, including pipetting, vortexing and centrifugation, whereas Xpert MRSA is largely self-contained and automated. Reducing manual input may go some way towards improving sensitivity by minimising errors introduced from the external environment. TwistDx have developed a second-generation fluorometer that removes some manual steps, including automated mixing through the use of magnetic beads, but the machine was not available for use with this thesis work.

PART 1 FINAL DISCUSSION: RPA-BASED DETECTION OF MRSA

The RPA-based diagnostic assay, TwistAmp MRSA, offers a rapid, simple and accurate alternative to PCR-based systems such as BD GeneOhm MRSA and Xpert MRSA that currently lead the market in molecular detection of MRSA. The work presented in Chapter 3 of this thesis has shown that the performance of TwistAmp MRSA is comparable to that of commercial assays. However in the UK, false negative MRSA isolates were reported with the assay due to the presence of novel MREJ types identified among epidemic MRSA clones. False negative results were also obtained for recently described SCC*mec* types IX-XI, while certain variants of existing SCC*mec* types appeared to cause detection problems for the assay. This highlights the problem of ever-increasing SCC*mec* variation that is faced by all assays targeting the MREJ. MREJ-based detection methods must therefore be continually evaluated and modified to keep abreast of such variation, particularly in areas where problematic MRSA strains are common. Testing of a more genotypically and geographically diverse range of MRSA strains is required to fully assess the ability of TwistAmp MRSA to detect most MRSA.

Despite comparable TwistAmp MRSA performance to current commercial assays and its ability to detect a wide range of MRSA strains, its clinical sensitivity was lower than expected from initial pre-clinical studies in the US and UK, and so Chapter 4 assessed potential reasons for this. Analytical sensitivity was found to be extremely high, at approximately 20 CFU/swab, and thus was not considered a cause of lower than expected clinical sensitivity. While there was some evidence for the benefit of incorporating lysis and filtration steps into the assay protocol, doing so would be, at present, unlikely to sufficiently improve assay performance. More comprehensive testing using clinical samples is required to fully assess and improve

TwistAmp MRSA performance, and thus bring its clinical sensitivity more into line with that of the leading commercial assay, Xpert MRSA.

An aspect of TwistAmp MRSA that was not explored in this thesis was the optimisation of its current primers and the multiplex assay as a whole. Further work on the assay would therefore involve investigating the possibility of splitting the multiplex into a two-tube system, for example with primers for MREJ types i-iii in one tube and primers for MREJ types iv, v and vii in a second tube. A new internal control would need to be designed for the second tube, and the addition of more MREJ types to each tube could be explored, for example novel MREJ d that was found to be the most common MREJ type among false negative isolates of the UK MRSA collection. Since primers for MREJ types iv, v and vii performed less well than those for types i-iii (data not shown), the former primers could be redesigned or an alternative *orfX* primer designed to improve their performance. A primer screen of 30-mers for MREJ types iv, v and vii could also be conducted to see if their performance is improved compared to the original 35-mers used in the multiplex assay.

RPA could also be used as the basis of other diagnostic assays for MRSA, for example for specific clones such as USA300 (ST8-MRSA-IV), the highly successful community-associated strain that predominates in the US. Targets for an RPA-based USA300 test could include the enterotoxin genes *sek* and *seq*, or the arginine catabolic mobile element (ACME), both of which would distinguish USA300 from its epidemic progenitor USA500 [Li et al. 2009].

Knowledge regarding the nature of MRSA clones that are disseminating globally is crucial for implementation of infection control strategies in both nosocomial and community settings [Boucher and Corey 2008, Chen et al. 2009].

Consequently, rapid characterisation of MRSA strains by SCC*mec* typing is an important issue [Boucher and Corey 2008]. With the advent of RPA as a novel alternative to PCR, it is possible to apply RPA to any area of MRSA epidemiology that uses PCR-based methods. As such, I began work on developing an RPA-based multiplex assay for SCC*mec* typing, as a rapid alternative to PCR-based SCC*mec* typing methods. I designed RPA primers (Eurogentec) and TwistAmp exo probes (Biosearch Technologies) for a two-tube assay that detects SCC*mec* types I-VIII using a four-dye system (see Appendix 3 for primer and probe sequences and the proposed multiplex format). Primers and probes were designed according to TwistDx recommendations [TwistDx Ltd 2009b] and using Primer-BLAST. Primers and probes were also tested for secondary structures using AutoDimer [Vallone and Butler 2004], and tested for specificity using prototypic strains for SCC*mec* types I-VIII in singleplex TwistAmp exo reactions (Table 3.1; see Appendix 3 for figures demonstrating specificity). Singleplex TwistAmp exo reactions were analysed in real-time using the Twista machine for FAM- and TAMRA-labelled probes, as well as the second-generation fluorometer developed by TwistDx, capable of detecting FAM-, HEX- and ROX-labelled probes.

Although these singleplex reactions detected these SCC*mec* types, due to time constraints, it was not possible to combine the primers and probes into an optimal multiplex format, or validate the assay using a collection of previously characterised, geographically, temporally, and genotypically diverse MRSA. Further work is therefore required to fully develop the SCC*mec* typing method, but it is clear that it would provide a rapid alternative to current methods, giving results within 20 minutes. A machine capable of detecting four dyes and adapted for RPA reaction conditions (i.e. constant temperature) would be required, such as the ABI 7500 real-

time PCR system (Applied Biosystems), which would also allow the method to be high-throughput - up to 48 samples (48 two-tube reactions) in one run.

The proposed RPA-based SCC*mec* typing method would detect two loci for each of the SCC*mec* types I-VI and VIII, and one locus for type VII, in addition to the *mecA* locus for all types. Because of the lack of discriminatory power associated with targeting just one locus for an SCC*mec* type, a confirmatory test would be necessary for SCC*mec* type VII, for example targeting *ccrC8*. The method of Boye et al. [2007] used in this thesis for SCC*mec* typing, as well as that of Zhang et al. [2005], are easy to use multiplex PCR assays for SCC*mec* types I-V, but they detect only a single locus for the majority of the SCC*mec* types, and therefore lack discriminatory power. The RPA-based method would offer superior discriminatory power in this respect.

A disadvantage of all SCC*mec* typing methods, including the RPA-based method proposed here, is that they determine different structural properties of the element. A single universal assay for the determination of SCC*mec* type therefore needs to be developed, that is based on a universally accepted nomenclature. In 2006, Chongtrakool et al. [2006] proposed a novel nomenclature based on the *ccr* genes (indicated by a number) and the *mec* complex (indicated by an uppercase letter). Application of this nomenclature results in SCC*mec* type 1A (type I), type 2A (type II), type 3A (type III), type 2B (type IV), type 4B (type VI) and type 5C (type V and VII). In addition, the nomenclature designates the differences in the J1 and J2-J3 regions by numbers such that for example type IVb is type 2B.2.1. The *ccr* genes and J regions are also numbered in chronological order according to their time of discovery [Chongtrakool et al. 2006]. Based on this novel nomenclature, Kondo et al. [2007] developed a PCR scheme using five multiplex PCR reactions, but the need for

multiple multiplex PCR reactions makes the method time consuming and thus not ideal for routine applications [Deurenberg and Stobberingh 2008].

Recently, Chen et al. [2009] developed a rapid molecular beacon real-time PCR (MB-PCR) assay for SCC*mec* typing, based on the established definition of SCC*mec* types, i.e. the combination of *mec* and *ccr* complexes, and by following the recommendations of the IWG-SCC [2009]. The assay consists of two multiplex panels, the combination of which results in two targets for each SCC*mec* type. The assay can detect types I-VI and the recently described type VIII. Up to 96 isolates can be classified within 3-4 hours, including DNA isolation, PCR cycling, and analysis [Chen et al. 2009]. This novel assay is faster, more robust and more sensitive than previously published typing schemes. The use of molecular beacons in the assay has obvious advantages over current typing methods that would also be seen with the proposed RPA probe-based method, namely speed and specificity.

RPA is a rapid and simple nucleic acid amplification technology that can easily be applied to any method that traditionally uses PCR. In the field of molecular characterisation of MRSA, an RPA-based real-time SCC*mec* typing method such as the one proposed in this thesis, would obviate the need for lengthy thermal cycling and electrophoretic analysis of amplicons, providing an attractive, potentially high-throughput alternative to the popular typing method of Milheirico, Oliveira and de Lencastre [2007a], and the most recently described real-time method of Chen et al. [2009]. In molecular diagnostics for MRSA, RPA performs comparably to PCR-based detection methods, and TwistAmp MRSA has the potential to provide a fast, simple and cheap alternative to current commercial assays.

PART 2 INTRODUCTION: MRSA IN ORANGE COUNTY, CALIFORNIA

Orange County (OC), California, provides a unique and diverse population from which to draw valuable data regarding MRSA epidemiology. OC is the sixth largest US County according to 2011 estimates [US Census Bureau 2011a]. This metropolitan county covers 790 square miles of land and has a population of just over 3 million people [US Census Bureau 2010], isolated on three sides by the ocean to the west, forest to the east, and miles of undeveloped land to the south. Traffic is considered a major barrier to driving north into Los Angeles County for routine healthcare. It is not only an ethnically diverse county, with a population comprising the following persons: 44% non-Hispanic White, 34% Hispanic, 18% Asian, 2% Black and 2% other race; but is also an economically diverse one, with 10% of the population living below the poverty line [US Census Bureau 2010]. OC has 39 hospitals and 74 nursing homes (Table II.1) [OSHPD 2011]. The hospitals range from small, long term, acute care facilities that care for chronically ill patients, to large academic medical centres. Nursing homes, which include sub-acute and chronic care facilities, range from small facilities with as few as ten licensed beds for long-term care, to large facilities with more than 300 beds.

Table II.1 Size of healthcare facilities in OC, CA [OSHPD 2011].

Licensed Beds	Acute Hospitals	Nursing Homes
<100	10	48
100-199	14	21
200-299	9	5
300-399	2	0
400-499	2	0
500+	2	0
Total	**39**	**74**

MRSA is a major global cause of morbidity and mortality, imposing serious economic costs on patients and healthcare facilities [Abramson and Sexton 1999, Cosgrove et al. 2003, Engemann et al. 2003, Cosgrove et al. 2005, Klein, Smith and Laxminarayan 2007, Shurland et al. 2007]. A better understanding of the frequency and genetic diversity of healthcare-associated and community-associated MRSA strains in both hospital and nursing home reservoirs may help to inform infection control strategies to prevent MRSA transmission and disease in the US. In collaboration with researchers at the University of California, Irvine (UCI), MRSA isolates were collected from OC hospitals and nursing homes in the first population-based countywide study of MRSA strain diversity and distribution in healthcare facilities.

In the US, MRSA carriage (both asymptomatic and symptomatic) is estimated at 6-12% in general hospital patient populations and 9-24% in intensive care units (ICUs) [Huang et al. 2007a, Robicsek et al. 2008, Lucet et al. 2009]. HA-MRSA has long been the primary cause of MRSA infections, but community-associated MRSA

(CA-MRSA), which often causes infections among healthy children and young adults with no exposure to the healthcare setting, is becoming increasingly prevalent. The first reports of MRSA isolated from patients with no identifiable risk factors came from Australia and the US in the 1990s [Udo, Pearman and Grubb 1993, Herold et al. 1998, CDC 1999]. Since then CA-MRSA prevalence has rapidly increased, with reports of CA-MRSA infection from virtually every geographic region of the world [Tristan et al. 2007a, Wallin, Hern and Frazee 2008]. The incidence of life-threatening invasive infections owing to CA-MRSA is increasing, and CA-MRSA appears to be particularly virulent among children [Moellering 2006]. Moreover, CA-MRSA has caused outbreaks in the hospital setting [O'Brien et al. 1999, Saiman et al. 2003, Bratu et al. 2005] with some reports suggesting it may be replacing HA-MRSA [Seybold et al. 2006, Patel et al. 2008, Popovich, Weinstein and Hota 2008, D'Agata et al. 2009].

The predominant community-associated MRSA clone in the US is now USA300 (defined by *spa* typing and multilocus sequence typing (MLST) as t008 and ST8 respectively), having rapidly disseminated and replaced USA400 (t128/ST1) since its isolation in 2000. USA300 has several characteristics that may offer a selective advantage over other MRSA clones, both community-associated (e.g. USA400) and healthcare-associated (e.g. USA100 (t002/ST5)). These advantages include (i) a smaller SCC*mec* element (usually type IV) than those of healthcare-associated strains (usually SCC*mec* types I-III), which is more readily transmissible and may be an advantage in terms of DNA replication speed; (ii) fewer antibiotic resistance genes than healthcare-associated strains, resulting in a fitness benefit due to the carriage of smaller or fewer genes; and (iii) a higher growth rate *in vitro* that may lead to successful colonization by outcompeting healthcare-associated strains [Okuma et al. 2002, D'Agata et al. 2009]. Furthermore, the linkage of ACME with SCC*mec* type IV

in USA300 likely confers increased fitness and/or pathogenicity [Diep et al. 2008b]. Finally, greater expression of regulatory genes associated with the virulence factors PVL and α-haemolysin has been shown in USA300 versus USA400 isolates, which may contribute to the invasiveness of USA300 [Montgomery et al. 2008]. However, there is evidence that CA-MRSA do not need PVL to cause nosocomial infections [Regev-Yochay et al. 2005, David et al. 2006a, Gould et al. 2009, Otter and French 2011].

In addition, as CA-MRSA strains move into the healthcare setting and are exposed to nosocomial antibiotic pressure, they have developed greater antibiotic resistance. In one US study, USA300 isolates classified as healthcare-associated were significantly more likely to be ciprofloxacin-resistant than CA-MRSA USA300 isolates [Huang et al. 2006], and another study reported a USA300 isolate with intermediate vancomycin susceptibility and reduced daptomycin susceptibility from a hospital in San Francisco in 2007 [Graber et al. 2007]. As CA-MRSA strains continue to encroach on healthcare MRSA reservoirs, they may come to resemble the antibiotic resistance profiles of HA-MRSA, as well as behave more like HA-MRSA clinically [Davis et al. 2006, Benoit et al. 2008, Moore et al. 2009, Otter and French 2011]. While it is not clear if CA-MRSA cause more severe disease in the healthcare setting and whether they are more transmissible than HA-MRSA, their higher fitness and growth rate could lead to increasing prevalence in hospitals.

Chapters 5 and 6 of Part 2 of my thesis focus on MRSA in OC hospitals, in order to determine the frequency of hospital-associated and community-associated clones, particularly USA300, among adult and paediatric inpatients, and to gain a better understanding of the nature of the hospital MRSA reservoir by assessing the diversity and distribution of MRSA across 30 hospitals. This knowledge may better

inform infection control strategies employed in hospitals.

Very little is known about the diversity and distribution of MRSA in long-term care facilities. A recent longitudinal analysis by Tattevin et al. [2009] attributed an increasing incidence of MRSA infections in a long-term care facility in San Francisco, CA, to two clonal groups - ST5-MRSA-II and ST8-MRSA-IV. The study highlights the need for further investigation into the epidemiology of MRSA in long-term care facilities in order to minimise further MRSA transmission, particularly since such facilities, long thought to be a reservoir for nosocomial MRSA clones, are now emerging as an important reservoir for the community associated clone USA300 (ST8-MRSA-IV), and could play a role in the emergence of multidrug-resistant USA300 [Tattevin et al. 2009]. Chapter 7 therefore assesses the diversity and distribution of all carriage MRSA collected from residents of 25 nursing homes in OC, in order to gain a better understanding of the nature of the MRSA reservoir in these unique facilities.

Note: All work presented in Chapter 5 has been published previously, and co-authors are listed in Appendix 6 as part of the official citation. Apart from study design and isolate collection, I performed all work, including laboratory methods and molecular typing, statistical analyses, and manuscript writing. For Chapters 6 and 7, apart from study design and isolate collection which were performed by my collaborators at UCI and the Orange County Public Health Laboratory, I performed all work, including laboratory methods and molecular typing, statistical analyses, and writing.

CHAPTER 5: DIFFERENCES IN MRSA STRAINS ISOLATED FROM PAEDIATRIC AND ADULT HOSPITAL INPATIENTS

5.1 INTRODUCTION

The phenotypic and genotypic differences between HA- and CA-MRSA strains have been well documented [Ma et al. 2002, Okuma et al. 2002, Eady and Cove 2003, Diep et al. 2006a, Bassetti, Nicco and Mikulska 2009], yet there are few studies that have directly explored the differences in MRSA strains isolated from adults and those isolated from children. Park et al. [2007] previously compared a small number of adult and paediatric MRSA isolates in a South Korean hospital and found a predominance of CA-MRSA isolates among children. A better understanding of the frequency of community- versus healthcare-associated MRSA clones among adults and children, and in particular the USA300 clone, may inform strategies to prevent transmission and disease.

Children may have different exposures to MRSA, as they constitute a largely healthy population that is most likely to incur MRSA infection through skin and soft tissue injuries related to sports and other play activities [Frei et al. 2010]. This is in contrast to the chronically and critically ill adult population, which frequents hospitals and may encounter healthcare-associated MRSA strains more readily. Furthermore, children may experience different antimicrobial drug selection pressure compared to that of adults due to differences in common disease syndromes and different guidance on antibiotic therapy [David et al. 2006b, Park et al. 2007].

Defining the characteristics of MRSA strains in adults and children would provide insight into the spread of MRSA strains, particularly since there is growing evidence that community and healthcare MRSA reservoirs are mixing [Kourbatova et al. 2005, Seybold et al. 2006, Maree et al. 2007, Liu et al. 2008, Popovich, Weinstein

and Hota 2008]. Furthermore, few studies of adult or paediatric MRSA strains have involved a population-based sample of strains. We conducted a prospective cohort study of inpatients in a large metropolitan county to characterise differences in paediatric versus adult MRSA strains.

5.2 METHODS

5.2.1 Study

A population-based, prospective collection of clinical isolates of MRSA from 30 hospitals in OC, California, was conducted. This study was approved by the Institutional Review Board of the University of California Regents.

5.2.2 Isolate collection

Clinical (non-screening) isolates of MRSA from unique adult patients (≥18 years of age) and unique paediatric patients (<18 years of age) were collected from hospital microbiology laboratories. Hospitals were instructed to collect MRSA isolates from unique patients up to a total of 100 isolates or for a duration of 12 months, whichever came first. In order to have a representative sample of Orange County MRSA isolates, we limited isolates in this study to those collected for a uniform duration of time from adult hospitals. Since the largest adult hospitals reached 100 isolates over a 5-month period, we restricted the period of all adult isolate collections to 5 months. All paediatric hospitals required a 12-month collection period. Nearly all adult isolates were collected between December 2008 and April 2009. Paediatric isolates were collected between October 2008 and September 2009. Isolates from patients not admitted to hospitals were excluded from the study. Samples were batched and delivered to the Orange County Public Health Laboratory

using soy agar slants. For the repeated confirmation of MRSA, isolates were plated on selective media for MRSA (BD CHROMagar). MRSA strains were stored at −65°C in 15% glycerol Brucella broth.

5.2.3 Specimen data and hospital characteristics

Specimen data, including patient age in years, specimen source (wound, blood, urine, sputum, or other), specimen location (ICU or non-ICU), and time of specimen collection with respect to admission date (hospital onset, ≥3 days after admission; community onset, <3 days after admission), were collected. Hospital characteristics were obtained from a California hospital dataset [OSHPD 2005], which included annual admissions, hospital type (acute care versus long-term acute care (LTAC) facility), percentage of Medicaid-insured patients, and percentage of Hispanic patients. Population estimates of adults and children in OC were obtained from the 2010 US Census [US Census Bureau 2011b].

5.2.4 Laboratory methods and molecular typing

All strains were shipped to me for *spa* typing, and stored at −80°C. Cells were harvested on blood agar plates (Oxoid) and incubated at 37°C overnight. DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit. DNA samples were eluted in 200µl of elution buffer (10 mM Tris-Cl, 0.5 mM EDTA (pH 9.0)) and stored at −20°C. Following sequencing of the *spa* region, *spa* types were determined using Ridom StaphType v2.1 (Ridom GmbH) [Harmsen et al. 2003]. To assess *spa* type diversity and relatedness, cluster analysis of *spa* types was performed separately for adult and paediatric isolates using the Based Upon Repeat Pattern (BURP) algorithm, a built-in feature of the StaphType software [Mellmann et al. 2007]. MLST and *Sma*I

PFGE were performed on a subset of the isolates (*n* = 171) to confirm MRSA strain types, according to methods described previously [Pfaller 1998, Enright et al. 2000]. This subset included one isolate of each *spa* type and, for the ten most common *spa* types, one isolate from each of the hospitals in which these *spa* types were present. Isolates were selected using a random number generator. I performed MLST, but collaborators at the University of Iowa performed PFGE. For PFGE, DNA profiles were analyzed using BioNumerics software (version 5.0, 2007; Applied Maths). PFGE types were defined using a similarity coefficient of 78%, and USA100 to USA800 strains were used as references.

5.2.5 Statistical analyses

Annual adult and paediatric population estimates of hospitalised patients with clinical MRSA cultures were calculated by *spa* type, accounting for the duration of countywide collection. I further calculated the percentage of MRSA strains from adult versus paediatric patients that were due to the most common *spa* types (t008, t242, t002) and compared them using χ^2 tests. Specimen data for t008, t242 and t002 isolates were compared using χ^2 or Fisher's exact tests and, for patient age, the Wilcoxon Mann-Whitney test. Simpson's index of diversity ($1-D$) was used to compare the genetic diversities of MRSA strains among adults and children. $1-D$ gives an unbiased measure of the probability of drawing two different *spa* types given the distribution of *spa* types in a sample [Grundmann et al. 2010]. The 95% confidence intervals (CIs) were calculated as described previously [Grundmann, Hori and Tanner 2001]. I conducted bivariate tests to evaluate the association of *spa* type t008 with individual variables, including age (adult/paediatric), specimen source (specifically wound and blood), time of specimen collection (community or hospital

onset), and ward type (non-ICU/ICU). I also tested hospital-level variables, including annual admissions (greater or less than 10,000), LTAC facility, percentage of Hispanic patients, and percentage of Medicaid-insured patients. For multivariate analyses, variables with a *p* value of <0.1 were entered into a generalized linear mixed model clustered by hospital and were retained at an α value of ≤ 0.05 (xtmelogit, STATA release 11, StataCorp. 2009).

5.3 RESULTS

A total of 1,124 adult and 159 paediatric MRSA isolates were collected over the 5- and 12-month periods, respectively. A summary of the characteristics of the clinical MRSA strains collected is shown in Table 5.1. The median age of adults was 67 years (interquartile range (IQR), 50 to 81 years) and that of children was 2 years (IQR, 1 to 9 years).

t008, t242 and t002 were the predominant *spa* types in OC, accounting for 83% of all isolates (Table 5.2). The distribution of these *spa* types among adults (t008, 41%; t242, 23%; t002, 19%) was significantly different from that among children (t008, 69%; t242, 9%; t002, 6%) ($\chi^2=52.29$, $p<0.001$). Annual population estimates of clinical inpatient MRSA cultures were 119/100,000 adults and 22/100,000 children. Annual estimates by *spa* type were 48/100,000 adults and 15/100,000 children for t008, 27/100,000 adults and 2/100,000 children for t242, and 22/100,000 adults and 1/100,000 children for t002.

Table 5.1 Characteristics of clinical MRSA strains isolated from adult and paediatric patients.

	No. (%) of isolates		
Characteristic	**Adult**[a]	**Paediatric**[b]	**Total/Overall**
Number of MRSA isolates	1124 (87.6)	159 (12.4)	1283 (100)
Specimen source[c]			
Wound/Abscess	488 (43.4)	81 (55.9)	569 (44.8)
Sputum	331 (29.4)	27 (18.6)	358 (28.2)
Urine	109 (9.7)	4 (2.8)	113 (8.9)
Blood	104 (9.3)	7 (4.8)	111 (8.8)
Other[d]	92 (8.2)	26 (17.9)	118 (9.3)
ICU collection[e]	187 (16.7)	17 (11.8)	204 (16.1)
Hospital onset	399 (35.5)	40 (25.2)	439 (34.2)

[a] Collected for 5 months from hospitals serving adults.

[b] Collected for 12 months from hospitals serving children.

[c] Fourteen missing paediatric entries.

[d] According to brief notes in the dataset, 'other' specimen sources included the following anatomical locations or types of specimens:
5 ear; 5 eye; 3 buttock; 2 each of finger, leg, pleural, and skin; and 1 each of gastrointestinal, sinus, perineum, spleen, and umbilical for paediatric specimen sources and 8 leg; 7 foot, knee, and medical device related; 6 groin; 5 abdominal, spinal, and stool; 4 gastric; 4 hand; 3 back, pleural, and tissue; 2 each of ankle, body fluid, buttock, ear, eye, stump, synovial fluid, and unknown; and 1 each of drainage, gallbladder, hip, humerus, ileal crest, lung, pancreatic fluid, skin, and stoma for adult specimen sources.

[e] Nineteen missing entries (4 adult and 15 paediatric).

According to MLST, the t008 isolates in this study were the prototypic community clone USA300 (t008/ST8), and the t002 isolates were the prototypic hospital clone USA100 (t002/ST5), with t242 isolates identified as ST5 (Table 5.2). Comparison of t242 and t002 isolates for the following parameters revealed no significant difference: the proportion from each specimen source, the proportion of hospital and community onset, the proportion collected on ICU and non-ICU wards, and the age distribution of patients (all $p>0.05$). Conversely, t008 isolates were significantly different from t242 and t002 isolates in the same tests ($p<0.001$). t242 and t002 isolates shared the most common specimen source, sputum (34% and 38%, respectively), whereas wounds were the most common specimen source of t008 isolates (56%). PFGE of a sample of t242 and t002 isolates showed them to be predominantly USA100 isolates (data not shown).

BURP analysis of the *spa* types clustered the majority of adult isolates (97%) into three *spa* clonal complexes (*spa*-CC) and most paediatric isolates (96%) into two *spa*-CCs (Figure 5.1). *spa* types were clustered with either t008 (*spa*-CC008; community-associated strains) or t002 (*spa*-CC002; healthcare-associated strains), but in adults, a further *spa*-CC with founder t324 was identified (*spa*-CC324). Isolates in this *spa*-CC were characterised as ST72. For both adult and paediatric MRSA isolates, MLST results showed that all isolates in *spa*-CC008 were ST8 and all isolates in *spa*-CC002 were either ST5 or a SLV, ST105 (*spa* types t045, t088 and t1791 for the latter). According to the BURP algorithm, *spa* types that differ from all other *spa* types in the sample by more than 4 repeats, and thus which cannot be clustered into a *spa*-CC, are termed singletons. For adults, 10 (11.2%) *spa* types (40 (3.6%) isolates) were classified as singletons, and for children 4 (18.2%) *spa* types (6 (3.8%) isolates) were classified as singletons. *spa* types of less than five repeats in length were

excluded from the BURP analysis because no reliable evolutionary history can be inferred from 'short' *spa* types [Mellmann et al. 2007]. For adults, two (2.2%) *spa* types (two (0.2%) isolates) were excluded and for children, one (4.5%) *spa* type (one (0.6%) isolate) was excluded. The estimated genetic diversity of MRSA isolates was significantly higher among adults than among children ($1-D$ = 75% versus 51%) (Table 5.2).

Table 5.2 Ten most frequently found *spa* types among adult and paediatric patients in OC, California[a].

Rank	Adult patients					Paediatric patients				
	***spa* type**	**MLST**	**No. of isolates**	**% of isolates**	**Cumulative %**	***spa* type**	**MLST**	**No. of isolates**	**% of isolates**	**Cumulative %**
1	t008	8	457	40.7	40.7	t008	8	110	69.2	69.2
2	t242	5	260	23.1	63.8	t242	5	14	8.8	78
3	t002	5	211	18.8	82.6	t002	5	9	5.7	83.7
4	t024	8	19	1.7	84.3	t024	8	3	1.9	85.5
5	t037	8	15	1.3	85.6	t045	5	2	1.3	86.8
6	t127	1	14	1.3	86.8	t068	8	2	1.3	88.1
7	t088	105	12	1.1	87.9	t2689	8	2	1.3	89.3
8	t1737	5	11	1	88.9	t324	72	2	1.3	90.6
9	t306	5	6	0.5	89.4	t622	8	2	1.3	91.8
10	t126	72	5	0.4	89.9	13 others		1 each	0.6 each	100

[a] The total numbers of *spa* types were 89 for adult patients and 22 for paediatric patients. Simpson's index of diversity (1−*D*) values were 75% (95% CI, 73%, 76%) for adult patients and 51% (95% CI, 41%, 60%) for paediatric patients. MLST = multilocus sequence type.

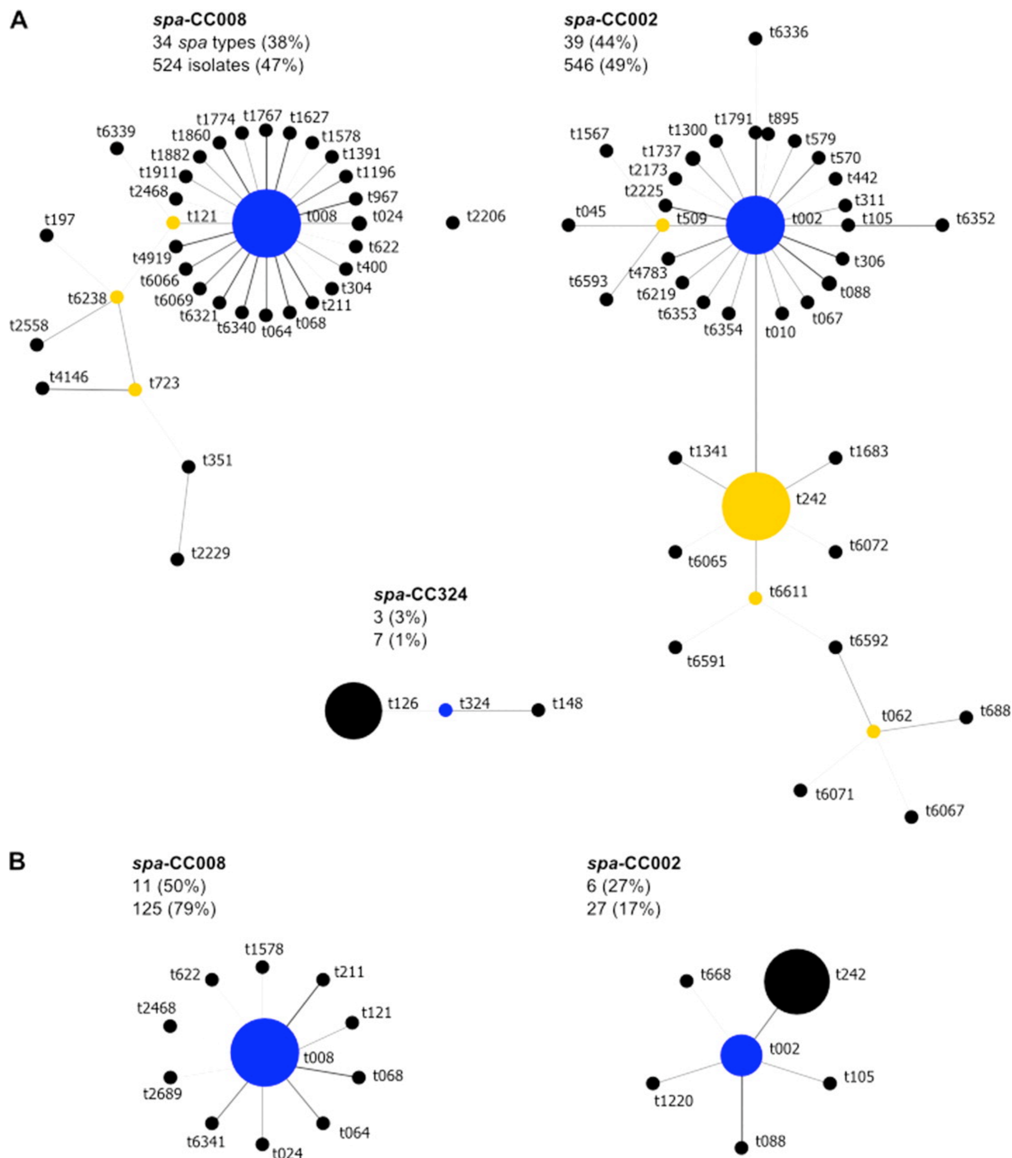


Figure 5.1 Relatedness of *spa* types among adult (A) and paediatric (B) MRSA isolates according to the Based Upon Repeat Pattern (BURP) algorithm. Clusters of linked *spa* types correspond to *spa* clonal complexes (*spa*-CCs). *spa* types are clustered into a *spa*-CC when their repeat patterns differ by no more than 4 repeats. The BURP algorithm sums up 'costs' (a measure of relatedness based on the repeat pattern) to define a founder score for each *spa* type in a *spa*-CC. The founder (blue node) is the *spa* type with the highest founder score in its *spa*-CC, and the subfounder (yellow node) is the *spa* type with the second highest founder score. *spa*-CC008 has founder t008. Each node represents a *spa* type. The node size represents the number of clustered strains that belong to that *spa* type. The shading of the branches represents the costs (similarities in repeat patterns) between two *spa* types; the darker the branch, the lower the cost (more similar repeat patterns).

In bivariate analyses, paediatric patients, wound specimens, isolation in a non-ICU ward, community onset timing of collection, and isolation from a hospital with >10,000 annual admissions were associated with t008 (USA300) isolates (Table 5.3). In addition, admission to a hospital with a high proportion of Medicaid-insured patients or a high proportion of Hispanic patients was linearly associated with the recovery of t008 isolates. In multivariate analyses, isolates from paediatric patients, wounds, non-ICU wards, and hospitals with a high proportion of Medicaid-insured patients remained significantly associated with *spa* type t008 (Table 5.4). Isolates from hospitals with a high proportion of Hispanic patients were significantly more likely to be t008 isolates (20% higher odds of being a *spa* type t008 isolate per 10% increase in numbers of Hispanic patients). However, this finding was collinear with hospitals with a high proportion of Medicaid-insured patients and thus was removed from the multivariate model.

Table 5.3 Bivariate analyses of variables associated with *spa* type t008.

Variable	% of t008 isolates		χ^2	*p*
	Those with characteristic	Those without characteristic		
Individual Variables				
Paediatric	69.81	40.75	47.67	<0.001
Community onset	48.10	37.13	14.09	<0.001
Non-ICU	47.17	27.45	27.00	<0.001
Blood specimen	40.54	44.30	0.58	0.446
Wound specimen	60.04	32.34	96.28	<0.001
Hospital-level Variables				
>10,000 annual admissions	38.40	51.52	22.00	<0.001
% Medicaid-insured patients[a]			1.34 (1.21-1.48)	<0.001
% Hispanic patients [a]			1.29 (1.15-1.44)	<0.001
LTAC facility	35.21	44.88	2.54	0.111

[a] Odds ratio per 10% increase.

Table 5.4 Multivariate analysis of variables associated with *spa* type t008.

Variable	Odds ratio	SE	95% CI	*p*
Patient/isolate characteristic				
Wound specimen	2.64	0.34	2.06, 3.39	<0.001
Paediatric	2.07	0.52	1.26, 3.40	0.004
Non-ICU	1.77	0.32	1.24, 2.54	0.002
Hospital characteristic				
% Medicaid-insured patients[a]	1.24	0.06	1.13, 1.35	<0.001

[a] Odds ratio per 10% increase.

5.4 DISCUSSION

A prospective cohort study of inpatients in a large metropolitan county in California was conducted, collecting all clinical MRSA isolates from 30 hospitals in order to characterise differences in paediatric versus adult MRSA strains. To my knowledge, this is the first study to assess adult and paediatric MRSA isolates from a population-based sample across a large region.

Countywide, adult and paediatric clinical MRSA isolates were dominated by three *spa* types, two of which were consistent with the prototypic community- and healthcare-associated clones prevalent in the US (t008 (USA300) and t002 (USA100)). t008 (USA300) was the most common single clone among both adult and paediatric isolates. Nevertheless, t008 comprised a large majority of paediatric isolates, whereas adult isolates were nearly equally divided among community- and healthcare-associated clones. Most other *spa* types were shown by BURP to be related to these two dominant clones. The two *spa* clonal complexes *spa*-CC008 and *spa*-CC002 can therefore be thought of as two distinct groups of isolates representing the major community- and healthcare-associated MRSA strains prevalent in the US.

Interestingly, t242/ST5 was slightly more common than t002/ST5 among both adult and paediatric isolates, despite the predominance of the t002/ST5 hospital clone in the US. Given the similarities of t242 and t002 isolates in this study, and the fact that t242 differs from t002 by only one nucleotide (resulting in a different *spa* repeat pattern by one *spa* repeat), t242/ST5 presumably represents a minor variant of USA100 that has become prevalent in OC hospitals. t242 has been reported infrequently in the literature [Kinnevey et al. 2010, Johnson et al. 2007, Weese, Avery and Reid-Smith 2010], with just one study reporting t242 at an endemic level in an Italian hospital [Parlato et al. 2009].

The additional *spa*-CC identified among adult isolates included a community-onset isolate identified as t324/ST72, an invasive community-associated MRSA clone reported in elderly patients in South Korea from 2006 to 2007, just before our isolate collection began [Lee et al. 2010]. According to the US Census Bureau, 17.9% of the OC population is Asian, approximately 2.9% of which is Korean [US Census Bureau 2011b].

There was significantly more genetic diversity among adult MRSA isolates than among paediatric isolates. This could simply represent the greater time that healthcare-associated clones have had to diversify at the *spa* locus than community-associated clones, which have emerged only in the past two decades. The greater MRSA diversity among adults could also be due to different degrees of contact; for example, adults may have more diverse MRSA encounters (travel, work, social venues, and healthcare facilities) than young children (schools and day care centres).

The population estimates of clinical MRSA isolates in OC show that there was a 6-fold-higher frequency of inpatient MRSA clinical cultures among adults than among children. This pattern was consistent among the three most common *spa* types t008, t242 and t002, and is likely a combination of more frequent hospitalisations among adults (many of whom were elderly, with a median age of 67 years) and more frequent MRSA carriage.

In multivariate analyses, the community-associated MRSA clone t008 (USA300) was associated with paediatric patients. In contrast to adults, children are often healthier and are more likely to encounter MRSA in the community through exposure to high-density environments, such as schools, day care centres, camps and sporting activities, where close contact may facilitate the spread of community MRSA strains. In agreement with data from previous studies, I found that USA300 was

associated with wounds, which is the most common presentation for hospitalization due to community-acquired MRSA infection [Fridkin et al. 2005, Bassetti, Nicco and Mikulska 2009]. USA300 was also associated with hospitals that treat a large fraction of Medicaid-insured patients, suggesting that community MRSA infections may be more prevalent among patients from economically disadvantaged or high-density areas.

USA300 was also associated with isolation from non-ICU wards, suggesting that this community strain is occurring in healthier hosts or is producing infections that are less severe than those caused by traditional healthcare-associated strains. Nevertheless, there is ample evidence that community strains are capable of producing fulminant infections [Frazee et al. 2005, Miller et al. 2005, Seybold et al. 2006]. An understanding of what component of invasiveness is due to host comorbidities versus pathogen virulence factors is an area of active research.

Interestingly, I did not find that the isolation of t008 was associated with community onset clinical isolates (clinical culture isolated less than 3 days after admission). This finding is likely due to the fact that the majority of healthcare-associated carriage or infection is found on readmission to hospitals [Klevens et al. 2007]. It could also be explained by community-associated strains that have become endemic in some hospitals [Seybold et al. 2006, Popovich, Weinstein and Hota 2008].

Community- and healthcare-associated MRSA strains are becoming increasingly difficult to distinguish epidemiologically as community-associated strains continue to penetrate hospital MRSA reservoirs. Furthermore, it remains unclear whether community clones are adding to or replacing traditional healthcare MRSA strains [Bootsma et al. 2006a, Hota et al. 2007, Popovich, Weinstein and Hota 2008, D'Agata et al. 2009]. The implication of the blurred line between community-

and healthcare-associated MRSA strains may be that efforts to control MRSA transmission within hospitals will not be effective in controlling community influx into hospitals. Simultaneous community strategies to limit MRSA spread are needed. However, much is still unknown about the acquisition and transmission of CA-MRSA, so improved knowledge is needed to better guide infection control strategies. Further studies are needed to ascertain whether community strategies to reduce transmission in children and young adults would produce benefits across the entire age spectrum.

One limitation of this study is that few individual-level characteristics were available. Also, this study did not account for the different policies in place at each hospital with regard to when to obtain clinical cultures. These differences could affect MRSA detection at each hospital and, possibly, the type of MRSA strains isolated, if clinical cultures were more likely to be obtained for sicker, older patients. Moreover, the results could have been affected by the potential seasonality of MRSA infections and infection types due to the different collection periods for adult and paediatric isolates (largely winter and spring for adult collections, compared to all seasons for paediatric collections). Seasonality of *S. aureus* infections, particularly skin infections, has been observed in paediatric and adult patients in temperate and tropical environments, with a predominance of infections during summer and autumn [Loffeld et al. 2005, Szczesiul et al. 2007, Van De Griend et al. 2009, Mermel, Machan and Parenteau 2011]. A recent study in Rhode Island found a two- to three-fold-increased incidence of MRSA infections (both CA- and HA-MRSA) in paediatric patients during the second two quarters of the year, over the last decade [Mermel, Machan and Parenteau 2011]. However, in the same study, adult CA-MRSA infections showed less seasonal variation than did paediatric infections, and no variation was observed

among adult HA-MRSA infections. Some studies observed no significant seasonality of *S. aureus* infections, but those studies focused on bacteremia [Morin and Hadler 2001, Perencevich et al. 2008]. The collection of both adult and paediatric MRSA isolates for the same time period i.e. twelve months, would have accounted for any potential seasonality effects and/or other factors that could affect the type and diversity of MRSA strains isolated.

Mandatory screening of high-risk inpatients was not in place in California until 2009; therefore, my population estimates are likely underestimates. In addition, my estimates should not be construed as measures of MRSA infection among inpatients. Clinical isolates often represent carriage without infection. Finally, my estimates of the index of diversity for adult and paediatric MRSA isolates may have been influenced by differing sample sizes [Grundmann, Hori and Tanner 2001].

In conclusion, this study found that in a large county, MRSA isolates from hospitalised children were more likely to be *spa* type t008 (USA300). This community-associated *spa* type was associated with children, wounds, non-ICU care, and admission to a hospital with a high percentage of Medicaid-insured patients. Despite the association of t008 isolates with children, t008 was still the most common *spa* type among adult patients, suggesting that community-based interventions are needed to stem the influx of t008 isolates into hospitals. The study also found evidence for a prevalent variant of the USA100 clone (t242/ST5), which has not been reported elsewhere. While community- and hospital-associated MRSA reservoirs have begun to merge, significant differences remain in paediatric versus adult patient populations, which may provide an impetus for different age-based strategies to reduce transmission and disease.

CHAPTER 6: DIVERSITY OF MRSA STRAINS ISOLATED FROM INPATIENTS OF 30 HOSPITALS

6.1 INTRODUCTION

HA-MRSA has long been the primary cause of MRSA infections, but CA-MRSA, which often causes infections among healthy children and young adults with no exposure to the healthcare setting, has become increasingly prevalent across the globe, particularly in the US [Udo, Pearman and Grubb 1993, Herold et al. 1998, CDC 1999, Eady and Cove 2003, Mongkolrattanothai et al. 2003, Francis et al. 2005, Gonzalez et al. 2005a, Miller et al. 2005, Moran et al. 2005, Moellering 2006, Tristan et al. 2007a, Wallin, Hern and Frazee 2008, Otter and French 2010, Otter and French 2011]. While well documented in the community, there is increasing evidence that CA-MRSA is penetrating healthcare MRSA reservoirs [O'Brien et al. 1999, Saiman et al. 2003, Bratu et al. 2005, Kourbatova et al. 2005, David et al. 2006a, Gonzalez et al. 2006, Otter and French 2006, Saunders et al. 2007, Boyce 2008, Otter and French 2008, Sonnevend et al. 2012]. CA-MRSA has caused outbreaks in the hospital setting since 2003, often in paediatrics and obstetrics where HA-MRSA prevalence is low and community influx of patients without prior healthcare exposure is common [Otter and French 2011]. Furthermore, some reports suggest CA-MRSA may be replacing HA-MRSA [Seybold et al. 2006, Maree et al. 2007, Patel et al. 2008, Popovich, Weinstein and Hota 2008, D'Agata et al. 2009].

Most prior studies of CA-MRSA penetration into hospital reservoirs involve a single centre. Regional evaluation of healthcare facilities may provide further information about the extent of reservoir mixing of CA-MRSA and HA-MRSA strains across community and academic healthcare facilities, as well as paediatric hospitals and LTAC facilities. A prospective, population-based study of clinical

MRSA isolates across nine medical centers in San Francisco, California, found that USA300 was the predominant clone in both the community and hospital setting [Liu et al. 2008]. While this city-based study collected almost 4000 MRSA isolates, only a fifth of these were selected for molecular analysis, with the primary goal to determine clonal groupings based upon the isolate collection date (hospital onset or community onset). Further comprehensive evaluations of the diversity of isolates within and across clonal complexes will provide valuable information about how exact strain types are evolving and being shared across facilities. It is also unclear how much hospital onset disease is caused by CA-MRSA. Given the increasing dominance of USA300 and the growing evidence that community and healthcare MRSA reservoirs are mixing [Kourbatova et al. 2005, Seybold et al. 2006, Maree et al. 2007, Liu et al. 2008, Popovich, Weinstein and Hota 2008], a better understanding of the frequency and diversity of community- and healthcare-associated MRSA clones in hospitals may inform strategies to prevent MRSA transmission and disease in the US. We conducted a prospective cohort study of inpatients in a large metropolitan county to investigate the frequency and genetic diversity of MRSA at a population level.

6.2 METHODS

6.2.1 Study

As described in section 5.2.1.

6.2.2 Isolate collection

Clinical (non-screening) isolates of MRSA from unique patients were collected from hospital microbiology laboratories by my collaborators, between October 2008 and April 2010. Hospitals were instructed to collect non-blood MRSA

isolates from unique patients up to a total of 100 or for a duration of 12 months, whichever came first. In addition, hospitals were instructed to collect all blood isolates from unique patients until collection ended. Isolates from patients not admitted to hospital were excluded from the study. Samples were then processed and stored by the Orange County Public Health Laboratory as described in section 5.2.2.

6.2.3 Specimen data and hospital characteristics

Specimen data, hospital characteristics and the OC population estimate were obtained as described previously (section 5.2.3) [Hudson et al. 2012].

6.2.4 Laboratory methods and molecular typing

All strains were shipped to me for *spa* typing, and processed as before (section 5.2.4). To assess *spa* type diversity and relatedness, cluster analysis of *spa* types was performed using the Based Upon Repeat Pattern (BURP) algorithm, a built-in feature of the StaphType software [Mellmann et al. 2007]. MLST was also performed on a subset of the isolates (n=284) to confirm MRSA strain types, according to methods described previously [Enright et al. 2000]. This subset was selected as described in section 5.2.4.

6.2.5 Statistical analyses

I calculated the number of hospitalized patients with MRSA clinical cultures among both the total population of OC and total annual admissions across all 30 hospitals, accounting for duration of isolate collection within each hospital. The number of community onset MRSA clinical cultures among the total OC population was also calculated. χ^2 tests were performed to compare isolate characteristics

(community onset versus hospital onset, and number of isolates belonging to different *spa*-CCs) between hospitals. One-sample z-tests for equality of proportions were conducted, to compare isolate characteristics (community onset versus hospital onset, and number of isolates belonging to different *spa*-CCs) within each hospital.

I also used Simpson's index of diversity ($1-D$) to estimate inter- and intra-hospital genetic diversity of the MRSA strains collected, as well as the genetic diversity of the two major *spa*-CCs, and genetic diversity among hospital and community onset isolates. $1-D$ gives an unbiased measure of the probability of drawing two different *spa* types given the distribution of *spa* types in a sample [Grundmann et al. 2010]. 95% confidence intervals (CIs) were calculated as described previously [Grundmann, Hori and Tanner 2001]. For comparison of diversity indices, a significant difference ($p<0.05$) was determined by non-overlapping 95% CIs.

I computed Pearson's correlation coefficients to determine the relationship between hospital-level and isolate variables, and genetic diversity. Due to the small sample size (28 hospitals; two were excluded as they collected <10 MRSA isolates and thus their diversity estimates were unreliable) and the number of potential predictor variables for genetic diversity, variables were considered for entry into a bootstrapped multiple linear regression model based on a combination of their correlation coefficient and current knowledge regarding their association with MRSA. Only variables with $p<0.1$ in correlation tests were considered for the exploratory model. I also tested the correlation between community onset and *spa*-CC008 isolates. All statistical tests were performed using STATA (release 11, StataCorp 2009).

6.3 RESULTS

6.3.1 Overview

Between October 2008 and April 2010, 2,246 clinical MRSA isolates were collected from 30 OC hospitals. Annual population incidence of clinical inpatient MRSA isolates in OC was estimated at 86/100,000 people (88/10,000 admissions). Annual population incidence of clinical inpatient MRSA isolates in OC that were community onset was estimated at 60/100,000 people (62/10,000 admissions). Most clinical MRSA isolates were isolated from wounds or abscesses (47%), in non-intensive care units (non-ICUs; 84%), and were community onset (72%). Median patient age was 64 (IQR, 44-79; 13 missing values). Table 6.1 gives a summary overview of the participating hospitals and isolate characteristics.

Table 6.1 Summary of the 30 participating hospitals and the clinical MRSA isolates from hospital inpatients in OC, CA.

Characteristic	Value
Hospital characteristics (Median (IQR[a]))	
Annual admissions	7868 (2819-16157)
% Hispanic patients	19.2 (11.4-32.9)
% Medicaid-insured patients	15.1 (5.8-34.6)
N MRSA isolates per hospital per month	4.7 (2.5-11)
N *spa* types per hospital	14 (7-17)
N LTAC-facilities[b] (No. of isolates (%))	6 (132 (5.9))
Overall isolate characteristics (No. of isolates (%))	
MRSA isolates	2246 (100)
Specimen source[c]	
Sputum	596 (26.7)
Wound/Abscess	1047 (47)
Blood	213 (9.5)
Urine	189 (8.5)
Other	184 (8.3)
Intensive care unit collection	374 (16.7)
Hospital onset	627 (27.9)

[a] IQR = interquartile range.

[b] LTAC = long-term acute care.

[c] 17 missing values.

6.3.2 spa typing and MLST

Among the 2,246 MRSA isolates collected, 134 *spa* types were identified, including one non-typeable (NT) isolate and 28 *spa* types (1.6% of all isolates) that did not match any known *spa* sequence. These novel *spa* sequences were automatically submitted to the Ridom SpaServer via the Ridom StaphType software and were assigned new *spa* types. The isolate with the NT *spa* type was re-tested to confirm the result was not due to a processing error, and the sequence quality was deemed excellent by the StaphType software. The NT *spa* type bore closest resemblance to t008, with a missing nucleotide in the ninth repeat, making the repeat 23-bp long. This is surprising since it would put the *spa* coding region out of frame although others have reported *spa* repeats with an unexpected length [Rothganger 2010]. The NT *spa* type was submitted to Ridom for their records. The three most common *spa* types were t008, t242 and t002, representing 83% of all isolates collected (Table 6.2).

BURP analysis of the *spa* types clustered 96% of isolates into two large *spa*-CCs and 1.2% of isolates into six smaller *spa*-CCs (Figure 6.1). 78% of *spa* types were clustered into either *spa*-CC242 (founder t242) or *spa*-CC008 (founder t008), including 18 and 8 novel *spa* types, respectively. Under the BURP algorithm, *spa* types that differ from all other *spa* types in the sample by more than 4 repeats cannot reasonably be clustered into a *spa*-CC, and are termed singletons. Nine *spa* types (56 isolates) were classed as singletons, including two novel *spa* types. Six isolates represented six *spa* types that were less than five repeats in length and were excluded from BURP analysis because no reliable evolutionary history can be inferred from 'short' *spa* types [Mellmann et al. 2007]. The NT isolate could not be included in the

BURP algorithm. Estimated genetic diversity of MRSA in OC hospitals using *spa* typing was high, at 72% (Table 6.2).

Table 6.2 Ten most frequently found *spa* types among isolates from OC hospital inpatients[a].

Rank	*spa* type	MLST	Freq	%	Cumulative %
1	t008	8	1034	46	46
2	t242	5	478	21.3	67.3
3	t002	5	347	15.4	82.8
4	t024	8	33	1.5	84.2
5	t037	8	25	1.1	85.4
6	t045	5	22	1.0	86.3
7	t088	105	21	0.9	87.3
8	t127	474[b]	18	0.8	88.1
9	t306	5	14	0.6	88.7
10	t1737	5	12	0.5	89.2
-	Other	-	242	10.8	100.0

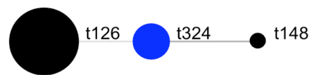
[a] The total number of *spa* types was 134, including one non-typeable isolate. Simpson's index of diversity ($1-D$) value was 72% (95% CI, 70%, 73%). MLST = multilocus sequence type.

[b] t127 isolates were also ST1 and ST1900, both SLVs of ST474.

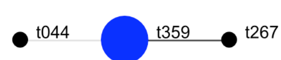
***spa*-CC242**
54 *spa* types (40.3%)
979 isolates (43.6%)

***spa*-CC008**
50 (37.3%)
1,175 (52.3%)

***spa*-CC324**
3 (2.2%)
10 (0.5%)

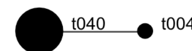


***spa*-CC359**
3 (2.2%)
4 (0.2%)

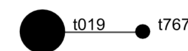


***spa*-CCNF1**
2 (1.5%)
5 (0.2%)

***spa*-CCNF2**
2 (1.5%)
3 (0.1%)



***spa*-CCNF3**
2 (1.5%)
3 (0.1%)



***spa*-CCNF4**
2 (1.5%)
2 (0.1%)

Figure 6.1 Relatedness of *spa* types among hospital MRSA isolates according to the Based Upon Repeat Pattern (BURP) algorithm. Clusters of linked *spa* types correspond to *spa* clonal complexes (*spa*-CCs). *spa* types are clustered into a *spa*-CC when their repeat patterns differ by no more than 4 repeats. The BURP algorithm sums up 'costs' (a measure of relatedness based on the repeat pattern) to define a founder score for each *spa* type in a *spa*-CC. The founder (blue node) is the *spa* type with the highest founder score in its *spa*-CC, and the subfounder (yellow node) is the *spa* type with the second highest founder score. *spa*-CC008 has founder t008, and *spa*-CCNF refers to a *spa*-CC with no founder. Each node represents a *spa* type. The node size represents the number of clustered strains that belong to that *spa* type. The shading of the branches represents the 'costs' (similarities in repeat patterns) between two *spa* types; the darker the branch, the lower the cost (more similar repeat patterns).

To confirm strain types, 284 isolates were selected for MLST. Among the 23 unique sequence types (STs) identified, ST5 (45%), ST8 (38%) and ST105 (4%) were the three most common, with the vast majority of isolates (90%) belonging to one of two major MLST CCs: CC5 (50%; four STs) and CC8 (40%; three STs) (Table 6.3). The remaining 10% of isolates comprised sporadic incidences of both HA- and CA-MRSA clones, but mostly the latter (7.4%). According to MLST, t008 isolates were the prototypic community clone USA300 (t008/ST8) and t002 isolates were the prototypic hospital clone USA100 (t002/ST5), with t242 isolates identified as ST5 (Tables 6.2 and 6.3). *spa* type t242 differs from t002 by one *spa* repeat, as a result of a single nucleotide difference. The non-typeable *spa* isolate was ST8, with 64% of the novel *spa* types being ST5 and 36% ST8.

Table 6.3 Relatedness of MLST sequence types (STs) among 284 hospital MRSA isolates according to the eBURST algorithm.

CC (no. of isolates)[a]	MLST	Associated *spa* types[b]
CC5 (142)	5	t242, t002, t045
	105	t088, t045
	225	t045
	840	t088
CC8 (114)	8	t008, t024, t037
	239	t037
	576	t1635
CC474 (9)	474	t127
	1900	t127
	1	t127
CC NF1 (4)	45	t004, t026, t040
	1811	t1081
CC NF2 (3)	59	t3424, t976
	87	t216
CC NF3 (2)	36	t018
	30	t019
Singletons (10)[c]	72	t126, t148, t324
	22	t005
	12	t160
	88	t5916
	97	t359
	188	t189
	635	t044

[a] CC = clonal complex. All members of a CC share identical alleles at six of the seven loci with at least one other member of the CC.

[b] Only the three most common *spa* types are listed if more than three associated with that ST.

[c] STs with allelic profiles that share less than six of their seven loci with all other STs in the dataset.

6.3.3 Inter-hospital differences

The estimated genetic diversity of MRSA between hospitals ranged from 33% to 79% (Figure 6.2). Percentage of blood specimens isolated per hospital and the median age of patients that specimens were collected from were positively correlated with genetic diversity within hospitals (r = 0.57, $p<0.01$ and r = 0.78, $p<0.001$ respectively; Table 6.4). Significant negative correlations were found between genetic diversity of hospital MRSA isolates and the percentage of Medicaid-insured patients (r = -0.57, $p<0.01$), Hispanic patients (r = -0.38, $p = 0.04$) and wound/abscess specimens (r = -0.65, $p<0.001$) per hospital (Table 6.4). Percentage of Hispanic patients and percentage of Medicaid-insured patients were highly correlated (r = 0.85, $p<0.001$), and since both are markers for patients from economically disadvantaged/high-density areas, the former was not considered for entry into the bootstrapped linear regression model. Only percentage of blood specimens and median patient age remained significantly correlated to genetic diversity in the exploratory regression model (Table 6.5).

The three most common *spa* types, t008, t242 and t002, accounted for 65-95% of isolates at each hospital, showing that these *spa* types are consistently dominant across OC hospitals. Since MLST has shown that t008 isolates are the community-associated clone USA300 and t002 isolates the healthcare-associated clone USA100, I can infer that the two major *spa*-CCs containing these strains each represent community-associated (*spa*-CC008) and healthcare-associated *spa* types (*spa*-CC242, which also included t002). The proportion of *spa*-CC008 isolates compared to *spa*-CC242 isolates varied significantly between hospitals ($\chi^2 = 250.57$, df = 29, $p<0.001$) (Figure 6.2). Five hospitals (16.7%) had significantly more *spa*-CC242 isolates, whereas fifteen hospitals (50%) had significantly more *spa*-CC008 isolates ($p<0.01$).

Diversity of *spa* types among *spa*-CC008 (1−*D* = 22% (95% CI, 19-26%)) was significantly lower than diversity among *spa*-CC242 (1−*D* = 63% (95% CI, 61-66%)).

6.3.4 Community onset versus hospital onset MRSA

MRSA *spa* type genetic diversity was significantly higher among hospital onset isolates (1−*D* = 75% (95% CI, 73-77%)) than among community onset isolates (1−*D* = 70% (95% CI, 68-72%)). The proportion of community onset and hospital onset isolates was also significantly different between hospitals ($\chi^2 = 127.4$, df = 29, $p<0.001$); all but two hospitals isolated significantly more community onset MRSA ($p<0.001$), with seven hospitals only isolating community onset MRSA. The remaining two hospitals showed no significant difference in the proportions of community onset and hospital onset MRSA ($p>0.01$). No significant correlation was found between the proportions of community onset isolates per hospital and the proportions of *spa*-CC008 isolates per hospital (r = 0.08, $p = 0.69$). Among hospital onset isolates, 42.3% belonged to *spa*-CC008 (18.2-100% per hospital, ignoring community onset only hospitals), while 39.4% of community onset isolates were *spa*-CC242 (6.9%-82.9% per hospital).

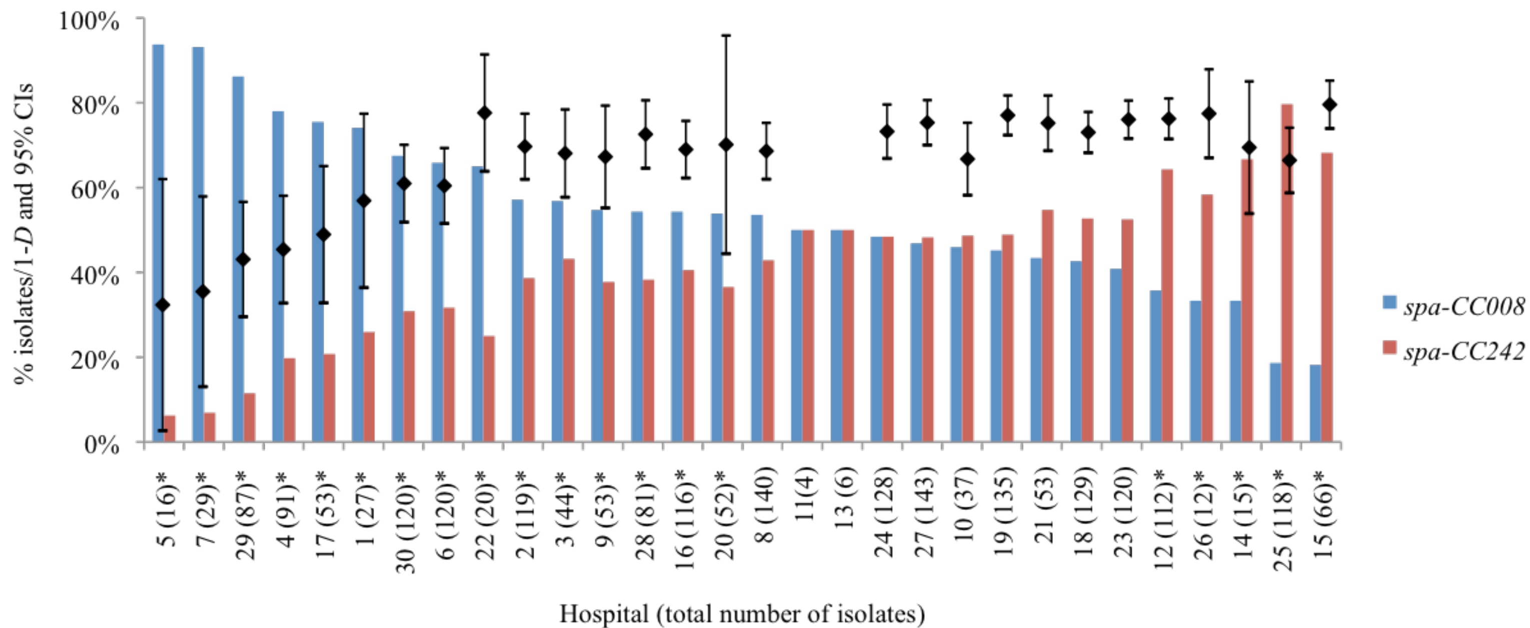


Figure 6.2 Proportion of isolates belonging to *spa*-CC242 versus *spa*-CC008, by hospital. *indicates a significant difference at the 99% level in the proportion of isolates belonging to *spa*-CC242 and *spa*-CC008 at that hospital. The black bars show the point estimates and 95% confidence intervals of hospital-specific genetic diversity expressed as Simpson's index of diversity ($1-D$) of *spa* types (as a percentage). Diversity indices for hospitals 11 and 13 were excluded from the figure as these hospitals had *spa* type data on less than ten isolates. Diversity indices with non-overlapping 95% CIs were considered significantly different ($p<0.05$).

Table 6.4 Correlation of hospital and isolate variables with hospital genetic diversity.

Variable	r	*p*[a]
Hospital variable		
Annual admissions	0.28	0.15
% Medicaid-insured patients	-0.57	<0.01
% Hispanic patients	-0.38	0.04
LTAC-facility[b]	0.002	0.99
Isolate/patient variable		
% Non-ICU[c] isolate collection	-0.27	0.16
% Community onset	-0.21	0.29
% Wound/abscess specimens	-0.65	<0.001
% Blood specimens	0.57	<0.01
Median age	0.78	<0.001

[a] Variables with $p<0.1$ were considered for exploratory multivariate analyses.

[b] LTAC = long-term acute care.

[c] Non-Intensive care unit (Non-ICU).

Table 6.5 Multivariate analysis of variables associated with hospital genetic diversity.

Variable	Coefficient	Bootstrap SE	Normal-based 95% CI	*p*
% Blood specimens	0.82	0.24	0.35, 1.29	<0.01
Median age	0.44	0.08	0.28, 0.60	<0.001

6.4 DISCUSSION

A prospective cohort study of inpatients in a large metropolitan county was conducted, collecting all clinical MRSA isolates from 30 hospitals in order to investigate the frequency and genetic diversity of MRSA at a population level. To my knowledge, this is the first study to assess MRSA isolates from a population-based sample across a large region. While Liu et al. [2008] conducted a large population-based study of clinical MRSA isolates in both hospital inpatients and outpatients, they sampled from a single city and characterized only 20% of all MRSA isolates collected. Our countywide study was more comprehensive, encompassing 30 hospitals and characterizing all inpatient clinical MRSA isolates (over 2000).

Countywide, three *spa* types dominated clinical MRSA isolates. USA300 (t008/ST8), the prototypic community-associated clone prevalent in the US, was the most common clone, making up just under half of all clinical MRSA isolates. USA100 (t002/ST5), the prototypic healthcare-associated clone, was also common, but interestingly, t242/ST5 isolates were slightly more common than t002/ST5 isolates. Given the clinical similarities of t242 and t002 isolates found in our previous study (Chapter 5) [Hudson et al. 2012], and that pulsed field gel electrophoresis showed a sample of t242 and t002 isolates to be predominantly USA100, t242/ST5 likely represents a minor variant of USA100 that has become prevalent in OC hospitals (Chapter 5) [Hudson et al. 2012]. t242 has been reported sporadically elsewhere, but was endemic in one hospital in Italy [Kinnevey et al. 2010, Parlato et al. 2009, Johnson et al. 2007, Weese, Avery and Reid-Smith 2010].

Most *spa* types were closely related to either the USA300 or USA100 clone, creating two *spa*-CCs each representing CA- and HA-MRSA strains. The remaining unrelated *spa* types were clustered into six small *spa*-CCs representing several

community- and healthcare-associated clones, but occurred only sporadically. Four of the six smaller *spa*-CCs were community-associated. The largest represented ST72, an invasive community-associated clone that was reported in elderly patients in South Korea just before our isolate collection began [Lee et al. 2010]. According to the US Census Bureau, 17.9% of the OC population is Asian of which approximately 2.9% are Korean [US Census Bureau 2011b]. ST72 strains belong to the USA700 clone and have also been reported in Australia and Europe [Monecke et al. 2011]. The other three community-associated *spa*-CCs represented clones including USA1000 (ST59), USA1100 (ST30/ Southwest Pacific clone) and a rare CA-MRSA clone (ST97) only reported once before in the US [Chung et al. 2004] and recently as a clone transmitted among neonates [Udo et al. 2011], although this clone was isolated from two adults in this study.

The remaining two small *spa*-CCs represented the hospital-associated clones USA600 (ST45/Berlin clone) and the pandemic HA-MRSA clone EMRSA-15 (ST22), however the latter has recently been reported in the community setting [Mollaghan et al. 2010]. Among the few isolates not belonging to a *spa*-CC was the HA-MRSA clone USA200 (ST36/EMRSA-16), isolates representing a pandemic HA-MRSA clone (ST239) and isolates representing strains of MLST CC1, a CA-MRSA lineage that includes USA400. Most isolates of this latter group were *spa* type t127 and ST474, a SLV of ST1. ST1/t127 is one of the most common CA-MRSA strains in the UK [Otter et al. 2009], but to my knowledge has not been reported in the US previously. While there is MRSA diversity in the OC population, USA300 and USA100 continue to dominate, with most diversity caused by their close *spa*-type relatives.

Overall genetic diversity of MRSA in OC was relatively high, but heterogeneous between hospitals. This variation in diversity was mostly non-significant, with all hospitals dominated by the three most common *spa* types t008, t242 and t002. Only one regional study of *spa* type diversity has been performed previously, in Europe. This study found that *spa* type genetic diversity of MRSA causing invasive infections is much higher in Europe (94%), ranging from 62% to 91% between countries, indicating the presence of less dominant MRSA *spa* types than in OC [Grundmann et al. 2010]. Invasive infections are traditionally caused by HA-MRSA, which could explain the higher diversity seen in the European study. However, HA-MRSA in OC still exhibited low diversity (63%) in comparison.

Diversity was significantly lower among *spa*-CC008 isolates than *spa*-CC242 isolates, indicating overall MRSA diversity in OC is driven by HA-MRSA, perhaps simply due to the greater time healthcare-associated strains have had to diversify compared to community-associated strains. Genetic diversity was significantly associated with older patient age and isolation of MRSA from blood specimens. HA-MRSA are typically associated with older patients, whereas CA-MRSA are associated with children and young adults (Chapter 5) [Hudson et al. 2012]. Blood infection is more commonly associated with hospital onset MRSA, which are more likely to be healthcare-associated strains.

Genetic diversity was also significantly lower among community onset MRSA than hospital onset MRSA, but this difference was marginal. Furthermore, the lack of correlation between *spa*-CC008 isolates (CA-MRSA) and community onset suggests that isolation of a community-associated strain does not imply community onset. Indeed, among community onset isolates (obtained within the first two days of hospitalization), large numbers of HA-MRSA strains were found, and, conversely,

among hospital onset isolates, there were large numbers of CA-MRSA strains. In fact, nearly half of hospital onset isolates were CA-MRSA strains. This suggests full mixing of CA-MRSA and HA-MRSA reservoirs among the majority of hospitals in OC.

The vast majority of MRSA isolates were obtained within the first two days of hospitalization, suggesting that MRSA hospital reservoirs are mainly maintained by importation. Over a third of all community onset isolates were *spa*-CC242, which could partly be explained by the fact that healthcare-associated carriage or infection is often found on readmission to hospitals [Tacconelli et al. 2004, Klevens et al. 2007]. A history of healthcare exposure however does not exclude the possibility of MRSA acquisition and onset in the community [Klevens et al. 2007].

The high penetration of CA-MRSA among hospital-onset isolates highlights the needs for community-based strategies to be implemented in an effort to address the MRSA epidemic in the community and minimize the ability of community-associated MRSA strains to become endemic in hospitals. The consequences of CA-MRSA continuing to infiltrate the healthcare setting include 1) the emergence of multidrug resistant CA-MRSA due to nosocomial antibiotic pressure [Kardas-Sloma et al. 2011], 2) the potential of increased virulence of healthcare-associated infections due to PVL-positive CA-MRSA strains, although studies suggest CA-MRSA are clinically similar to HA-MRSA once in the healthcare setting [Moore et al. 2009], and 3) the risk of hospital outbreaks due to the influx of CA-MRSA from the ever-expanding community reservoir [D'Agata et al. 2009, Skov and Jensen 2009, Kardas-Sloma et al. 2011].

A limitation of this study was that few individual level characteristics were available. Also, variation among hospitals in obtaining clinical cultures could not be

accounted for. Screening cultures were excluded since mandatory screening of high-risk inpatients was not in place in California until 2009 and capture would have been inconsistent across facilities. Therefore, my population estimate of MRSA isolates from hospital inpatients is likely an underestimate. In addition, my estimate should not be construed as a measure of MRSA infection among inpatients. Clinical isolates often represent carriage without infection. Finally, my estimates of the indices of diversity for hospital onset and community onset MRSA isolates may have been influenced by differing sample sizes [Grundmann, Hori and Tanner 2001].

In conclusion this study found that in a large county, CA-MRSA strains accounted for 56% of community onset isolates and 42% of hospital onset isolates. No correlation was found between community onset isolates and CA-MRSA, providing strong regional evidence that community and healthcare MRSA reservoirs have fully mixed. Genetic diversity of MRSA was still driven by HA-MRSA, with a highly prevalent, previously unreported USA100 variant found across all OC hospitals. Community-based MRSA strategies are needed to stem the influx of community-associated strains, particularly USA300, into the healthcare setting.

CHAPTER 7: DIVERSITY OF MRSA STRAINS ISOLATED FROM RESIDENTS OF 25 NURSING HOMES

7.1 INTRODUCTION

Residence in a nursing home, which typically provides long-term care for chronically ill and/or elderly people, is a well-established risk factor for MRSA carriage and infection [Hsu et al. 1988, Bradley 1997, O'Sullivan and Keane 2000, Eveillard et al. 2008], and MRSA carriage in nursing home residents is associated with increased mortality [Suetens et al. 2006]. Nursing homes represent a unique and important MRSA reservoir. People colonized with MRSA tend to introduce the organism into nursing homes via the hospital setting, and MRSA can also be transported back into hospitals and the community from the nursing home. The reservoir represented by colonized patients is often large due to the high MRSA prevalence in nursing homes, sometimes higher than 30%, which increases the risk of MRSA transmission in these facilities [Eveillard et al. 2008, Eveillard and Joly-Guillou 2009, Li, Arnsberger and Miller 2010]. Furthermore, once colonized, nursing home residents seem to carry the same MRSA strain for prolonged periods of time; asymptomatic colonization has been reported to last anything from 3 months to 3 years [Bradley et al. 1991, Sanford et al. 1994]. Studies suggest that multiple strains, not a single strain, circulate within nursing homes [Bradley et al. 1991, Fraise et al. 1997, Eveillard et al. 2008].

Not only does the complex operational structure of nursing homes, that act as both a healthcare setting and a resident's home, make it difficult for standard MRSA control practices to be implemented in these facilities, but a standardized MRSA control strategy for nursing homes is yet to be agreed on, largely due to the lack of studies aimed at identifying appropriate strategies [Hughes, Smith and Tunney 2008].

There is also a general dearth of studies, particularly regional ones, investigating the makeup of the nursing home MRSA reservoir. A study of 60 nursing homes in Belgium identified hospital care, co-morbidities and a lack of coordinated MRSA surveillance and control activities as risk factors for MRSA carriage in nursing home residents [Denis et al. 2009a]. It also found that the predominant MRSA strains among nursing home residents were identical to those found in hospital inpatients, highlighting the need for synergistic infection control between nursing homes and hospitals [Denis et al. 2009a]. A better understanding of the frequency and diversity of nursing home MRSA strains and predictors thereof, will help to form strategies for minimizing MRSA transmission and infection in nursing homes, and thus reduce the impact of the nursing home MRSA reservoir on hospitals.

Assessing the extent to which CA-MRSA has penetrated the nursing home reservoir is also of interest. CA-MRSA has become increasingly dominant in recent years, and USA300 in particular has several characteristics that may offer a selective advantage over HA-MRSA, including higher transmissibility and increased pathogenicity [Okuma et al. 2002, Diep et al. 2008b]. There is also growing evidence that community and healthcare reservoirs are mixing [Kourbatova et al. 2005, Seybold et al. 2006, Maree et al. 2007, Liu et al. 2008, Popovich, Weinstein and Hota 2008] (Chapter 6).

We conducted a prospective study of MRSA isolates in nursing home residents in a large metropolitan county to investigate the frequency and genetic diversity of MRSA in these facilities, and thus gain a better understanding of the nature of the nursing home MRSA reservoir.

7.2 METHODS

7.2.1 Study

A population-based prospective study of carriage (symptomatic and asymptomatic) isolates of MRSA from 26 nursing homes in OC, California, was conducted. This study was approved by the Institutional Review Board of the University of California Regents.

7.2.2 Isolate collection

Carriage isolates of MRSA from unique residents were collected from participating nursing homes between January 2009 and April 2011. Each nursing home was instructed to swab the nares of 100 consecutive residents upon admission (within three days of arrival), and 100 residents on a single day (point prevalence screening), using bilateral nares swabs (BD Culture Swabs, Fisher Scientific). For nursing homes with a low bed turnover, fewer residents were screened (30-50). For nursing homes with an average length of stay in years, admission screening was not performed. Swabs were cultured for MRSA using selective media (BD CHROMagar). MRSA strains were stored at −65°C in 15% glycerol Brucella broth.

7.2.3 Specimen data and nursing home characteristics

Specimen data including swab type (admission or point prevalence), swab day since admission, room type (shared or single resident room) and whether the swabbed resident had prior MRSA, were collected. Demographic and co-morbidity data for participating nursing homes were derived from the Centers for Medicare and Medicaid Services (CMS) Long Term Care Minimum Data Set for 2009 [CMS 2009],

and included annual admissions, and the percentage of residents with the following characteristics: under 65 and over 85 years old, male, non-white, Hispanic, education less than high school, admitted from hospital, history of MRSA, diabetes, fecal incontinence, skin lesions and medical devices (which included tracheostomy, ventilator and dialysis devices).

7.2.4 Laboratory methods and molecular typing

I processed all strains in the laboratory according to methods described previously, including *spa* typing of all isolates, assignment to *spa* clonal complexes (*spa*-CCs), and MLST of a subset (n = 138) of isolates (Chapter 6).

7.2.5 Statistical analyses

I conducted one- and two-sample z-tests for equality of proportions to compare *spa*-CCs within each nursing home and overall MRSA carriage at admission versus MRSA point prevalence, respectively. I also used Simpson's index of diversity ($1-D$) to estimate inter- and intra-nursing home genetic diversity of the MRSA strains collected, as well as the genetic diversity of the two major *spa*-CCs and genetic diversity among the admission and point prevalence isolates. $1-D$ gives an unbiased measure of the probability of drawing two different *spa* types given the distribution of *spa* types in a sample [Grundmann et al. 2010]. Confidence intervals (95% CIs) were calculated as described previously [Grundmann, Hori and Tanner 2001]. For comparison of diversity indices, a significant difference ($p<0.05$) was determined by non-overlapping 95% CIs. χ^2 tests compared *spa*-CCs between nursing homes and between MRSA admission and point prevalence isolates. I computed Pearson's

correlation coefficients to determine the relationship between nursing home and isolate variables, and genetic diversity.

Due to the small sample size of nursing homes (21, since one nursing home did not isolate any MRSA and four were excluded in this analysis as they collected <10 MRSA isolates and thus their diversity estimates were unreliable) and the large number of potential predictor variables for genetic diversity, I considered variables for entry into a bootstrapped multiple linear regression model based on a combination of their correlation coefficient and current knowledge regarding their association with MRSA. Only variables with $p<0.1$ in correlation tests were considered for the exploratory model. All statistical tests were performed using STATA (release 11, StataCorp 2009).

7.3 RESULTS

7.3.1 Overview

Between January 2009 and April 2011, 3,806 nasal swabs were taken from residents of 26 OC nursing homes either on admission or for estimating MRSA point prevalence. Of these, 837 swabs (22%) isolated MRSA. One nursing home did not isolate any MRSA. Overall admission prevalence was 16%, and point prevalence was significantly higher at 27% ($p<0.001$). The majority of the 837 MRSA isolates were from point prevalence testing (68%), from residents with no prior history of MRSA (76%), and from residents sharing a room (95%). Median swab day since admission was 53 (IQR, 4-265). A third of all admissions swabs were collected at day 4 since some nursing homes could not swab earlier. Table 7.1 gives a summary overview of the 25 nursing homes and the characteristics of their MRSA isolates.

Table 7.1 Summary of the 25 nursing homes and 837 MRSA carriage isolates from nursing home residents in OC, CA.

Characteristic	**Value**
Nursing home characteristics	Median (IQR[a])
Annual admissions	264 (144-520)
% Residents under 65 years old	20 (4-39)
% Male	40.8 (31.6-48.2)
% Education less than high school	23.8 (7.4-30.3)
% Hispanic residents	11.9 (3.7-23)
% Non-white residents	15 (7.8-21.7)
% Residents admitted from hospital	82.2 (58.5-93.8)
% Diabetes	27.1 (23.4-42.1)
% Fecal incontinence	43.8 (29.2-54.8)
% Skin lesions	72.7 (50.7-86.5)
% Devices	2.2 (1.4-7.1)
% Residents with MRSA history	12 (6-19)
MRSA admission prevalence	16 (10.2-22)
MRSA point prevalence	26.7 (19-34)
N *spa* types per nursing home	5 (4-8)
Overall MRSA isolate characteristics	No. of isolates (%)
MRSA isolates	837 (100)
Admissions swab	269 (32.1)
Resident had prior MRSA	201 (24.0)
Resident shared room	795 (95.0)

[a] IQR = interquartile range.

7.3.2 spa typing and MLST

Of the 837 MRSA isolates collected, 835 were *spa* typed. Two isolates could not be *spa* typed as one did not grow upon culturing and a *spa* PCR product was not obtained from the other. Among the 835 MRSA isolates, 60 *spa* types were identified, including nine novel *spa* types (1.4% of all isolates) with hitherto unknown *spa* repeat sequences. One isolate that was non-typeable (NT) by *spa* typing was identical to *spa* type t002, except for two extra nucleotides in the third repeat, making the repeat 26-bp long and putting the *spa* coding region out of frame. A clinical MRSA isolate from an OC hospital inpatient was similarly NT, and others have reported *spa* repeats of unexpected length (see also Chapter 6) [Rothganger 2010]. The three most common *spa* types were t242, t008 and t002, representing 83% of all isolates collected (Table 7.2).

BURP analysis of the *spa* types clustered 94% of isolates into two large *spa*-CCs and 3% of isolates into two smaller *spa*-CCs (Figure 7.1). Half of all *spa* types were clustered into *spa*-CC002 (predicted founder t002) and 20% into *spa*-CC008 (founder t008), including six and one novel *spa* type(s), respectively. Under the BURP algorithm, singletons are *spa* types that differ from all other *spa* types in the sample by more than 4 repeats, and thus cannot reasonably be clustered into a *spa*-CC. Ten *spa* types (17 isolates) were classed as singletons, including one novel *spa* type. Since no reliable evolutionary history can be inferred from short *spa* types [Mellmann et al. 2007], two isolates representing two *spa* types (t026 and t8606) were excluded from BURP analysis. The NT isolate could also not be included in the BURP analysis. Estimated genetic diversity of MRSA in OC nursing homes using *spa* typing was high, at 77% (Table 7.2).

Table 7.2 Ten most frequently found *spa* types among 835 MRSA isolates from OC nursing home residents[a].

Rank	***spa* type**	MLST	Freq	%	Cumulative %
1	t242	5	273	32.7	32.7
2	t008	8	222	26.6	59.3
3	t002	5	195	23.4	82.6
4	t127	474	12	1.4	84.1
5	t306	5	11	1.3	85.4
6	t088	105	10	1.2	86.6
7	t037	239	7	0.8	87.4
8	t024	8	6	0.7	88.1
9	t068	8	6	0.7	88.9
10	t548	5	6	0.7	89.6
-	Other	-	87	10.4	100.0

[a] The total number of *spa* types was 60, including one non-typeable isolate. Simpson's index of diversity ($1-D$) value was 77% (95% CI, 75%, 78%). MLST = multilocus sequence type.

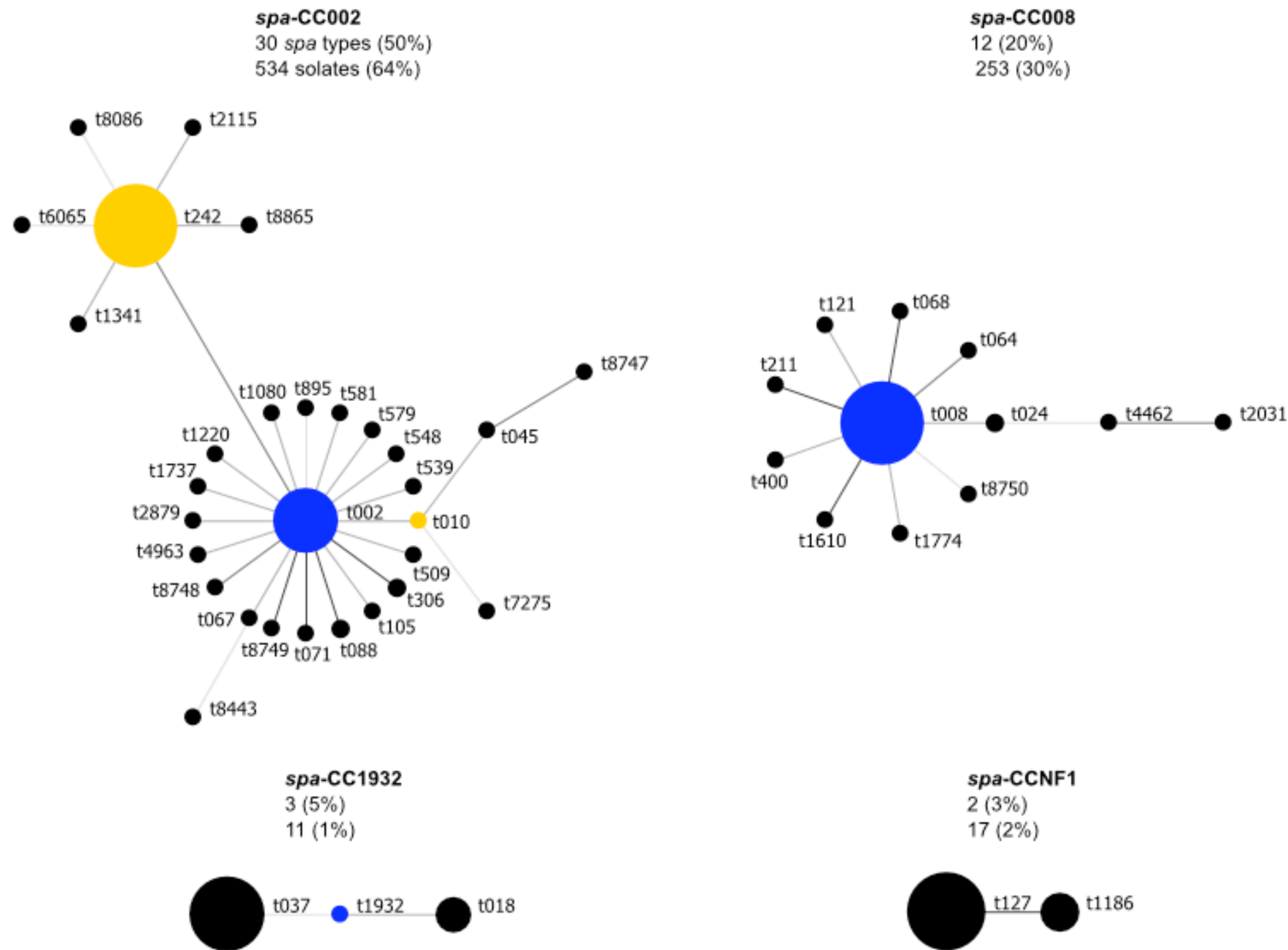

spa-CC002
30 spa types (50%)
534 solates (64%)
t8086
t2115
t6065
t242
t8865
t1341
t8747
t1080
t895
t581
t579
t548
t539
t1220
t1737
t2879
t002
t010
t045
t4963
t8748
t067
t8749
t071
t088
t105
t306
t509
t7275
t8443
spa-CC008
12 (20%)
253 (30%)
t121
t068
t064
t211
t008
t024
t4462
t2031
t400
t8750
t1610
t1774
spa-CC1932
3 (5%)
11 (1%)
t037
t1932
t018
spa-CCNF1
2 (3%)
17 (2%)
t127
t1186

Figure 7.1 Relatedness of *spa* types among nursing home MRSA isolates according to the Based Upon Repeat Pattern (BURP) algorithm. Clusters of linked *spa* types correspond to *spa* clonal complexes (*spa*-CCs). *spa* types are clustered into a *spa*-CC when their repeat patterns differ by no more than 4 repeats. The BURP algorithm sums up ‘costs’ (a measure of relatedness based on the repeat pattern) to define a founder score for each *spa* type in a *spa*-CC. The founder (blue node) is the *spa* type with the highest founder score in its *spa*-CC, and the subfounder (yellow node) is the *spa* type with the second highest founder score. *spa*-CC008 has founder t008, and *spa*-CCNF refers to a *spa*-CC with no founder. Each node represents a *spa* type. The node size represents the number of clustered strains that belong to that *spa* type. The shading of the branches represents the ‘costs’ (similarities in repeat patterns) between two *spa* types; the darker the branch, the lower the cost (more similar repeat patterns).

To confirm strain types, 138 isolates were selected for MLST. Among the 15 unique sequence types (STs) identified, ST5 (54%) and ST8 (28%) were the most predominant, with the majority of isolates belonging to one of two major MLST CCs: CC5 (60%; five STs) and CC8/239 (29%; two STs) (Table 7.3). The remaining 11% of isolates comprised sporadic incidences of both HA-MRSA (4%) and CA-MRSA (7%) clones. According to MLST, t008 isolates were the prototypic community clone USA300 (ST8/t008) and t002 isolates were the prototypic hospital clone USA100 (ST5/t002). t242 isolates, which differ from t002 isolates by one *spa* repeat as a result of a single nucleotide difference, were identified as ST5 (Tables 7.2 and 7.3). The NT *spa* isolate and one novel, singleton *spa* type were ST105, with seven of the other novel *spa* types being ST5 and one ST8.

Since the founders of the two large *spa*-CCs represent a community-associated clone (the predominant US clone USA300 for *spa*-CC008) and a healthcare-associated clone (the dominant US hospital clone USA100 for *spa*-CC002), *spa*-CC008 effectively represents CA-MRSA and *spa*-CC002 HA-MRSA. The two smaller *spa*-CCs each represented HA-MRSA and CA-MRSA, and the remaining 2.4% of isolates that could not be assigned to a *spa*-CC also included both HA-MRSA and CA-MRSA.

Table 7.3 Relatedness of MLST sequence types (STs) among 138 nursing home MRSA isolates according to the eBURST algorithm.

CC (no. of isolates)[a]	MLST	Associated *spa* types[b]
CC5 (83)	5	t002, t242, t306, 28 others
	105	t088, t002, t8444
	221	t002
	1011	t895
	1510	t242
CC8/239 (40)	8	t008, t024, 12 others
	239	t037
CC474/1900 (6)	474	t127, t1186
	1900	t127
Singletons (9)[c]	45	t026, t040, t736
	36	t018, t1932
	59	t437
	88	t5916
	188	t189
	217	t032

[a] CC = clonal complex. All members of a CC share identical alleles at six of the seven loci with at least one other member of the CC.

[b] Only the three most common *spa* types are listed if more than three associated with that ST.

[c] STs with allelic profiles that share less than six of their seven loci with all other STs in the dataset.

7.3.3 Differences among nursing homes

The estimated genetic diversity of MRSA within nursing homes ranged from 43% to 84% (Figure 7.2), but due to relatively small numbers these differences were mostly non-significant, with the clear exception of hospital 23 that exhibited significantly higher diversity (84%) than nine other nursing homes. Four nursing homes collected <10 MRSA isolates and so their genetic diversities could not be reliably estimated. For the 21 remaining nursing homes, MRSA genetic diversity within nursing homes was positively correlated with the percentage of residents admitted from hospital (r = 0.52, $p = 0.02$), percentage of residents with diabetes (r = 0.57, $p < 0.01$), percentage of residents with skin lesions (r = 0.46, $p = 0.03$), MRSA admission prevalence (r = 0.50, $p = 0.03$) and MRSA point prevalence (r = 0.47, $p = 0.03$), and negatively correlated with the percentage of residents under 65 years old (r = -0.57, $p<0.01$) and the percentage of male residents (r = -0.43, $p = 0.05$) (Table 7.4). The percentage of residents with skin lesions was positively correlated with the percentage of residents admitted from hospital (r = 0.84, $p<0.001$) and negatively correlated with the percentage of residents under 65 (r = -0.58, $p<0.01$). Skin lesions are very common among elderly people, and are often caused by disease or trauma that requires hospital treatment. The percentage of residents with skin lesions showed the weakest correlation with genetic diversity and thus was not considered for entry into the bootstrapped linear regression model. Similarly, the percentage of male residents and the percentage of residents under 65 were highly correlated (r = 0.88, $p<0.001$). Since age was more strongly correlated with MRSA diversity than gender, and differences in MRSA strain types have been observed between age groups in our previous study (Chapter 5) [Hudson et al. 2012], the percentage of male residents was not considered for entry into the multiple regression model. Finally, point prevalence

was highly correlated with admission prevalence (r = 0.74, $p<0.001$) and diabetic residents (r = 0.69, $p<0.001$), thus only admission prevalence was considered for regression model entry. Only the percentage of residents 65 or over and the percentage of diabetic residents remained significant predictors of *spa* type genetic diversity in the exploratory regression model (Table 7.5).

The three most common *spa* types - t242, t008 and t002 - accounted for 55-96% of isolates at each of 23 nursing homes. Two further nursing homes only isolated t008, although the sample sizes were <10. Twelve nursing homes isolated mostly t242 (36-63%). The proportion of *spa*-CC008 isolates, representing community-associated *spa* types, compared to *spa*-CC002 isolates, representing healthcare-associated *spa* types, varied significantly between nursing homes (χ^2 = 69.2, df = 24, $p<0.001$) (Figure 7.2). Two nursing homes (8%) had significantly more *spa*-CC008 isolates, and 16 nursing homes (64%) had significantly more *spa*-CC002 isolates ($p<0.01$). Diversity of *spa* types among *spa*-CC008 isolates ($1-D$ = 23% (95% CI, 12-33%)) was significantly lower than diversity among *spa*-CC002 isolates ($1-D$ = 60% (95% CI, 58-63%)).

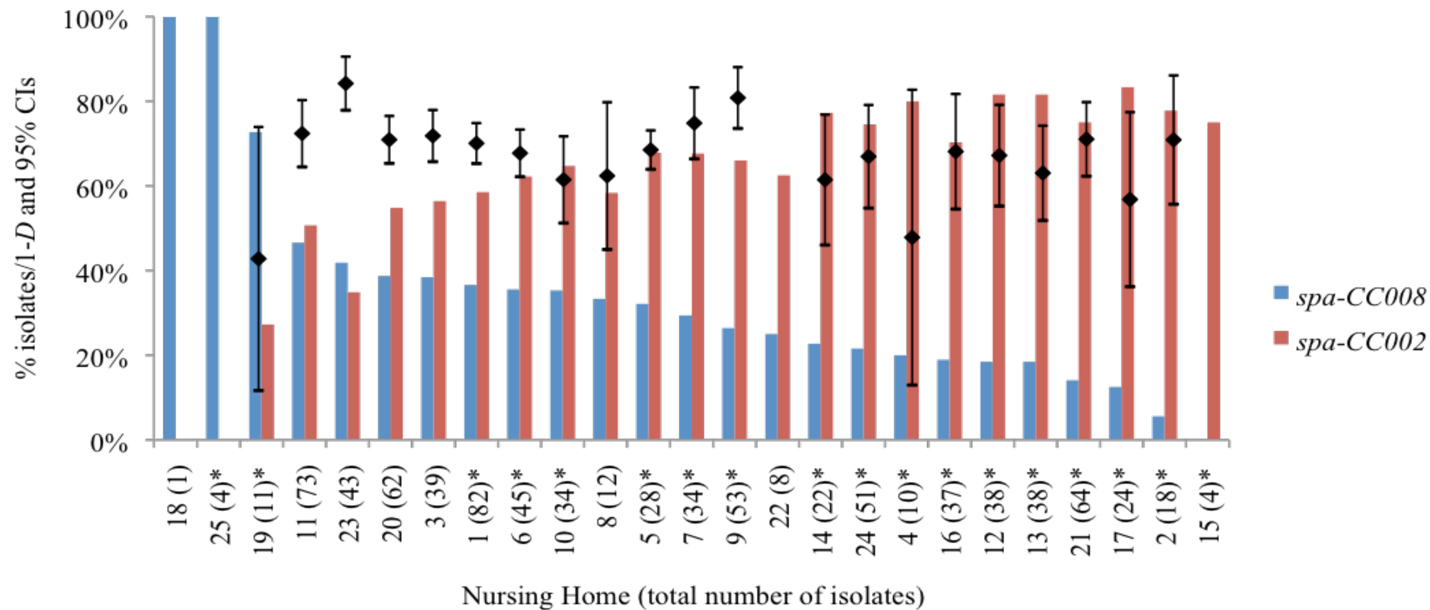


Figure 7.2 Proportion of isolates belonging to *spa*-CC002 versus *spa*-CC008, by nursing home. *indicates a significant difference at the 99% level in the proportion of isolates belonging to *spa*-CC002 and *spa*-CC008 at that nursing home. The black bars show the point estimates and 95% confidence intervals of nursing home-specific genetic diversity expressed as Simpson's index of diversity ($1-D$) of *spa* types (as a percentage). Diversity indices for nursing homes 15, 18, 22 and 25 were excluded from the figure as these nursing homes had *spa* type data on less than ten isolates. Diversity indices with non-overlapping 95% CIs were considered significantly different ($p<0.05$).

Table 7.4 Correlation of nursing home and isolate variables with nursing home genetic diversity.

Variable	r	p^a
Nursing home variable		
Annual admissions	0.21	0.36
% Residents under 65 years old	-0.57	<0.01
% Male	-0.43	0.05
% Education less than high school	0.36	0.11
% Hispanic residents	-0.04	0.88
% Non-white residents	0.24	0.29
% Residents admitted from hospital	0.52	0.02
% Diabetes	0.57	<0.01
% Fecal incontinence	0.05	0.82
% Skin lesions	0.46	0.03
% Devices	-0.29	0.20
% Residents with MRSA history	-0.23	0.31
MRSA admission prevalence	0.50	0.03
MRSA point prevalence	0.47	0.03
Isolate/resident variable		
% Prior MRSA	-0.07	0.77
% Residents shared room	-0.13	0.58
Swab within 3 days of admission	0.20	0.39

[a] Variables with $p<0.1$ were considered for exploratory multivariate analyses.

Table 7.5 Multivariate analysis of variables associated with nursing home genetic diversity.

Variable	Coefficient	Bootstrap SE	Normal-based 95% CI	*p*
% Diabetic residents	0.41	0.11	0.20, 0.62	<0.001
% Residents under 65	-0.22	0.07	-0.37, -0.08	<0.01

7.3.4 Admissions versus Point Prevalence MRSA

MRSA *spa* type genetic diversity was not significantly different among isolates collected at admission ($1-D$ = 76% (95% CI, 74-79%)) versus isolates collected during point prevalence testing ($1-D$ = 77% (95% CI, 75-79%)). No significant correlation was found between admission prevalence and the proportion of *spa*-CC008 isolates (r = -0.20, p = 0.34), however nursing homes with a higher percentage of residents admitted from hospital had significantly lower percentages of *spa*-CC008 isolates (r = -0.58, p<0.01). Proportions of *spa*-CC008, *spa*-CC002 and other isolates were not significantly different between admissions and point prevalence MRSA (26%, 68% and 6% among admissions MRSA versus 32%, 62% and 6% among point prevalence MRSA, respectively, χ^2 = 3.3, df = 2, p = 0.2).

7.4 DISCUSSION

A prospective collection of carriage isolates of MRSA from 26 nursing homes in OC, CA, was conducted. The study investigated the frequency and genetic diversity of MRSA in these little-studied healthcare facilities, to better inform nursing home-based infection control strategies. This is the first study to assess MRSA isolates in nursing homes at a population level and across a large region.

Countywide, nursing home carriage MRSA isolates were dominated in approximately equal proportions by three strains: the predominant community-associated clone in the US, USA300 (ST8/t008); the healthcare-associated clone USA100 (ST5/t002); and ST5/t242 isolates, likely a minor variant of USA100 that has become prevalent in OC healthcare facilities (Chapters 5 and 6) [Hudson et al. 2012]. ST5/t242 isolates were slightly more common than USA300 and USA100

however, representing a third of all carriage MRSA isolates. The same three strains dominated in OC hospitals, but in this setting USA300 was the most common clone (Chapter 6).

As in OC hospitals, most *spa* types were closely related to either USA300 or USA100, creating two large *spa*-CCs each representing CA-MRSA and HA-MRSA strains. The remaining, sporadically occurring *spa* types were both community-associated and healthcare-associated, representing several known clones. Of the two smaller *spa*-CCs, one represented strains of the CA-MRSA lineage MLST CC1, with most isolates typed as ST474/t127. ST474 is a single-locus variant (SLV) of ST1, and ST1/t127 is a common CA-MRSA strain in the UK [Otter et al. 2009]. ST474/t127 isolates were also found among OC hospital inpatients (Chapter 6), but no ST1/t127 isolates have yet been identified in the US. The other small *spa*-CC, *spa*-CC1932, represented HA-MRSA and included the epidemic clone USA200/EMRSA-16 (ST36) and the pandemic clone ST239.

The isolates that could not be assigned to a *spa*-CC included both HA-MRSA and CA-MRSA. The healthcare-associated strains were USA600/Berlin clone (ST45) and ST217/t032, a SLV of ST22, the pandemic HA-MRSA clone EMRSA-15 that has also recently been reported in the community [Mollaghan et al. 2010]. The community-associated strains were USA1000 (ST59), ST188 (a double-locus variant of ST1 and ST474 reported sporadically in Australia and Asia [Nimmo and Coombs 2008, Peck et al. 2009, Ghaznavi-Rad et al. 2010a]) and ST88, a clone closely related to CC1 that has been reported in several countries, particularly Nigeria, but has not been previously reported in the US [Ghebremedhin et al. 2009, Monecke et al. 2011]. It is clear that USA300 and USA100 dominate healthcare facilities in OC, in line with the MRSA picture seen nationwide. However, it would be interesting to investigate

whether the USA100 variant seen in this county is also more common than USA100 elsewhere in the US, particularly since t242 has been reported rarely in the literature [Kinnevey et al. 2010, Parlato et al. 2009, Johnson et al. 2007, Weese, Avery and Reid-Smith 2010].

Overall genetic diversity of MRSA in OC nursing homes was significantly higher than that seen in OC hospitals (Chapter 6). The higher proportion of HA-MRSA strains present in nursing homes likely drives this, which could be a result of the high proportion of residents directly admitted to nursing homes from a number of different OC hospitals. Diversity was significantly lower among *spa*-CC008 (CA-MRSA) isolates than *spa*-CC002 (HA-MRSA) isolates, probably due to the greater time HA-MRSA have had to diversify compared to CA-MRSA. This suggests that MRSA diversity in OC is driven by healthcare-associated strains.

In exploratory analyses, greater MRSA genetic diversity was significantly associated with older resident age and diabetic residents. Diabetic foot ulcers are a known risk factor for MRSA, and in particular HA-MRSA, with MRSA found to be present in 10-30% of diabetic wounds [Goldstein, Citron and Nesbit 1996, Tentolouris et al. 1999, Shankar et al. 2005, Wang et al. 2010]. Diabetic complications such as neuropathy, osteomyelitis and peripheral vascular disease may result in a prolonged hospital stay, increasing the exposure of diabetic people to HA-MRSA [Wang et al. 2010]. Older age is a well-established risk factor for HA-MRSA, as elderly patients tend to be sicker and require hospital treatment. Older age was a significant predictor of genetic diversity in OC hospitals (Chapter 6), and was associated with non-t008 strains in a study comparing adult and paediatric OC inpatients (Chapter 5) [Hudson et al. 2012].

Genetic diversity was not significantly different between admissions MRSA and MRSA collected during point prevalence, with no correlation found between *spa*-CC008 isolates (CA-MRSA) and admissions MRSA. In fact, over two-thirds of admissions MRSA belonged to *spa*-CC002 (HA-MRSA). The vast majority of residents in this study were admitted to nursing homes directly from hospital and thus were not recently exposed to the community MRSA reservoir, thus reducing the likelihood of isolating a community-associated strain at admission. Community-associated strains are present in nursing homes, although to a lesser extent than in hospitals (Chapter 6), with the majority of nursing homes isolating significantly more HA-MRSA. This is probably due to the resident demographic - older, sicker people generally have a history of healthcare exposure and thus tend to have HA-MRSA, which is associated with more invasive infections and serious illness. The long-term care provided by nursing homes means that the turnover rate in nursing homes is far lower than the patient turnover rate in hospitals. This results in a lower frequency of possible introductions of MRSA from outside the healthcare setting.

The CA-MRSA seen among residents upon admission to nursing homes and during point prevalence sampling likely comes from the hospital MRSA reservoir, in which it is clearly becoming dominant (Chapter 6). The slightly higher proportion of CA-MRSA from point prevalence sampling compared to admission sampling and consequently the lower proportion of HA-MRSA, although non-significant, possibly suggests that CA-MRSA could be more easily spread in nursing homes than HA-MRSA, perhaps due to a more community-like environment that CA-MRSA is well-adapted to, e.g. communal areas and shared rooms. It could also possibly mean that additional exposures to CA-MRSA are occurring via contact with visitors and/or healthcare workers. Given the non-significant result however, it could just be due to

the different sample of residents that were tested at point prevalence versus at admission (some residents would have been tested at both times, while others would have been tested only at point prevalence and not at admission, and vice versa).

The significantly higher point prevalence of MRSA compared to admissions prevalence may be due to the transmission of MRSA imported by residents upon admission, evidenced by the lack of significant change in genetic diversity or proportions of CA-MRSA and HA-MRSA from admissions to point prevalence. Factors influencing transmission in nursing homes and predictors of MRSA carriage at point prevalence have been reported previously [Reynolds et al. 2011].

A limitation of this study was that few individual-level characteristics were available and so facility-level data were primarily used. Also, only a single site was swabbed for MRSA. Although nasal screening is thought to detect the majority of MRSA carriers [Manian et al. 2002, Lucet et al. 2003], anatomical sites such as the rectum and throat have been shown to be important in the detection of MRSA carriage [Eveillard et al. 2006, Batra et al. 2008, Eveillard et al. 2008]. The point prevalence study design also limited the accuracy of carriage estimates. In using genetic diversity as the outcome, the sample size was reduced considerably, and thus multivariate analysis of predictor variables could only be exploratory. Also, the diversity indices estimated for admissions MRSA versus point prevalence MRSA, and *spa*-CC008 isolates versus *spa*-CC002 isolates, may have been influenced by differing sample sizes [Grundmann, Hori and Tanner 2001].

In conclusion this study found that in a large county, the diversity of carriage MRSA isolates among nursing home residents, although heterogeneous between facilities, was significantly higher than the diversity among clinical MRSA isolates from hospital inpatients in the same county. MRSA diversity in both hospitals and

nursing homes appears to be driven by HA-MRSA. The proportions of older, diabetic residents were significant predictors of nursing home MRSA diversity. Nursing home strains were dominated by a healthcare-associated, OC-prevalent variant of USA100 (ST5/t242). The CA-MRSA strain USA300 was the second most common clone isolated from the nares of nursing home residents, suggesting substantial penetration of community-associated strains into the nursing home reservoir, however to a lesser extent than seen in the hospitals, where USA300 was predominant. Nursing home MRSA burden appeared to be largely due to importation of diverse strains from hospitals and subsequent transmission of these imported strains, leading to high MRSA point prevalence. One of the OC nursing homes in this study was previously reported to have an MRSA point prevalence of 52% [Reynolds et al. 2011]. Nursing homes therefore represent a significant reservoir for MRSA, and as such a consensual, regionally implemented control strategy tailored to this unique setting is required. However, due to the complexity of MRSA control in these facilities, further studies evaluating the contribution of nursing homes to regional MRSA transmission are needed before developing such a strategy.

PART 2 FINAL DISCUSSION: MRSA DIVERSITY IN ORANGE COUNTY, CALIFORNIA

In collaboration with researchers at the University of California, Irvine (UCI), MRSA isolates were collected from OC hospitals and nursing homes in the first population-based countywide study of MRSA strain diversity and distribution in healthcare facilities. The aim of this work was to gain a better understanding of the frequency and genetic diversity of HA-MRSA and CA-MRSA in hospital and nursing home MRSA reservoirs in the hope that such knowledge would better inform regional infection control strategies.

While MRSA diversity was observed among OC healthcare facilities, three strains dominated both hospital and nursing home MRSA reservoirs in this metropolitan county: the increasingly prevalent community-associated clone USA300, a surprisingly common variant of the hospital-associated clone USA100, and USA100 itself. USA300 accounted for almost half of all MRSA isolates collected from OC hospitals and over a quarter of isolates from OC nursing homes. Despite significantly higher numbers of USA300 in hospitalised children, USA300 was still the most common strain among adult inpatients, and the second most common strain among nursing home residents. No correlation was found between CA-MRSA and community onset among hospital MRSA isolates, with 42% of hospital onset isolates CA-MRSA, 39% of community onset isolates HA-MRSA, suggesting that community and healthcare MRSA reservoirs are mixing. Other US studies have reported such mixing. The Centers for Disease Control and Prevention ABC surveillance system for invasive MRSA infections found that the USA300 clone accounted for 22% of community onset HA-MRSA infection and 16% of hospital onset HA-MRSA infection in 2004-2005 [Klevens et al. 2007]. It also found that 23% of CA-MRSA

infections were caused by USA100. Another study involving a single urban hospital and its associated outpatient clinics in Atlanta, GA, found that previous hospitalization was a risk factor for community onset MRSA infections, 99% of which were caused by USA300 [King et al. 2006]. Finally, among cases of hospital onset MRSA bacteraemia between 2000 and 2006 in a single hospital in Chicago, IL, community-genotype strains caused an increasing proportion of cases from 24% to 49% while the proportion of hospital-genotype strains decreased [Popovich, Weinstein and Hota 2008].

Because of their changing epidemiology and increasingly indistinguishable clinical characteristics, the definitions of HA- and CA-MRSA need to be revised in order to better inform infection control strategies [Popovich and Weinstein 2009, David and Daum 2010, McCarthy et al. 2010]. The predominance of community onset isolates among hospital inpatients highlights the need for community-based interventions to stem the influx of community-associated strains, particularly the highly successful USA300 clone, into the healthcare setting.

In addition to the *spa* typing and MLST I performed as part of my thesis, PFGE was also performed on the OC MRSA isolates by my collaborators. PFGE results suggest that the USA300 strain may be diverging (data not shown). While these typing methods for characterising MRSA isolates are useful for identifying preliminary differences among strains, they are also limited. For example, MLST evaluates housekeeping genes, so while excellent at unambiguously assigning a strain, it is unlikely to capture recent or subtle changes in USA300 strains. Whole genome sequencing would allow us to characterise this predominant MRSA strain in OC on a finer scale, helping us to elucidate the rapidly evolving differences in this strain and thus better understand its epidemiology and pathogenicity. In collaboration with the

Wellcome Trust Sanger Institute, all USA300 strains from the large prospective study of MRSA in OC are currently being whole genome sequenced using NGS technology, allowing us to assess the extent to which USA300 has spread across the 30 OC hospitals and into the 25 OC nursing homes included in the study, and to possibly determine its major transmission pathways. Other collaborators working with the *spa* typing data from this OC MRSA project found that hospitals that share patients share strains [Ke et al. 2012]. Integrating genome data into the network models they have created will help to quantify USA300 spread between hospitals.

Work linking this part of my thesis with that involving my industrial sponsor, TwistDx, could involve using the various MRSA strains identified in the OC study to test the performance of their MRSA diagnostic assay, TwistAmp MRSA. Also, MREJ typing all MRSA isolates collected from OC hospitals and nursing homes would allow comparison to MREJ typing data from the KC MRSA isolates in Chapter 3, as well as MRSA isolates from the UK collection, to form a better picture of the frequencies and diversity of MREJ types.

I am currently involved in the development of OC-Maps, with collaborators from the Department of Infectious Disease Epidemiology, Imperial College London. OC-Maps will be a web-based interactive mapping tool for the interrogation of the geographic distribution of different *spa* types isolated from hospitals in OC. It will be based on the publicly accessible mapping tool SRL-Maps (http://www.spatialepidemiology.net/SRL-Maps) [Grundmann et al. 2010]. OC-Maps will be countywide, covering 30 hospitals, whereas SRL-Maps spans a continent covering 450 hospitals, so it is unlikely that any clear geographical patterns could be visualized using the former. However, it will offer an elegant way to summarise the *spa* typing and related clinical data both across and within hospitals in OC, CA and

aid the formation of hypotheses regarding the spread of MRSA strains within this county.

All hospital locations will be represented as placemarks on a Google map. Clicking on a placemark will display, below the map, all *spa* types identified at that hospital including frequencies (Figure II.1). The number of isolates found elsewhere (if any) for each of these *spa* types, along with the number of hospitals each *spa* type has been isolated from, would also be displayed. There will also be the option of viewing the countywide distribution of any *spa* type, and placemarks on the map will be colour-coded on the basis of the percentage of isolates of that *spa* type at each hospital, with the number of isolates shown inside the placemark (Figure II.1). Graphical charts will be displayed showing either *spa*-type specific, hospital-specific or overall age distributions, specimen types, onset and ICU/non-ICU isolate collection (Figure II.1). This tool, once developed, would allow the identification and mapping of strains with particular public health importance, for example USA300.

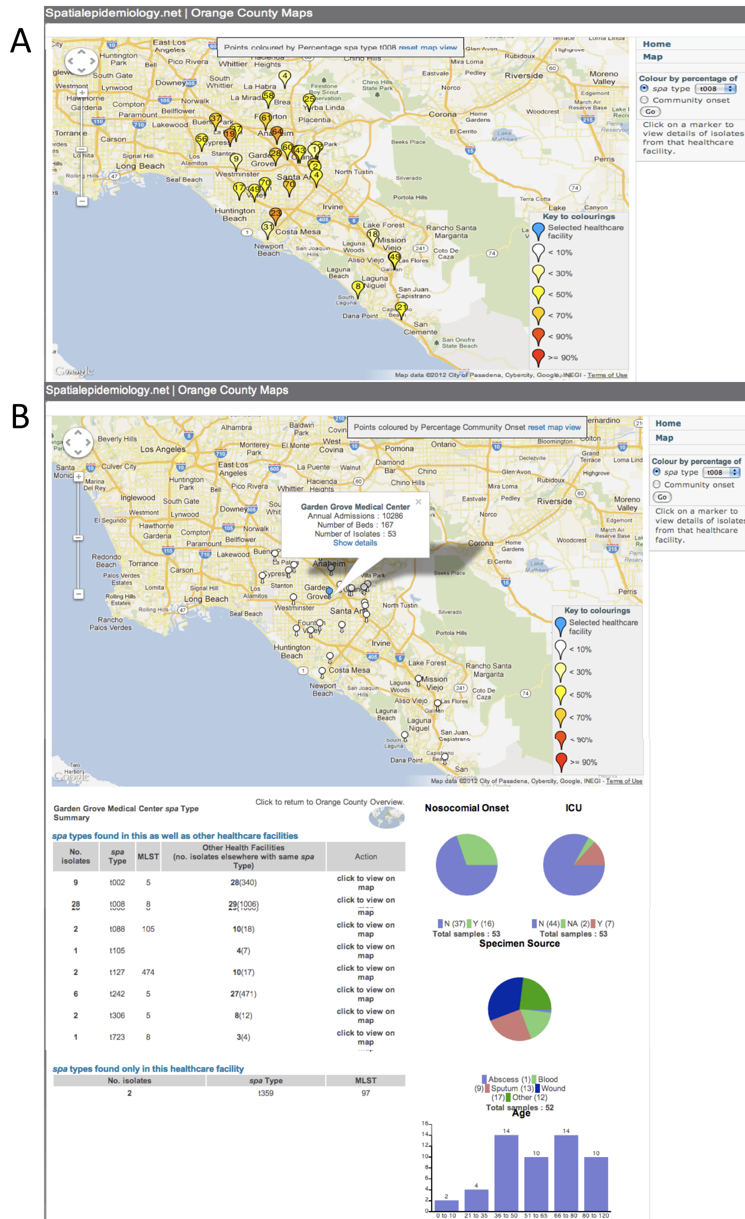


Figure II.1 Example of what OC-Maps will look like. **A** Location of hospitals isolating *spa* type t008 viewed using OC-Maps. Each placemark is colour-coded to show the percentage of t008 isolates at each hospital, and the total number of MRSA isolates isolated from each hospital are shown inside the placemarks. **B** Information specific to the hospital selected (blue) is shown below the map. For the hospital selected, the pie charts show the proportion of nosocomial onset MRSA, proportion of MRSA isolated from an ICU and the proportions of each specimen type MRSA were isolated from. The bar chart displays patient age distribution.

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APPENDICES

APPENDIX 1: LABORATORY MRSA COLLECTION

Table Laboratory collection of MRSA isolates.

Clone	No. of isolates	Geographic spread	Clone name(s)
ST1-MRSA-IV	3	US, Australia	WA MRSA-1, USA400
ST5-MRSA-1	3	UK, Slovenia	EMRSA-3
ST5-MRSA-II/ST5-GISA-II	5	Finland, Ireland, UK, Japan, US	New York/Japan clone/GISA, USA100
ST5-MRSA-III	1	Belgium	
ST5-MRSA-IV	2	France, US	Paediatric clone, USA800
ST8-MRSA-I	1	Australia	
ST8-MRSA-II	3	UK, Ireland, US	Irish-1
ST8-MRSA-III	1	UK	EMRSA-7
ST8-MRSA-IV	4	US, France, UK	EMRSA-2, -6, USA300, -500
ST22-MRSA-IV	3	Ireland, UK	EMRSA-15, Barnim
ST30-MRSA-IV	1	Australia	Southwest Pacific clone, USA1100
ST36-MRSA-II	3	Finland, UK	EMRSA-16, USA200, Irish AR7.0
ST-36-MRSA-IV	1	UK	
ST45-MRSA-IV	4	Finland, Germany, Sweden	Berlin clone
ST59-MRSA-V	1	France	Taiwan clone (ST59-MRSA-V(5C2&5), t437)
ST80-MRSA-IV	3	France, Sweden, Algeria	European CA-MRSA clone
ST93-MRSA-IV	2	Australia	Queensland clone
ST156-MRSA-IV	1	Finland	
ST157-MRSA-III	1	Poland	
ST225-MRSA-II	1	US	
ST228-MRSA-I	1	Germany	Southern Germany Clone
ST231-MRSA-II	1	US	
ST235-MRSA-I	1	UK	Scottish GISA isolate
ST239-MRSA-III	3	Holland, Portugal, Germany	Portugese/Brazilian, Vienna, EMRSA-1, -4, -11
ST247-MRSA-I	2	Portugal, Belgium	EMRSA-5, -17, Iberian
ST250-MRSA-I	2	UK, Switzerland	First MRSA, Archaic clone
ST254-MRSA-I	1	UK	
ST254-MRSA-IV	1	Germany	EMRSA-10, Hannover
ST280-MRSA-IV	1	UK	
Total	**57**	**16 countries**	

APPENDIX 2: DNA QUANTIFICATION PROTOCOL USING THE TWISTA MACHINE

1. Prepare 2X SYBR Gold solution

   In a 15ml Falcon tube prepare a 2X SYBR Gold working solution:

- Pipette 5ml of TE buffer into the tube
- Add 1µl SYBR Gold to the TE
- Vortex and protect from light

2. Prepare 2ng/µl DNA solution

   This step may vary depending on the concentration of the genomic DNA.

   Example using 200µg/ml (200ng/µl) DNA stock:

   $\frac{200ng/\mu l}{2ng/\mu l} = 100$-fold dilution required

- Add 10µl of stock DNA to 990µl TE buffer.
- Vortex solution for 1 minute to ensure DNA is properly mixed.

  Must be used on day of preparation.

3. Reaction setup

   For each strip of 8 PCR tubes, tubes 1-4 are used for the standard curve and tubes 5-8 for the samples. Samples should be tested in duplicate.

   The range of quantification is between 0.1 and 0.7ng/µl final concentration, consequently the sample must be diluted down to that range. For gel purified PCR products or miniprep plasmid DNA a 1:100 or 1:50 final dilution can be used. If the sample mV value obtained is too low or too high the sample should be retested at a different dilution.

Contents of tubes for standards:

Tube	1	2	3	4
TE Buffer (µl)	50	45	25	15
2ng/µl DNA (µl)	0	5	25	35
2x SYBR Gold solution (µl)	50	50	50	50
Final DNA concentration after addition of 2x SYBR Gold solution (ng/µl)	0	0.1	0.5	1

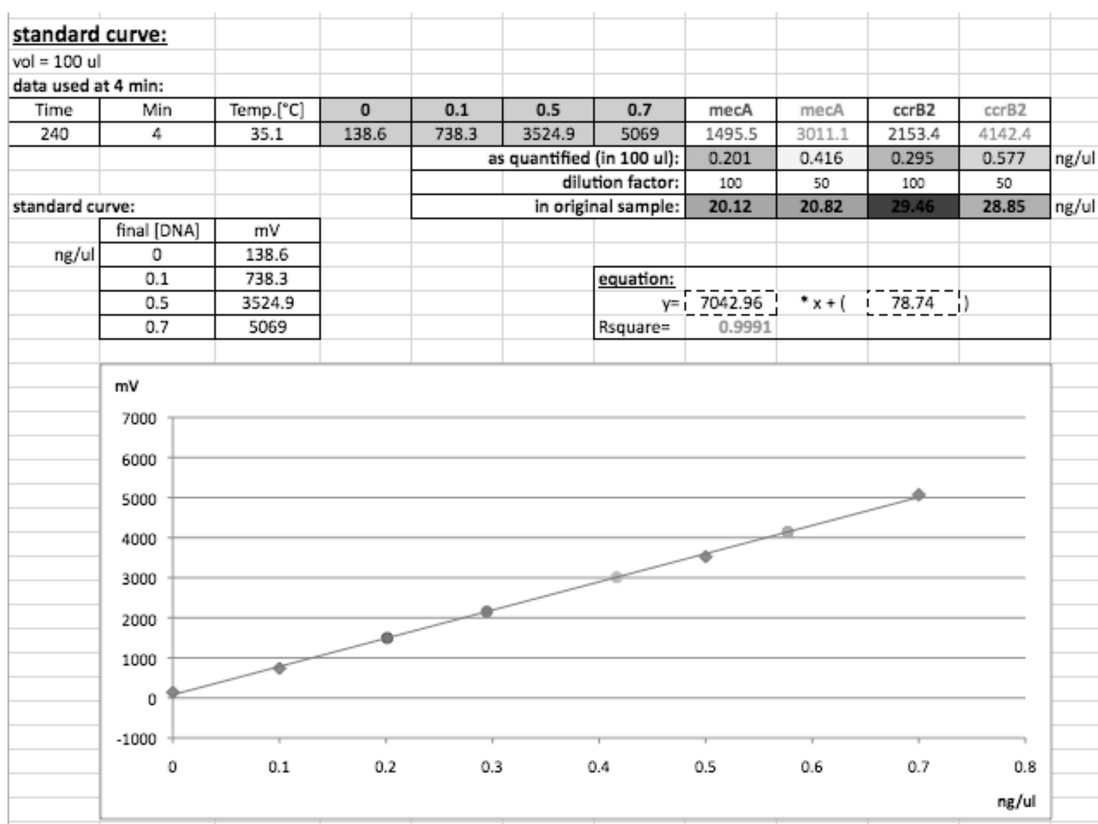
Contents of tubes for samples:

Tubes 5-8 should be filled according to the required dilutions in the following table.

Tube	5	6	7	8
Dilution	1:25	1:50	1:100	1:200
Sample (µl)	4	2	1	0.5
TE Buffer (µl)	46	48	49	49.5
2x SYBR Gold solution (µl)	50	50	50	50

4. Starting the reaction

- After 50µl of 2X SYBR Gold solution has been added to all eight tubes, seal using caps.
- Vortex and spin tubes, place in Twista machine and begin data acquisition (8 mins at 35°C).
- Analyse the data using the DNA quantification template. Example output:



APPENDIX 3: RPA-BASED MULTIPLEX SCCMEC TYPING ASSAY

Table Primer and probe sequences for the RPA-based SCC*mec* multiplex assay (continued on next page).

Target	Sequence (5' → 3')	Amplicon size (bp)	Specificity (SCC*mec* type)
mecA	Forward TCCAGATTACAACTTCACCAGGTTCAACTCAA		
	Reverse TTGTAACGTTGTAACCACCCCAAGATTTATCTT	145	All
	Probe ATAACAAAACATTAGACGATAAAACAAG-T(TAMRA)-dSpacer-T(BHQ-2)-AAAATCGATGGTAAAG		
mecI	Forward ACAAAGGCGGTTTCAATTCACTTGTCTTAAAC		
	Reverse ATTCAACGACTTGATTGTTTCCTCTGTTTTCT	158	II, III, VIII
	Probe TGTAGAAAAAGAAGATCTATCACAAGA-T(ROX)-GA-dSpacer-A-T(BHQ-2)-AGAATAATTGAGAAA		
mecC2	Forward ATGCAGTATACGGTTCATATCGATGATCAAATTA		
	Reverse CTTGTACCTCTCCTGCATATTCTGGTTGTAAC	139	V
	Probe GAGTGTACCAAAAAGTTTAAAATTTTGCC-T(FAM)-dSpacer-T(BHQ-1)-GGAAGATAAAATTCC		
IS1272	Forward GCAAATACATGGAGGTCAATATGGAAACAAAA		
	Reverse AGAATCACTTTTAACATCATTTTAGGATGGTACGA	156	I, IV, VI
	Probe TTTTACTGTAAATGATATTGTTGAAACAA-T(HEX)-dSpacer-CC-T(BHQ-1)-GACAATGAATTCGA		

ccrB1	Forward TGAACGTATGGAAGTCAAAAATTCAACAGGCA		
	Reverse GGAAGATTGCCTTGATAATAGCCCTCTAAAGC	150	I
	Probe GCAAGTTTTTCCGAATTCGAAAGAAA-T(ROX)-ACA-dSpacer-T(BHQ-2)-TTTAGAGAATATTTAC		
ccrB2	Forward ACCACGGTCAGCGTATATATCTTTAACTTCAA		
	Reverse TGCAACAACTTAAAACAAAACGTGTCGGTATC	158	II, IV
	Probe TATCAACAGAAATGCAAAGCACAGAAGGT-T(HEX)-dSpacer-T(BHQ-1)-AGTATCGACGGACAAA		
ccrB4	Forward TGCAAACGGATGGTTACAGTATTCAWGGTCAAT		
	Reverse CTTTTAACATACGTTGTAATTCWGGACGTTG	150	VI, VIII
	Probe ACGACTTCATAACCTTGAAATTGACAATAC-T(TAMRA)-C-dSpacer-G-T(BHQ-2)-AAGTTGATTYAATTGACC		
ccrC	Forward CATCCAATGCTTCTGGAGAAGTACTCGTTACA		
	Reverse TGATAATTTGTAGCCTAAAACACGACCGGTGA	155	III, V, VII
	Probe TGGAGAAGTACTCGTTACAATGTTTGGGT-T(FAM)-dSpacer-A-T(BHQ-1)-AGGATCTATMGAACG		

Table Multiplex format for the RPA-based SCC*mec* typing assay. + denotes a positive result for that SCC*mec* type.

	Tube 1				**Tube 2**			
Target	*mecA*	*mecI*	*mecC2*	*IS1272*	*ccrB1*	*ccrB2*	*ccrB4*	*ccrC*
SCC*mec* type								
I	+			+	+			
II	+	+				+		
III	+	+						+
IV	+			+		+		
V	+		+					+
VI	+			+			+	
VII	+							+
VIII	+	+					+	

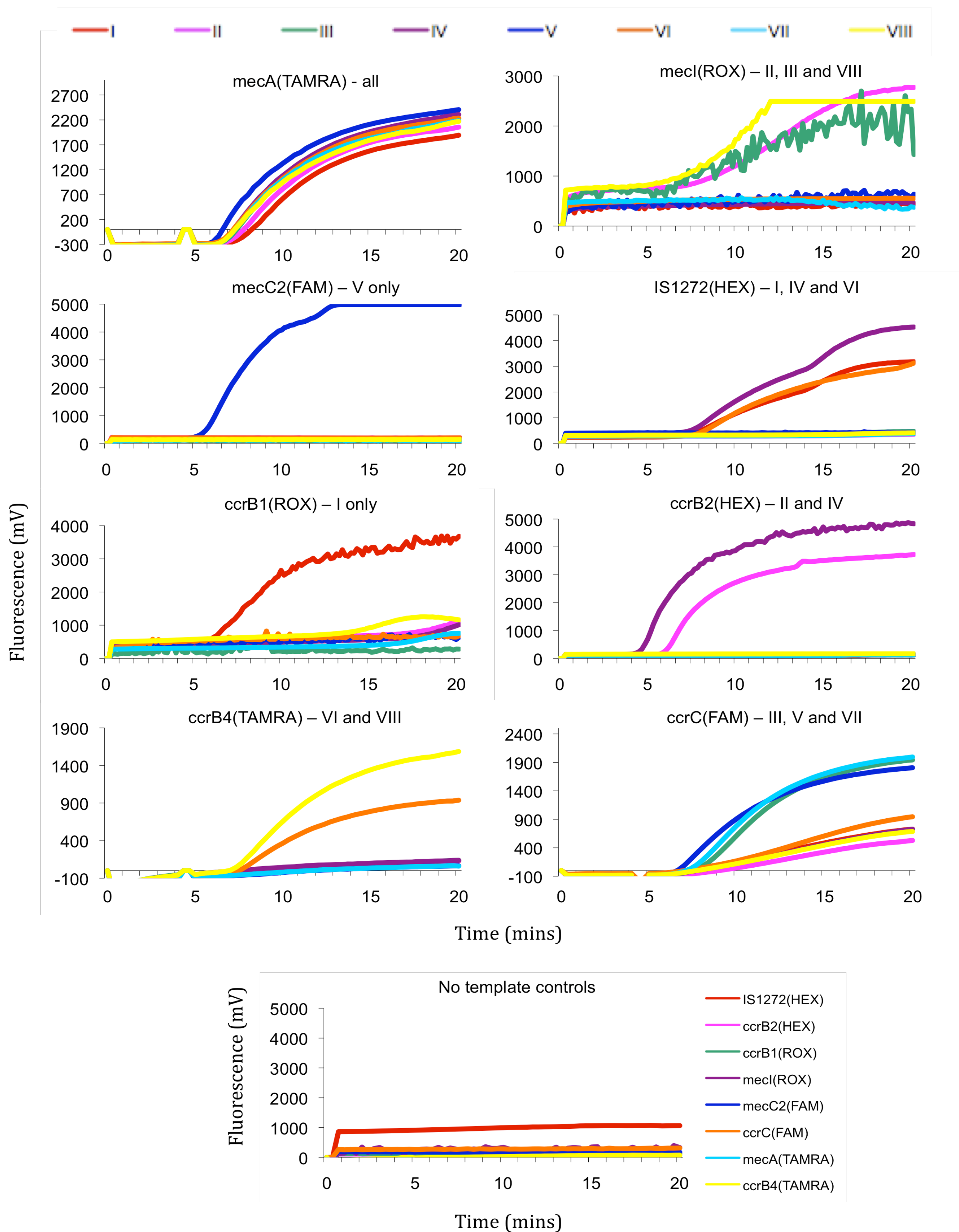


Figure Singleplex TwistAmp exo reactions for each primer pair and probe of the SCC*mec* multiplex assay, to test their specificity. 1µl of DNA template (~9k copies/µl) was added to 50µl TwistAmp exo reactions. Note that the ccrB4 fluorescence is weak, and that the jagged lines, particularly in mecI, were thought to be a result of the magnetic mixing in the pre-production, second-generation fluorometer (the magnetic bead sometimes blocked the fluorescence reader).

APPENDIX 4: SPA TYPES BY OC HOSPITAL

Table *spa* type frequencies by hospital for the 2,246 clinical MRSA isolates collected from 30 hospitals in Orange County, CA.

Hospital	*spa* type	Frequency	%
1	t008	17	63.0
	t242	4	14.8
	t002	2	7.4
	t024	1	3.7
	t306	1	3.7
	t622	1	3.7
	t955	1	3.7
	Total	27	100.0
2	t008	60	50.4
	t242	21	17.6
	t002	14	11.8
	t024	4	3.4
	t045	3	2.5
	t088	3	2.5
	t127	3	2.5
	t306	2	1.7
	t324	2	1.7
	t010	1	0.8
	t1774	1	0.8
	t1911	1	0.8
	t211	1	0.8
	t6069	1	0.8
	t6071	1	0.8
	t688	1	0.8
	Total	119	100.0

3	t008	21	47.7
	t002	12	27.3
	t242	5	11.4
	t024	2	4.5
	t045	1	2.3
	t1882	1	2.3
	t6072	1	2.3
	t723	1	2.3
	Total	44	100.0
4	t008	66	72.5
	t242	11	12.1
	t024	2	2.2
	t5654	2	2.2
	t002	1	1.1
	t005	1	1.1
	t068	1	1.1
	t088	1	1.1
	t105	1	1.1
	t1220	1	1.1
	t1578	1	1.1
	t216	1	1.1
	t2689	1	1.1
	t668	1	1.1
	Total	91	100.0
5	t008	13	81.3
	t024	1	6.3
	t068	1	6.3
	t088	1	6.3
	Total	16	100.0
6	t008	71	59.2
	t002	19	15.8

	t242	16	13.3
	t088	3	2.5
	t2468	2	1.7
	t723	2	1.7
	t044	1	0.8
	t121	1	0.8
	t2104	1	0.8
	t211	1	0.8
	t324	1	0.8
	t622	1	0.8
	t852	1	0.8
	Total	120	100.0
7	t008	23	79.3
	t002	2	6.9
	t211	2	6.9
	t121	1	3.4
	t1610	1	3.4
	Total	29	100.0
8	t008	70	50.0
	t242	27	19.3
	t002	22	15.7
	t024	3	2.1
	t045	3	2.1
	t088	2	1.4
	t127	2	1.4
	t1737	2	1.4
	t026	1	0.7
	t037	1	0.7
	t068	1	0.7
	t126	1	0.7
	t1300	1	0.7
	t4783	1	0.7

	t548	1	0.7
	t6321	1	0.7
	t6352	1	0.7
	Total	140	100.0
9	t008	28	52.8
	t002	9	17.0
	t242	6	11.3
	t088	2	3.8
	t127	2	3.8
	t306	2	3.8
	t359	2	3.8
	t105	1	1.9
	t723	1	1.9
	Total	53	100.0
10	t008	17	45.9
	t242	10	27.0
	t002	8	21.6
	t126	1	2.7
	t127	1	2.7
	Total	37	100.0
11	t242	2	50.0
	t008	1	25.0
	t2689	1	25.0
	Total	4	100.0
12	t242	40	35.7
	t008	31	27.7
	t002	20	17.9
	t1774	3	2.7
	t045	2	1.8
	t088	2	1.8

	t1737	2	1.8
	t010	1	0.9
	t064	1	0.9
	t068	1	0.9
	t1196	1	0.9
	t121	1	0.9
	t1683	1	0.9
	t2032	1	0.9
	t311	1	0.9
	t579	1	0.9
	t6068	1	0.9
	t622	1	0.9
	t6591	1	0.9
	Total	112	100.0
13	t008	2	33.3
	t002	1	16.7
	t024	1	16.7
	t242	1	16.7
	t570	1	16.7
	Total	6	100.0
14	t002	5	33.3
	t008	4	26.7
	t088	2	13.3
	t242	2	13.3
	t024	1	6.7
	t1737	1	6.7
	Total	15	100.0
15	t002	20	30.3
	t242	19	28.8
	t008	9	13.6
	t037	6	9.1

	t018	3	4.5
	t010	1	1.5
	t067	1	1.5
	t088	1	1.5
	t1737	1	1.5
	t304	1	1.5
	t530	1	1.5
	t548	1	1.5
	t6066	1	1.5
	t6868	1	1.5
	Total	66	100.0
16	t008	56	48.3
	t242	27	23.3
	t002	17	14.7
	t045	2	1.7
	t127	2	1.7
	t018	1	0.9
	t024	1	0.9
	t037	1	0.9
	t064	1	0.9
	t121	1	0.9
	t2054	1	0.9
	t2558	1	0.9
	t5160	1	0.9
	t6070	1	0.9
	t622	1	0.9
	t6352	1	0.9
	t842	1	0.9
	Total	116	100.0
17	t008	37	69.8
	t002	5	9.4
	t242	5	9.4

	t1081	2	3.8
	t024	1	1.9
	t190	1	1.9
	t622	1	1.9
	t688	1	1.9
	Total	53	100.0
18	t008	50	38.8
	t242	40	31.0
	t002	19	14.7
	t127	4	3.1
	t045	3	2.3
	t018	1	0.8
	t024	1	0.8
	t068	1	0.8
	t121	1	0.8
	t160	1	0.8
	t1791	1	0.8
	t2229	1	0.8
	t306	1	0.8
	t442	1	0.8
	t548	1	0.8
	t6066	1	0.8
	t6073	1	0.8
	t688	1	0.8
	Total	129	100.0
19	t008	50	37.0
	t242	34	25.2
	t002	22	16.3
	t064	5	3.7
	t976	3	2.2
	t088	2	1.5
	t127	2	1.5

	t6065	2	1.5
	t6340	2	1.5
	t010	1	0.7
	t024	1	0.7
	t045	1	0.7
	t1220	1	0.7
	t126	1	0.7
	t1300	1	0.7
	t2302	1	0.7
	t4695	1	0.7
	t4919	1	0.7
	t509	1	0.7
	t586	1	0.7
	t622	1	0.7
	t723	1	0.7
	Total	135	100.0
20	t008	26	50.0
	t242	9	17.3
	t002	6	11.5
	t024	2	3.8
	t306	2	3.8
	t018	1	1.9
	t037	1	1.9
	t062	1	1.9
	t267	1	1.9
	t3424	1	1.9
	t570	1	1.9
	t6337	1	1.9
	Total	52	100.0
21	t008	19	35.8
	t242	15	28.3
	t002	10	18.9

	t304	2	3.8
	t306	2	3.8
	t018	1	1.9
	t045	1	1.9
	t121	1	1.9
	t1578	1	1.9
	t6212	1	1.9
	Total	53	100.0
22	t008	8	40.0
	t242	4	20.0
	t2468	2	10.0
	t002	1	5.0
	t024	1	5.0
	t037	1	5.0
	t6238	1	5.0
	t967	1	5.0
	t976	1	5.0
	Total	20	100.0
23	t008	42	35.0
	t242	35	29.2
	t002	20	16.7
	t037	7	5.8
	t1860	3	2.5
	t024	2	1.7
	t105	2	1.7
	t126	1	0.8
	t1627	1	0.8
	t1737	1	0.8
	t2173	1	0.8
	t6072	1	0.8
	t6338	1	0.8
	t6339	1	0.8

	t6354	1	0.8
	t895	1	0.8
	Total	120	100.0
24	t008	58	45.3
	t242	24	18.8
	t002	20	15.6
	t1737	4	3.1
	t037	3	2.3
	t062	3	2.3
	t1341	2	1.6
	t306	2	1.6
	t6353	2	1.6
	t024	1	0.8
	t1084	1	0.8
	t121	1	0.8
	t127	1	0.8
	t197	1	0.8
	t2225	1	0.8
	t4146	1	0.8
	t6067	1	0.8
	t6219	1	0.8
	t6592	1	0.8
	Total	128	100.0
25	t242	62	52.5
	t002	21	17.8
	t008	19	16.1
	t024	2	1.7
	t045	2	1.7
	t1341	2	1.7
	t1683	2	1.7
	t040	1	0.8
	t1737	1	0.8

	t1791	1	0.8
	t189	1	0.8
	t2032	1	0.8
	t3746	1	0.8
	t509	1	0.8
	t6593	1	0.8
	Total	118	100.0
26	t002	5	41.7
	t008	4	33.3
	t019	1	8.3
	t045	1	8.3
	t242	1	8.3
	Total	12	100.0
27	t008	59	41.3
	t002	33	23.1
	t242	21	14.7
	t105	4	2.8
	t024	2	1.4
	t037	2	1.4
	t045	2	1.4
	t067	2	1.4
	t126	2	1.4
	t6068	2	1.4
	t004	1	0.7
	t018	1	0.7
	t064	1	0.7
	t088	1	0.7
	t121	1	0.7
	t148	1	0.7
	t211	1	0.7
	t2164	1	0.7
	t2293	1	0.7

	t306	1	0.7
	t351	1	0.7
	t400	1	0.7
	t6611	1	0.7
	t895	1	0.7
	Total	143	100.0
28	t008	37	45.7
	t242	17	21.0
	t002	11	13.6
	t037	3	3.7
	t024	2	2.5
	NT	1	1.2
	t040	1	1.2
	t045	1	1.2
	t064	1	1.2
	t1578	1	1.2
	t1911	1	1.2
	t2063	1	1.2
	t306	1	1.2
	t447	1	1.2
	t4919	1	1.2
	t767	1	1.2
	Total	81	100.0
29	t008	65	74.7
	t002	5	5.7
	t242	4	4.6
	t1635	2	2.3
	t211	2	2.3
	t024	1	1.1
	t127	1	1.1
	t1767	1	1.1
	t1892	1	1.1
	t2104	1	1.1
	t2115	1	1.1

	t6869	1	1.1
	Total	87	100.0
30	t008	71	59.2
	t002	17	14.2
	t242	16	13.3
	t622	3	2.5
	t019	1	0.8
	t024	1	0.8
	t027	1	0.8
	t088	1	0.8
	t121	1	0.8
	t1391	1	0.8
	t1567	1	0.8
	t1677	1	0.8
	t197	1	0.8
	t304	1	0.8
	t450	1	0.8
	t6336	1	0.8
	t6341	1	0.8
	Total	120	100.0

APPENDIX 5: SPA TYPES BY OC NURSING HOME

Table *spa* type frequencies by nursing home for the 835 carriage MRSA isolates collected from 25 nursing homes in Orange County, CA.

Nursing Home	*spa* type	Frequency	%
1	t002	30	36.6
	t008	29	35.4
	t242	16	19.5
	t018	2	2.4
	t037	1	1.2
	t2031	1	1.2
	t437	1	1.2
	t4963	1	1.2
	t895	1	1.2
	Total	82	100.0
2	t242	8	44.4
	t002	5	27.8
	t008	1	5.6
	t189	1	5.6
	t2049	1	5.6
	t548	1	5.6
	NT	1	5.6
	Total	18	100.0
3	t008	14	35.9
	t002	12	30.8
	t242	9	23.1
	t189	2	5.1
	t067	1	2.6
	t400	1	2.6
	Total	39	100.0

4	t002	7	70.0
	t008	1	10.0
	t1610	1	10.0
	t242	1	10.0
	Total	10	100.0
5	t242	10	35.7
	t008	9	32.1
	t002	8	28.6
	t1341	1	3.6
	Total	28	100.0
6	t002	18	40.0
	t008	15	33.3
	t242	10	22.2
	t1610	1	2.2
	t8606	1	2.2
	Total	45	100.0
7	t242	13	38.2
	t002	8	23.5
	t008	7	20.6
	t8750	2	5.9
	t037	1	2.9
	t088	1	2.9
	t1341	1	2.9
	t211	1	2.9
	Total	34	100.0
8	t242	6	50.0
	t008	4	33.3
	t1341	1	8.3

	t736	1	8.3
	Total	12	100.0
9	t242	19	35.9
	t306	8	15.1
	t008	7	13.2
	t068	6	11.3
	t002	4	7.6
	t8748	2	3.8
	t024	1	1.9
	t026	1	1.9
	t127	1	1.9
	t189	1	1.9
	t548	1	1.9
	t6065	1	1.9
	t723	1	1.9
	Total	53	100.0
10	t242	17	50.0
	t008	12	35.3
	t002	3	8.8
	t045	1	2.9
	t2115	1	2.9
	Total	34	100.0
11	t008	33	45.2
	t002	13	17.8
	t242	13	17.8
	t088	5	6.9
	t037	2	2.7
	t306	2	2.7
	t539	2	2.7
	t010	1	1.4

	t024	1	1.4
	t105	1	1.4
	Total	73	100.0
12	t242	19	50.0
	t002	9	23.7
	t1774	5	13.2
	t008	1	2.6
	t121	1	2.6
	t306	1	2.6
	t8747	1	2.6
	t8865	1	2.6
	Total	38	100.0
13	t242	20	52.6
	t002	9	23.7
	t008	7	18.4
	t548	1	2.6
	t8749	1	2.6
	Total	38	100.0
14	t002	12	54.6
	t008	5	22.7
	t010	4	18.2
	t242	1	4.6
	Total	22	100.0
15	t002	3	75.0
	t1932	1	25.0
	Total	4	100.0
16	t242	19	51.4
	t008	7	18.9

	t002	3	8.1
	t5916	3	8.1
	t071	2	5.4
	t032	1	2.7
	t509	1	2.7
	t579	1	2.7
	Total	37	100.0
17	t242	15	62.5
	t002	3	12.5
	t024	3	12.5
	t8086	2	8.3
	t127	1	4.2
	Total	24	100.0
18	t008	1	100.0
	Total	1	100.0
19	t008	8	72.7
	t002	2	18.2
	t242	1	9.1
	Total	11	100.0
20	t008	24	38.7
	t002	19	30.7
	t242	13	21.0
	t127	2	3.2
	t037	1	1.6
	t040	1	1.6
	t1080	1	1.6
	t1220	1	1.6
	Total	62	100.0

21	t242	30	46.9
	t002	12	18.8
	t008	9	14.1
	t127	7	10.9
	t088	2	3.1
	t045	1	1.6
	t2879	1	1.6
	t509	1	1.6
	t581	1	1.6
	Total	64	100.0
22	t002	2	25.0
	t008	2	25.0
	t242	2	25.0
	t548	1	12.5
	t8444	1	12.5
	Total	8	100.0
23	t008	13	29.6
	t002	7	15.9
	t1186	5	11.4
	t242	4	9.1
	t4462	4	9.1
	t037	2	4.6
	t548	2	4.6
	t5916	2	4.6
	t064	1	2.3
	t088	1	2.3
	t2229	1	2.3
	t8443	1	2.3
	Total	43	100.0
24	t242	27	51.9

	t008	9	17.3
	t002	6	11.5
	t045	2	3.9
	t018	1	1.9
	t024	1	1.9
	t088	1	1.9
	t127	1	1.9
	t1737	1	1.9
	t400	1	1.9
	t7275	1	1.9
	Total	51	100.0
25	t008	4	100.0
	Total	4	100.0

APPENDIX 6: PUBLICATIONS

Hudson LO, Murphy CR, Spratt BG, Enright MC, Terpstra L, Gombosev A, et al. 2012. Differences in Methicillin-Resistant *Staphylococcus aureus* Strains Isolated from Paediatric and Adult Patients from Hospitals in a Large County in California. J Clin Microbiol. **50**: 573-9.

Ke W, Huang SS, Hudson LO, Elkins KR, Nguyen CC, Spratt BG, et al. 2012. Patient sharing and population genetic structure of methicillin-resistant *Staphylococcus aureus*. Proc Natl Acad Sci U S A. **109**: 6763-8.

The above manuscripts are presented in their published form on the following pages:

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Differences in Methicillin-Resistant *Staphylococcus aureus* Strains Isolated from Pediatric and Adult Patients from Hospitals in a Large County in California

Lyndsey O. Hudson,[a] Courtney R. Murphy,[b] Brian G. Spratt,[a] Mark C. Enright,[c] Leah Terpstra,[d] Adrijana Gombosev,[d] Paul Hannah,[e] Lydia Mikhail,[e] Richard Alexander,[e] Douglas F. Moore,[e] and Susan S. Huang[d]

Department of Infectious Disease Epidemiology, Imperial College London, London, United Kingdom[a]; School of Social Ecology, and Division of Infectious Diseases, University of California Irvine School of Medicine, Irvine, California, USA[b]; AmpliPhi Biosciences, Colworth Science Park, Sharnbrook, Bedfordshire, United Kingdom[c]; Division of Infectious Diseases and Health Policy Research Institute, University of California Irvine School of Medicine, Irvine, California, USA[d]; and Orange County Health Care Agency, Santa Ana, California, USA[e]

Studies of U.S. epidemics of community- and health care-associated methicillin-resistant *Staphylococcus aureus* (MRSA) suggested differences in MRSA strains in adults and those in children. Comprehensive population-based studies exploring these differences are lacking. We conducted a prospective cohort study of inpatients in Orange County, CA, collecting clinical MRSA isolates from 30 of 31 Orange County hospitals, to characterize differences in MRSA strains isolated from children compared to those isolated from adults. All isolates were characterized by *spa* typing. We collected 1,124 MRSA isolates from adults and 159 from children. Annual Orange County population estimates of MRSA inpatient clinical cultures were 119/100,000 adults and 22/100,000 children. *spa* types t008, t242, and t002 accounted for 83% of all isolates. The distribution of these three *spa* types among adults was significantly different from that among children ($\chi^2 = 52.29$; $P < 0.001$). Forty-one percent of adult isolates were of t008 (USA300), compared to 69% of pediatric isolates. In multivariate analyses, specimens from pediatric patients, wounds, non-intensive care unit (ICU) wards, and hospitals with a high proportion of Medicaid-insured patients were significantly associated with the detection of t008 strains. While community- and health care-associated MRSA reservoirs have begun to merge, significant differences remain in pediatric and adult patient populations. Community-associated MRSA *spa* type t008 is significantly more common in pediatric patients.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major global cause of morbidity and mortality, imposing serious economic costs on patients and hospitals (1, 6, 7, 13, 26, 53). Prior to the mid-1990s, MRSA was largely a health care-associated pathogen, causing infection predominantly in people with frequent or recent contact with health care facilities (health care-associated MRSA [HA-MRSA]). In the United States, the rates of MRSA carriage (both asymptomatic and symptomatic) are estimated to be 6 to 12% in general hospital patient populations and 9 to 24% in intensive care unit populations (ICUs) (23, 32, 50). Although HA-MRSA has long been the primary cause of MRSA infections, community-associated MRSA (CA-MRSA), which often causes infections among healthy children and young adults with no exposure to the health care setting, is becoming increasingly prevalent. The first reports of MRSA isolated from patients with no identifiable risk factors came from Australia and the United States in the 1990s (5, 21, 56). Since then, the prevalence of CA-MRSA has rapidly increased, with reports of CA-MRSA infection from virtually every geographic region of the world (55, 59). The incidence of life-threatening invasive infections owing to CA-MRSA is increasing, and CA-MRSA appears to be particularly virulent among children (38). Moreover, CA-MRSA has caused outbreaks in the hospital setting (4, 41, 51), with some reports suggesting that it may be replacing HA-MRSA (8, 46, 49, 52).

In the United States, the predominant community-associated MRSA clone is now USA300 (defined by *spa* typing and multilocus sequence typing [MLST] as t008 and ST8, respectively), having rapidly disseminated and replaced USA400 (t128/ST1) since its appearance in 2000. USA300 has several characteristics that may offer a selective advantage over other MRSA clones, both community associated (e.g., USA400) and health care associated (e.g., USA100 [t002/ST5]). These advantages include (i) a smaller staphylococcal cassette chromosome *mec* (SCC*mec*) element (usually type IV) than those of health care-associated strains (usually SCC*mec* types I to III), which is more readily transmissible and may be an advantage in terms of the DNA replication speed; (ii) fewer antibiotic resistance genes than health care-associated strains, resulting in a fitness benefit due to the carriage of smaller or fewer genes; and (iii) a higher growth rate, which may lead to successful colonization by outcompeting health care-associated strains (8, 43). Furthermore, the linkage of an arginine catabolic mobile element with SCC*mec* type IV in USA300 likely confers increased fitness and/or pathogenicity (11). Finally, high levels of expression of regulatory genes associated with the virulence factors Panton-Valentine leukocidin and alpha-toxin have been shown for USA300 versus USA400 isolates, which may contribute to the invasiveness of USA300 (39).

The phenotypic and genotypic differences between HA- and

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Address correspondence to Brian G. Spratt, b.spratt@imperial.ac.uk.
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CA-MRSA strains have been well documented (2, 10, 12, 33, 43), yet there are few studies that have directly explored the differences in MRSA strains isolated from adults and those isolated from children. Park et al. (44) previously compared a small number of adult and pediatric MRSA isolates in a South Korean hospital and found a predominance of CA-MRSA isolates among children. A better understanding of the frequency of community- versus health care-associated MRSA clones among adults and children, and in particular the USA300 clone, may inform strategies to prevent transmission and disease.

Children may have different exposures to MRSA, as they constitute a largely healthy population that is most likely to incur MRSA infection through skin and soft tissue injuries related to sports and other play activities (16). This is in contrast to the chronically and critically ill adult population, which frequents hospitals and may encounter health care-associated MRSA strains more readily. Furthermore, children may experience different antimicrobial drug selection pressure compared to that of adults due to differences in common disease syndromes and different guidance on antibiotic therapy (9, 44).

Defining the characteristics of MRSA strains in adults and children would provide insight into the spread of MRSA strains, particularly since there is growing evidence that community and health care MRSA reservoirs are mixing (28, 30, 34, 49, 52). Furthermore, few studies of adult or pediatric MRSA strains have involved a population-based sample of strains. We conducted a prospective cohort study of inpatients in a large metropolitan county to characterize differences in pediatric and adult MRSA strains.

MATERIALS AND METHODS

Study. We conducted a population-based, prospective collection of clinical isolates of MRSA from 30 of 31 hospitals in Orange County, CA. This study was approved by the Institutional Review Board of the University of California Regents.

Isolate collection. Clinical (nonscreening) isolates of MRSA from unique adult patients (≥18 years of age) and unique pediatric patients (<18 years of age) were collected from hospital microbiology laboratories. Hospitals were instructed to collect MRSA isolates from unique patients up to a total of 100 isolates or for a duration of 12 months, whichever came first. In order to have a representative sample of Orange County MRSA isolates, we limited isolates in this study to those collected for a uniform duration of time from adult hospitals. Since the largest adult hospitals reached 100 isolates over a 5-month period, we restricted the period of all adult isolate collections to 5 months. All pediatric hospitals required a 12-month collection period. Nearly all adult isolates were collected between December 2008 and April 2009. Pediatric isolates were collected between October 2008 and September 2009. Isolates from patients not admitted to hospitals were excluded from the study. Samples were batched and delivered to the Orange County Public Health Laboratory using soy agar slants. For the repeated confirmation of MRSA, isolates were plated onto selective medium for MRSA (BD CHROMagar). MRSA strains were stored at −65°C in 15% glycerol Brucella broth.

Specimen data and hospital characteristics. Specimen data, including patient age in years, specimen source (wound, blood, urine, sputum, or other), specimen location (ICU or non-ICU), and time of specimen collection with respect to admission date (hospital onset [HO], ≥3 days after admission; community onset [CO], <3 days after admission), were collected. Hospital characteristics were obtained from a California hospital data set (42), which included annual admissions, hospital type (acute care versus long-term acute care [LTAC] facility), percentage of Medicaid-insured patients, and percentage of Hispanic patients. Population estimates of adults and children in Orange County were obtained from the 2010 U.S. Census (57).

Laboratory methods and molecular typing. All strains were shipped to Imperial College London in the United Kingdom for *spa* typing and stored at −80°C. Cells were harvested on blood agar plates (Oxoid) and incubated at 37°C overnight. DNA was extracted by using a Qiagen DNeasy Blood & Tissue kit. DNA samples were eluted in 200 μl of elution buffer (10 mM Tris-Cl, 0.5 mM EDTA [pH 9.0]) and stored at −20°C. Following the sequencing of the *spa* region, *spa* types were determined by using Ridom StaphType v2.1 (Ridom GmbH, Würzburg, Germany) (20). To assess *spa* type diversity and relatedness, cluster analysis of *spa* types was performed separately for adult and pediatric isolates by using the Based upon Repeat Pattern (BURP) algorithm, a built-in feature of the StaphType software (35). MLST and SmaI pulsed-field gel electrophoresis (PFGE) were performed on a subset of the isolates ($n = 171$), to confirm MRSA strain types, according to methods described previously (14, 48). This subset included one isolate of each *spa* type and, for the 10 most common *spa* types, one isolate from each of the hospitals in which these *spa* types were present. Isolates were selected by using a random number generator. For PFGE, DNA profiles were analyzed by using BioNumerics software (version 5.0, 2007; Applied Maths). PFGE types were defined using a similarity coefficient of 78%, and USA100 to USA800 strains were used as references.

Statistical analyses. Annual adult and pediatric population estimates of hospitalized patients with clinical MRSA cultures were calculated by *spa* type, accounting for the duration of countywide collection. We further calculated the percentage of MRSA strains from adult versus pediatric patients that were due to the most common *spa* types (t008, t242, and t002) and compared them by using χ^2 tests. Specimen data for t008, t242, and t002 isolates were compared by using χ^2 or Fisher's exact tests and, for patient age, the Wilcoxon Mann-Whitney test. Simpson's index of diversity ($1 - D$) was used to compare the genetic diversities of MRSA strains among adults and children. $1-D$ gives an unbiased measure of the probability of drawing two different *spa* types given the distribution of *spa* types in a sample (19). The 95% confidence intervals (CIs) were calculated as described previously (18). We conducted bivariate tests to evaluate the association of *spa* type t008 with individual variables, including age (adult/pediatric), specimen source (particularly wound and blood), time of specimen collection (community or hospital onset), and ward type (non-ICU/ICU). We also tested hospital-level variables, including annual admissions (greater or less than 10,000), LTAC facility, percentage of Hispanic patients, and percentage of Medicaid-insured patients. For multivariate analyses, variables with a P value of <0.1 were entered into a generalized linear mixed model clustered by hospital and were retained at an α value of ≤0.05 (xtmelogit, STATA release 11, 2009; Stata Corp.).

RESULTS

A total of 1,124 adult and 159 pediatric MRSA isolates were collected over the 5- and 12-month periods, respectively. A summary of the characteristics of the clinical MRSA strains collected is shown in Table 1. The median age of adults was 67 years (interquartile range [IQR], 50 to 81 years), and that of children was 2 years (IQR, 1 to 9 years).

t008, t242, and t002 were the predominant *spa* types in Orange County, accounting for 83% of all isolates (Table 2). The distribution of these *spa* types among adults (t008, 41%; t242, 23%; t002, 19%) was significantly different from that among children (t008, 69%; t242, 9%; t002, 6%) ($\chi^2 = 52.29$; $P < 0.001$). Annual population estimates of clinical inpatient MRSA infections were 119/100,000 adults and 22/100,000 children. Annual estimates by *spa* type were 48/100,000 adults and 15/100,000 children for t008, 27/100,000 adults and 2/100,000 children for t242, and 22/100,000 adults and 1/100,000 children for t002.

According to MLST, the t008 isolates in our study were of the

TABLE 1 Characteristics of clinical MRSA strains isolated from adult and pediatric patients

Characteristic	No. (%) of isolates		
	Adult[a]	Pediatric[b]	Total/overall
Total MRSA isolates	1,124 (87.6)	159 (12.4)	1,283 (100)
Specimen source of[c]:			
Wound/abscess	488 (43.4)	81 (55.9)	569 (44.8)
Sputum	331 (29.4)	27 (18.6)	358 (28.2)
Urine	109 (9.7)	4 (2.8)	113 (8.9)
Blood	104 (9.3)	7 (4.8)	111 (8.8)
Other[d]	92 (8.2)	26 (17.9)	118 (9.3)
Intensive care unit collection[e]	187 (16.7)	17 (11.8)	204 (16.1)
Hospital onset	399 (35.5)	40 (25.2)	439 (34.2)

[a] Collected for 5 months from hospitals serving adults.
[b] Collected for 12 months from hospitals serving children.
[c] Fourteen missing pediatric entries.
[d] According to brief notes in the data set, "other" specimen sources included the following anatomical locations or types of specimens: 5 ear; 5 eye; 3 buttock; 2 each of finger, leg, pleural, and skin; and 1 each of gastrointestinal, sinus, perineum, spleen, and umbilical for pediatric specimen sources and 8 leg; 7 foot, knee, and medical device related; 6 groin; 5 abdominal, spinal, and stool; 4 gastric; 4 hand; 3 back, pleural, and tissue; 2 each of ankle, body fluid, buttock, ear, eye, stump, synovial fluid, and unknown; and 1 each of drainage, gallbladder, hip, humerus, ileal crest, lung, pancreatic fluid, skin, and stoma.
[e] Nineteen missing entries (4 adult and 15 pediatric).

prototypic community clone USA300 (t008/ST8), and the t002 isolates were of the prototypic hospital clone USA100 (t002/ST5), with t242 isolates being identified as ST5 (Table 2). Comparison of t242 and t002 isolates for the following parameters revealed no significant difference: the proportion from each specimen source, the proportion of hospital and community onset, the proportion collected in ICU and non-ICU wards, and the age distribution of patients (all $P > 0.05$). Conversely, t008 isolates were significantly different from t242 and t002 isolates in the same tests ($P < 0.001$). t242 and t002 isolates shared the most common specimen source, sputum (34% and 38%, respectively), whereas wounds were the most common specimen source of t008 isolates (56%). PFGE of a sample of t242 and t002 isolates showed them to be predominantly USA100 isolates (data not shown).

BURP analysis of the *spa* types clustered the majority of adult isolates (97%) into three *spa* clonal complexes (*spa*-CCs) and most pediatric isolates (96%) into two *spa*-CCs (Fig. 1). *spa* types were clustered with either t008 (*spa*-CC008; community-associated strains) or t002 (*spa*-CC002; health care-associated strains), but in adults, a further *spa*-CC with founder t324 was identified (*spa*-CC324). Isolates in this *spa*-CC were characterized as ST72 isolates. For both adult and pediatric MRSA isolates, MLST results showed that all isolates in *spa*-CC008 were of ST8 and that all isolates in *spa*-CC002 were of either ST5 or a single-locus variant, ST105 (*spa* types t045, t088, and t1791 for the latter). According to the BURP algorithm, *spa* types that differ from all other *spa* types in the sample by more than 4 repeats, and thus which cannot be clustered into a *spa*-CC, are termed singletons. For adults, 10 (11.2%) *spa* types (40 [3.6%] isolates) were classified as singletons, and for children, 4 (18.2%) *spa* types (6 [3.8%] isolates) were classified as singletons. *spa* types of less than 5 repeats in length were excluded from the BURP analysis because no reliable evolutionary history can be inferred from "short" *spa* types (35). For adults, 2 (2.2%) *spa* types (2 [0.2%] isolates) were excluded, and for children, 1 (4.5%) *spa* type (1 [0.6%] isolate) was excluded. The estimated genetic diversity of MRSA isolates was significantly higher among adults than among children ($1-D = 75\%$ versus 51%) (Table 2).

In bivariate analyses, pediatric patients, wound specimens, isolation in a non-ICU ward, community-onset timing of collection, and isolation from a hospital with >10,000 annual admissions were associated with t008 (USA300) isolates (Table 3). In addition, admission to a hospital with a high proportion of Medicaid-insured patients or a high proportion of Hispanic patients was linearly associated with the recovery of t008 isolates. In multivariate analyses, isolates from pediatric patients, wounds, non-ICU wards, and hospitals with a high proportion of Medicaid-insured patients remained significantly associated with *spa* type t008 (Table 4). Isolates from hospitals with a high proportion of Hispanic patients were significantly more likely to be t008 isolates (20% higher odds of being a *spa* type t008 isolate per 10% increase in numbers of Hispanic patients). However, this finding was collinear with hospitals with a high proportion of Medicaid-insured patients and thus was removed from the multivariate model.

DISCUSSION

We conducted a prospective cohort study of inpatients in a large metropolitan county in Californian, collecting all clinical MRSA

TABLE 2 Ten most frequently found *spa* types among adult and pediatric patients in Orange County, CA[a]

Rank	Adult patients					Pediatric patients				
	spa type	MLST	No. of isolates	% of isolates	Cumulative %	*spa* type	MLST	No. of isolates	% of isolates	Cumulative %
1	t008	8	457	40.7	40.7	t008	8	110	69.2	69.2
2	t242	5	260	23.1	63.8	t242	5	14	8.8	78
3	t002	5	211	18.8	82.6	t002	5	9	5.7	83.7
4	t024	8	19	1.7	84.3	t024	8	3	1.9	85.5
5	t037	8	15	1.3	85.6	t045	5	2	1.3	86.8
6	t127	1	14	1.3	86.8	t068	8	2	1.3	88.1
7	t088	105	12	1.1	87.9	t2689	8	2	1.3	89.3
8	t1737	5	11	1	88.9	t324	72	2	1.3	90.6
9	t306	5	6	0.5	89.4	t622	8	2	1.3	91.8
10	t126	72	5	0.4	89.9	13 others		1 each	0.6 each	100

[a] The total numbers of *spa* types were 89 for adult patients and 22 for pediatric patients. Simpson's index of diversity (1-*D*) values were 75% (95% CI, 73%, 76%) for adult patients and 51% (95% CI, 41%, 60%) for pediatric patients. MLST, multilocus sequence type.

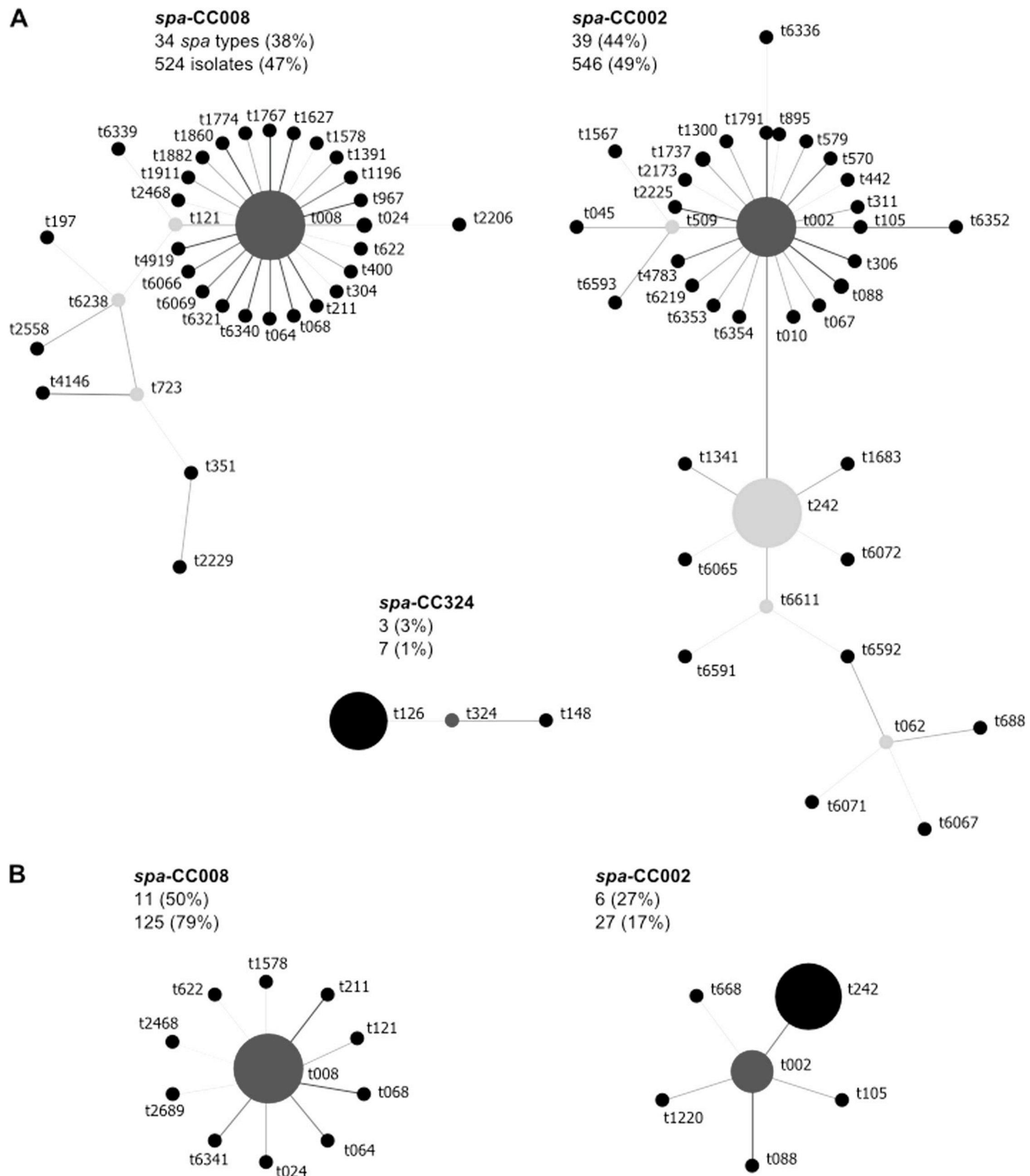

FIG 1 Relatedness of *spa* types among adult (A) and pediatric (B) MRSA isolates according to the Based upon Repeat Pattern (BURP) algorithm. Clusters of linked *spa* types correspond to *spa* clonal complexes (*spa*-CCs). *spa* types are clustered into a *spa*-CC when their repeat patterns differ by no more than 4 repeats. The BURP algorithm sums up "costs" (a measure of relatedness based on the repeat pattern) to define a founder score for each *spa* type in a *spa*-CC. The founder (blue node) is the *spa* type with the highest founder score in its *spa*-CC, and the subfounder (yellow node) is the *spa* type with the second highest founder score. *spa*-CC008 has founder t008. Each node represents a *spa* type. The node size represents the number of clustered strains that belong to that *spa* type. The shading of the branches represents the costs (similarities in repeat patterns) between two *spa* types; the darker the branch, the lower the cost (more similar repeat patterns).

isolates from 30 of 31 hospitals in order to characterize differences in pediatric and adult MRSA strains. To our knowledge, this is the first study to assess adult and pediatric MRSA isolates from a population-based sample across a large region.

Countywide, adult and pediatric clinical MRSA isolates were dominated by three *spa* types, two of which were consistent with the prototypic community- and health care-associated clones prevalent in the United States (t008 [USA300] and t002 [USA100]). t008 (USA300) was the most common single clone among both adult and pediatric isolates. Nevertheless, t008 comprised a large majority of pediatric isolates, whereas adult isolates were nearly equally divided among community- and health care-associated clones. Most other *spa* types were shown by BURP to be related to these two dominant clones. The two *spa* clonal complexes *spa*-CC008 and *spa*-CC002 can therefore be thought of as two distinct groups of isolates representing the major

TABLE 3 Bivariate analyses of variables associated with *spa* type t008

Variable	% of t008 isolates			
	Those with characteristic	Those without characteristic	χ^2	*P*
Individual				
Pediatric	69.81	40.75	47.67	<0.001
Community onset	48.10	37.13	14.09	<0.001
Non-ICU	47.17	27.45	27.00	<0.001
Blood specimen	40.54	44.30	0.58	0.446
Wound specimen	60.04	32.34	96.28	<0.001
LTAC	35.21	44.88	2.54	0.111
Hospital level				
>10,000 annual admissions	38.40	51.52	22.00	<0.001
Medicaid-insured patients[a]			1.34 (1.21–1.48)	<0.001
Hispanic patients[a]			1.29 (1.15–1.44)	<0.001

[a] Odds ratio per 10% increase.

community- and health care-associated MRSA strains prevalent in the United States.

Interestingly, t242/ST5 was slightly more common than t002/ST5 among both adult and pediatric isolates, despite the predominance of the t002/ST5 hospital clone in the United States. Given the similarities of t242 and t002 isolates in this study, and the fact that t242 differs from t002 by only one nucleotide (resulting in a different *spa* repeat pattern by one *spa* repeat), t242/ST5 presumably represents a minor variant of USA100 that has become prevalent in Orange County hospitals. t242 has been reported infrequently in the literature (24, 25, 60), with just one study reporting t242 at an endemic level in an Italian hospital (45).

The additional *spa* clonal complex identified among adult isolates included a community-onset isolate identified as a t324/ST72 isolate, an invasive community-associated MRSA clone reported for elderly patients in South Korea from 2006 to 2007, just before our isolate collection began (29). According to the U.S. Census Bureau, 17.9% of the Orange County population is Asian, approximately 2.9% of which is Korean (57).

There was significantly more genetic diversity among adult MRSA isolates than among pediatric isolates. This could simply represent the greater time that health care-associated clones have had to diversify at the *spa* locus than community-associated clones, which have emerged only in the past 2 decades. The greater MRSA diversity among adults could also be due to different degrees of contact; for example, adults may have more diverse MRSA encounters (travel, work, social venues, and health care facilities) than young children (schools and day care centers).

TABLE 4 Multivariate analysis of variables associated with *spa* type t008

Variable	Odds ratio	SE	95% CI	*P*
Patient/isolate characteristic				
Wound specimen	2.64	0.34	2.06, 3.39	<0.001
Pediatric	2.07	0.52	1.26, 3.40	0.004
Non-ICU	1.77	0.32	1.24, 2.54	0.002
Hospital characteristic				
% Medicaid-insured patients[a]	1.24	0.06	1.13, 1.35	<0.001

[a] Odds ratio per 10% increase.

The population estimates of clinical MRSA isolates in Orange County show that there was a 6-fold-higher frequency of inpatient MRSA clinical cultures among adults than among children. This pattern was consistent among the three most common *spa* types, t008, t242, and t002, and is likely a combination of more frequent hospitalizations among adults (many of whom were elderly, with a median age of 67 years) and more frequent MRSA carriage.

In multivariate analyses, community-associated MRSA clone t008 (USA300) was associated with pediatric patients. In contrast to adults, children are often healthier and are more likely to encounter MRSA in the community through exposure to high-density environments, such as schools, day cares, camps, and sporting activities, where close contact may facilitate the spread of community MRSA strains. In agreement with data from previous studies, we found that USA300 was associated with wounds, which is the most common presentation for hospitalization due to community-acquired MRSA infection (2, 17). USA300 was also associated with hospitals that treat a large fraction of Medicaid-insured patients, suggesting that community MRSA infections may be more prevalent among patients from economically disadvantaged or high-density areas.

USA300 was also associated with isolation from non-ICU wards, suggesting that this community strain is occurring in healthier hosts or is producing infections that are less severe than those caused by traditional health care-associated strains. Nevertheless, there is ample evidence that community strains are capable of producing fulminant infections (15, 37, 52). An understanding of what component of invasiveness is due to host comorbidities versus pathogen virulence factors is an area of active research.

Interestingly, we did not find that the isolation of t008 was associated with community-onset clinical isolates (clinical culture isolated less than 3 days after admission). This finding is likely due to the fact that the majority of health care-associated carriage or infection is found upon readmission to hospitals (27). It could also be explained by community-associated strains that have become endemic in some hospitals (49, 52).

Community- and health care-associated MRSA strains are becoming increasingly difficult to distinguish epidemiologically as community-associated strains continue to penetrate hospital MRSA reservoirs. Furthermore, it remains unclear whether com-

munity clones are adding to or replacing traditional health care-associated MRSA strains (3, 8, 22, 49). The implication of the blurred line between community- and health care-associated MRSA strains may be that efforts to control MRSA transmission within hospitals will not be effective in controlling community influx into hospitals. Simultaneous community strategies to limit MRSA spread are needed. However, much is still unknown about the acquisition and transmission of CA-MRSA, so improved knowledge is needed to better guide infection control strategies. Further studies are needed to ascertain whether community strategies to reduce transmission in children and young adults would produce benefits across the entire age spectrum.

One limitation of our study is that few individual-level characteristics were available. Also, our study did not account for the different policies in place at each hospital with regard to when to obtain clinical cultures. These differences could affect MRSA detection at each hospital and, possibly, the type of MRSA strains isolated, if clinical cultures were more likely to be obtained for sicker, older patients. Moreover, our results could have been affected by the potential seasonality of MRSA infections and infection types due to the different collection periods for adult and pediatric isolates (largely winter and spring for adult collections, compared to all seasons for pediatric collections). A seasonality of *S. aureus* infections, particularly skin infections, has been observed in pediatric and adult patients in temperate and tropical environments, with a predominance of infections during summer and autumn (31, 36, 54, 58). A recent study in Rhode Island found a 2- to 3-fold-increased incidence of MRSA infections (both CA- and HA-MRSA) in pediatric patients during the second two quarters of the year over the last decade (36). However, in that same study, adult CA-MRSA infections showed less seasonal variation than did pediatric infections, and no variation was observed among adult HA-MRSA infections. Some studies observed no significant seasonality of *S. aureus* infections, but those studies focused on bacteremia (40, 47). The collection of both adult and pediatric MRSA isolates for the same time period, i.e., 12 months, would have accounted for any potential seasonality effects and/or other factors that could affect the type and diversity of MRSA strains isolated.

Mandatory screening of high-risk inpatients was not in place in California until 2009; therefore, our population estimates are likely underestimates. In addition, our estimates should not be construed as measures of MRSA infection among inpatients. Clinical isolates often represent carriage without infection. Finally, our estimates of the index of diversity for adult and pediatric MRSA isolates may have been influenced by differing sample sizes (18).

In conclusion, our study found that in a large county, MRSA isolates from hospitalized children were more likely to be of *spa* type t008 (USA300). This community-associated *spa* type was associated with children, wounds, non-ICU care, and admission to a hospital with a high percentage of Medicaid-insured patients. Despite the association of t008 isolates with children, t008 was still the most common *spa* type among adult patients, suggesting that community-based interventions are needed to stem the influx of t008 isolates into hospitals. We also found evidence for a prevalent variant of the USA100 clone (t242/ST5), which has not been reported elsewhere. While community- and hospital-associated MRSA reservoirs have begun to merge, significant differences remain in pediatric and adult patient populations, which may provide an impetus for different age-based strategies to reduce transmission and disease.

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We do not have an association that might pose a conflict of interest.

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SEE COMMENTARY

Patient sharing and population genetic structure of methicillin-resistant *Staphylococcus aureus*

Weixiong Ke[a], Susan S. Huang[b], Lyndsey O. Hudson[c], Kristen R. Elkins[b], Christopher C. Nguyen[b], Brian G. Spratt[c], Courtney R. Murphy[b], Taliser R. Avery[d], and Marc Lipsitch[a,e,1]

[a]Department of Epidemiology and [e]Center for Communicable Disease Dynamics and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115; [b]Division of Infectious Diseases and Health Policy Research Institute, Irvine School of Medicine, University of California, Irvine, CA 92617; [c]Department of Infectious Disease Epidemiology, Imperial College London, London W2 1PG, United Kingdom; and [d]Department of Population Medicine, Harvard Pilgrim Health Care Institute and Harvard Medical School, Boston, MA 02215

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Rates of hospital-acquired infections, specifically methicillin-resistant *Staphylococcus aureus* (MRSA), are increasingly being used as indicators for quality of hospital hygiene. There has been much effort on understanding the transmission process at the hospital level; however, interhospital population-based transmission remains poorly defined. We evaluated whether the proportion of shared patients between hospitals was correlated with genetic similarity of MRSA strains from those hospitals. Using data collected from 30 of 32 hospitals in Orange County, California, multivariate linear regression showed that for each twofold increase in the proportion of patients shared between 2 hospitals, there was a 7.7% reduction in genetic heterogeneity between the hospitals' MRSA populations (permutation *P* value = 0.0356). Pairs of hospitals that both served adults had more similar MRSA populations than pairs including a pediatric hospital. These findings suggest that concerted efforts among hospitals that share large numbers of patients may be synergistic to prevent MRSA transmission.

Methicillin-resistant *Staphylococcus aureus* (MRSA), one of the most common and virulent nosocomial pathogens, is also an increasingly important cause of community-acquired disease. MRSA strains, particularly those associated with hospitals, are often resistant to multiple antibiotics, limiting treatment options. *S. aureus* is carried asymptomatically in ∼30% of healthy adults and is shown to be a major cause of invasive disease among hospitalized patients (1); MRSA makes up a growing proportion of nosocomial *S. aureus* infections in many countries. The circulation of a small number of MRSA clones that characterizes the current epidemic is thought to be mainly the result of between-patient transmission rather than de novo appearance of resistance in patients exposed to antibiotics (2), because the appearance of a new MRSA strain requires acquisition of a *mec* resistance element, a relatively rare event. The level of genetic variation occurring in *S. aureus* within identifiable clonal lineages allows the use of genetic markers to track transmission of these lineages and sublineages (3, 4).

The prevalence of MRSA varies considerably both within and between countries (5, 6). About 30% of the *S. aureus* causing bloodstream infections is methicillin resistant in the United Kingdom, whereas that proportion is ∼1% in The Netherlands and Scandinavian countries (7). Among countries with high endemic MRSA infection rates, the proportion is highest in large teaching hospitals (6, 8), where the highest frequency of new emerging MRSA clones has also been reported (9–12). The proposed reasons include increased antibiotic use and increased prevalence of medical procedures and serious medical conditions associated with MRSA acquisition and disease (13). Because MRSA can be carried asymptomatically for a long time (14), readmission could introduce a previously acquired strain into a new hospital (15). Thus, failure of one hospital's infection control could in principle affect the prevalence of MRSA in other hospitals that share patients with it (16). Previous studies have suggested that patient transfer or patient referral patterns (17) could affect the prevalence of MRSA in hospitals (1, 2, 16, 18, 19), on the basis of theoretical arguments and observations that clones of MRSA appear in neighboring hospitals. Population genetics can provide a test of the hypothesis that patient sharing plays an important role in MRSA dynamics: If so, one might expect that hospitals that share large numbers of patients would also tend to share genetically similar populations of MRSA.

In the current study, we sought to investigate whether the pattern of genetic relatedness among MRSA isolates from hospitals within Orange County (OC), California, was consistent with a significant role for patient sharing in determining the population of MRSA strains within a hospital. OC is well suited for this study because it is the fifth largest county in the United States, and it has relatively low population flow from three of its four sides. A finding that MRSA isolates from hospitals that share more patients tend to be related to one another would provide an independent line of evidence for the importance of patient transfer in spreading MRSA from hospital to hospital. For *S. aureus* genotyping, we used the *spa* locus, which encodes protein A, a species-specific protein known for its IgG binding capacity (3). This locus features highly polymorphic internal regions due to short tandem repeats (STRs) (20) and therefore serves as a good target for molecular genotyping (*spa* typing). This genotyping method has been demonstrated to be useful in researching transmission, outbreaks, or geographic distributions (3, 21, 22). Using *spa* typing, we sought evidence on how MRSA strains "travel" with patient flow, to infer how patient transfer might influence MRSA spread among hospitals.

Results

Summary of Overall Approach. We used Wright's *F* statistics to measure genetic heterogeneity between MRSA populations in hospitals and groups of hospitals and used pairwise regression analysis supplemented by group-level analysis as our methods, as described in *Materials and Methods*.

In the pairwise regression analysis, we calculated the heterozygosity of each pair of hospitals (H_R) (for each pair we regard the two hospitals as a group) and the heterozygosity of each single hospital within a pair (H_S). F_{SR} was calculated for each pair of hospitals accordingly and was used as our response variable to measure the genetic dissimilarity between a pair of hospitals. A positive coefficient for a predictor of F_{SR} indicates that hospitals are more divergent from one another, whereas a negative coefficient indicates that hospitals are more similar to one another.

Author contributions: W.K., S.S.H., B.G.S., and M.L. designed research; W.K., C.R.M., and M.L. performed research; L.O.H. contributed new reagents/analytic tools; W.K., L.O.H., K.R.E., C.C.N., C.R.M., and T.R.A. analyzed data; and W.K., S.S.H., B.G.S., C.R.M., and M.L. wrote the paper.

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[1]To whom correspondence should be addressed. E-mail: mlipsitc@hsph.harvard.edu.

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The distribution of F_{SR} is shown in Fig. S1*A*. We found that the distribution of this pairwise F_{SR} is significantly skewed, so we log-transformed this variable in the regression analysis, and the new distribution is shown in Fig. S1*B*. This procedure was also carried out for the main predictor variable, patient flow, as shown in Fig. S1 *C* and *D*.

In group-level analysis, we used the F_{ST} statistics calculated from heterozygosity of all 30 hospitals (H_T) and heterozygosity of each individual hospital (H_S) to measure heterogeneity or the reduction of heterozygosity from the total population level to the individual hospital level. For this study, $F_{ST} = 0.0853$, and H_T and H_S from which it is calculated = 0.719 and 0.658, respectively. As F_{RT} measures the reduction of heterozygosity when hospitals are grouped compared with the total 30 hospitals, this result implies that the best possible grouping (that is, each single hospital is viewed as one group) could do no better than to achieve an $F_{RT} = 0.0853$ ($F_{RT} = F_{ST}$).

Predictors of Similarity Between Pairs of Hospitals. ***Individual categorizing variables.*** Table S1 shows the characteristics of all 30 hospitals. For pairwise analyses described below, dichotomous variables for private insurance proportion, Medicaid coverage, hospital size, and proportion Hispanic were created to reflect above-cut point or below-cut point values.

Pairwise analysis of similarity. For each of the 435 hospital pairs, we calculated and log-transformed F_{SR} to get log-F_{SR}, serving as a measure of population differentiation between them. We performed multivariate regression on the relationship of log-F_{SR} to log-transformed patient flow and to pairwise geographic distances between hospitals, average isolates collected, and the dichotomous variables mentioned above. The *P* values presented in all of our regression analyses are multiple Mantel test permutation *P* values, used to account for dependency between observations involving pairs of hospitals. The results are presented in Table 1. Greater patient flow between a pair of hospitals (log flow) was associated with reduced pairwise F_{SR}, i.e., greater similarity in MRSA populations between hospitals (coefficient = −0.115, *P* value = 0.0356). Another predictor of similarity in MRSA populations was for both to be nonpediatric (ped00, coefficient = −0.801, *P* value = 0.0448). Univariate analyses are shown in Table S2.

In addition, we calculated Pearson correlation coefficients for each pair of the 13 variables to assess collinearity of predictors. We found relatively large correlation between the proportion of Hispanic patients and Medicaid coverage (coefficient for ethnicity11 and medicaid11 = 0.581 and for ethnicity00 and medicaid00 = 0.564), and between the average number of isolates collected and hospital size (coefficient for SSize and size11 = 0.616 and for SSize and size00 = −0.680). This result suggests that these variables might have had small and insignificant effects in our models due to collinearity. We thus performed multivariate analyses with each (group) of these variables removed in turn, as shown in Tables S3–S6. The results of these models were consistent with those in the primary analysis. In these alternative models, the coefficient of log flow ranged from −0.110 to −0.134 (vs. −0.115 in the base model) and remained statistically significant. Exclusion of sample size led to a statistically significant increase in similarity between pairs of large hospitals, compared with pairs containing a small and a large hospital (Table S3). Exclusion of hospital size variables led to a statistically significant association between larger sample size and greater similarity between the hospitals (Table S4). Neither exclusion of the ethnicity variables (Table S5) nor exclusion of the Medicaid variables (Table S6) produced a statistically significant association with the other, but with Medicaid excluded there was a trend toward greater similarity of hospitals having >20% Hispanic patients.

Although the correlation between patient flow—our main variable of interest—and geographical distance was not as large (coefficient for log flow and dist = −0.378), we conducted another multivariate analysis with distance excluded, as shown in Table S7. The result suggested that by removing the distance predictor, a slightly greater degree of similarity was associated with greater patient flow (coefficient = −0.128).

Two of the hospitals have fewer isolates available (hospital 21, six isolates; hospital 29, four isolates). To verify that our results were not driven by them, we removed them from the dataset and ran the full multivariate regression again. The coefficient on patient flow was almost unchanged (−0.114) although the Mantel *P* value increased to 0.053 (Table S8).

As we hypothesized, our results based on the full multivariate model showed that hospitals sharing more patients have significantly more similar MRSA populations, after adjustment for other possible confounders. For each factor of 2 increase in patient flow (see *Materials and Methods* for definition), there is an associated $1–2^{-0.115}$ = 7.7% reduction in pairwise F_{SR} between the hospitals, whereas the interquartile range of hospital pairs for log flow was −11.51 to −8.74, which corresponds to a 19.8% reduction in pairwise F_{SR}. Another variable was also statistically associated with increased similarity of MRSA populations: Hospitals that both served adult patients tended to have populations that were more similar to one another. Visual inspection suggested that none of these results were driven by individual outliers.

Predictors of Similarity at the Group Level. ***Estimating grouping efficiency.*** We used group-level analysis to supplement our results in pairwise analysis. This method divides hospitals into groups by a given criterion and calculates F_{RT} for the grouping. F_{RT} here measures the reduction of heterozygosity caused by grouping relative to all hospitals without grouping and serves as a mea-

Table 1. Results of pairwise multivariate analysis

Variable no.	Variable name	Description	Coefficient	*P* value*
1	Log_flow	Log-transformed pairwise patient flow	−0.115	**0.0356**
2	SSize	Average sample size of MRSA isolates provided by a pair of hospitals	−0.010	0.2474
3	Medicaid11	Indicator of both hospitals having Medicaid >10%	−0.342	0.2476
4	Medicaid00	Indicator of both hospitals having Medicaid no more than 10%	−0.191	0.5510
5	Private11	Indicator of both hospitals having private insurance >35%	0.041	0.8742
6	Private00	Indicator of both hospitals having private insurance no more than 35%	0.081	0.7068
7	Size11	Indicator of both hospitals having annual admission >10,000	−0.133	0.7198
8	Size00	Indicator of both hospitals having annual admission no more than 10,000	−0.026	0.9404
9	Ethnicity11	Indicator of both hospitals having >20% Hispanic patients	−0.077	0.8102
10	Ethnicity00	Indicator of both hospitals having no more than 20% Hispanic patients	−0.166	0.5714
11	Ped00	Indicator of both hospitals being mainly nonpediatric	−0.801	**0.0448**
12	Ped11	Indicator of both hospitals being pediatric	0.368	0.5022
13	Dist	Distance between a pair of hospitals in kilometers	0.004	0.7066

*Permutation *P* values were calculated by multiple Mantel test permutation. *P* values that reached significance are in boldface type.

surement of between-group heterogeneity—that is, a meaningful grouping should give a higher F_{RT}. In group analysis, "T" refers all 30 hospitals (MRSA population), "R" refers to a group of hospitals, and "S" refers to a single hospital.

We first show the evaluation criteria for groupings. In Fig. 1, the red line gives the theoretical best F_{RT} any grouping can achieve, and the "X"s and error bars show how well random groupings can perform. We used a genetic algorithm to search for groupings with nearly the best possible results attainable for a given number of groups, and we constructed random groups to give the null distribution of F_{RT}; see *Materials and Methods* for details of these approaches.

Clearly, with more groups it is possible to obtain a higher value of F_{RT}; in the limiting case of 30 groups each representing one hospital, we would have had $F_{RT} = F_{ST}$. Thus, we want a grouping scheme to have high F_{RT} while also having a relatively low number of groups.

Fig. 1 also gives evaluations of grouping by a few individual criteria (corresponding to some of the predictors in pairwise analysis). Roughly, these results agree with that from the pairwise analysis. Details can be found in Table S9, and the result of network grouping is described below.

Grouping by the information theoretic approach (network grouping). This method creates groups of hospitals such that there is more frequent patient sharing within each group than between groups. The method used to create groups within the patient-sharing network uses an information theoretic approach (23) that creates groups on the basis of minimizing the expected description length of an idealized random walk on the network; for details see ref. 23. The four groups are illustrated in Fig. S3.

Given this grouping scheme, the H_R, or the heterozygosity on groups, is found to be 0.705, and we calculated $F_{RT} = 0.0204$ (Fig. 1), with P value = 0.050. At four groups of hospitals, the maximum F_{RT} by genetic algorithm is 0.0448, and thus the grouping is achieving 45.5% of the best achieved by the GA.

Discussion

This study used patient sharing data together with *spa* genotyping of MRSA strains to analyze how genetic similarity of MRSA depends on patient sharing networks in Orange County hospitals.

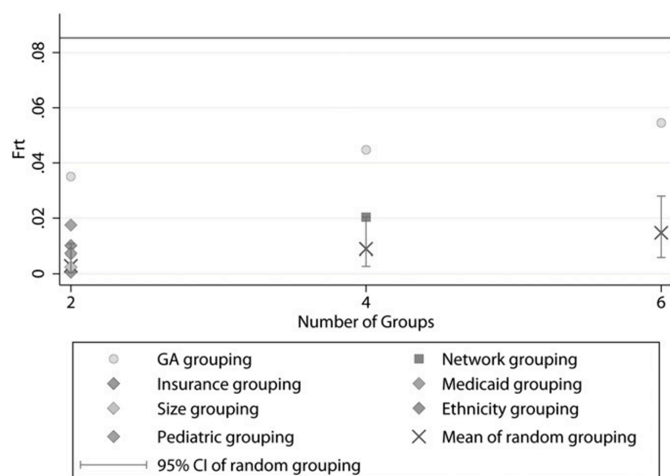

Fig. 1. F_{RT}s by various grouping methods. The uppermost horizontal line represents the maximum value F_{RT} can achieve under any condition (which is F_{ST} of the total population of 30 hospitals). F_{RT}s attained by the genetic algorithm (GA) serve as an estimate of the largest F_{RT} attainable in practice by numerical optimization. The mean and 95% confidence interval (CI) of random grouping display the distribution of randomly generated F_{RT}s at a given number of groups, serving as an estimation of "background". Other symbols of various colors stand for F_{RT}s of different groupings, the detailed description of which can be found in the main text.

In the United States, patient sharing is driven by both the patient and the health provider (24). Patients may choose services at different locations and are influenced by many factors, including their insurance policy, which may restrict patients' choice of hospitals. Changes in insurance policies, transfers to more advanced hospitals for better care, and other reasons might cause patient moves between hospitals. Many theoretical studies have addressed the association between MRSA prevalence and patient referral between hospitals (for example, see refs. 2 and 15), and a positive correlation has been predicted. We address this question from a bacterial population genetic perspective (25, 26), using systematic samples from hospitals within a single county.

We found that the extent of patient sharing between hospitals predicts the extent of genetic similarity between isolates of MRSA obtained from them. Using both pairwise and group-level analysis, we found that the more patients were shared between hospitals, the more similar their MRSA appeared at the *spa* locus. Regression analysis for pairs of hospitals showed significantly more similarity between MRSA from pairs of hospitals that shared more patients, after accounting for other potential predictors including physical distance. Meanwhile, our group-level analysis found that the grouping that classified hospitals on the basis of patient sharing gave an F_{RT} that was significantly better than that of randomly generated grouping schemes.

Agent-based models using these data have found that outbreaks in one hospital could translate to increases in MRSA burden in another hospital (27). The finding that greater patient sharing is associated with greater genetic similarity of MRSA strains, after adjusting for possible confounders, supports the idea that patients track contagious pathogens across hospitals. This result is important given the perhaps unexpectedly large volume of patient sharing that occurs during routine medical care in US hospitals (17).

Patients in Orange County tend to be admitted to hospitals close to their homes. As a result, it is likely that similarities in MRSA strains found in patients who reside near one another could be caused by shared exposure to the same hospitals, as well as by transmission within the community. The finding that hospitals caring for adult patients had more similar MRSA strains than pediatric hospitals may be a further indicator (beyond our findings about patient flow) that MRSA genotypes segregate with patient sharing patterns, since pediatric and adult medical care is segregated in the United States. More definitive studies showing reduction in MRSA burden and strain similarities following regional hospital collaboratives are needed to further understand the contagious impact of sharing patients and the magnitude of prevention that is achievable.

To date, despite a number of theoretical studies suggesting the possible benefits of interventions coordinated among groups of hospitals sharing patients and the possible "externalities" of high MRSA rates in one hospital increasing those in neighboring hospitals, policies such as Medicare reimbursement treat MRSA infections as a problem of the individual hospital, with the effect, as has been argued, that "current Medicare rules subsidize MRSA pollution" (ref. 28, p. 163–182). A possible reason for this seeming disconnect between modeling evidence and policy is the lack of direct empirical evidence that populations of MRSA in one hospital can be traced to sharing of patients from other hospitals at the local level. Many prior empirical studies have documented the spread of clones between hospitals, regions, or countries or have shown that individuals with MRSA colonization are transferred between hospitals. Other studies, which showed that referral hospitals had the highest rates of MRSA infection, did not disentangle whether this association was due to greater numbers of transferred patients, sicker patients, or other factors. This study provides rigorous evidence for the role of patient sharing within a local area in leading to measurable changes in the MRSA population in individual hospitals over a sustained period; moreover, the genetic

evidence provided here is an independent line of evidence that confirms the importance of patient sharing. This finding implies that there is hope for synergistic impact to reduce MRSA, with concerted efforts by hospitals to implement prevention strategies together. Had we found that MRSA strains were indiscriminately found throughout all hospitals, this result would have suggested that a county-wide approach to MRSA containment would be necessary. Instead, it appears possible that targeted approaches might well produce substantial impact when applied to a small group of hospitals that are strongly connected by patient sharing.

There are several limitations to our study. First, our measure of patient sharing considered transfer of patients between hospitals, in both directions and regardless of their MRSA colonization status. A more directly relevant measure, if it were available, would be the transfer of patients colonized with MRSA from each hospital to the other (19). On the other hand, the demonstration here that overall patient transfer is a predictor of MRSA similarity between hospitals suggests that overall patient transfer is an adequate surrogate for the effect of sharing of MRSA-colonized patients, at least for analyses of this type. A related limitation is that whereas 92% of adults in the data set for patient transfer had identifiers (thus only 8% were untracked), the majority (86%) of children lacked such identifiers. However, of these, 63% were <6 mo of age, and a large proportion of these would have been hospitalized at birth and would not have been readmitted (17). Second, the current study did not assess the impact of strains categorized as hospital-onset (HO)-MRSA vs. community-onset (CO)-MRSA (3). We did not assess this distinction partly because community and healthcare reservoirs are mixing and because, at a hospital level, there were often too few strains to make these types of evaluations. Moreover, all strains were treated equally, assessing only whether they were distinguishable by our typing method. Third, patient sharing data could be used in different ways that take into account patient sharing directedness, time of transfer, and length of stay in hospitals, which might provide other interesting findings but were not implemented due to data limitations. Fourth, unmeasured or residual confounding of the association between patient flow and MRSA population similarity is a possibility, as in all such studies. A potential confounder of particular concern is that hospitals that transfer many patients may also draw from the same patient population, so that similarity of the catchment populations leads to importation of similar strains, which could explain MRSA population similarities independent of any causal effect of patient sharing. To address this problem, we included in our model a variable for distance between the two hospitals; compared with a model omitting distance (Table S7), the baseline model (Table 1) had a similar, but slightly smaller effect of patient sharing. Moreover, distance between hospitals was not a significant predictor in the baseline model, nor was it significant in univariate analysis, whereas patient sharing was. In addition, we included variables for shared demographic characteristics of patients, to further eliminate spurious associations with patient sharing that are in fact caused by similar patient populations. Nonetheless, because none of these variables perfectly captures similarities in patient populations, it is possible that the association between patient sharing and genetic similarity of MRSA remains biased by some of these factors. Specific *spa* types associated with pediatric and adult patients in this population have been described recently (29). Fifth, although the *spa* genotyping method used in the current study is widely used as a fast and reliable genotyping technique for *S. aureus*, we might obtain more meaningful results if we used higher-resolution typing systems. Finally, we noticed that some of our hospitals have relatively fewer isolates available. Although the exclusion of these hospitals did not qualitatively affect the results of our pairwise analysis, we found that removing hospitals 21 and 29 made our network grouping result insignificant. The main reason for this loss of statistical significance is that each hospital has a *spa* type that is rare among all hospitals (appearing only in the hospitals that are in the same networking group), and its removal, combined with the fact that these hospitals have fewer isolates, made the distribution of random F_{RT}s generated by random grouping higher. Although we preserved these hospitals in group-level analysis as we believe these rare isolates indicated within-group similarity, more isolates from these hospitals, if possible, are strongly desired.

In summary, we found that patient sharing patterns across hospitals are likely to be correlated with MRSA genetic heterogeneity, along with several other hospital characteristics. This study is a unique regional analysis of a relatively enclosed large metropolitan region of 3 million people. It performs a comprehensive analysis of whether hospitals that share patients also share MRSA strains. It provides evidence of local ecosystems within a single region that are associated with shared patients and suggests that certain groups of local hospitals could make concerted and synergistic efforts to reduce the prevalence of important resistant pathogens and reduce healthcare-associated disease.

Materials and Methods

Study. We conducted a population-based, prospective collection of clinical isolates of MRSA from 30 of 32 hospitals in OC, California as described elsewhere (17). The geographical distribution of these hospitals is shown in Fig. S4. This study was approved by the Institutional Review Board of the University of California Regents.

Isolate collection, specimen- and hospital-level data, and laboratory methods are described in *SI Text*.

Measuring the Genetic Similarity in MRSA Between Pairs or Groups of Hospitals. We adopted a standard measure of genetic similarity: Wright's *F* statistics (30). *F* statistics detect population substructure measured by a given genetic locus of interest. One first calculates the "heterozygosities" of this locus on different levels; here, heterozygosity corresponds to the probability that two randomly chosen isolates will differ at the locus of interest. Three hierarchical levels of population were used in this study: (*i*) subpopulations (S) refer to the bacteria isolated from a single hospital, (*ii*) "regions" (R) refer to the bacteria from a subset (group) of hospitals less than the 30 total hospitals in our study, and (*iii*) the total population (T) refers to all bacteria included in our study. Note that in pairwise analysis, we only have R (a pair of hospitals) and S (a single hospital)—T is not used in the pairwise analysis. If there is any population substructure, then the heterozygosity calculated for the total population will be higher than the weighted average of that calculated for each group individually. In this report, we use the term "heterogeneity" to refer to high *F* statistics implying genetic differentiation between different populations.

Formally, heterozygosity of a population (in terms of one genetic locus) is defined as one minus the sum of squared allele frequencies. Let p_i ($i = 1, 2, \ldots$) represent the frequency of allele i; then the heterozygosity of this locus is given by

$$H = 1 - \sum_{i=1}^{k} p_i^2,$$

where H stands for heterozygosity, and k is the total number of alleles present.

Here, in the total population T (the MRSA population of all hospitals involved) we calculate the heterozygosity of this total population (H_T), using

$$H_T = 1 - \sum_{i=1}^{k} p_{Ti}^2$$

with each allele's frequency (p_{T_i}) and the number of different alleles (k) in the total population. H_S, the heterozygosity of subpopulations (individual hospitals) is calculated similarly to H_T, except that first, we do the calculation restricted to each subpopulation and then calculate H_S as the average of all of the computed subpopulation heterozygosities,

$$H_S = \frac{1}{n} \sum_{i=1}^{n} H_{Si}$$

$$H_{Si} = 1 - \sum_{j=1}^{k_i} p_{ij}^2,$$

where n is the total number of subpopulations, H_{Si} is the heterozygosity of subpopulation i, k_i is the number of different alleles in subpopulation i, and p_{ij} is allele j's frequency in subpopulation i.

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Between these two levels, another level, the regional heterozygosity H_R (in the current study, a region means a group of hospitals we classified), is also calculated similarly: We take a group of hospitals and compute the heterozygosity of that group. Afterward, H_R is just the weighted average of all these regional heterozygosities, with the number of hospitals in each group being the weights. It can be shown that $H_T \geq H_R \geq H_S$ (equal signs hold when there is no population substructure). Then, the F statistics we used are defined as

$$F_{SR} = \frac{H_R - H_S}{H_R}$$

$$F_{RT} = \frac{H_T - H_R}{H_T}$$

$$F_{ST} = \frac{H_T - H_S}{H_T}.$$

Measurement of MRSA Population Similarity—Pairwise Analysis of Hospitals. ***Potential individual predictors.*** To assess patient demographic factors that might account for genetic similarity of MRSA found in pairs of hospitals, we defined the following dichotomous variables. For dichotomized proportions, the subscript zero indicates a proportion less than or equal to the break point. Hospitals were classified for whether they were or had the following:

Over 35% of patients privately insured
Over 10% of patients on Medicaid
Over 20% of patients Hispanic
Over 10,000 admissions per year
Pediatric hospital (vs. adult).

Pairs of hospitals were classified as 00, 01/10, or 11 on each of these variables, and genetic similarity was assessed for hospitals that were similar on these variables (00 or 11) compared with pairs containing hospitals that were different (01/10).

The predictor of primary interest was patient flow. Using previously published data (17) on the number of times any patient was transferred between two hospitals (including possible multiple transfers of the same patient or discharge from the first before admission to the second, with an intervening stay at home), the flow of patients from hospital A to hospital B, T_{AB}, was defined as the proportion of hospital B's patients in a year who had a previous stay in hospital A during the year. The average flow between hospital A and B was then defined as $(T_{AB} + T_{BA})/2$ and was used in our analyses. A more detailed definition can be found in *SI Text*.

Linear regression analysis. We used multivariate linear regression to assess the predictors of genetic similarity between the MRSA populations in pairs of hospitals and used univariate regression for each single variable as a supplement. The response variable that measures heterogeneity was pairwise F_{SR}—the reduction in heterozygosity when two hospitals are viewed as a whole. We log-transformed these two variables to obtain normally distributed data. As some of the hospital pairs have flow = 0, we added 0.00001 (~50% of the smallest available data) to all flow data to perform the log transformation. To account for other possible predictors, we adjusted for the demographic variables described in the previous section by also using two indicator variables for each demographic variable. Standard regression P values do not account for the dependence among the observations induced by the fact that the response variables are genetic "distances" between pairs of hospitals. To adjust for this nonindependence, we performed multiple Mantel permutation tests (univariate Mantel test for univariate analyses) to generate permutation P values for all regression analyses and referred to these P values as permutation P values, as described elsewhere (31). Briefly, we constructed a genetic distance matrix for our response variable—pairwise F_{SR} from our data, with each element in the matrix—and d_{ij} corresponds to the log-F_{SR} of hospitals i and j and comes from the row of data that records the pairwise information of these two hospitals (i.e., patient flow, distances, etc.). Then we shuffled this matrix by each hospital—in other words, we shuffled the rows and columns in the same way 5,000 times, and the resulting matrices were flattened and paired back with predictor variables to conduct 5,000 regressions. Two-tailed P values were calculated from the distribution of t statistics of corresponding coefficients generated.

In addition, for each pair of hospitals, we also adjusted for (*i*) sample size, by using the average number of *spa*-typed isolates of the two hospitals, and (*ii*) distance between the two hospitals, calculated on the basis of their longitudinal and latitudinal data, in kilometers.

Univariate plots of the response vs. individual predictors were checked visually for outliers.

Measurement of MRSA Population Similarity—Analysis of Groups of Hospitals. As a complementary approach, we considered whether grouping the hospitals into a small number of groups on the basis of the demographic characteristics used above of their patient populations or, of more direct interest, on the basis of their patterns of patient sharing, would create groups that captured some of the population genetic structure of the MRSA in the hospitals. To assess this possibility, we sought both to assess the extent to which the best possible grouping could create groups that are genetically homogeneous (the value of F_{RT} obtained by an optimal grouping) and to assess how much genetic structure would be captured in randomly constructed groupings of all 30 hospitals (the range of F_{RT} values obtained by random groupings). The first assessment was done by using a genetic algorithm (GA) to give an approximate numerical value because an exhaustive/exact method is computationally infeasible, and the second assessment was done by creating groupings in which hospitals were randomly assigned to group membership.

GA—Evaluation of grouping efficiency. To establish a standard for the possible extent to which any grouping scheme of hospitals could define genetically similar MRSA populations ("grouping scheme" or "grouping" refers to a specific group assignment of all hospitals), we attempted to find the groupings of hospitals (using no information about the hospitals themselves) that maximized F_{RT}—the measure of genetic heterogeneity between groups—by using a GA. We implemented this method by generating random groupings at a given number of groups and evaluating F_{RT} for each grouping and then evolving the groupings by enriching and combining groupings with high F_{RT}. The goal was to approximate the optimal groupings at a given number of groups to serve as a reference of maximum F_{RT}. This method was repeated for groupings with two, four, or six groups (we primarily used two and four groups, and the result of six groups was used to show the trend of F_{RT} when the number of groups increases). These groupings are referred to as GA groupings. Details of this algorithm can be found in *SI Text* and Fig. S2.

Evaluation of null distribution of F_{RT}. To evaluate whether groupings of hospitals based on any given measurement contain information about the genetic structure of the MRSA population (F_{RT}) greater than expected by chance alone, we created a null distribution of F_{RT} for randomly chosen groupings that divided hospitals into two, four, and six groups. For each given number of groups, we randomly generated 15,000 groupings and tabulated the distribution of F_{RT} from these results (these groupings are referred to as random groupings of k groups). We obtained the mean and 95% coverage interval of F_{RT}s for these random groupings. The P value (double sided) of a given F_{RT} for a particular grouping is defined by the proportion of randomly generated F_{RT}s that are of equal distance or farther away from the mean than it is.

Grouping of hospitals by prespecified categories. We first grouped all hospitals using categorizing variables specified in *Measurement of MRSA Population Similarity—Pairwise Analysis of Hospitals* , including private insurance proportion, Medicaid coverage, size (annual admission), ethnicity (Hispanic), and pediatric vs. adult-only hospitals (the groups are named by their grouping criteria).

Grouping of hospitals by the information theoretic approach (network grouping). For patient sharing, we used the algorithm of ref. 23 to identify "modules" or neighborhoods within the network of hospitals on the basis of patient flow. To do so, we considered each hospital as a "node" in the network and between each hospital constructed an undirected edge representing patient sharing with weight determined by the proportion of shared patients, as given in *Measurement of MRSA Population Similarity—Pairwise Analysis of Hospitals*. The algorithm defines neighborhoods—roughly speaking—as sets of nodes in which an imaginary random walker, traversing edges of the network with probabilities proportional to the edge weight, would be much more likely to stay within a set. Thus, sets of nodes that are well connected with one another will tend to be in the same group; in our case, sets of hospitals that share many patients with one another will tend to be in the same group, and sets of hospitals that do not share many patients with one another will tend to be in different groups. Technical details of this method are given in *SI Text*, which summarizes the account given in ref. 23. This method is referred to as network grouping.

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