Genomics of Golden Staph: Discovering genomic markers of transmission of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is an important, predominantly human pathogen with a high burden of disease. *S. aureus* infections result in significant morbidity and mortality globally, owing in part to the wide spectrum of diseases which can be attributed to the pathogen (Tong *et al*., 2015).

The significant burden of *S. aureus* diseases across the community necessitates the development of tools for epidemiological investigations and a variety of infection control interventions, particularly within the healthcare setting (Dufkova *et al*., 2022). Bacterial subtyping is well established as an epidemiological tool and can be used in epidemiological investigations to determine details of microbial transmission (Sandora *et al*., 2014).

Epidemiological investigations and typing efforts to date, largely focus on MRSA, where incidence rates of around 10 per 1000 hospital admissions are reported from whole of hospital studies (Al-Talib *et al*., 2010). Binary typing of MRSA by multiplex PCR reverse line blot assay (mPCR/RLB) was previously developed (O'Sullivan *et al*., 2012).

In this thesis, targets were selected as the first step in developing a binary typing assay for methicillin-sensitive *Staphylococcus aureus*. An mPCR/RLB assay was then developed for use in prospective typing studies of MSSA. Subsequently, the assay has been employed in three different healthcare and community settings. Namely, in the typing of methicillin-sensitive *S. aureus* (MSSA) causing bloodstream infections in New South Wales; in typing MSSA responsible for both infection and colonisation from all sources at a single healthcare facility; and amongst players from a professional sporting team amidst an outbreak of skin and soft tissue infection.

Peer reviewed publications arising during the course of this thesis.

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Presentations arising

M. Brown, M. V. N. O'Sullivan, M. Ramsperger, F. Zhou, G. L. Gilbert. Selection of Targets for Binary Typing of Methicillin –Sensitive *Staphylococcus aureus* by Multiplex PCR and Reverse Line Blot Assay. Poster presentation, StaphPath conference, Adelaide, South Australia, Australia, 2017.

M. Brown, C. Li, M. Ramsperger, G. L. Gilbert, M. V. N. O'Sullivan. Molecular epidemiology of methicillin-sensitive *Staphylococcus aureus* isolated from a hospital in western Sydney in 2017. Poster presentation, International Symposium of Staphylococci and Staphylococcal Infections conference, Copenhagan, Denmark, 2018.

M. Brown, C. Li, M. Ramsperger, G. L. Gilbert, M. V. N. O'Sullivan. Molecular epidemiology of methicillin-sensitive *Staphylococcus aureus* causing bloodstream infections in New South Wales in 2017. Poster presentation, International Symposium of Staphylococci and Staphylococcal Infections conference, Copenhagan, Denmark, 2018.

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Abbreviations Used in this Thesis

Organisms

C. difficile	*Clostridioides difficile*
CA-MRSA	Community-acquired Methicillin-resistant *Staphylococcus aureus*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
MTBC	*Mycobacterium tuberculosis* complex
S. aureus	*Staphylococcus aureus*
VRE	Vancomycin-resistant Enterococcus

General Abbreviations

AuSeTTS	Automated selection of typing target subsets
BD	Becton, Dickinson and Company
BLAST	Basic local alignment search tool
bp	Base pair
CC	Clonal complex
CG-MLST	Core genome multi-locus sequence typing
Cnr	Corner
COA	Coagulase gene
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
egc	Enterotoxin gene cluster
ICPMR	Institute of Clinical Pathology and Medical Research
LAMP	Loop-mediated isothermal amplification
MALDI-TOF	Matrix assisted laser desorption ionisation - time of flight
MLST	Multi-locus sequence typing
MLVA	Multiple-locus variable number tandem repeat analysis
mPCR/RLB	Multiplex PCR-based reverse line blot
NICU	Neonatal intensive care unit
NSW	New South Wales
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PFT	Pulsed-field types
PVL	Panton-Valentine leukocidin
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
SCC	Staphylococcal cassette chromosome
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
SEF	Staphylococcal enterotoxin F
SNP	Single nucleotide polymorphism
SPA	Staphylococcal protein A
SSTI	Skin and soft tissue infections
ST	Sequence type

subsp	Subspecies
™	Trade mark
TSST	Toxic shock syndrome toxin
VNTR	Variable number of tandom repeats
WG-MLST	Whole genome multi-locus sequence typing
WGS	Whole genome sequencing

Units of Measurement

µL	Microlitre
C	Celsius
ng	Nanogram

Chapter 1. Review of Literature

1.1 *Staphylococcus aureus* causes a significant burden of disease.

Staphylococcus aureus is a Gram-positive coccus from the Staphylococcaceae family of the order Bacillales. The bacterium is most closely related to *S. argenteus* and *S. schweitzeri* and collectively these are now referred to as the *S. aureus* complex (Becker et al., 2019). Owing to an extraordinarily high number of toxins and other virulence determinants the organism can produce, there is a broad spectrum of diseases in which *S. aureus* can be implicated. (Cheung et al., 2021).

S. aureus is an important, predominantly human pathogen with a high burden of disease. *S. aureus* infections result in significant morbidity and mortality globally, owing in part to the wide spectrum of diseases which can be attributed to the pathogen (Tong *et al*., 2015). The organism can be found as commensal flora in the nares, amongst other sites. Persistent nasal colonisation is seen in around 10 – 20% of the population with up to 50% of people being identified as intermittent carriers (Lowy, 1998). Nasal carriage is a well-defined risk factor for subsequent infection in most patient groups studied (Kluytmans and Wertheim, 2005).

S. aureus-related skin and soft tissue infections (SSTI) are common. Community-associated methicillin-resistant *S. aureus* (MRSA) alone has become the most frequent cause of cases of SSTI presenting to emergency departments in the United States (Moran *et al*., 2006). The burden of community-associated methicillin-sensitive *S. aureus* (MSSA) in SSTI is also significant. *S. aureus* is a leading cause of bacteraemia, endocarditis and other serious invasive diseases including osteoarticular and pleuropulmonary infections (Tong *et al*., 2015)

S. aureus, including MRSA, is responsible for notably high rates of infection in indigenous peoples, including Native Americans, Pacific Islanders and Australian Aboriginals (Turnidge, 2009). The incidence of *S. aureus* bacteraemia among Australian Aboriginals in the Northern Territory's top end at 172 cases per 100,000 population was found to be more than five times higher than the incidence in the non-Aboriginal population in that same area and time (Tong *et al*., 2009). Some of the earliest

detections of community-associated MRSA (CA-MRSA) were from remote indigenous communities in the Kimberly region of Western Australia (Riley and Rouse, 1995).

Nosocomial *S. aureus* infections are common. Much of the literature focuses specifically on MRSA and incidence rates of around 10 infections per 1000 hospital admissions are reported from whole of hospital studies (Al-Talib *et al*., 2010). Nosocomial MSSA bacteraemia has been found to occur in patients with a shorter median hospital stay than patients who develop MRSA bacteraemia, at 4 days as opposed to 12 days (Abramson and Sexton, 1999). This study also found that while both infections significantly prolong length of stay, MRSA bacteraemia results in an approximate threefold increase in associated costs compared with MSSA bacteraemia.

Due largely to environmental factors, colonisation and non-invasive *S. aureus* diseases are common in certain groups, such as for those people in prisons. In an incarcerated population in New York State, colonisation rates for MRSA are up to 10 times higher than for the general population (Mukherjee *et al*., 2014). Colonisation and disease are also more prevalent within contact sporting teams (Brancaccio *et al*., 2020).

The significant burden of *S. aureus* diseases across the community necessitates the development of tools for epidemiological investigations and a variety of infection control interventions, particularly within the healthcare setting (Dufkova *et al*., 2022).

1.2 Bacterial subtyping is a valuable epidemiological tool.

While determining the source of a given infection can be challenging, guided interventions can interrupt outbreaks and prevent infections. Bacterial subtyping can be used in epidemiological investigations to determine details of microbial transmission (Sandora *et al*., 2014). At the most basic level, if two isolates are found different by a subtyping method, they are less likely to be from a common source and more likely unrelated. Bacterial subtyping is well established as an

epidemiological tool, and a variety of subtyping methods have been employed on a wide array of bacterial pathogens in various community, healthcare, and even agricultural settings. Subtyping has evolved over time from phenotypic methods to predominantly molecular methods, initially PCR based and subsequently sequence based. Refer to Table 1 for summary of typing methods.

1.2.1 Bacterial subtyping in the community setting

For nearing 100 years, *Salmonella enterica* subsp. *enterica* has been subtyped by phenotypic, agglutination based serotyping methods using the Kauffmann White Scheme (Wattiau *et al*., 2011). While this typing still informs outbreak investigations, serotype prediction based on whole genome sequencing (WGS) may soon supersede this method. In a study of 1,041 Salmonella isolates from food, feed or environmental swabs, WGS and an *in silico* tool for serotype prediction found agreement for 899 isolates (86.4%); assigned a different serotype for 80 isolates (7.7%); but could not assign a serotype for 62 isolates (5.9%) (Ibrahim and Morin, 2018).

Phenotypic subtyping *Streptococcus pneumoniae* by capsular serotyping, or the Quellung reaction, can elucidate valuable information around vaccine efficacy. This allows for vaccine impact studies (Habib *et al*., 2014) and can guide future vaccine formulation. The method currently remains the gold standard although new molecular methods are emerging. Loop-mediated isothermal amplification (LAMP), a molecular method which does not require extensive reagents or equipment, has been used to detect the 13 common capsular types included in the pneumococcal conjugate vaccine, directly from blood and CSF samples (Takano *et al*., 2019). The sensitivity of the LAMP assay was found to be 10 -100 copies per reaction.

Spacer oligonucleotide typing, or spoligotyping, is a molecular, hybridisation-based assay that detects variability in the direct repeat region in the DNA of *Mycobacterium tuberculosis* complex (MTBC). Spoligotyping is reasonably rapid and may be considered in management of patients with tuberculosis in a clinical setting. However, given the very long incubation times associated with tuberculosis the method is most useful in comprehensive continuous prospective community surveillance programs.

Spoligotyping has shown a 100% negative predictive value, but overestimates isolates with identical fingerprints by around 50% as compared to *IS*6110-based restriction fragment length polymorphism (RFLP) typing (Gori *et al*., 2005). Increasingly, WGS is being used to characterise the molecular epidemiology of MTBC in low incidence settings (Dale *et al*., 2022); and in primary diagnosis coupled with detection of drug resistance (Katale *et al*., 2020).

1.2.2 Bacterial subtyping in a nosocomial context

Subtyping is also widely used in monitoring certain nosocomial infections. Molecular typing methods have been widely employed for *Clostridioides difficile*. Ribotyping in the hospital setting can identify hypervirulent strains including O27 and inform both infection control interventions and treatment regimens (Merrigan *et al*., 2010). In combination with epidemiological data, molecular typing by way of multi-locus sequence typing (MLST) can detect distinct lineages within outbreaks of *C. difficile* infection (Walker *et al*., 2012).

Following the emergence of vancomycin-resistant enterococci (VRE) in Taiwan from 2003 – 2010, when the prevalence of vancomycin resistance increased from 2% to 16% in community hospitals and 3% to 21% in medical centres, pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing were used (Lee *et al*., 2013). The study found one MLST type, 414, was predominant and that inter-hospital spread in Taiwan was indicated.

The broad utility of bacterial subtyping is thus well established, and the potential benefits of subtyping *S. aureus* isolates in a variety of circumstances and settings is evident. More broadly, screening and surveillance programs for MRSA are shown to be cost effective. One such study assessed three differing protocols for screening / laboratory processes and found in the American setting at least, they are cost beneficial (Nyman *et al*., 2011). To date however, much of the interest in the epidemiology of *S. aureus* and subsequently the typing of strains, has focussed on MRSA. This is particularly evident in a healthcare associated setting.

1.3 Subtyping of *S. aureus* has largely focussed on MRSA.

To date, subtyping of *S. aureus* has largely focussed on MRSA, and for many methods employed, almost exclusively so. There are a variety of methods which are useful, or potentially useful, for the subtyping of MSSA and MRSA, including: PFGE, staphylococcal protein A sequencing (SPA typing) and multiple locus variable-number tandem repeat analysis (MLVA). Sequence-based binary typing has shown utility, including multiplex PCR-based reverse line blot assay (mPCR/RLB) (O'Sullivan *et al*., 2012) (see below). Whole genome sequencing-based approaches (WGS) are also increasingly employed. Despite this, most evaluations have focussed on their utility in the molecular epidemiology of MRSA.

PFGE is an epidemiological molecular typing method which involves subjecting total bacterial DNA to restriction enzyme digestion, then observing the migrating fragments of nucleic acid products in agarose gel, by way of electrophoresis, to generate a fingerprint, or macrorestriction fragment profile. The method requires development and use of standardised pathogen-specific protocols and has traditionally been a gold standard for international surveillance programs. The *Sma*I restriction enzyme is most used for PFGE typing of MRSA. In the development of a national database in the United States in 2003 it was found that 93% of MRSA isolates clustered into 8 major pulsed-field types (PFT), designated as USA100- USA800. (McDougal *et al*., 2003). The same study found that only 57% of MSSA isolates had PFGE patterns which clustered in those 8 PFTs. Data from an advanced medical emergency centre in Japan (Hidaka *et al*., 2013) showed PFGE was employed to monitor infection control interventions which included use of intranasal mupirocin ointment and screening of new inpatients. Total MRSA isolations, including of the predominant outbreak PFGE type decreased significantly, albeit following 6 months of interventions. A known shortcoming of the standard PFGE protocol is for multilocus sequence type (ST) 398 MRSA isolates, which are non-typeable by the method due to resistance to digestion by the *Sma*I restriction enzyme. *Cfr*9I, a neoschizomer of *Sma*I has been optimised and employed to investigate ST398 MRSA isolates (Bosch *et al*., 2010). The study

found that all previously non-typeable MRSA can be typed with the alternate *Cfr*9I based PFGE scheme.

spa typing is a single locus typing method which is rapid, reproducible, and portable (Hallin *et al*., 2009). *spa* typing involves sequencing a polymorphic region (X) of the protein A gene (*spa*) which contains a variable number of 24-bp repeats and is found in all strains of *S. aureus*. An established, standardised nomenclature and database are employed for staphylococcal *spa* typing. A 2013-2017 study from two hospitals in South Africa found a single *spa* type accounted for around a quarter of isolates in each of the two hospitals, t037 and t045 respectively. (Strasheim *et al*., 2021). Supplementary WGS revealed a low frequency of single nucleotide polymorphisms (SNP) of ≤20 differences, leading the authors to conclude uninterrupted transmission and persistence of these two *spa* types. Conversely, a 2021 study which characterised all *S. aureus* isolated during routine surveillance in a neonatal intensive care unit (NICU) (Grohs *et al*., 2021) did not detect any evidence of MRSA outbreaks. MRSA colonisation rates were 2% amongst the 1556 hospitalised patients during the study period. Six MRSA *spa* types were found in ≥ 2 infants while an additional 5 MRSA *spa* types were found only in individuals. The authors concluded that MSSA was a greater concern for their NICU, and interventions should be designed around that.

Multiple-locus variable-number tandem-repeat analysis (MLVA) involves simultaneous amplification of multiple variable-number tandem repeat (VNTR) loci, and analysis by electrophoresis. In assessing the utility of MLVA for grouping MRSA isolates into known epidemiologically linked clusters, several approaches have been tested. A study published in 2007 from the Centers for Disease Control and Prevention and Rollins School of Public Health (Tenover *et al*., 2007) found that visual analysis using any band difference to establish an MLVA type was not successful. In that study, which used previously established criteria (Sabat *et al*., 2003) where any band change was classified as a distinct MLVA type, Isolates known to be epidemiologically linked or highly related by PFGE did not group together. Epidemiologically defined outbreak strains of USA300-0114 were separated into four

different clusters. BioNumerics software (bioMérieux, Marcy-l'Étoile, France) was employed to assess potential relatedness cut-offs of >80% and >75%. >75% relatedness showed utility as a screening test for isolates collected over a relatively short period of time in a single hospital facility. But, for either percent cut-off assessed, type strains USA300 and USA500 were indistinguishable, suggesting MLVA may have difficulty grouping the ST8 isolates, which also includes the Iberian clone. A study published in 2010 from Spain, looked at 292 MRSA isolates which had previously been characterized by PFGE, MLST and SCC *mec* type to evaluate if MLVA could predict MRSA clones (Rivero-Perez *et al*., 2010). The study found MLVA could predict MRSA clones previously identified by PFGE/MLST-SCC*mec* typing and had utility in hospitals where resources are not available for MLST or PFGE typing.

Staphylococcal cassette chromosome *mec* (SCC*mec*) typing differentiates MRSA into at least 11 SCC*mec* types (I-XI) based on PCR amplification of targets in the *ccr* gene and the *mec* gene complexes. Some correlation has been found between SCC*mec* type and antimicrobial susceptibility patterns. A study in Iran found very high rates of resistance to clindamycin and ciprofloxacin among SCC*mec* type III isolates (Moosavian *et al*., 2018). Methods such as SCC*mec* typing that focus on aspects of the organism related to methicillin-resistance or that are otherwise unique to MRSA are simply not applicable for MSSA investigations.

Whole-genome sequencing (WGS) has recently emerged as the mainstay of bacterial typing, including for MRSA. The two main analysis methods are core genome single-nucleotide polymorphism (SNP) analysis and whole genome MLST (WG-MLST). A 2016 study used whole-genome SNP to retrospectively investigate a suspected point source in a series of surgical site infections over a one-year period. (Roe *et al*., 2016). The suspected source of transmission was a surgical team member who was involved in all surgeries. Informative SNPs between the surgical team member's isolate and the surgical site infection isolates ranged from 283 to 'thousands', excluding the surgical team member as the source. As part of a larger national surveillance program in the Netherlands between 2008 and 2019, a subset of 4798 MRSA isolates were subjected to WG-MLST (Schouls *et al*., 2023). WG-MLST

profiles partitioned into 35 distinct groups (differing from each other in ≥1000/2567 WG-MLST loci). Genogroups closely matched classical MLST clonal complexes. Refer to Table 1 for summary of typing methods.

1.4 Methods previously employed in typing MSSA.

While the bulk of studies involving typing of *S. aureus* isolates to date have focussed on MRSA, several typing methods have been employed in investigations of MSSA. Many of these methods have proven sufficiently discriminatory to allow findings related to clustering of isolates or even identifying potential chains of transmission.

Phage-typing, in which bacterial strains are assessed for lysis mediated by certain bacteriophages, has long been employed in a variety of settings as a typing tool for *S. aureus*, and its use pre-dates the emergence of MRSA. Phage-typing of *S. aureus* isolates originating from humans generally employed a standard international typing set of 23 phages. However, with this approach a significant number of isolates may remain non-typable, necessitating either complementary typing methods or the inclusion of additional phage. The standard typing set for human-origin isolates is seemingly of limited value in typing *S. aureus* from other sources (Kibenge *et al*., 1982). The authors found 74.2% of their poultry-sourced isolates could however be typed when using the Shimizu avian *S. aureus* phage standard set. Whilst phage-typing is not technically difficult, the requirement for a typing set of phages, which may vary depending on the source of isolates being studied, and the high percentage of non-typeable strains means the method has largely been superseded.

PCR to detect prophage patterns has also been employed to type clinical *S. aureus* isolates (Dini *et al*., 2019). In 126 clinical *S. aureus* isolates, of which 81 were MSSA, 112 strains contained at least one of the 17 different prophage types detected in the study. All bar one of the 17 prophage types, SGL, was detected in both MRSA and MSSA.

PFGE was used to interrogate maternal-infant perinatal transmission of *S. aureus* including MSSA (Pinter *et al*., 2009). 43/304 mothers from the study period were colonised with MSSA; 25/252 evaluable infants were colonised with MSSA. Five mother-infant pairs had PFGE concordant MSSA strains. The authors concluding that about 20% of instances of perinatal *S. aureus* colonisation could be accounted for by maternal-infant transmission but that most instances of *S. aureus* perinatal infections resulted from colonisation that occurred after discharge from the nursery. PFGE has traditionally been a gold standard for subtyping internationally and is inexpensive and not technically difficult. However, its use does require the development of target-species specific protocols. In comparison to sequence-based typing, PFGE has been described as simply 'counting the number of chapters' rather than 'comparing all the words in a book' (WSDH, 2019). As a result, PFGE is slowly being replaced by sequence-based methods.

As part of a larger study which also included MRSA, PFGE was compared with MLVA for typing of 63 clinically significant *S. aureus* isolates (Chung *et al*., 2012). In this study, PFGE and MLVA yielded similar Simpson's diversity indices ([D]) – indicating similar discriminatory power. However, the overall concordance between the two methods was low. PFGE type was a better predictor of MLVA type than MLVA was for PFGE type. The authors noted that the inclusion in the study of MSSA isolates (n=15), which are known to be more polyclonal than MRSA, may explain the lower Wallace coefficient of PFGE predicting MLVA type when compared to previous studies. Wallace's coefficient is an indicator of the agreement between partitions which can be adjusted for the agreement due to chance alone.

An improved MLVA typing system interrogating sixteen VNTR loci in two multiplexed PCRs was developed (Sobral *et al*., 2012). 145 *S. aureus* isolates from a variety of animal species, veterinary disease conditions, food sources and food poisoning events were tested. A strong correlation was shown with MLST, allowing for prediction of MLST CC based on MLVA. The isolates were assigned to 12 known MLST clonal complexes (CCs) and a few singletons. Half of the isolates belonged to four CCs previously mostly associated with animals (CC9, CC97, CC133 and CC398); The remaining 8 CCs (CC1,

CC5, CC8, CC15, CC25, CC30, CC45, CC51) representing 46% of the isolates are common in humans. Interestingly, isolates deemed responsible for food poisoning showed a CC distribution typical of human isolates and markedly different from the animal isolates, suggesting a predominantly human origin. Also, MSSA strains belonging to CC398 were identified in poultry, which had previously been described only once. The authors were unsure whether these sensitive strains were circulating in poultry prior to the emergence of CC398 MRSA in pigs or whether part, or all of the SCC*mec* cassette had been spontaneously excised so that the strains reverted to a sensitive phenotype. MLVA is a useful typing method to differentiate fast-evolving strains in an outbreak setting (these strains may appear the same by PFGE). However, there is a moderate degree of skill required by the technician and species-specific protocols are required.

Spa typing has been employed to expand characterisation of MSSA isolates in neonatal intensive care units (NICU) in studies which also investigated MRSA (Grohs *et al*., 2021). This study found that amongst the 14% of hospitalised infants who were colonised with MSSA, 36 infants harboured unique MSSA *spa* types, while 30 MSSA *spa* types were found in $\geq$ 2 infants. The most isolated MSSA *spa* type in this study, MSSA-t279, was not associated with increased morbidity or mortality in that study, but was strongly associated with mupirocin resistance, prompting a re-evaluation of mupirocin-based decolonisation protocols within the NICU.

In a large study of skin and soft tissue infections (SSTI) in Hong Kong, MSSA was isolated from 105/298 (35%) of patients presenting with SSTI (Ho *et al*., 2008). 24 *spa* types were identified amongst the 105 isolates with 17 isolates being of a unique *spa* type. The most common *spa* type, t034 was isolated from 5 patients. Panton-Valentine leukocidin (PVL) was detected in at least one isolate of 12/24 *spa* types identified. There was no correlation observed with *spa* type and antimicrobial resistance.

A small cross-sectional study from Iran using *spa* typing, focussed exclusively on MSSA (Saffari *et al*., 2020). Haemodialysis patients and health-care workers from the dialysis unit, were screened for nasal carriage of MSSA. Nasal carriage rates were found to be 24.4% amongst the 41 patients included and

the study and 18.8% amongst the 11 health-care workers. 8 different *spa* types were detected amongst the 12 isolates of MSSA. One of the *spa* types identified, t-084 was encountered in both patient and health-care worker groups, alarming the study investigators, and alerting them to the possibility of strain circulation between staff and patients. The authors did not however comment on the prevalence of this strain within the general community. *Spa* typing can be slow and relatively expensive given it is a sequencing-based approach and can lack sufficient discriminatory power for many applications given a single locus is interrogated.

Coagulase gene typing (COA typing) is a method which interrogates a heterogenous tandem repeat region at the 3' -end of the coagulase gene. PCR is performed, followed by *Alu*I restriction enzyme digestion with analysis by RFLP. Thirty MSSA were included in a larger study of 240 *S. aureus* isolates from a single hospital in Sapporo, Japan (Kobayashi *et al*., 1995). Of the 13 *Alu*I restriction patterns detected in the study (A-M), only 1 (E) was not represented by any MSSA strains. The MSSA strains were diverse, and no restriction pattern had more than 8 representative isolates, the authors concluding that none predominated. This contrasted to the MRSA identified in the study in which two of the restriction patterns (A, B) accounted for 182 and 17 isolates each, or 87% and 8% of MRSA respectively. COA typing is an easy to perform and inexpensive subtyping method, however there is a lot of heterogeneity noted in MSSA (as with other typing methods). There is limited evidence of the method being successfully employed in investigations surrounding MSSA.

Enterotoxin production is implicated in the pathogenicity of *S. aureus* diseases, including in food poisoning. A 1983 study from Lyon, France evaluated the production of enterotoxins A, B, C and F in 403 *S. aureus* isolates including 29 strains implicated in food poisoning. 62% of the strains implicated in food poisoning were found to be enterotoxigenic as were 51% of the remaining 374 clinical isolates. Staphylococcal enterotoxin A (SEA) was the most detected, in 81 isolates, followed by staphylococcal Enterotoxin B (SEB), in 57 isolates. This study also sought correlation of enterotoxin type with phage type and found strains producing staphylococcal Enterotoxin F (SEF) were mostly phage group I. More

recently, enterotoxin gene cluster (*egc)* polymorphism has been used to subtype *S. aureus* (Blaiotta *et al*., 2006). Thirty-five strains were analysed by PCR REA (restriction endonucleases analysis) of the *egc* operon in combination with *spa* typing. Of the 7 REA *egc* groups detected; 1 and 7 were most common with 11 and 10 isolates respectively. The 11 REA *egc* group 1 isolates were of 3 *spa* types (t002; t209 and t164) while the 10 REA *egc* group 7 isolates were of 9 different *spa* types. Enterotoxin typing, whilst no longer routinely employed, is a useful method as it can help profile important virulence factors in *Staphylococcus aureus*.

Increasingly, WGS is employed in typing of MSSA in a variety of settings. A 2022 study from a large acute hospital in Ireland used core genome MLST (CG-MLST) to interrogate 406 MSSA from clinical samples, screening swabs, healthcare workers and environmental samples (Kinnevey *et al*., 2022). 183/406 MSSA isolates segregated into 59 related isolate groups (≤24 CG-MLST allelic differences), while 223 were deemed unrelated. There were strong indications of strain transmission in a particular unit (Ward H). Another study found whole-genome SNP analysis particularly discriminatory (Durand *et al*., 2018). A group of CC8 Lyon clone isolates collected from the Perigeux region shared the same *spa* type and toxin profile but were shown by SNP to include genetically distant strains (110 SNPs). The considerable expense, and potentially lengthy turn-around-times for sequencing based-assays means it is not yet feasible to employ these methods prospectively on large numbers of isolates. Nonetheless, sequenced based typing assays are clearly emerging as the gold standard typing method, not just for *S. aureus* but bacteria in general. Refer to Table 1 for summary of typing methods.

Conventional, generally species-specific typing methods have traditionally been the mainstay in bacterial epidemiology studies. Resolution of epidemiological typing has been improved by the development of typing methods employing molecular analysis of microbial DNA. However, new typing methods are often applied without critical evaluation of their performance characteristics (Struelens, 1996). In assessing prospective typing methods, six performance criteria have been proposed by the authors. These criteria are: typeability, stability, discriminatory power, epidemiologic

concordance and typing system concordance. Typeability describes the proportion of strains which can be assigned to any type, with the remainder being non-typable by the method. Reproducibility is the ability of the assay to assign the same type to a given isolate on independent assay runs. Stability measures the variability of results generated by the typing method over time, with in vitro or in vivo changes in the target strain. Discriminatory power assesses the likelihood of the assay assigning different types to unrelated strains. This can be expressed by a formula known as the Simpson index of diversity (Hunter, 1990). Concordance is assessed in prospective typing methods with respect to how results align with both epidemiological data (epidemiologic concordance) and with independent typing systems (typing system concordance). Furthermore, in this endeavour, the adjusted Wallace coefficient measures the congruence between two typing methods, accounting for chance agreement (Severiano *et al*., 2011).

Table 1: Summary of typing methods.

Category	Method	Target Organisms	Comments
Phenotypic	Serotyping	*Salmonella enterica* (Kauffman White scheme for latex agglutination)*; Streptococcus pneumoniae* (Quellung reaction) and others	A large set of expensive antisera is required. Non-typeable exist. Soon to be superseded by emerging sequence-based methods.
	Phage typing	*Staphylococcus aureus* and others	Standard protocols and phage libraries are required. Method is highly species specific
Genotypic - no amplification	Pulsed-field gel electrophoresis (PFGE)	Vancomycin resistant enterococci; *Staphylococcus aureus* and others	Moderate technical skills required for gel lane comparison. Whole-genome sequencing is increasingly used
Genotypic – amplification (product length)	Multiple-locus variable-number tandem repeat analysis (MLVA)	*Staphylococcus aureus; Salmonella typhimurium* and others	Based on variable copy numbers of tandem repeats. Loci selected for discriminatory power.
Genotypic – amplification (probe-based)	Reverse line blot assay (RLB)	MRSA and others	Inexpensive, technically basic method which can allow more targeted use of expensive sequencing methods.
	Spoligotyping	*Mycobacterium tuberculosis* complex	Negative predictive value is good, yet the method overcalls isolate fingerprints as identical. This leads to a poor positive predictive value. Whole-genome sequencing is increasingly used to characterise the complex.
Genotypic – amplification (sequence-based)	Spa typing	*Staphylococcus aureus*	Single locus typing system, which is rapid, reproducible and portable
	Ribotyping	*Clostridioides difficile*	Hypervirulent strains like O27 are easily identified. Whole genome sequencing largely superseded this method. Supplemental epidemiological data required to detect lineages within outbreaks.
Genotypic (sequence-based)	whole-genome sequencing	Various	Currently expensive, requiring significant equipment and other infrastructure.

1.5 The development of a new binary typing assay.

Binary typing of MRSA by multiplex PCR reverse line blot assay (mPCR/RLB) has recently been developed as an effective high-throughput strain typing method, suitable for routine, prospective use as part of a hospital infection control programme, which affords an opportunity for targeted interventions (O'Sullivan *et al*., 2012). The method allows a non-selective approach to typing, where all MRSA strains encountered in the laboratory can be typed with this rapid, cheap, discriminatory, and high-throughput method. This mPCR/RLB was found to have similar discriminatory power to that of PFGE ([D] = 0.994, versus 0.987) and higher than for *spa* typing ([D] = 0.926) Notwithstanding the challenges posed by the more heterogeneous MSSA, it is evident there is potential for development of a binary typing assay suitable for MSSA to detect transmission events and outbreaks.

1.6 Aims of this Thesis

The aims of this thesis are:

- To describe the burden of MSSA infections and the methods that have been employed in subtyping to date.
- To develop a new binary typing assay tailored for subtyping of MSSA in the form of an mPCR/RLB assay.
- To assess this newly developed subtyping assay against standard, published criteria.
- Prospectively employ this new binary typing method in a broad range of settings.

Chapter 2. Assay Design, Development and Methods

2.1 Introduction

Binary typing of MRSA by multiplex PCR reverse line blot assay (mPCR/RLB) has been developed (O'Sullivan et al., 2012). This method was optimised for MRSA with respect to discriminatory power and the inclusion of other informative targets. Particularly, the inclusion of six SCCmec elements holds no relevance for the typing of MSSA strains. This chapter describes the development of a new assay specifically targeted at MSSA strains, from the selection of targets, through to the development and optimisation of the assay. The assay is subsequently assessed against consensus guidelines.

2.2 Methods

2.2.1 Selection of Targets for Binary Typing of Methicillin-Sensitive *Staphylococcus aureus* by Multiplex PCR and Reverse Line Blot Assay

We previously established binary typing of methicillin-resistant *S. aureus* by multiplex reverse line blot (mPCR/RLB) as an effective high-throughput strain typing methods, suitable for routine, prospective use as part of a hospital infection control programme, which affords an opportunity for targeted interventions (O'Sullivan *et al*., 2012). Here we describe the selection of binary targets as the first step in developing a similar typing method, specific for methicillin-sensitive *S. aureus* (MSSA).

A reference library of 137 MSSA strains, collected between 2004-2005 and 2013-2014, were selected, representing isolates from a wide variety of infections as well as colonisation. The collection included 33 multi-locus sequence types. They were isolated mostly from the former Pathology West network of laboratories, now known as NSW Health Pathology, a pathology service provider covering a large geographical area of New South Wales. Repeat isolations of patients already included in the reference library were excluded. 80 potential targets for binary typing were identified from both a review of the literature (Coldea *et al*., 2013; Feng *et al*., 2008; Ho *et al*., 2008; Kobayashi *et al*., 1995; Monecke *et al*., 2013; Song *et al*., 2016; Tong *et al*., 2015; Suzuki *et al*., 2006) and the existing MRSA mPCR/RLB

assay. Whole genome sequencing (WGS) of isolates was performed using the Illumina NextSeq500 platform. Sequences were aligned to potential assay targets using the CLC Genomics Workbench 8.5.1 to identify the presence or absence of each target in each isolate. These results were analysed using the software package AuSeTTS (Automated Selection of Typing Target Subsets) (O'Sullivan *et al*., 2013) to identify the most discriminatory set of 20 targets for inclusion in the final binary typing assay and determine the maximum Simpson's index of diversity. Twenty is the maximum number of dual-probe targets which can be included on a 43-lane membrane format, allowing for the inclusion of controls.

2.2.2 Design principles for oligonucleotides of the mPCR/RLB assay.

In optimising sensitivity and specificity of the mPCR/RLB assay, an important criterion for the design of primers and probes is they must have similar reaction dynamics (Kong and Gilbert, 2006). The authors assert that the key to a successful mPCR/RLB assay is to minimise competition between primer pairs during amplification and hybridisation. Primers and probes should be between 18 – 30 base pairs in length, with melt temperatures of 58°C - 65°C. Also, ideally amplicon sizes are at the lower end of an 80 - 400 base pair window to limit the competitive disadvantage suffered by amplicons relative to length. Common with all molecular assays, primer dimer formation and strong secondary structure must be avoided. Some of the primers and probes used in the assay were from a previous publication (O'Sullivan *et al*., 2010). Other primers and probes were designed using a variety of available tools, including, sequence search database GenBank and BLAST search (NCBI, National Institutes of Health, Bethesda, MD) and OligoCalc oligonucleotide properties calculator version 3.27 (Northwestern University, Chicago, IL). Pooled controls were included to ensure consistency of probe performance. Oligonucleotide primer and probe sequences for the mPCR/RLB assay are shown in table 2.

Table 2: Oligonucleotide primer and probe sequences.

Target	Oligo Name	Oligo Sequence (5'-3')	Position	GenBank Accession	Product Size (bp)
nuc	nucSb	[btn]GCGATTGATGGTGATACGGTT	854574-854594	CP157524.1	278
	nucAp	[AmC6]CATTGGTTGACCTTTGTACATTAA	854598-854621		
	nucSp	[AmC6]GATGGAAAAATGGTAAACGAAG	854808-854829		
	nucAb	[btn]AGCCAAGCCTTGACGAACTAAAGC	854829-854852		
femA	femASb	[btn]ATAATAACGAGGTYATTGCAGCTTG	1370866 - 1370890	NZ_CP009361.1	232
	femASp	[AmC6]TTCAAATCGCGGTCCAGTRA	1370936 - 1370955		
	femAAp	[AmC6]TGGATCGATATGTAGGTATAGACARCG	1371024 - 1371050		
	femAAb	[btn]ATTACCTGTAATCTCGCCATCATGA	1371074 - 1371098		
mecA	mecAP4b	[btn]TCCAGATTACAACTTCACCAGG	44877-44898	CP157524.1	167
	mecAAp	[AmC6]CTAATGTTTTGTTATTTAACCCAATCAT	44824-44851		
	mecASp	[AmC6]GATGGTAAAGGTTGGCAAAAA	44780-44800		
	mecAP7b	[btn]CATTTACCACTTCATATCTTGTAACG	44731-44756		
eta	etaSb	[btn]CGGGAAATTCTGGATCAGGTATAT	936859-936882	LS483311.1	129
	etaSp	[AmC6]AGTGTCTCATCTTGATAGAGAGCA	936923-936946		
	etaAp	[AmC6]TGCTAGAATGTATACCAACTAATTCTCC	936894-936921		
	etaAb	[btn]CGCTTGACATAATTCCCAATACC	936966-936988		
etb	etbSb	[btn]TACCACCTAATACCCTAATAATCCAA	2702366 - 2702391	CP140702.1	369
	etbSp	[AmC6]GAGACAGTGCATTAAATGAATAAYTTT	2702269 - 2702295		
	etbAp	[AmC6]GATTTCTTCTGCGCTGTATTCTT	2702042 - 2702064		
	etbAb	[btn]CATTATCCGTAATGTGTGTATAAAGC	2701972 - 2701997		
sea	seaSb	[btn]CCTTTGGAAACGGTTAAAACG	2047518 - 2047538	CP157303.1	204
	seaSp	[AmC6]GGAGTTGGATCTTCAAGCAAGACG	2047471 - 2047494		
	seaAp	[AmC6]TCTGAACCTTCCCATCAAAAAC	2047412 - 2047433		

	seaAb	[btn]TTGAATACTGTCCTTGAGCACC	2047334 - 2047355		
sec	secSb	[btn]GCTCAAGAACTAGACATAAAAGCTAGG	199452-199478	CP140697.1	271
	secSp	[AmC6]AACGRCAATACTTTTTGGTATGAT	199560-199583		
	secAp	[AmC6]CTTCACWCTTTTAGAATCAACCG	199651-199673		
	secAb	[btn]TCAAAATCGGATTAACATTATCC	199701-199723		
sed	sedSb	[btn]CTAGTTTGGTAATATCTCCTTTAAACG	1014904 - 1014878	CP155060.1	321
	sedSp	[AmC6]TAAAGCCAATGAAAACATTGATTCA	1014852 - 1014876		
	sedAp	[AmC6]CTTTTATTTTCTCCTATTATTGGATTTTT	1014745 - 1014773		
	sedAb	[btn]CAATTAATGCTATATCTTATAGGGTAAACATC	1014583 - 1014614		
see	seeSb	[btn]ACAGATGCATTTAATGGAAAAATAC	2041282 - 2041306	CP155060.1	128
	seeSp	[AmC6]CTTCTTCTGGTGATTCGGT	2041239 - 2041257		
	seeAp	[AmC6]TCCTTGAGCACCAAATAAATC	2041211 - 2041231		
	seeAb	[btn]TCTATATATCCTCAACTGTGTATCTGG	2041178 - 2041204		
lukS-PVL	pvlSb	[btn]TTTTAGGCTCAAGACAAAGCAAC	1569732 - 1569754	CP157310.1	132
	pvlAp	[AmC6]TACCTCTGGATAACACTGGCATTTT	1569674 - 1569698		
	pvlSp	[AmC6]CTTCAATCCAGAATTTATTGGTGT	1569649 - 1569672		
	pvlAb	[btn]TTTGCAGCGTTTTGTTTTCG	1569622 - 1569641		
tsst	tsstSb	[btn]AAGCCAACATACTAGCGAAGGAAC	1232542 - 1232565	CP097311.1	208
	tsstSp	[AmC6]TGGYGTTACAAATACTGAAAAATTACC	1232587 - 1232613		
	tsstAp	[AmC6]TTATCGAACTTTGGCCMATACTTT	1232668 - 123691		
	tsstAb	[btn]GTATTTGAGTTAGCTGATGACGAA	1232727 - 1232750		
bla	blaSb	[btn]ACTGTTGTTAAAACTAAWGCACTTGC	845854-845879	CP155452.1	290
	blaSp	[AmC6]ACAGCAATGTGTTCAAATTTTC	846080-846101		

	blaAp	[AmC6]TGTAATCGATTGCAAATAARTHATAGGAG	845921-845949		
	blab	[btn]ATGGAAATAAAAAGTCAGCCCG	846123-846144		
dfrK	dfrKsb	[btn]ATTGCTGCGATGGATAAGAATAG	42795-42817	CP125862.1	197
	dfrKAp	[AmC6]AGGATTCCCAAGGACTGG	42849-42866		
	dfrKSp	[AmC6]CTGTCAGGTAAGGCTCTTCC	42933-42952		
	dfrKAb	[btn]AAAGGTAAACCCCTTATCTCTCGTC	42968-42992		
ermC	ermCsb	[btn]ACTTGTTGATCACGATAATTTCC	2772682 - 2772704	CP140688.1	184
	ermCSp	[AmC6]AACATAAGTACGGATATAATACGCAA	2772579 - 2772604		
	ermCAp	[AmC6]GCAATATATCCTTGTTTAAAACTTGG	2772658 - 2772683		
	ermCAb	[btn]TAGCAAACCCGTATTCCACG	2772520 - 2772539		
coa	coaSb	[btn]AGGTTGTTGGAAAAGGTATTAAGGG	267379-267403	CP154290.1	205
	coaSp	[AmC6]TCCTCGTACAGATTTAAAAATGG	267483-267505		
	coaAp	[AmC6]AGATTTCTTTCTCTCCAAAAGATATTG	267409-267435		
	coaAb	[btn]AGTGCTCTCTTTAATGCATCCTGTA	267560-267584		
splE	splESb	[btn]ATGTATGAGTCAACAGGAAAAGTGC	1946495 - 1946519	CP154290.1	129
	splESp	[AmC6]GCAACTCAGGTTCAGCTGT	1946428 - 1946446		
	splEAp	[AmC6]CATCAGTAATAATCATGTTGCCTT	1946462 - 1946485		
	splEAb	[btn]CAAAGTGAACACCTACAACTTCGTA	1946390 - 1946414		
see	seeSb	[btn]ACAGATGCATTTAATGGAAAAATAC	2041282 - 2041306	CP155060.1	128
	seeSp	[AmC6]CTTCTTCTGGTGATTCGGT	2041239 - 2041257		
	seeAp	[AmC6]TCCTTGAGCACCAAATAAATC	2041211 - 2041231		
	seeAb	[btn]TCTATATATCCTCAACTGTGTATCTGG	2041178 - 2041204		
Tn554 *tnpB*	N046Sb	[btn]TGCTTCAATTTCCACTCTCG	58297-58316	CP157524.1	127
	N046Ap	[AmC6]CAAGTTCAGAGAGTACACCAATTAA	58317-58341		
	N046Sp	[AmC6]GTCTTTGGGCAATATCACTTACATA	58380-58404		

	N046Ab	[btn]TGAACGTGGATAGCTTTTCC	58405-58424		
ϕMu50B SAV0881	SAV0881Sb	[btn]TGCTTGTTGTCATATCGCC	1154373-1154391	CP152457.1	199
	SAV0881Ap	[AmC6]TGTTTTGGTAACTAGCCACTGTATAGATA	1154392-1154420		
	SAV0881Sp	[AmC6]TCAAATTTCTTTTTGAATAGTAAGTCAGA	1154522-1154550		
	SAV0881Ab	[btn]CCTAGCTTGTATGTCTGCGCTA	1154551-1154572		
ϕPV83 ORF 2	PV83ORF-2Sb	[btn]GGCGCTTCTTCTTACAGGAG	2107583-2107602	CP134532.1	446
	PV83ORF-2Ap	[AmC6]CATTGTTAGATATTTATATGGTATGTAACCTAAAA	2107548-2107582		
	PV83ORF-2An	GATAATCTTGTTTTTTTCACTAACTAAACCTAT	2107515-2107547		
	PV83ORF-2Sn	TGTTTAATAACAACGGTAAACCAGTATTT	2107218-2107246		
	PV83ORF-2Sp	[AmC6]ATAGTTATTAAAGACTTTGAAAACAGAATCATT	2107185-2107217		
	PV83ORF-2Ab	[btn]GAATTATAGGTTTTAAGTTCACCCTCTTC	2107156-2107184		
ϕ11 nt 4427-5251	phi11-4563Sb	[btn]GATATGCAAGATCAGACAATGCC	343453-343475	CP140675.1	519
	phi11-4610Ap	[AmC6]CCTCGCTATCAACATGATTTCTAAT	343476-343500		
	phi11-4632An	CTAAATTGGTGCGTCAGTTTGT	343501-343522		
	phi11-5026Sn	CAAACTACTACACGAAGCTAGACTACAAC	343916-343944		
	phi11-5055Sp	[AmC6]GAAAAGTAAATAAACAGTGGGTGCTTTA	343945-343972		
	phi11-5103Ab	[btn]CTCTTGCCCATGTGTTCTGAG	343973-343993		
ϕSLT ORF 257	SLTorf257Sb	[btn]GTGTTATCGCTATGAGTGGTGAC	1601652-1601674	CP157524.1	340
	SLTorf-257Ap	[AmC6]TTAAAAAACTATTTTTGTGCATAAAAATAGT	1601621-1601651		

	SLTorf-257An	GTCATAACCCATGAATTATGAATCA	1601596 - 1601620		
	SLTorf-257Sn	TTAGGAGCTAATGAAATAGCTGCTAGTA	1601384 - 1601411		
	SLTorf-257Sp	[AmC6]TCTCTAAAGAGCAATATAAGCGTTTC	1601358 - 1601383		
	SLTorf-257Ab	[btn]CTTTAAATCTTCTGGGACGTTCTC	1601334 - 1601357		
ϕN315 SA1801	SA1801Ab	[btn]CAATCAGCGGTCGAGAACT	2073671 - 2073689	CP155060.1	516
	SA1801Ap	[AmC6]GAGTCTTAACCTCTAATGCTTGATGA	2073645 - 2073670		
	SA1801An	CATTCTTTCAAACCATTTTTTGTATG	2073619 - 2073644		
	SA1801Sn	CGCAGATTGTTTGAGTGGTTA	2073217 - 2073237		
	SA1801Sp	[AmC6]CGTCAAAACGGATTCCTTATTAAA	2073193 - 2073216		
	SA1801Ab	[btn]TTATAATCCACACCCTTGCG	2073173 - 2073192		
ϕMu50A SAV1974	SAV1974Sb	[btn]GCCACAAGAAAAGGCAGTG	2165194 - 2165212	CP157310.1	217
	SAV1974Ap	[AmC6]TGCTTACAGCTACATCTGTTTTGAT	2165169 - 2165193		
	SAV1974An	CGTTTTACTACTTACACCACTACGG	2165113 - 2165137		
	SAV1974Sp	[AmC6]GATATGAGTAACTTTGGTCGGAGTC	2164995 - 2165019		

2.2.3 Development and Optimisation of the mPCR/RLB Assay

2.2.3.1 Extraction of target DNA

Extraction of DNA from target isolates was performed using the Qiagen EZ1 Advanced XL platform and the Qiagen DNA Blood extraction kit. Manufacturer's instructions were followed (QIAGEN). An optional mechanical step involving the addition of glass beads and use of a vortex was omitted. (QIAGEN)(QIAGEN). Initially, extraction was attempted without a prior enzyme extraction step, aiming for a final DNA concentration of 25-75ng/µL. Gel electrophoresis using the BIO-RAD Molecular Imager

Gel Doc XR system and manufacturer's protocols (BIO-RAD) ()showed this method was unsuccessful. Fluorometric quantification using the Invitrogen Qubit Fluorometer, as per the manufacturer's instructions, showed a peak DNA concentration of only 4.03ng/µL was achieved with bacterial suspensions of varying turbidity ranging from 2.0 to 5.0 McFarland. A pre-extraction enzyme incubation step using Lysozyme and Lysostaphin was introduced. Subsequent use of a restriction endonuclease showed a well sheared DNA profile, and quantification using the Invitrogen Quant-iT ™ PicoGreen™ dsDNA Reagent and Kit, as per the manufacturer's instructions, showed DNA concentration of 56.4 – 71.2ng/µL, within the target range. In prospective typing of MSSA, DNA lysates were prepared of a single, isolated colony, using a McFarland 0.5 suspension, boiled at 100°C for 10 minutes before freezing at -20°C, which was found to produce sufficient DNA for use in the assay.

2.2.3.2 Singleplex PCR and hybridisation

Single PCR was performed using each of the target primer sets on two isolates with known expected positive results for those targets. MangoMix™ PCR master mix was used as per the manufacturer's instructions (Meridian). Single 25µL PCR reactions were set up containing components as shown in table 3. MSSA mPCR/RLB master mix components. PCR was performed using the thermocycler profile shown below in table 4. MSSA mPCR thermocycler profile.

Table 3: MSSA singleplex master mix components

Mastermix Component	Volume
MangoMix	12.5µL
10µM primer (forward)	0.5µL
10µM primer (reverse)	0.5µL
H_2O	6.5µL
Template DNA	5µL

Table 4: MSSA mPCR reaction thermocycler profile.

Number of cycles	Temperature	Time
1	95°C	5 minutes
35	95°C	30 seconds
	58°C	30 seconds
	72°C	30 seconds
1	72°C	10 minutes
1	4°C	Hold

Gel electrophoresis showed successful PCR amplification of all targets. Hybridisation was performed as per the RLB laboratory operating procedures described in this chapter section 2.4.1. Successful hybridisation results were achieved for all targets.

2.2.3.3 Multiplex PCR and hybridisation

Multiplex PCR/RLB was performed on a panel of isolates selected to include at least one expected positive for each target of the assay. MangoMix™ PCR master mix was used as per the manufacturer's

instructions (Meridian). Single multiplexed 25µL PCR reactions were set up containing the following components:

Table 5: MSSA mPCR master mix components

Mastermix Component	**Volume**
MangoMix	12.5µL
100µM primer (forward) x 27	0.05µL x 27 = 13.5 µL
100µM primer (reverse) x 27	0.05µL x 27 = 13.5 µL
H_2O	4.8µL
Template DNA	5µL

PCR was performed using the same thermocycler profile described in Chapter 2.2. Hybridisation was performed as per the RLB laboratory operating procedures described in Chapter 5.

2.3 Results

Within the reference set of 137 isolates, 121 unique binary types were distinguishable. Refer to Table 7 for binary profiles. The maximum Simpson's diversity index of 0.9977 (95%CI, 0.9960 to 0.9993) was achieved with a subset of just 24 of the 80 targets. Targets selected were varied and included toxin genes, antimicrobial resistance markers and phage derived open reading frames. When reading the mPCR/RLB assay, hybridisation is scored numerically as either a 2, 1 or 0 (for strong, faint or no visualised hybridisation respectively). A target is considered positive if both probes for that target show hybridisation, regardless of the intensity of hybridisation. If a single probe shows hybridisation, the target is only considered positive if that hybridisation is deemed strong. Figure 1. Shows the parameters used in the AuSeTTs analysis, including total number of candidate targets interrogated and targets which were excluded because they were present in all reference isolates or absent in all

reference isolates or 100% concordant with another candidate target. Figure2. Illustrates the threshold at which the addition of further targets does not increase the discriminatory power of the assay. Table1. Shows that two different combinations of 24 candidate targets are equally able to achieve the maximum discrimination of the group of 137 isolates into 121 unique binary types. Next, incorporation of these targets to an mPCR/RLB platform was undertaken with the aim producing an inexpensive and high throughput method for typing of MSSA. Figure3. Shows such an RLB assay, in which chemiluminescence is used to visualise hybridisation, and generate a binary profile.

Table 6: Parameters and summary output of AuSeTTS analysis.

Parameters	Summary Output
Number of isolates used in the dataset	137
Number of potential targets identified in the dataset	80
Number of non-informative targets (either all positive or all negative for all isolates in the collection)	38
Number of targets excluded, fully concordant with another target in the dataset	2
Number of potentially informative targets for analysis	40
Number of targets required to achieve maximum Simpson's index of diversity	24
Number of different binary types that could be distinguished	121
Number of isolates associated with the most common binary type	4 (3%)

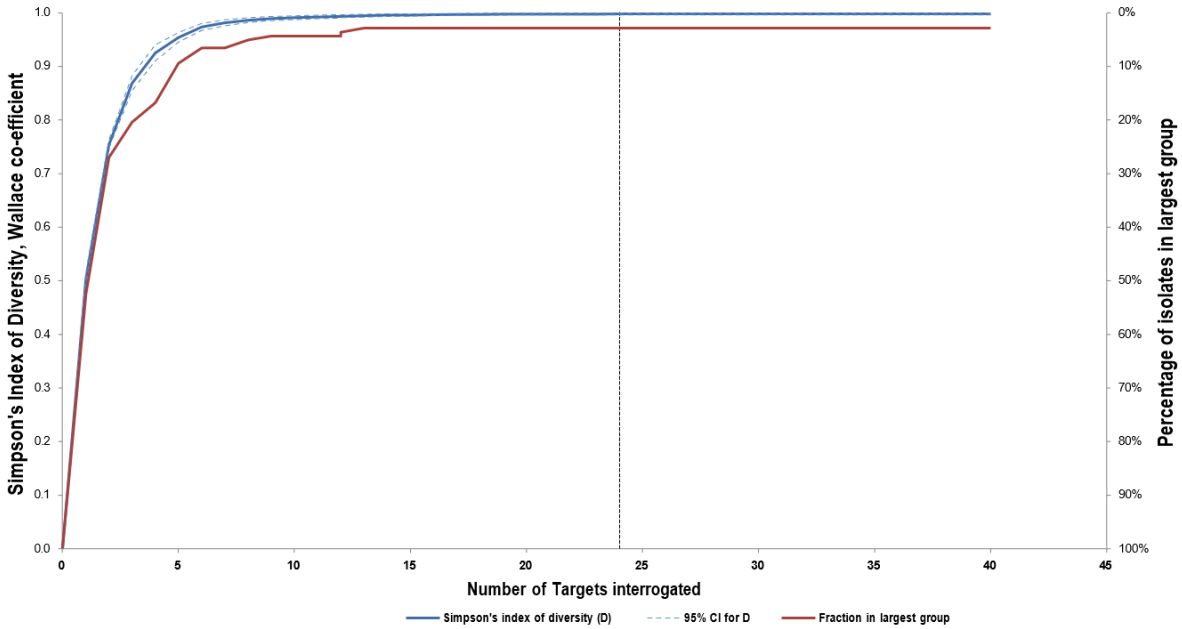

Figure 1: Graph illustrating Simpson's Index of Diversity and targets interrogated.

The data analysis showed that any one of two different combinations of 24 targets could be used to produce the maximal discrimination in the dataset. (Table 1).

Table 7: Targets selected by the AuSeTTs software for the binary typing system.

Target type	Targets present in both combinations	Targets present in one of the two combinations (combination number)
Toxin genes:	*sea, sec, sed, eta, see, etb* nt550-750	
Phage-derived open reading frames:	φMu50B SAV0881, φPV83 ORF2, φMu50B SAV0858, φ11 nt4427-5251, φSLT ORF257, φN315 SA1801, φMu50A SAV1974	
Antimicrobial resistance genes	*cad*B, *erm*C, *dfr*K, *amp* nt001-200	
Others	*coa* (coagulase), *coa* nt1800-2000, *splE* (exoprotein), *spl*E nt330-530, phage integrase, Tn554tnpB (transposon)	*ccr*AB (SCC*mec* element) (1) CQ002 (SCC*mec* element) (2)

Several changes were subsequently incorporated into the final 43 lane mPCR/RLB assay format to accommodate 20 informative and/or discriminatory targets. Non-discriminatory controls, both

positive and negative, were also included (*nuc, fem*A and *mec*A). AuSeTTS was rerun with several targets of potentially clinical interest force included (TSST and *lukS*-PVL). In the final analysis for 20 discriminatory targets; ϕMu50B, SAV0858, *cad*B, *amp* and phage integrase are removed and *bla* was an addition. For the final format of the mPCR/RLB, Simpson's index of diversity is calculated at 0.991 (95% CI 0.987 – 0.996).

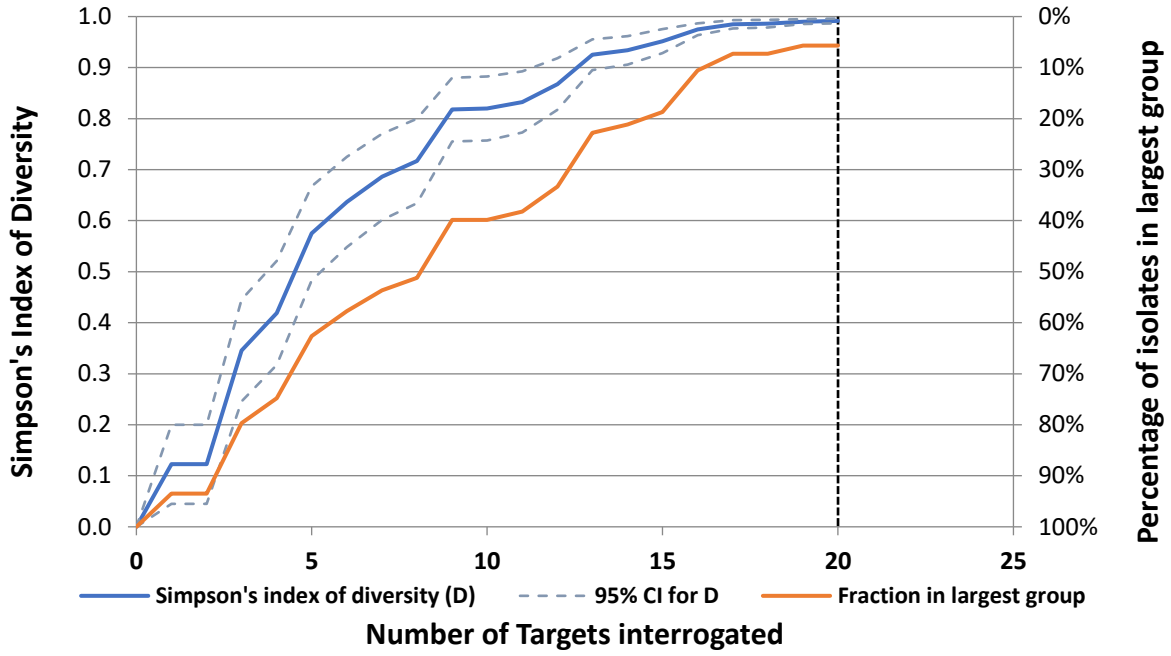

Figure 2: Graph indicating Simpson's Index of diversity and targets interrogated for the final MSSA mPCR/RLB format.

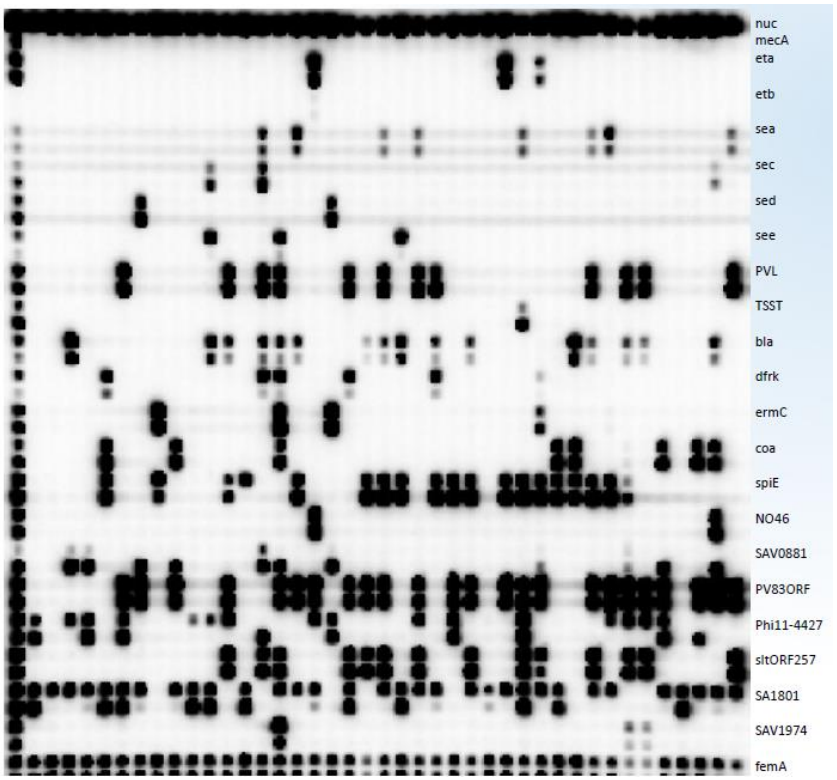

Figure 3: MSSA mPCR/RLB assay showing hybridisation. Lane 1 (left) = pooled control. Lanes 2 to 43 = study isolates.

Successful hybridisation results were achieved for all targets in the multiplexed reaction. Initially an exposure time of 8 minutes was selected for film development, however this was found to produce very intense hybridisation spots with some smudging and other artefact. Five minutes exposure produced a clearer result but with some smudging still evident. Three minutes exposure time was found to produce clear film with hybridisation spots of an appropriate intensity, and this was adopted for the protocol.

2.4 Discussion

2.4.1 Assessment of Assay against Consensus Guidelines

Consensus guidelines have been developed to assess potential sub-typing assays against a series of criteria (Struelens, 1996). The author notes that new subtyping assays are often introduced without thorough assessment of eithertechnical performance or convenience. Technical performance criteria

include typeability, reproducibility, stability, discriminatory power, epidemiologic concordance and typing system concordance. Convenience criteria include flexibility, rapidity, accessibility, and ease of use. Here they are discussed with respect to the MSSA mPCR/RLB assay.

2.4.1.1 Performance Criteria

2.4.1.1.1 Typeability

Rationale: The typeability of the assay is a measure of what proportion of strains interrogated by the typing system which can be assigned a type.

Methods: This can be expressed by the formula $T = N^1/N$ where T (typeability) should approach 1. N^1 is the number of strains assigned a type and N is the total number of strains tested.

Results: The typeability of the MSSA mPCR/RLB assay is assessed as 1 given all isolates used in both the development of the assay and the prospective application of it in Chapters 3, 4 and 5 had a binary type generated.

Implications: It is worthwhile qualifying here that even if none of the informative or discriminatory targets are positive and the positive and negative controls are as expected a binary profile is generated, simply one containing many zeros. For this reason, binary typing assays in general will score well in typeability measures.

2.4.1.1.2 Reproducibility

Rationale: The reproducibility of the assay is a measure of its ability to assign the same subtype to a given strain when it is tested on separate, independent assay runs.

Method: This can be expressed by the formula $R = N^t/N$ where R (reproducibility) should be >0.95. N^t is the number of strains assigned an identical type on repeat testing and N is the total number of strains tested. Important considerations in developing reproducibility include the use of standardised protocols and equipment, along with the impact of subjectivity if it exists in category

assignment. This is a consideration for the visual reading / category assignment used in the mPCR/RLB assay.

Results: In calculating the reproducibility of the assay, a control strain, and 16 clinical strains were used. The Mu3 control strain was included on 9 assays run between 19/05/2017 and 21/02/2018. After this, a pooled positive control was run on each assay. This pooled positive was not considered appropriate to include in assessment of reproducibility given there is potentially multiple positive strains within the pool for any given target. A bias towards reproducibility is likely in that scenario. A single digit difference in the binary profile for the control strain was recorded for the *splE* anti-sense probe at position 28 (underlined) on the run from 14/02/2018 (binary profile: 110000 111100 000011 110000 000<u>1</u>11 110010 00101111). Note that on review that the hybridisation at this point was weak. All other targets were identical across the 9 runs. For other duplicate tested isolates, which were assayed between two and four times each, there were two further instances of binary type discordance. These both differed however, by a single target. The reproducibility in this data set of 45 binary profiles is calculated as 0.93, slightly below the target of >0.95.

Implictions: The reproducibility being slightly below the target of >0.95 is considered acceptable given that discordant results differed only by a single target. These isolates are still easily recognised as highly similar.

2.4.1.1.3 Stability

Rationale: A stable typing assay can recognise the relatedness of a given strain to a common ancestor despite the genomic variation that occurs either during laboratory processes (*in vitro* stability) or during dissemination within nature (*in vivo* stability).

Methods: This can be expressed by the formula $S = N_t/N$ where S (stability) should approach 1. N_t is the number of strains assigned an identical type on repeat testing and N is the total number of tests performed in the assessment of stability.

Results: *In vitro* stability, assessed with a control strain and 12 clinical isolates is calculated as 0.94, and *in vivo* stability, assessed using a total of 28 isolates collected from 13 patients separated by either ≥28 days or from a different collection site is calculated as 0.93.

Implications: The MSSA mPCR/RLB assay is relatively stable and can recognise relatedness.

2.4.1.1.4 Discriminatory Power

Rationale: A discriminatory assay will assign a different type to unrelated strains which have been sampled randomly from the studied population.

Methods: A reference library of 137 MSSA strains chosen specifically to represent a diverse, unrelated population included 89 unique binary types when interrogated with the mPCR/RLB assay.

Results: The Simpson's index of diversity was calculated by the AuSeTTs software package as 0.991 (95%CI 0.987 – 0.996). The most common binary type accounted for 6% of isolates.

Implications: The MSSA mPCR/RLB assay is sufficiently discriminatory assign different types to a large proportion of isolates from a diverse library.

2.4.1.1.5 Epidemiologic Concordance

Limited epidemiological data is available for the dataset to allow assessment of epidemiologic concordance. No spatial clustering was identified in chapters 3 and 4 of this thesis. A potentially contributing factor to this lack of identified spatial clustering, is the lack of visibility around bed movements of patients that may have been admitted to the hospital through the emergency department, after collection of a sample. Of the 491 isolates included for analysis in chapter 4, 422 were collected in the emergency department and subsequently excluded from analysis for spatial clustering. Epidemiological concordance is illustrated in 12 patients, with isolates from different collection sites being concordant for 23 of 25 of binary profiles (93%).

2.4.1.1.6 Typing System Concordance

Rationale: Assessing the level of concordance of a newly developed assay with established methods is a valuable tool in assessing the usefulness of the assay.

Methods: Of the 137 reference library isolates, 124 underwent Illumina HiSeq™ whole genome sequencing. *In silico* MLST types were determined using CLC genomics workbench.

Results: Twenty-eight MLST types were identified belonging to 13 clonal complexes. ST5 was the most identified MLST type with 24 isolates (19.35%). AuSeTTs was used to calculate the adjusted Wallace coefficient for binary type predicting MLST, which was 0.597 (95% CI 0.444 – 0.749). See table 9. *In silico* binary profiles for comparison of WGS and mPCR/RLB results. Within the 137 reference library isolates, 10 targets were detected by WGS only and two targets where positive by mPCR/RLB only. All remaining targets were concordant.

Implications: Significant heterogeneity in MSSA is a likely contributing factor to the low coefficient, although this remains unproven.

2.4.1.2 Convenience Criteria

2.4.1.2.1 Flexibility

The mPCR/RLB method exhibits significant flexibility as a subtyping tool. Here we have modified an assay originally developed for subtyping of MRSA (O'Sullivan *et al*., 2012) and adapted it to MSSA. This involved removing targets which were either non-discriminatory or not relevant and replacing them with alternate informative and/or discriminatory ones. It is likely that this new assay could be utilised on MRSA isolates, however this was not formally assessed. The incorporation of SCC*mec* targets would likely lead to a more informative and discriminatory assay for MRSA. Whilst intra-species flexibility is established, the assay has also been used successfully on a variety of other bacterial species including *Streptococcus pneumoniae* (Kong *et al*., 2006) and Vancomycin resistant Enterococci. Beyond intra- and inter-species flexibility the method has also been successfully employed in higher order taxonomic

groups, albeit mostly for identification at the species level rather than subtyping. A method was developed for identifying dermatophytes from nail, skin, and hair samples (Bergmans *et al*., 2008). Also, tick-borne pathogens such as *Theileria, Babesia, Anaplasma* and *Ehrlichia* species have been identified in cattle from the Maghreb region of Africa (Iqbal *et al*., 2013).

2.4.1.2.2 Rapidity

The mPCR/RLB takes two days to perform in standard laboratory working hours. DNA Extraction and PCR can be performed on day one, and the RLB hybridisation can be done within six hours on day two. In a scenario where results were considered urgent the test can even be performed on one day with extended hours of operation (10 – 12 hours total time). In comparison, whole genome sequencing based methods, which are now the gold standard in subtyping take at least a week in a best-case scenario (including extraction, library preparation, sequencing, and analysis). There is great advantage in using the mPCR/RLB method given the superior turnaround time. Being able to implement targeted interventions based on subtyping results available >3 days earlier could lead to significantly improved outcomes.

2.4.1.2.3 Accessibility

The mPCR/RLB assay is a readily accessible tool for laboratories to employ and has significantly reduced consumable and equipment costs compared with many favoured and emerging typing systems, particularly those based on whole-genome sequencing. Equipment required is basic and inexpensive, and includes: a hybridisation oven, glass rolling bottles, a mini-blotter, nylon separating mesh, foam cushions and a rocking platform. An X-ray film exposure cassette, film cartridge and developer will also be required for the visualising the hybridisation. The assay is described as versatile and easily transferrable to other laboratories. (Kong and Gilbert, 2006) mPCR/RLB Primers and probes may be ordered lyophilised, allowing for convenient shipping and storage at room temperature.

2.4.1.2.4 Ease of Use

The mPCR/RLB is an easy-to-use assay which can be readily deployed in multi-centre studies with the use of standardised protocols. Whilst it is important to follow the numerous steps accurately, there is little in terms of technical difficulty in the performance of the mPCR, the hybridisation or indeed interpretation of the assay. Simple to follow laboratory operating procedures have been developed to cover the preparation of an RLB membrane and the production of required solutions, the mPCR/RLB assay protocol and the analysis of results. Prerequisite knowledge for potential users is described (Kong and Gilbert, 2006) and is limited to basic computer skills, and basic molecular biology skills (extraction of DNA, conventional PCR, gel electrophoresis and preparation of buffers and solutions).

In conclusion, WGS sequencing and subsequent analysis of a reference library of MSSA isolates has identified useful targets for a binary typing assay. Considering the performance criteria which have been assessed, the mPCR/RLB is found to be fit for purpose as a tool for prospective use in the binary typing of MSSA. The applicability of this assay does not likely extend to MRSA due to the omission of the SCC*mec* targets which are informative and discriminatory for that population. This was not formally assessed. Reproducibility, as calculated at 0.93, is noted to be below the target of >0.95 (Struelens, 1996). The three discordant binary profiles differed by only a single target and are considered highly similar. Further investigation may be warranted for highly similar binary profiles. The MSSA mPCR/RLB performs similarly on assessment on consensus performance criteria to the previously developed MRSA mPCR/RLB assay (O'Sullivan *et al*., 2012), MLST 0.993 (0.986 – 1.000).

Binary typing using the mPCR/RLB will provide an inexpensive, timely and discriminatory assay for strain-based surveillance of nosocomial MSSA infection and colonisation. Routine, prospective application of this assay in the hospital setting will reduce the need for more expensive methods such as WGS and afford new insights into the epidemiology of MSSA infections.

Chapter 3. Molecular epidemiology of methicillin-sensitive *Staphylococcus aureus* causing blood stream infections in New South Wales in 2017

In this Chapter, the utility of the mPCR/RLB binary typing assay in epidemiological investigations of methicillin-sensitive *Staphylococcus aureus* (MSSA) is investigated prospectively in the hospital setting. Specifically, 213 MSSA strains isolated from blood cultures collected at ten hospitals in New South Wales in 2017, were typed using the assay. Duplication of some content with Chapter 4. *Molecular epidemiology of methicillin-sensitive Staphylococcus aureus isolated from a hospital in western Sydney in 2017* is acknowledged. Research papers forming the basis of these two chapters were prepared for submission simultaneously.

3.1 Statement of contribution

My contribution to this publication is assay and study design, experimental work including mPCR/RLB and whole genome sequencing with analysis relating to the selection of targets, data analysis and preparation of manuscript. MOS and GG contributed with study design and manuscript preparation, and MR contributed with supervision and assistance of whole genome sequencing and analysis.

3.2 Molecular Epidemiology of Methicillin-Sensitive *Staphylococcus aureus* causing blood stream infections in New South Wales in 2017

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Abstract

Objective: *Staphylococcus aureus* (*S. aureus*) bloodstream infection is a clinically important disease that is often associated with healthcare and carries significant mortality risk. We report the findings from a study of methicillin-sensitive *Staphylococcus aureus* (MSSA) causing bacteraemia in New South Wales in 2017. Understanding the molecular epidemiology of MSSA bacteraemia will afford insight into genomic markers of interest and illuminate potential chains of transmission.

Methods: All MSSA isolated from blood cultures collected at 10 hospitals in metropolitan and regional New South Wales in 2017 were included in the study (n= 213). Binary typing was performed using a multiplex PCR based reverse line blot assay (mPCR/RLB).

Results: All available isolates were successfully binary typed, with 134 unique binary types identified. The most prevalent binary type accounted for 4.69% of isolates (n=10). Toxic shock syndrome toxin (TSST) was detected in 11.9% of isolates (n=25), Panton-Valentine leucocidin (PVL) was detected in 2.86% of isolates (n=6).

Conclusions: A diverse population of MSSA was responsible for bacteraemia at 10 hospitals in New South Wales in 2017. Binary typing revealed three temporospatial clusters of several rare binary types at the facility level.

Introduction

We have previously established binary typing of methicillin-resistant *Staphylococcus aureus* (MRSA) by multiplex PCR reverse line blot (mPCR/RLB) as an effective high-throughput strain typing method, suitable for routine, prospective use as part of a hospital infection control programme, which affords an opportunity for targeted interventions (O'Sullivan *et al*., 2012). A similar mPCR/RLB assay, developed and optimised for methicillin sensitive *Staphylococcus aureus* (MSSA) has been developed. In this study, the MSSA mPCR/RLB assay is prospectively applied in the typing of MSSA isolated from blood cultures collected at 10 hospitals in metropolitan Sydney and regional New South Wales. *Staphylococcus aureus* is a common causative agent of bacteraemia, with significant impacts for both patients and the healthcare sector. A pairwise-matched nested case control study from Duke University Medical Centre (Abramson and Sexton, 1999) found a median length of stay of 4 days for nosocomial primary MSSA bacteraemia and an attributable median total cost US$9661. While these metrics are more favourable than the authors found for MRSA, which were a mean length of stay of 12 days and an attributable median total cost of $27,083, they are significant. Better understanding of the molecular epidemiology of MSSA bacteraemia will help elucidate potential transmission events, identify markers of interest, and inform potential interventions.

Methods

Two hundred and thirteen MSSA strains isolated from blood cultures collected at 10 hospitals in metropolitan Sydney and regional New South Wales in 2017 were prospectively typed using the MSSA mPCR/RLB assay. Cases were excluded from the study if the patient had been culture positive in a two-week period prior. Three isolates which qualified for inclusion in the study were not stored and subsequently unable to be binary typed. Blood cultures were collected using the BD Bactec™ FX system as per standard clinical protocols and bottles were incubated for 5 days. Flagged positive

bottles indicating Gram positive cocci suggestive of staphylococci had PCR performed using the BD Max™ StaphSR assay for a presumptive identification of *Staphylococcus aureus* and to indicate methicillin susceptibility. Positive bottles were also sub-cultured onto standard laboratory including Columbia base horse blood agar, and chocolatised blood agar for work-up. Following incubation, identification of suspect colonies was confirmed using the Bruker™ MALDI-TOF biotyper, and formal susceptibilities determined using the PMIC-84 panel on the BD Phoenix™ system. Confirmed MSSA were stored at -70°C in cryostorage media containing skim milk, glucose, and glycerol, and, also had DNA extracted on the Qiagen EZ1 platform using the blood DNA extraction kit and manufacturer's instructions. Multiplex PCR/RLB was performed on isolates as per standard laboratory operating procedures. MangoMix™ PCR master mix was used as per the manufacturer's instructions. Oligonucleotide sequences for primers and probes used in the mPCR/RLB assay can be found in **Appendix 1. Oligonucleotide primers and probes**. Analysis of the RLB membrane was performed as per standard laboratory operating procedures.

Results were analysed for clustering. Temporospatial clusters were defined as two or more isolates with the same binary type collected less than or equal to 28 days apart, from different patients at the same healthcare facility or ward. Spatial clusters were defined as two or more isolates from different patients on the same ward, excluding the emergency department. Binary type clusters were defined as groups of isolates from different patients belonging to a rare binary type representing 4 or fewer isolates. Less common binary types are less likely to be temporo-spatially clustered by chance alone.

Results

All available isolates were successfully binary typed by the MSSA mPCR/RLB assay. **Figure 5** shows, for a subset of the isolates, the mPCR/RLB membrane with hybridisation visualised at the intersection of PCR products and membrane linked probes. A total of 134 unique binary types were identified.

Simpson's index of diversity was calculated using the AuSeTTS software package (O'Sullivan *et al*., 2013) as 0.992 (95%CI 0.989 – 0.995). The most prevalent binary profile, designated as binary type 152, accounted for 4.69% of isolates. Refer to Table 7 for binary profiles.

There were 24 clusters of rare binary type represented by 4 or fewer isolates. Three of these rare binary type clusters contained two isolates each from the same facility with collection dates within 28 days. These were not spatially linked at the ward level, being collected in the emergency department. The toxic shock syndrome toxin gene (t*st*) was detected in 11.9% of isolates (n=25) and the *lukS* gene encoding Panton-Valentine leucocidin (PVL) was detected in 2.86% of isolates (n=6). Exfoliative toxin gene detection was uncommon with a single *eta* detected. The prevalence of staphylococcal enterotoxin genes encoding enterotoxins A, C, D and E were 14.76%; 5.25%; 8.57% and 14.29% respectively. Resistance markers *dfrK* and *ermC* were found in 1.43% and 4.29% of isolates respectively.

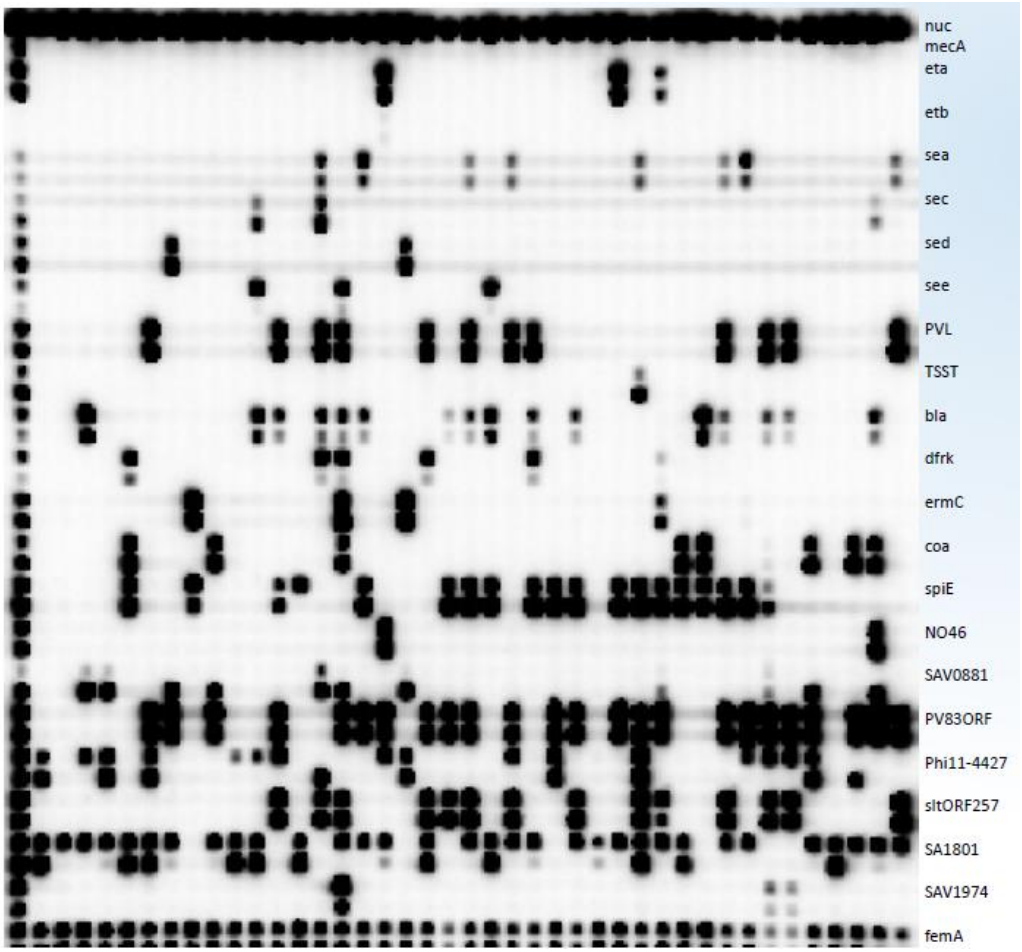

Figure 4: MSSA mPCR/RLB assay showing hybridisation. Lane 1 (left) = pooled control. Lanes 2 to 43 = study isolates. Target presence is visualised as a hybridisation spot at the junction of mPCR product and biotinylated probes.

Discussion

A diverse population of MSSA was responsible for bacteraemia at 10 hospitals in metropolitan Sydney and regional New South Wales in 2017. A total of 134 unique binary types were identified, with a Simpson's index of Diversity >99%. All 20 targets on the assay were shown to be contributory to the discrimination of the isolates as indicated by **Figure 6**, where the cumulative Simpson's index of diversity for increasing target number is visualised.

Of the 24 clusters of rare binary types detected, three contained two isolates each from the same facility with collection dates within 28 days. These could be considered temporospatial clusters at the facility level, however there was no identified spatial association for these clusters within the hospital. A limitation of the dataset is a lack of visibility about bed movements within the hospital following

sample collection. This may mask potential clusters of transmission. Collection of data regarding movement of patients through the hospital would enable greater granularity of cluster analysis. For these potential clusters, whole-genome sequencing with single-nucleotide polymorphism analysis could be performed to explore relatedness of the isolates with more granularity and confirm transmission events.

For the most commonly identified binary type, binary type 152 (n=9), collection dates were spread across the study period, from the 16th of January 2017 to the 7th of October 2017. The 9 binary type 152 isolates were also collected from 6 out of the 10 hospitals represented in the study. 4 of the 9 binary type 152 isolates did come from the one hospital, which is the largest facility in the dataset, however these were each collected in a different calendar month of the study period.

The mPCR/RLB assay reveals information on the toxin profile of the bacteraemia causing MSSA isolates, with the gene encoding toxic shock syndrome toxin being detected in around 12% of isolates. In a 2019 study from South Korea reported a high early mortality rate in patients with toxic shock syndrome toxin 1-producing clonal complex 5 MRSA of the New York/ Japan epidemic clone. (Kim *et al*., 2019). The gene encoding Panton-Valentine leucocidin (PVL) was found in 6 isolates. PVL production has been linked to specific clinical syndromes such as necrotising pneumonia. A case control study from the University of Massachusetts in 2022 found 29% of 1681 sequences *S. aureus* genomes contained the genes that encode for PVL (Qu *et al*., 2022). The authors noted the limited data on the clinical and epidemiological features of PVL positive *S. aureus* bacteraemia in their study. Exfoliative toxins were uncommon with a single detection of *eta*, and no *etb* detected. Exfoliative toxins are typically associated with skin and soft tissue infections like staphylococcal scalded skin syndrome, and the incidence is reported to be increasing (Azarian *et al*., 2021). Detection of genes encoding staphylococcal enterotoxins were common (38.39% of isolates carried at least one enterotoxin gene). Heat-stable staphylococcal enterotoxins are implicated in a wide variety of

diseases including food poisoning (Ortega *et al*., 2010), though significance of these enterotoxins in bacteraemia is less clear.

A limited amount of information regarding antimicrobial susceptibilities can be garnered from the binary typing results. *dfrK* and *ermC*, which are markers of trimethoprim and macrolide/lincosamide resistance, were found in less than 5% of isolates.

The mPCR/RLB assay is a readily accessible tool for laboratories to employ and has significantly reduced consumable and equipment costs compared with many favoured and emerging typing systems, particularly those based on whole-genome sequencing. Equipment required is basic and inexpensive, and includes: a hybridisation oven, glass rolling bottles, a mini-blotter, nylon separating mesh, foam cushions and a rocking platform. An X-ray film exposure cassette, film cartridge and developer will also be required for the visualising the hybridisation. The assay is described as versatile and easily transferrable to other laboratories. (Kong and Gilbert, 2006) mPCR/RLB Primers and probes may be ordered lyophilised, allowing for convenient shipping and storage at room temperature.

The mPCR/RLB is an easy-to-use assay which can be readily deployed in multi-centre studies with the use of standardised protocols. Whilst it is important to follow the numerous steps accurately, there is little in terms of technical difficulty in the performance of the mPCR, the hybridisation or indeed interpretation of the assay. Simple to follow laboratory operating procedures have been developed to cover the preparation of an RLB membrane and the production of required solutions, the mPCR/RLB assay protocol and the analysis of results. Prerequisite knowledge for potential users is described (Kong and Gilbert, 2006) and is limited to basic computer skills, and basic molecular biology skills (extraction of DNA, conventional PCR, gel electrophoresis and preparation of buffers and solutions).

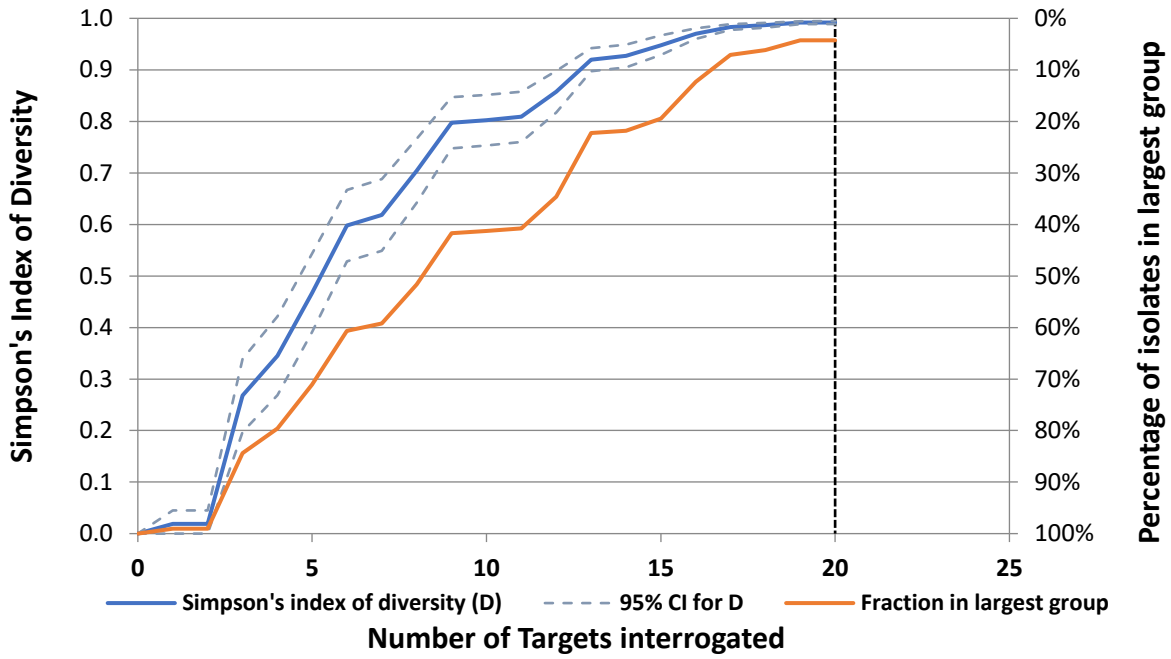

Figure 5: Simpson's index of diversity and percentage in largest group vs number of targets included in analysis.

Conclusions

A diverse population of MSSA was responsible for 213 cases of bacteraemia at 10 hospitals in metropolitan Sydney and regional New South Wales in 2017. There was evidence in this dataset of limited temporospatial clustering of rare binary types. Targeted application of whole genome sequencing with single nucleotide polymorphism (SNP) analysis and collection of patient movement data at the ward level would help elucidate these potential clusters further. A variety of toxins and antimicrobial resistance markers that are of potential significance were found in these strains.

The MSSA mPCR/RLB assay is an inexpensive and rapid typing method, with results able to be generated within 2 days. This compares favourably with WGS and other subtyping methods. The mPCR/RLB method is best employed as a screen to identify potential clusters and allow for the more targeted application of methods like WGS, avoiding unnecessary and costly sequencing of strains. Given the basic equipment and expertise requirements, the assay can be used to track MSSA not just within a single facility but across multi-centre studies.

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Disclosure Statement

The authors have no conflicts of interest or competing interests to disclose.

Appendix 1. Oligonucleotide primers and probes

Target	Oligo Name	Oligo Sequence (5'-3')	Position	GenBank Accession	Product Size (bp)
nuc	nucSb	[btn]GCGATTGATGGTGATACGGTT	854574-854594	CP157524.1	278
	nucAp	[AmC6]CATTGGTTGACCTTTGTACATTAA	854598-854621		
	nucSp	[AmC6]GATGGAAAAATGGTAAACGAAG	854808-854829		
	nucAb	[btn]AGCCAAGCCTTGACGAACTAAAGC	854829-854852		
femA	femASb	[btn]ATAATAACGAGGTYATTGCAGCTTG	1370866-1370890	NZ_CP009361.1	232
	femASp	[AmC6]TTCAAATCGCGGTCCAGTRA	1370936-1370955		
	femAAp	[AmC6]TGGATCGATATGTAGGTATAGACARCG	1371024-1371050		
	femAAb	[btn]ATTACCTGTAATCTCGCCATCATGA	1371074-1371098		
mecA	mecAP4b	[btn]TCCAGATTACAACTTCACCAGG	44877-44898	CP157524.1	167
	mecAAp	[AmC6]CTAATGTTTTGTTATTTAACCCAATCAT	44824-44851		
	mecASp	[AmC6]GATGGTAAAGGTTGGCAAAAA	44780-44800		
	mecAP7b	[btn]CATTTACCACTTCATATCTTGTAACG	44731-44756		
eta	etaSb	[btn]CGGGAAATTCTGGATCAGGTATAT	936859-936882	LS483311.1	129
	etaSp	[AmC6]AGTGTCTCATCTTGATAGAGAGCA	936923-936946		
	etaAp	[AmC6]TGCTAGAATGTATACCAACTAATTCTCC	936894-936921		
	etaAb	[btn]CGCTTGACATAATTCCCAATACC	936966-936988		
etb	etbSb	[btn]TACCACCTAATACCCTAATAATCCAA	2702366-2702391	CP140702.1	369
	etbSp	[AmC6]GAGACAGTGCATTAAATGAATAAYTTT	2702269-2702295		
	etbAp	[AmC6]GATTTCTTCTGCGCTGTATTCTT	2702042-2702064		
	etbAb	[btn]CATTATCCGTAATGTGTGTATAAAGC	2701972-2701997		

sea	seaSb	[btn]CCTTTGGAAACGGTTAAAACG	2047518-2047538	CP157303.1	204
	seaSp	[AmC6]GGAGTTGGATCTTCAAGCAAGACG	2047471-2047494		
	seaAp	[AmC6]TCTGAACCTTCCCATCAAAAAC	2047412-2047433		
	seaAb	[btn]TTGAATACTGTCCTTGAGCACC	2047334-2047355		
sec	secSb	[btn]GCTCAAGAACTAGACATAAAAGCTAGG	199452-199478	CP140697.1	271
	secSp	[AmC6]AACGRCAATACTTTTTGGTATGAT	199560-199583		
	secAp	[AmC6]CTTCACWCTTTTAGAATCAACCG	199651-199673		
	secAb	[btn]TCAAAATCGGATTAACATTATCC	199701-199723		
sed	sedSb	[btn]CTAGTTTGGTAATATCTCCTTTAAACG	1014904-1014878	CP155060.1	321
	sedSp	[AmC6]TAAAGCCAATGAAAACATTGATTCA	1014852-1014876		
	sedAp	[AmC6]CTTTTATTTTCTCCTATTATTGGATTTTT	1014745-1014773		
	sedAb	[btn]CAATTAATGCTATATCTTATAGGGTAAACATC	1014583-1014614		
see	seeSb	[btn]ACAGATGCATTTAATGGAAAAATAC	2041282-2041306	CP155060.1	128
	seeSp	[AmC6]CTTCTTCTGGTGATTCGGT	2041239-2041257		
	seeAp	[AmC6]TCCTTGAGCACCAAATAAATC	2041211-2041231		
	seeAb	[btn]TCTATATATCCTCAACTGTGTATCTGG	2041178-2041204		
lukS-PVL	pvlSb	[btn]TTTTAGGCTCAAGACAAAGCAAC	1569732-1569754	CP157310.1	132
	pvlAp	[AmC6]TACCTCTGGATAACACTGGCATTTT	1569674-1569698		
	pvlSp	[AmC6]CTTCAATCCAGAATTTATTGGTGT	1569649-1569672		
	pvlAb	[btn]TTTGCAGCGTTTTGTTTTCG	1569622-1569641		
tsst	tsstSb	[btn]AAGCCAACATACTAGCGAAGGAAC	1232542-1232565	CP097311.1	208
	tsstSp	[AmC6]TGGYGTTACAAATACTGAAAAATTACC	1232587-1232613		

	tsstAp	[AmC6]TTATCGAACTTTGGCCMATACTTT	1232668-123691		
	tsstAb	[btn]GTATTTGAGTTAGCTGATGACGAA	1232727-1232750		
bla	blaSb	[btn]ACTGTTGTTAAAACTAAWGCACTTGC	845854-845879	CP155452.1	290
	blaSp	[AmC6]ACAGCAATGTGTTCAAATTTTC	846080-846101		
	blaAp	[AmC6]TGTAATCGATTGCAAATAARTHATAGGAG	845921-845949		
	blaAb	[btn]ATGGAAATAAAAAGTCAGCCCG	846123-846144		
dfrK	dfrKsb	[btn]ATTGCTGCGATGGATAAGAATAG	42795-42817	CP125862.1	197
	dfrKAp	[AmC6]AGGATTCCCAAGGACTGG	42849-42866		
	dfrKSp	[AmC6]CTGTCAGGTAAGGCTCTTCC	42933-42952		
	dfrKAb	[btn]AAAGGTAAACCCCTTATCTCTCGTC	42968-42992		
ermC	ermCsb	[btn]ACTTGTTGATCACGATAATTTCC	2772682-2772704	CP140688.1	184
	ermCSp	[AmC6]AACATAAGTACGGATATAATACGCAA	2772579-2772604		
	ermCAp	[AmC6]GCAATATATCCTTGTTTAAAACTTGG	2772658-2772683		
	ermCAb	[btn]TAGCAAACCCGTATTCCACG	2772520-2772539		
coa	coaSb	[btn]AGGTTGTTGGAAAAGGTATTAAGGG	267379-267403	CP154290.1	205
	coaSp	[AmC6]TCCTCGTACAGATTTAAAAATGG	267483-267505		
	coaAp	[AmC6]AGATTTCTTTCTCTCCAAAAGATATTG	267409-267435		
	coaAb	[btn]AGTGCTCTCTTTAATGCATCCTGTA	267560-267584		
splE	splESb	[btn]ATGTATGAGTCAACAGGAAAAGTGC	1946495-1946519	CP154290.1	129
	splESp	[AmC6]GCAACTCAGGTTCAGCTGT	1946428-1946446		
	splEAp	[AmC6]CATCAGTAATAATCATGTTGCCTT	1946462-1946485		
	splEAb	[btn]CAAAGTGAACACCTACAACTTCGTA	1946390-1946414		

see	seeSb	[btn]ACAGATGCATTTAATGGAAAAATAC	2041282-2041306	CP155060.1	128
	seeSp	[AmC6]CTTCTTCTGGTGATTCGGT	2041239-2041257		
	seeAp	[AmC6]TCCTTGAGCACCAAATAAATC	2041211-2041231		
	seeAb	[btn]TCTATATATCCTCAACTGTGTATCTGG	2041178-2041204		
Tn554 *tnpB*	N046Sb	[btn]TGCTTCAATTTCCACTCTCG	58297-58316	CP157524.1	127
	N046Ap	[AmC6]CAAGTTCAGAGAGTACACCAATTAA	58317-58341		
	N046Sp	[AmC6]GTCTTTGGGCAATATCACTTACATA	58380-58404		
	N046Ab	[btn]TGAACGTGGATAGCTTTTCC	58405-58424		
ϕMu50B SAV0881	SAV0881Sb	[btn]TGCTTGTTGTCATATCGCC	1154373-1154391	CP152457.1	199
	SAV0881Ap	[AmC6]TGTTTTGGTAACTAGCCACTGTATAGATA	1154392-1154420		
	SAV0881Sp	[AmC6]TCAAATTTCTTTTTGAATAGTAAGTCAGA	1154522-1154550		
	SAV0881Ab	[btn]CCTAGCTTGTATGTCTGCGCTA	1154551-1154572		
ϕPV83 ORF 2	PV83ORF2Sb	[btn]GGCGCTTCTTCTTACAGGAG	2107583-2107602	CP134532.1	446
	PV83ORF2Ap	[AmC6]-CATTGTTAGATATTTATATGGTATGTAACCTAAAA	2107548-2107582		
	PV83ORF2An	GATAATCTTGTTTTTTTCACTAACTAAACCTAT	2107515-2107547		
	PV83ORF2Sn	TGTTTAATAACAACGGTAAACCAGTATTT	2107218-2107246		
	PV83ORF2Sp	[AmC6]-ATAGTTATTAAAGACTTTGAAAACAGAATCATT	2107185-2107217		
	PV83ORF2Ab	[btn]GAATTATAGGTTTTAAGTTCACCCTCTTC	2107156-2107184		
ϕ11 nt 4427-5251	phi11-4563Sb	[btn]GATATGCAAGATCAGACAATGCC	343453-343475	CP140675.1	519
	phi11-4610Ap	[AmC6]-CCTCGCTATCAACATGATTTCTAAT	343476-343500		
	phi11-4632An	CTAAATTGGTGCGTCAGTTTGT	343501-343522		
	phi11-5026Sn	CAAACTACTACACGAAGCTAGACTACAAC	343916-343944		

	phi11-5055Sp	[AmC6]GAAAAGTAAATAAACAGTGGGTGCTTTA	343945-343972		
	phi11-5103Ab	[btn]CTCTTGCCCATGTGTTCTGAG	343973-343993		
ϕSLT ORF 257	SLTorf257Sb	[btn]GTGTTATCGCTATGAGTGGTGAC	1601652-1601674	CP157524.1	340
	SLTorf257Ap	[AmC6]TTAAAAAACTATTTTTGTGCATAAAAATAGT	1601621-1601651		
	SLTorf257An	GTCATAACCCATGAATTATGAATCA	1601596-1601620		
	SLTorf257Sn	TTAGGAGCTAATGAAATAGCTGCTAGTA	1601384-1601411		
	SLTorf257Sp	[AmC6]TCTCTAAAGAGCAATATAAGCGTTTC	1601358-1601383		
	SLTorf257Ab	[btn]CTTTAAATCTTCTGGGACGTTCTC	1601334-1601357		
ϕN315 SA1801	SA1801Ab	[btn]CAATCAGCGGTCGAGAACT	2073671-2073689	CP155060.1	516
	SA1801Ap	[AmC6]GAGTCTTAACCTCTAATGCTTGATGA	2073645-2073670		
	SA1801An	CATTCTTTCAAACCATTTTTTGTATG	2073619-2073644		
	SA1801Sn	CGCAGATTGTTTGAGTGGTTA	2073217-2073237		
	SA1801Sp	[AmC6]CGTCAAAACGGATTCCTTATTAAA	2073193-2073216		
	SA1801Ab	[btn]TTATAATCCACACCCTTGCG	2073173-2073192		
ϕMu50A SAV1974	SAV1974Sb	[btn]GCCACAAGAAAAGGCAGTG	2165194-2165212	CP157310.1	217
	SAV1974Ap	[AmC6]TGCTTACAGCTACATCTGTTTTGAT	2165169-2165193		
	SAV1974An	CGTTTTACTACTTACACCACTACGG	2165113-2165137		
	SAV1974Sp	[AmC6]GATATGAGTAACTTTGGTCGGAGTC	2164995-2165019		

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Prepared for submission research paper ***Molecular epidemiology of methicillin-sensitive Staphylococcus aureus causing blood stream infections in New South Wales in 2017*** has shown a diverse population of MSSA causing bacteraemia in metropolitan Sydney, and regional New South Wales in 2017. The utility of the method as a first line screening tool for molecular epidemiology studies is established. Potential temporospatial clusters have been identified, warranting further investigation with whole genome sequence-based SNP analysis. The profile of toxin and antimicrobial susceptibility markers generated reveals insights into the molecular epidemiology of bacteraemia causing MSSA strains.

Chapter 4. Molecular epidemiology of methicillin-sensitive *Staphylococcus aureus* isolated from a hospital in western Sydney 2017

In this Chapter, the utility of the mPCR/RLB binary typing assay in epidemiological investigations of methicillin-sensitive *Staphylococcus aureus* (MSSA) is investigated prospectively in the hospital setting. MSSA strains isolated from any source at one metropolitan hospital in western Sydney, New South Wales, in 2017 were typed using the assay (including some blood culture isolates part of the study described in Chapter 3). Some further duplication of content with Chapter 4. *Molecular epidemiology of methicillin-sensitive Staphylococcus aureus isolated from a hospital in western Sydney in 2017* is acknowledged.

4.1 Statement of contribution

My contribution to this publication is assay and study design, experimental work including mPCR/RLB and whole genome sequencing with analysis relating to the selection of targets, data analysis and preparation of manuscript. MOS and GG contributed with study design and manuscript preparation, and MR contributed with supervision and assistance of whole genome sequencing and analysis.

4.2 Molecular Epidemiology of Methicillin-Sensitive *Staphylococcus aureus* isolated from a hospital in Western Sydney in 2017

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Abstract

Objective: *Staphylococcus aureus* is a clinically important bacterial species associated with a wide spectrum of diseases and asymptomatic carriage. We report the findings from a study of methicillin-sensitive *Staphylococcus aureus* (MSSA) isolated from a large metropolitan hospital in New South Wales in 2017. Understanding the molecular epidemiology of MSSA across healthcare facility will allow the identify likely transmission events and allow targeted interventions.

Methods: MSSA isolated from all sources collected at a large metropolitan hospital in western Sydney were included in the study (n= 602). Binary typing was performed using a multiplex PCR based reverse line blot assay (mPCR/RLB). Clusters were identified based on temporal, spatial/geographic (ward) and binary type criteria, and combinations of these criteria.

Results: All available isolates were successfully binary typed, with 254 unique binary types identified. The most prevalent binary types, 40, 130, and 152, accounted for 3.13% of isolates each. There were 79 clusters of uncommon binary types and 52 temporal clusters. Of these, 24 clusters were both of an uncommon binary type and temporally linked. There were no spatial clusters identified at the ward level, however the absence of patient tracking data means the presence of such clusters cannot be excluded.

The toxic shock syndrome toxin gene (t*st*) was detected in 9.74% of isolates (n=48) and the *lukS* gene encoding Panton-Valentine leucocidin (PVL) was detected in 22.40% of isolates (n=110). Exfoliative toxin gene detection was uncommon with et*a* detected in 3.67% of isolates (n=18), and et*b* detected in 0.41% of isolates (n=2). The prevalence of staphylococcal enterotoxin genes encoding enterotoxins A, C, D and E were 15.66%; 6.71%; 6.04% and 8.95% respectively. Resistance markers *dfrK* and *ermC* were found in 5.37% and 6.71% of isolates respectively.

Conclusions: A diverse population of MSSA was responsible for both a variety of infections and colonisation at a large metropolitan hospital in Western Sydney, New South Wales in 2017. Clusters

of binary types were identified by the assay including those of rare binary type, and/or temporally linked. No spatially linked clusters were identified. The mPCR/RLB assay has utility as a screening tool to allow a more focussed used of sequencing for whole genome analysis.

Introduction

We have previously established binary typing of methicillin-resistant *Staphylococcus aureus* (MRSA) by multiplex PCR reverse line blot (mPCR/RLB) as an effective high-throughput strain typing method, suitable for routine, prospective use as part of a hospital infection control programme, which affords an opportunity for targeted interventions (O'Sullivan *et al*., 2012). A similar mPCR/RLB assay, developed and optimised for methicillin sensitive *Staphylococcus aureus* (MSSA) has been developed. In this study, the MSSA mPCR/RLB assay is prospectively applied in the typing of MSSA isolated from isolated from a large metropolitan hospital in western Sydney, New South Wales in 2017. A similar whole-facility study in the Netherlands (van der Schoor *et al*., 2023) using *spa* typing found limited evidence of transmission. Better understanding of the molecular epidemiology of MSSA infections and colonisation will help elucidate potential transmission events, identify markers of interest, and inform potential interventions.

Methods

Four hundred and ninety-one MSSA strains isolated from all sources at a single large hospital in western Sydney, New South Wales in 2017 were prospectively typed using the MSSA mPCR/RLB assay. Cases were excluded from the study if the patient had been culture positive in a two-week period prior for the same collection site. One hundred and eleven isolates which qualified for inclusion in the study were not stored and subsequently unable to be binary typed. A variety of sample types were collected as per routine protocols. Culture was performed as per standard laboratory operating

procedures using agars including Columbia base horse blood agar, and chocolatised blood agar. Following incubation, identification of suspect colonies was confirmed using the Bruker™ MALDI-TOF biotyper. Confirmed MSSA were stored at -70°C in cryostorage media containing skim milk, glucose, and glycerol. DNA lysates were prepared of a single, isolated colony, using a McFarland 0.5 suspension, boiled at 100°C for 10 minutes before freezing at -20°C. Multiplex PCR/RLB was performed on isolates as per standard laboratory operating procedures. MangoMix™ PCR mastermix was used as per the manufacturer's instructions. Oligonucleotide sequences for primers and probes used in the mPCR/RLB assay can be found in **Appendix 1. Oligonucleotide primers and probes**. Analysis of the RLB membrane was performed as per standard laboratory operating procedures. Weak hybridisation is discounted if it is for one probe of a pair only. Two faintly visible hybridisations of probes are counted as a positive, as is a single strong reaction.

Data was interrogated for evidence of clustering. Temporal clusters were defined as two or more isolates with the same binary type collected less than or equal to 28 days apart. Spatial clusters were defined as two or more isolates from the same inpatient ward. The emergency department was not included in spatial cluster considerations, given the high likelihood of community acquisition of such strains. Binary type clusters were defined as groups of isolates belonging to a rare binary type with 5 or fewer representatives.

Results

All available isolates were successfully binary typed by the MSSA mPCR/RLB assay. See Table 7. **Figure 6** shows, for a subset of the isolates, the mPCR/RLB membrane with hybridisation visible at the junction of PCR products and membrane linked probes. A total of 254 unique binary types were identified. Simpson's index of diversity was calculated using the AuSeTTS software package (O'Sullivan

et al., 2013) as 0.993 (95%CI 0.991 – 0.994). The most prevalent binary profiles, designated as binary types 40, 130, and 152, each accounted for 3.13% of isolates.

There were 52 temporal clusters of two to seven isolates of the same binary type with collection dates separated by intervals less than or equal to 28 days. There were 79 clusters of uncommon binary types with less than or equal to five representative strains. Of these clusters, 24 were both of an uncommon binary type and temporally linked. The number of isolates in these clusters ranged from two to four.

There were no spatial clusters identified, with most isolates being from samples collected in the emergency department.

The toxic shock syndrome toxin gene (t*st*) was detected in 9.78% of isolates (n=48) and the *lukS* gene encoding Panton-Valentine leucocidin (PVL) was detected in 22.40% of isolates (n=110). Exfoliative toxin gene detection was uncommon with et*a* detected in 3.67% of isolates (n=18), and et*b* detected in 0.41% of isolates (n=2). The prevalence of staphylococcal enterotoxin genes encoding enterotoxins A, C, D and E were, 15.66%, 6.71%, 6.04, and 8.95% respectively. Resistance markers *dfrK* and *ermC* were found in 5.37% and 6.71% of isolates respectively.

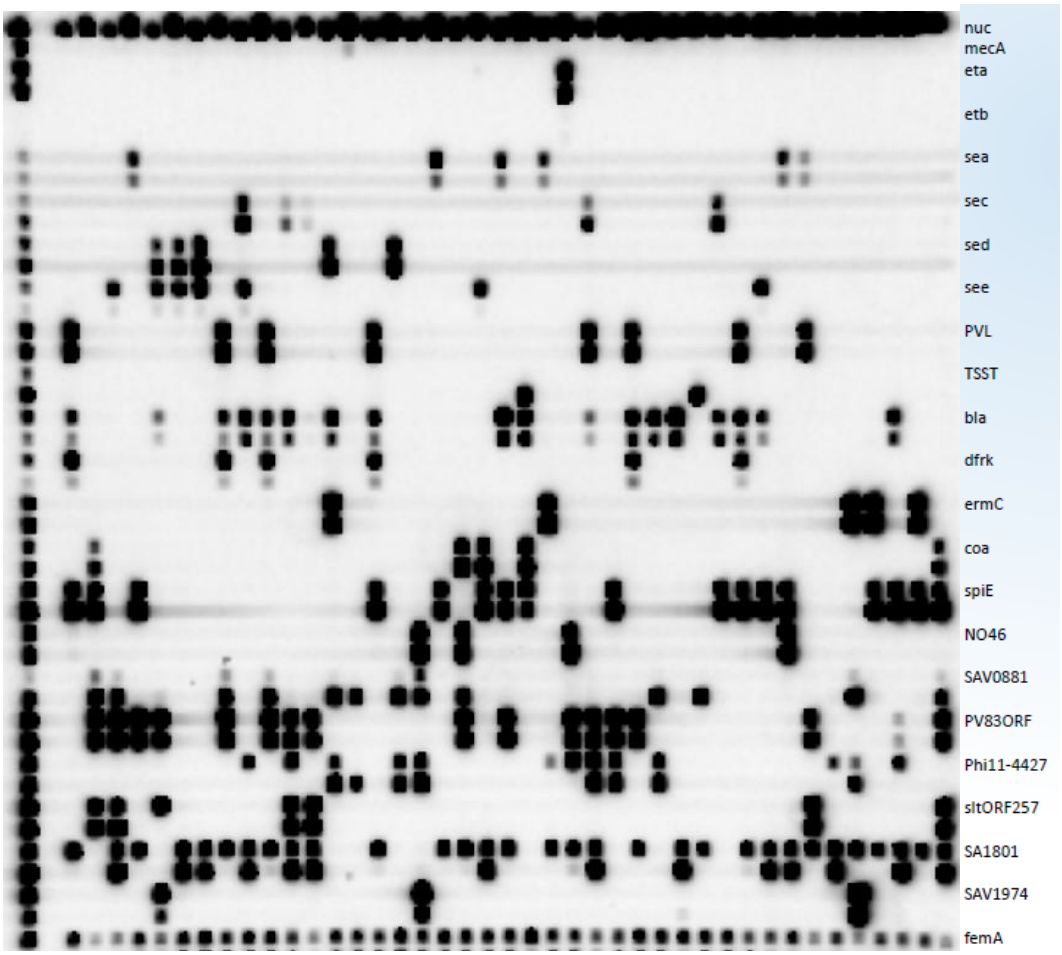

Figure 6: MSSA mPCR/RLB assay showing hybridisation. Lane 1 (left) = pooled control. Lane 2 = no template control. Lanes 3 to 43 = study isolates. Target presence is visualised as a hybridisation spot at the junction of mPCR product and biotinylated probes.

Discussion

A diverse population of MSSA was responsible for both a variety of infections and colonisation at a large metropolitan hospital in Western Sydney, New South Wales in 2017. A total of 254 unique binary types were identified, with a Simpson's index of Diversity >99%. All 20 targets on the assay were shown to be contributory to the discrimination of the isolates as indicated by **Figure 8**, where the cumulative Simpson's index of diversity for increasing target number is visualised.

The mPCR/RLB assay reveals information on the toxin profile of the all-source MSSA isolates, with the gene encoding toxic shock syndrome toxin being detected in around 10% of isolates. Toxic shock syndrome (TSS) emerged as a cause of concern in 1980 and some of the pathophysiology of the disease at the cellular and molecular levels remains to be elucidated (Arbuthnott, 1988). *lukS*, the gene encoding Panton-Valentine leucocidin (PVL) was found in around 25% of isolates. PVL production has been linked to particular clinical syndromes such as necrotising pneumonia. A case control study from the University of Massachusetts in 2022 found 29% of 1681 sequences *S. aureus* genomes contained the genes that encode for PVL (Qu *et al*., 2022). Exfoliative toxins were uncommon. Detection of genes encoding staphylococcal enterotoxins were common (38.39% of isolates carried at least one enterotoxin gene). Staphylococcal enterotoxins are implicated in a wide variety of diseases and immunological non-responsiveness to enterotoxin A is common (Kunstmann *et al*., 1989). The detection of *dfrK* and *ermC*, which are markers of trimethoprim and macrolide/lincosamide resistance in 5 – 10% of study isolates does not necessarily correlate with antimicrobial resistance, as other mechanisms may be involved.

The mPCR/RLB assay offers utility for even moderately equipped laboratories to employ, having significantly reduced consumable and equipment costs compared with many favoured and emerging typing systems, including those based on whole-genome sequencing. Required materials are basic,

and include: a hybridisation oven, a mini-blotter and glass rolling bottles, separating mesh and a rocking platform. An X-ray film exposure cassette, film cartridge and developer are also required for the visualising the hybridisation. The assay is described as versatile and easily transferrable to other laboratories. (Kong and Gilbert, 2006) mPCR/RLB Primers and probes may be ordered lyophilised, allowing for convenient shipping and storage at room temperature. Given the basic equipment and expertise requirements, the assay can be used to track MSSA not just within a single facility but across multi-centre studies.

There is little in terms of technical difficulty in the performance of the mPCR, the hybridisation or indeed interpretation of the assay. Simple to follow laboratory operating procedures have been developed to cover the preparation of an RLB membrane and the production of required solutions, the mPCR/RLB assay protocol and the analysis of results. Prerequisite knowledge for potential users is described (Kong and Gilbert, 2006) and is limited to basic computer skills, and basic molecular biology skills (extraction of DNA, conventional PCR, gel electrophoresis and preparation of buffers and solutions.

The criteria used in defining clusters, including the time between collection dates for which isolates are excluded as duplicate, and the number of isolates which are deemed to constitute a rare binary type may affect the findings of cluster analysis significantly. Considering combinations of cluster types, for example temporospatial clusters, increases the complexity of analysis involved. It is also worthwhile considering that isolates with a very similar binary profile, but not identical, may still constitute a cluster. This was evident in this dataset with several patients having multiple isolates that differed by a single binary target. This binary profile variation can be caused by *in vivo* or *in vitro* strain evolution. Evolutionary pressures can lead to altered binary profiles of target bacterial strains while they are either; living on a host or in the environment, or as an artefact of laboratory processes after sample collection.

For the identified clusters, whole-genome sequencing with single-nucleotide polymorphism analysis could be performed to explore relatedness of the isolates with more granularity and confirm clusters of transmission.

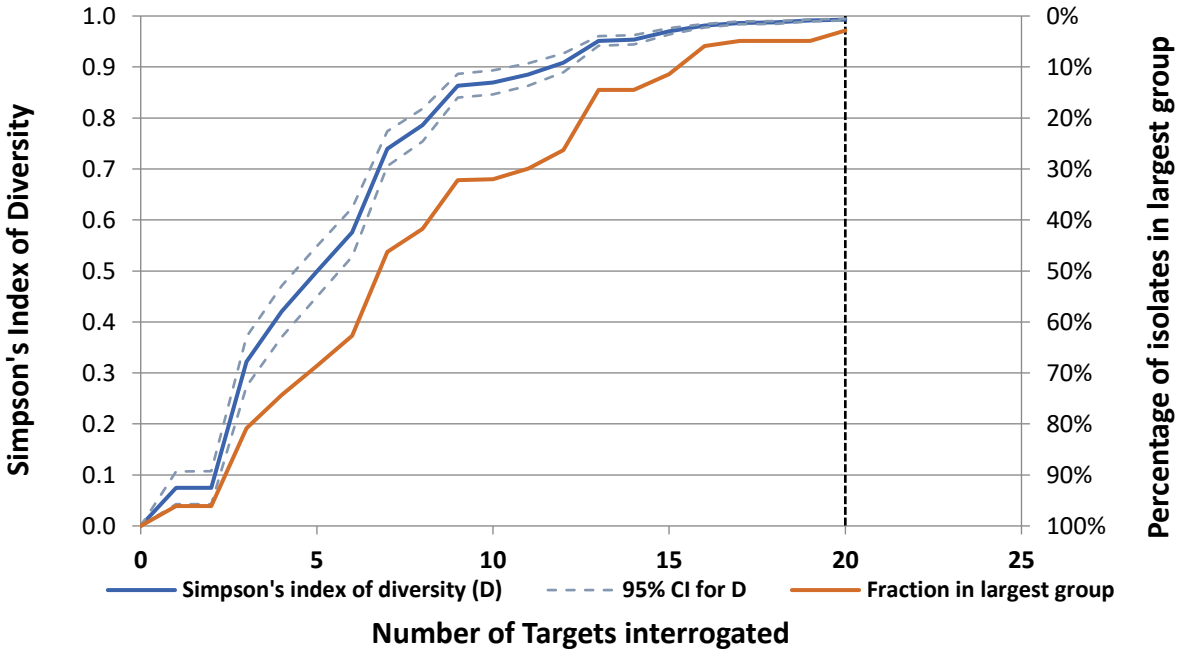

Figure 7: Simpson's index of diversity and percentage in largest group vs number of targets included in analysis.

Conclusions

A diverse population of MSSA was responsible for 491 infections and colonisations at a large hospital in western Sydney, New South Wales in 2017. There was evidence in this dataset of temporal and rare binary type clusters. A variety of toxins and antimicrobial resistance markers that are of potential significance were found in these strains. The MSSA mPCR/RLB assay is an inexpensive and rapid typing method, comparing favourably with WGS and other subtyping methods and has shown utility in the detection of potential clusters. Such clusters would warrant additional investigation, potentially including whole genome sequencing based SNP analysis and enhanced epidemiological data including on patient bed movements throughout their length of stay. These results also raise the intriguing possibility of using the assay, in potential future studies, as a tool for exploring bacterial phenotype/ disease phenotype relationships.

Acknowledgements

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Disclosure Statement

The authors have no conflicts of interest or competing interests to disclose.

Appendix 1. Oligonucleotide primers and probes

Target	Oligo Name	Oligo Sequence (5'-3')	Position	GenBank Accession	Product Size (bp)
nuc	nucSb	[btn]GCGATTGATGGTGATACGGTT	854574-854594	CP157524.1	278
	nucAp	[AmC6]CATTGGTTGACCTTTGTACATTAA	854598-854621		
	nucSp	[AmC6]GATGGAAAAATGGTAAACGAAG	854808-854829		
	nucAb	[btn]AGCCAAGCCTTGACGAACTAAAGC	854829-854852		
femA	femASb	[btn]ATAATAACGAGGTYATTGCAGCTTG	1370866-1370890	NZ_CP009361.1	232
	femASp	[AmC6]TTCAAATCGCGGTCCAGTRA	1370936-1370955		
	femAAp	[AmC6]TGGATCGATATGTAGGTATAGACARCG	1371024-1371050		
	femAAb	[btn]ATTACCTGTAATCTCGCCATCATGA	1371074-1371098		
mecA	mecAP4b	[btn]TCCAGATTACAACTTCACCAGG	44877-44898	CP157524.1	167
	mecAAp	[AmC6]CTAATGTTTTGTTATTTAACCCAATCAT	44824-44851		

	mecASp	[AmC6]GATGGTAAAGGTTGGCAAAAA	44780-44800		
	mecAP7b	[btn]CATTTACCACTTCATATCTTGTAACG	44731-44756		
eta	etaSb	[btn]CGGGAAATTCTGGATCAGGTATAT	936859-936882	LS483311.1	129
	etaSp	[AmC6]AGTGTCTCATCTTGATAGAGAGCA	936923-936946		
	etaAp	[AmC6]TGCTAGAATGTATACCAACTAATTCTCC	936894-936921		
	etaAb	[btn]CGCTTGACATAATTCCCAATACC	936966-936988		
etb	etbSb	[btn]TACCACCTAATACCCTAATAATCCAA	2702366-2702391	CP140702.1	369
	etbSp	[AmC6]GAGACAGTGCATTAAATGAATAAYTTT	2702269-2702295		
	etbAp	[AmC6]GATTTCTTCTGCGCTGTATTCTT	2702042-2702064		
	etbAb	[btn]CATTATCCGTAATGTGTGTATAAAGC	2701972-2701997		
sea	seaSb	[btn]CCTTTGGAAACGGTTAAAACG	2047518-2047538	CP157303.1	204
	seaSp	[AmC6]GGAGTTGGATCTTCAAGCAAGACG	2047471-2047494		
	seaAp	[AmC6]TCTGAACCTTCCCATCAAAAAC	2047412-2047433		
	seaAb	[btn]TTGAATACTGTCCTTGAGCACC	2047334-2047355		
sec	secSb	[btn]GCTCAAGAACTAGACATAAAAGCTAGG	199452-199478	CP140697.1	271
	secSp	[AmC6]AACGRCAATACTTTTTGGTATGAT	199560-199583		
	secAp	[AmC6]CTTCACWCTTTTAGAATCAACCG	199651-199673		
	secAb	[btn]TCAAAATCGGATTAACATTATCC	199701-199723		
sed	sedSb	[btn]CTAGTTTGGTAATATCTCCTTTAAACG	1014904-1014878	CP155060.1	321
	sedSp	[AmC6]TAAAGCCAATGAAAACATTGATTCA	1014852-1014876		
	sedAp	[AmC6]CTTTTATTTTCTCCTATTATTGGATTTTT	1014745-1014773		
	sedAb	[btn]CAATTAATGCTATATCTTATAGGGTAAACATC	1014583-1014614		

see	seeSb	[btn]ACAGATGCATTTAATGGAAAAATAC	2041282-2041306	CP155060.1	128
	seeSp	[AmC6]CTTCTTCTGGTGATTCGGT	2041239-2041257		
	seeAp	[AmC6]TCCTTGAGCACCAAATAAATC	2041211-2041231		
	seeAb	[btn]TCTATATATCCTCAACTGTGTATCTGG	2041178-2041204		
lukS-PVL	pvlSb	[btn]TTTTAGGCTCAAGACAAAGCAAC	1569732-1569754	CP157310.1	132
	pvlAp	[AmC6]TACCTCTGGATAACACTGGCATTTT	1569674-1569698		
	pvlSp	[AmC6]CTTCAATCCAGAATTTATTGGTGT	1569649-1569672		
	pvlAb	[btn]TTTGCAGCGTTTTGTTTTCG	1569622-1569641		
tsst	tsstSb	[btn]AAGCCAACATACTAGCGAAGGAAC	1232542-1232565	CP097311.1	208
	tsstSp	[AmC6]TGGYGTTACAAATACTGAAAAATTACC	1232587-1232613		
	tsstAp	[AmC6]TTATCGAACTTTGGCCMATACTTT	1232668-123691		
	tsstAb	[btn]GTATTTGAGTTAGCTGATGACGAA	1232727-1232750		
bla	blaSb	[btn]ACTGTTGTTAAAACTAAWGCACTTGC	845854-845879	CP155452.1	290
	blaSp	[AmC6]ACAGCAATGTGTTCAAATTTTC	846080-846101		
	blaAp	[AmC6]TGTAATCGATTGCAAATAARTHATAGGAG	845921-845949		
	blaAb	[btn]ATGGAAATAAAAAGTCAGCCCG	846123-846144		
dfrK	dfrKsb	[btn]ATTGCTGCGATGGATAAGAATAG	42795-42817	CP125862.1	197
	dfrKAp	[AmC6]AGGATTCCCAAGGACTGG	42849-42866		
	dfrKSp	[AmC6]CTGTCAGGTAAGGCTCTTCC	42933-42952		
	dfrKAb	[btn]AAAGGTAAACCCCTTATCTCTCGTC	42968-42992		
ermC	ermCsb	[btn]ACTTGTTGATCACGATAATTTCC	2772682-2772704	CP140688.1	184
	ermCSp	[AmC6]AACATAAGTACGGATATAATACGCAA	2772579-2772604		

	ermCAp	[AmC6]GCAATATATCCTTGTTTAAAACTTGG	2772658-2772683		
	ermCAb	[btn]TAGCAAACCCGTATTCCACG	2772520-2772539		
coa	coaSb	[btn]AGGTTGTTGGAAAAGGTATTAAGGG	267379-267403	CP154290.1	205
	coaSp	[AmC6]TCCTCGTACAGATTTAAAAATGG	267483-267505		
	coaAp	[AmC6]AGATTTCTTTCTCTCCAAAAGATATTG	267409-267435		
	coaAb	[btn]AGTGCTCTCTTTAATGCATCCTGTA	267560-267584		
splE	splESb	[btn]ATGTATGAGTCAACAGGAAAAGTGC	1946495-1946519	CP154290.1	129
	splESp	[AmC6]GCAACTCAGGTTCAGCTGT	1946428-1946446		
	splEAp	[AmC6]CATCAGTAATAATCATGTTGCCTT	1946462-1946485		
	splEAb	[btn]CAAAGTGAACACCTACAACTTCGTA	1946390-1946414		
see	seeSb	[btn]ACAGATGCATTTAATGGAAAAATAC	2041282-2041306	CP155060.1	128
	seeSp	[AmC6]CTTCTTCTGGTGATTCGGT	2041239-2041257		
	seeAp	[AmC6]TCCTTGAGCACCAAATAAATC	2041211-2041231		
	seeAb	[btn]TCTATATATCCTCAACTGTGTATCTGG	2041178-2041204		
Tn554 *tnpB*	N046Sb	[btn]TGCTTCAATTTCCACTCTCG	58297-58316	CP157524.1	127
	N046Ap	[AmC6]CAAGTTCAGAGAGTACACCAATTAA	58317-58341		
	N046Sp	[AmC6]GTCTTTGGGCAATATCACTTACATA	58380-58404		
	N046Ab	[btn]TGAACGTGGATAGCTTTTCC	58405-58424		
ϕMu50B SAV0881	SAV0881Sb	[btn]TGCTTGTTGTCATATCGCC	1154373-1154391	CP152457.1	199
	SAV0881Ap	[AmC6]TGTTTTGGTAACTAGCCACTGTATAGATA	1154392-1154420		
	SAV0881Sp	[AmC6]TCAAATTTCTTTTTGAATAGTAAGTCAGA	1154522-1154550		
	SAV0881Ab	[btn]CCTAGCTTGTATGTCTGCGCTA	1154551-1154572		

ϕPV83 ORF 2	PV83ORF2Sb	[btn]GGCGCTTCTTCTTACAGGAG	2107583-2107602	CP134532.1	446
	PV83ORF2Ap	[AmC6]-CATTGTTAGATATTTATATGGTATGTAACCTAAAA	2107548-2107582		
	PV83ORF2An	GATAATCTTGTTTTTTTCACTAACTAAACCTAT	2107515-2107547		
	PV83ORF2Sn	TGTTTAATAACAACGGTAAACCAGTATTT	2107218-2107246		
	PV83ORF2Sp	[AmC6]-ATAGTTATTAAAGACTTTGAAAACAGAATCATT	2107185-2107217		
	PV83ORF2Ab	[btn]GAATTATAGGTTTTAAGTTCACCCTCTTC	2107156-2107184		
ϕ11 nt 4427-5251	phi11-4563Sb	[btn]GATATGCAAGATCAGACAATGCC	343453-343475	CP140675.1	519
	phi11-4610Ap	[AmC6]CCTCGCTATCAACATGATTTCTAAT	343476-343500		
	phi11-4632An	CTAAATTGGTGCGTCAGTTTGT	343501-343522		
	phi11-5026Sn	CAAACTACTACACGAAGCTAGACTACAAC	343916-343944		
	phi11-5055Sp	[AmC6]GAAAAGTAAATAAACAGTGGGTGCTTTA	343945-343972		
	phi11-5103Ab	[btn]CTCTTGCCCATGTGTTCTGAG	343973-343993		
ϕSLT ORF 257	SLTorf257Sb	[btn]GTGTTATCGCTATGAGTGGTGAC	1601652-1601674	CP157524.1	340
	SLTorf257Ap	[AmC6]TTAAAAAACTATTTTTGTGCATAAAAATAGT	1601621-1601651		
	SLTorf257An	GTCATAACCCATGAATTATGAATCA	1601596-1601620		
	SLTorf257Sn	TTAGGAGCTAATGAAATAGCTGCTAGTA	1601384-1601411		
	SLTorf257Sp	[AmC6]TCTCTAAAGAGCAATATAAGCGTTTC	1601358-1601383		
	SLTorf257Ab	[btn]CTTTAAATCTTCTGGGACGTTCTC	1601334-1601357		
ϕN315 SA1801	SA1801Ab	[btn]CAATCAGCGGTCGAGAACT	2073671-2073689	CP155060.1	516
	SA1801Ap	[AmC6]GAGTCTTAACCTCTAATGCTTGATGA	2073645-2073670		
	SA1801An	CATTCTTTCAAACCATTTTTTGTATG	2073619-2073644		
	SA1801Sn	CGCAGATTGTTTGAGTGGTTA	2073217-2073237		

	SA1801Sp	[AmC6]CGTCAAAACGGATTCCTTATTAAA	2073193-2073216		
	SA1801Ab	[btn]TTATAATCCACACCCTTGCG	2073173-2073192		
ϕMu50A SAV1974	SAV1974Sb	[btn]GCCACAAGAAAAGGCAGTG	2165194-2165212	CP157310.1	217
	SAV1974Ap	[AmC6]TGCTTACAGCTACATCTGTTTTGAT	2165169-2165193		
	SAV1974An	CGTTTTACTACTTACACCACTACGG	2165113-2165137		
	SAV1974Sp	[AmC6]GATATGAGTAACTTTGGTCGGAGTC	2164995-2165019		

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Prepared for submission research paper ***Molecular epidemiology of methicillin-sensitive Staphylococcus aureus isolated from a hospital in western Sydney 2017*** has shown a heterogenous population of MSSA was responsible for colonisation and infections in a single healthcare facility in metropolitan Sydney in 2017. The utility of the method as a screening tool for molecular epidemiology studies in this setting is established. Application of the assay as part of an infection control screening program affords the opportunity to detect potential clusters and transmission pathways. Clustering was identified in this study and warrants further investigation. Enhanced epidemiological data would improve the utility of the assay in identifying clusters of potential transmission.

Chapter 5. Outbreak of community-acquired *Staphylococcus aureus* skin infections in an Australian professional football team

The potential value of the binary typing assay (mPCR/RLB) for epidemiological investigations of methicillin-sensitive *Staphylococcus aureus* is previously established in the hospital setting, both for clinical utility and infection prevention and control purposes (Chapter three: *Molecular epidemiology of methicillin-sensitive Staphylococcus aureus causing bloodstream infections in New South Wales in 2017* and chapter four *Molecular epidemiology of methicillin-sensitive Staphylococcus aureus isolated from a hospital in western Sydney in 2017,* respectively). This chapter presents a published study *Outbreak of community-acquired Staphylococcus aureus skin infections in an Australian professional football team* (Shaban, Li *et al* 2020) which demonstrates the utility of the assay beyond this context, in a community-based setting and used in combination with the MRSA mPCR/RLB assay. Given the significant burden of disease due to *Staphylococcus aureus*, such as skin and soft tissue infections, in the community and their associated morbidity, extending the use of the assay to this setting significantly increases the potential applicability of the mPCR/RLB binary typing assay.

5.1 Statement of contribution

My contribution to this publication is to assay and study design, conducting a site inspection of the football team's training facility, sample collection from players and staff with basic data collection, experimental work including culture, DNA extraction and the MSSA mPCR/RLB. I have also made contributions to the preparation of the manuscript.

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Original research

Outbreak of community-acquired *Staphylococcus aureus* skin infections in an Australian professional football team

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ABSTRACT

Objectives: Skin and soft tissue infections commonly affect athletes and can lead to cluster outbreaks if not managed appropriately. We report the findings of an investigation into an outbreak of community-acquired *Staphylococcus aureus* infection in an Australian professional football team.
Design: Retrospective cross-sectional study.
Methods: Nose, axilla, groin and throat swab were collected from 47 participants. MRSA and MSSA isolates underwent antibiotic susceptibility testing, binary typing and whole genome sequencing. Infection control practitioners (ICPs) investigated the training grounds for risk factors in the transmission of *S. aureus*.
Results: Almost half of the participants (n = 23, 48.9%) were found to be colonised with MSSA. An outbreak cluster of MRSA ST5 closely related to the fusidic acid-resistant New Zealand NZAK3 clone was identified in a group of four players. MSSA ST15 and MSSA ST291 strains were found to have colonised and spread between two and five players, respectively. All participants were advised to undergo decolonisation treatment consisting of 4% chlorhexidine body wash and mupirocin nasal ointment for ten days. The ICP team identified several unhygienic practices within the club's shared facilities that may have played a role in the transmission of *S. aureus*.
Conclusions: We report for the first time a community-associated *S. aureus* outbreak involving the highly successful fusidic acid-resistant MRSA ST5 clone in a professional football club associated with inadequate hygiene procedures. Management and prevention of *S. aureus* relies heavily on hygiene education and adherence to personal and environmental hygiene practices and policies.

Practical implications

- Evidence of fusidic acid-resistant New Zealand MRSA ST5 strain importation into Australia from New Zealand.
- Several poor hygiene practices within the club's shared facilities, including the use of the communal ice bath for sports therapy, were identified to be significant risk factors for transmission of *Staphylococcus aureus*.
- Skin and soft tissue infections commonly affect athletes and can lead to team outbreaks if not managed appropriately. As such, it is essential to educate players and staff about the importance of good personal and environmental hygiene practices and implement appropriate infection prevention and control policies.

1. Introduction

Staphylococcal skin and soft tissue infections are a well-documented complication of participation in sport activities.[1–3] Epidemiological studies have demonstrated that coaching staff and athletes in contact and non-contact sports have been implicated

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as carriers during outbreaks of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA).[4–7] CA-MRSA infections have been reported more frequently in athletes participating in contact sports such as wrestling, football, fencing, soccer and rugby due to frequent skin-to-skin contact.[8,9] Such outbreaks have been linked to contaminated ice baths, water bottles and shared equipment including towels and razors.[10,11]

On the basis of this evidence, the United States Centers for Disease Control and Prevention (CDC) issued guidelines for preventing staphylococcal infection amongst sports team members that instructs the athletes to: (i) cover all wounds; (ii) maintain good personal hygiene (e.g., showering and washing with soap after all practices and competitions); (iii) ensure availability of soap and water; (iv) avoid sharing of towels and personal items; (v) establish routine cleaning schedules for shared equipment; (vi) train coaches and athletes in recognition and first aid for wounds that are potentially infected; and (vii) report skin lesions and encourage coaches to assess athletes regularly for skin lesions.[12] Notwithstanding, staphylococcal infections in sports settings associated with poor hygiene practices are reported.[4,6,8,13] In this paper we report an outbreak of community-acquired *Staphylococcus aureus* skin infections in a professional Australian football team.

2. Methods

In July 2018, a team of infectious diseases, infection control and medical scientific staff from the local health district visited the training facility of the football team to investigate an outbreak of boils and other skin lesions in a team of professional football players. In total 47 participants, comprising of 17 professional staff members, 25 football players, and five other individuals (who belonged to the football team but did not provide their position to the research team), were swabbed using Sigma Transwab® swabs. Each participant was sampled twice, with separate swabs. Swab number one corresponded to a throat sample and swab number two corresponded to a combined sample from the nose, axilla and groin. Using a combined sample from these sites is standard and validated laboratory practice for *S. aureus* screening. A total of 94 samples were collected.

Processing of swabs for detection of methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA), and antimicrobial susceptibility profiles of islolates were undertaken at the Centre for Infectious Diseases and Microbiology Laboratory Services (CIDMLS), Institute for Clinical Pathology and Medical Research (ICPMR), Westmead Hospital. The BD Max™ StaphSR assay was utilised to screen for MSSA and MRSA by testing for the presence of *nuc* and *mecA/C* genes and the *SCCmec/orfX* junction. Samples positive on the BD Max assay were cultured to obtain isolates. Antimicrobial susceptibility testing was performed using the PMIC-84 panel on the BD Phoenix II automated microbiology system. This panel included Clindamycin, Cotrimoxazole, Ciprofloxacin, Erythromycin, Flucloxacillin, Gentamicin, and Fusidic Acid. Isolates were classified as MRSA if the oxacillin minimal inhibitory concentration (MIC) was >2 μg/mL and/or the cefoxitin MIC was >4 μg/mL.

Isolates of MRSA and MSSA underwent binary typing using the multiplex PCR-based reverse line blot (mPCR/RLB) assay technique as previously described.[14–17] Each mPCR amplification of two controls and 19 target genes was performed in a 15 μL reaction using the HotStarTaq® DNA polymerase kit (Qiagen) and PCR cycles.[16] Denatured PCR products were hybridised to the probe labelled membrane with streptavidin-peroxidase conjugate (Roche) and enhanced chemiluminescence (ECL) detection reagent to detect with the Bio-Rad ChemiDoc XRS+. Targets were assigned '1' (present) or '0' (absent, no probe signal) and results entered into a MRSA Access database and unique binary types derived from the 19 target genes.

Isolates that underwent whole genome sequencing were subcultured onto Columbia horse blood agar (HBA) and incubated overnight at 37 °C in 5% CO_2 to ensure purity before analysis. Several colonies were washed in PBS and genomic DNA extraction was performed using the Presto™ Mini gDNA Bacteria (Gene Target Solutions, Australia) for Gram-positive bacteria, as per the manufacturer's instructions, with the inclusion of 100 μg/mL Lysostaphin (L7386, Sigma) during lysis. DNA extracts were treated with 1U of RNase (Qiagen). Total DNA concentration was quantified using Picogreen (Invitrogen, Australia) and 1 ng/μL DNA was used to prepare DNA libraries employing the Nextera XT Library Preparation Kit (Illumina, Inc., CA, USA). Multiplexed libraries were sequenced using paired-end 150 bp chemistry on the NextSeq 500 (Illumina, Australia). Library construction and sequencing were performed by the Microbial Genomics Reference Laboratory, CIDMLS, ICPMR, NSW Health Pathology, Westmead Hospital. Whole genome sequencing (WGS) was performed on the Illumina NextSeq platform. Reads from isolates were *de novo* assembled and used to determine multilocus sequence typing (MLST) and to identify reference genomes for the ST5, ST15 and ST291 MLST groups, NZAK3 strain LT009690, CP022720 and AP017922, respectively. Reads from isolates were mapped to respective reference genomes using CLC Genomics Workbench 10.5.1 (CLC Bio, Aarhus, Denmark). Mobile genetic elements were identified using PHASTER (PHAge Search Tool Enhanced Release) and removed for core genomes Single nucleotide polymorphisms (SNPs) detection using CLC Genomics Workbench was based on the neighbourhood quality standard (NQS) algorithm. SNP distance matrix were generated using Geneious and transmission chains derived.

Ethics approval was obtained from the Western Sydney Local Health District Human Research Ethics Committee (1902-03 QA). As a retrospective, cross-sectional study, informed consent was not required. Swabbing and medical consultation were provided as part of routine clinical care. Please note that when a case number is used (e.g. case 38), the authors are referring the isolate obtained from participant number 38.

3. Results

Clinical microbiology diagnostic testing of the isolates (n = 94) using BD Max StaphSR assay platform and growth on selective MRSA/MSSA agar yielded four MRSA isolates from four different participants and 32 MSSA isolates from 23 different participants. 48.9% of the study cohort were found to be colonised with MSSA. Of the 23 participants found to be colonised with MSSA, 13 were football players, eight were staff members, and two were individuals associated with the team (did not provide their position to the research team). Seven MSSA isolates from six different players demonstrated an unusually resistant susceptibility profile, with resistance to ciprofloxacin, clindamycin, erythromycin, gentamicin, and cotrimoxazole identified. The four isolates identified as MRSA were sensitive to vancomycin, clindamycin, cotrimoxazole and erythromycin. Three of these MRSA isolates were resistant to fusidic acid.

We performed mPCR-RLB binary typing on the four isolates identified as MRSA. All four isolates were collected from football players. One isolate came from a combined swab sample from the nose, axilla and groin and the rest were from throat swabs. All four isolates were binary type (bt) 132352 and were positive for *nuc* and *mecA* genes (indicative of MRSA) and negative for the cytotoxin, Panton–Valentine leucocidin (*pvl*). The indistinguishable binary typing results indicated possible transmission of MRSA between the players. To investigate this further, WGS was performed.

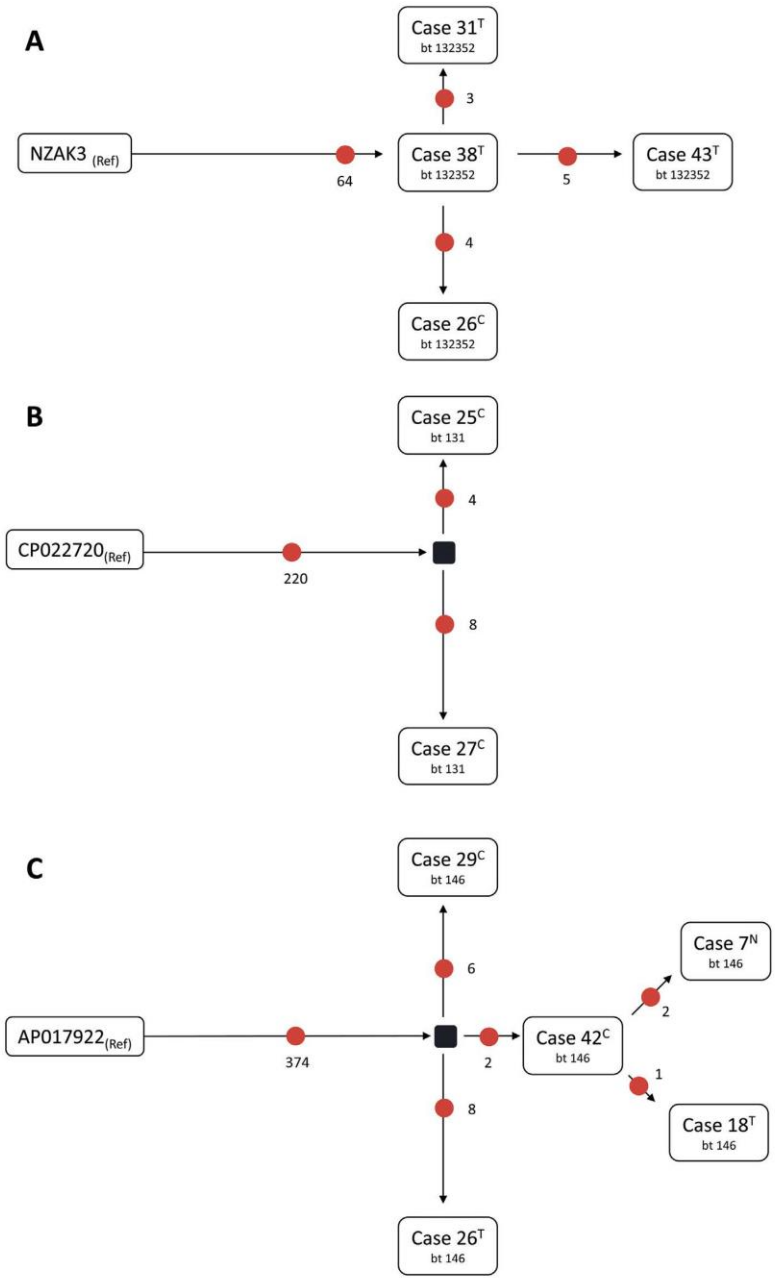

Fig. 1. Transmission chains from football players determined by whole genome sequencing and single nucleotide polymorphism (SNP) analysis: (A) MRSA ST5, (B) MSSA ST15 and (C) MSSA ST291. Number of SNPs are indicated by the numbers located adjacent to the red dots. Black squares indicated the unidentified common ancestor (e.g. clinical and/or environmental isolate) of the outbreak isolates. RLB binary typing (bt) numbers for each MRSA and MSSA isolate are indicated. [T] refers to throat only swab; [C] refers to combined nose, axilla and groin swab; [N] refers to nose only swab (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Comparative genomics and mapping of the WGS data of the four MRSA isolates to the reference NZAK3 ST5 strain revealed a high level of genetic similarity and conservation, thus confirming the outbreak isolates belonged to ST5 (Fig. 1A). Only 64 core genome single nucleotide polymorphisms (SNPs) separated the isolate from case 38 from the NZAK3 reference strain. Within the football players outbreak cluster only 12 SNPs separated the isolates, indicating direct transmission from case 38 to the other three cases (26, 31 and 43).

mPCR-RLB binary typing separated 32 MSSA isolates from 23 participants into 20 different binary types. 18 binary types were unique to a single isolate or isolates from 16 different participants.

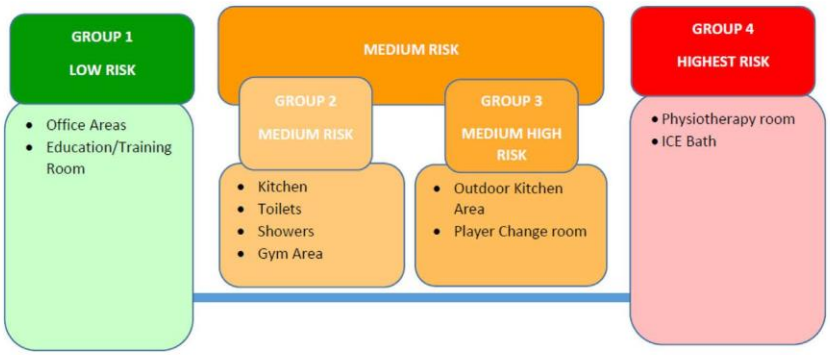

Fig. 2. Functional infection risk area of amenities.

However, there were two different binary types which included isolates from seven different participants. Isolates from case 25 (football player) and case 27 (participant did not provide their position to the research team) belonged to MSSA bt131 and isolates from cases 7, 18, 26, 29 and 42 (all football players) belonged to MSSA bt 146. All isolates were *nuc* and *femA* positive and negative for *mecA* (indicating methicillin sensitivity) and *pvl*. Case 26 yielded MSSA in a throat sample whereas the isolate from the combined nose, axilla and groin swab taken from the same player was identified as MRSA ST5. Binary typing results indicated that there were two different potential MSSA transmission events between players. This was further investigated with WGS and SNP analysis.

WGS confirmed the MSSA bt131 isolates as ST15 and the MSSA bt146 isolates as ST291 (see Fig. 1B–C). Two clusters of MSSA transmission were identified. The first involved two ST15 isolates, differing by 12 SNPs. The second involved ST291 isolates from five players, differing by 19 SNPs, and with strong evidence of transmission from case 42 to cases 18 and 7, differing only by a single SNP and two SNPs, respectively.

There were several potential contributing environmental factors for this outbreak including the sharing of personal equipment and the lack of routine cleaning procedures. However, the communal use of the physiotherapy table and ice bath, which were insufficiently cleaned and disinfected between each use, was observed to be significant risk factors for cross-contamination between players, and required immediate action (Fig. 2).

To reduce the risk of further transmission, all 47 participants underwent *S. aureus* decolonisation treatment for ten days.[18] Decolonisation treatment consisted of 4% chlorhexidine body wash and mupirocin nasal ointment. Infection control practitioners (ICP) assessed the training grounds of the football team and made several recommendations for improving overall hygiene. In particular, the ICP team recommended that the ice bath be replaced with an automated, filtered and chlorinated ice bath (pool) to allow multiple use or else for the ice bath to be emptied and refilled after each use and bleached daily. The ICP team also guided players and staff on improving personal and environmental hygiene by providing information on handwashing, usage of alcohol-based hand rubs, and cleaning of showers and toilets and the kitchen and lounge areas. It was also recommended to the football club that amenities be provided to the players in the locker room to avoid cross-contamination. Table 1 provides a summary of the identified infection control risk factors, and the outbreak management and infection prevention and control measures implemented to address the cause of the outbreak. It is important to note that at the time of the outbreak, the amenities were not purpose-built facilities for the team and were temporary and weathered, and the team management had attempted to adapt their use as safely as possible.

4. Discussion

This study reports an outbreak of community-acquired *S. aureus* in a professional football club. Colonisation and transmission of *S. aureus* is common in athletes.[10] This was supported by our study findings, with almost half of the 47 participants (n = 23, 48.9%) found to be colonised with methicillin-susceptible *S. aureus* (MSSA). Of these 23 participants, 13 were football players, eight were staff members, and two were individuals associated with the team (did not provide their position to the research team). Professional staff members in this study included coaches and physiotherapists. Due to their close contact with football players during training sessions, transmission of MSSA can occur directly via skin-to-skin contact or indirectly via contaminated objects including sports equipment, towels, and wound dressings/bandages.[12] However, our results show that there were two separate instances of MSSA ST15 and ST291 transmission between multiple football players and one participant whose position was not disclosed to the research team. While there was no report of skin and soft tissue infections arising from colonisation with these two MSSA strains, all were advised to undergo decolonisation treatment to prevent infection and reduce further spread.

Molecular typing and genomics analysis identified the acquisition and transmission of MRSA ST5 strain between four football players. MRSA ST1, MRSA ST5 and MSSA ST1 have emerged in recent years as the dominant fusidic acid-resistant MRSA clones in New Zealand as a result of the previously widespread use of topical fusidic acid.[19] A Danish group recently reported the emergence and expansion of fusidic acid-resistant MSSA ST1 clone in atopic dermatitis patients due to frequent topical treatment of skin infections with fusidic acid.[20] Fusidic acid-resistant MRSA ST5 has also been identified as the predominant clone responsible for increased resistance of *S. aureus* to fusidic acid in China.[21] As a result of its rapid emergence and international expansion and in a bid to prevent this highly successful clone from further spreading, there have been repeated calls for the restricted use of topical fusidic acid ointment.[22] Whole genome sequencing and core genome SNP analysis indicated that only 64 SNPs separated the reference New Zealand NZAK3 strain from the isolate from case 38, indicating close genetic similarity between the MRSA ST5 strains. 12 SNPs separated the outbreak cluster, indicating direct transmission between the players. During medical consultation, almost 90% of the participants (n = 42) stated that they had travelled overseas to various

Table 1
Identified infection control risks factors associated with the outbreak and the associated recommendation and remedies.

Identified infection control risk factor for cross-contamination	Recommendations and Remedies
1. Unhygienic use of the communal ice bath	Establish ice bath procedure to include empty and fill after each use; replace with filtered/chlorinated ice bath (pool), managed, monitored and validated
2. Cleaning products	Streamline and improve cleaning products (e.g. explore cleaning wipes)
	Cease topping up and decanting of cleaning spray bottles
	Cleaning and maintenance of cleaning equipment
	Review hand soap dispenser and product throughout
	Provide access to alcohol-based hand rubs
3. Inappropriate storage of equipment and use of facility areas and rooms	Review storage requirements and store equipment in designated room
	Disabled toilet area to be sorted or repurposed
	Cleaning, decluttering and organisation of changeroom/locker room and provide amenities to reduce cross-contamination of persons and items
4. Sharing of personal equipment	Towels on physiotherapy table should be changed and table cleaned in between each player
	No sharing of shower sponges
	Recovery boots to be cleaned between each player and after every use
5. Lack of routine cleaning procedures	Routine laundering of shower curtains
	Routine shampooing of lounge or replace with lounge surface that can be easily cleaned/wiped over
6. Limited education and promotion of good hygiene and infection prevention and control measures	Education to players and staff in basic infection prevention and control measures
	Improve general personal and environmental hygiene

countries, including New Zealand, for matches and training. The presence of MRSA ST5 in the team could potentially be a result of strain importation into Australia from overseas travel to New Zealand.

It was further reported to the infection control team that the football club were having problems managing recurrent *S. aureus* boils. Implementation of infection control program and hygiene education has been successful in reducing bacterial and viral burdens at athletic training rooms.[23] Our team of ICPs assessed the training grounds and current hygiene practices to establish potential risk factors for transmission. Athletes engaged in contact sports are at increased risk of being colonised with and spreading *S. aureus*.[24] Repeated skin-to-skin contact and compromised skin integrity (e.g. open wounds) increase risk of direct transmission from player to player or object to player. The importance of cleaning wounds and covering them with clean bandages was emphasised to the players and professional staff.[12,25] It was brought to the attention of the ICP team that towels used during physiotherapy sessions were not changed between players and the same towel was used amongst multiple players. It was strongly recommended to the professional staff to change the towel between players and to actively encourage players not to share towels and other personal hygiene items such as razors, as per the statement and guidelines released by the National Athletic Trainers' Association (NATA) on Community-Acquired MRSA Infections.[26] Studies have reported the use of therapeutic whirlpools to be risk factor for the transmission of MRSA due to inadequate disinfection practices.[4,27] The sharing of ice baths, towels and razors has previously been linked to a *S. aureus* outbreak in an English rugby team.[11] One of the other risk factors identified at the football club in this study was the current practices surrounding the use of the ice bath, which was determined to be manifestly unhygienic, with multiple players using the ice bath throughout the day. It was strongly recommended that the ice bath be replaced with an automated, filtered and chlorinated ice bath (pool) to allow multiple use between players.[28] In line with NATA's position statement on management of skin diseases, the club was advised that players with open wounds must avoid using the ice bath or any water facilities until the infections are cleared.[29]

This investigation only involved analysis of clinical samples and did not include samples from the environment. We were unable to collect environmental samples during the visit to the club. Collection of samples from the identified environmental risk factors such as the ice bath, towels, shower curtains, toilets and shared storage spaces in the locker room would have enabled a full epidemiological investigation of this community-acquired *S. aureus* outbreak. As such, we were unable to locate a specific point source(s) of contamination.

All participants in this study were advised to undergo *S. aureus* decolonisation treatment with 4% chlorhexidine body wash and mupirocin nasal ointment for a period of ten days. The IPC team completed a follow-up with the team and reported that there were no additional *S. aureus* skin lesions and the team had implemented several recommendations. As such, there was no indication that post-decolonisation screening was required to determine if decolonisation was successful. The use of the ice bath had ceased. The club purchased and installed a fully automated, filtered, chlorinated and monitored ice bath. The inability to make structural changes to the facility continues to have challenges. This further supports the design of purpose-built sport training facilities that promote the general wellbeing and health of players and staff and reduce risk of transmission of infections within communal sporting environments. At the time of writing, it was reported that the football team had moved into a new purpose-built training facility.

5. Conclusion

Skin and soft tissue infections commonly affect athletes and can lead to team outbreaks if not managed appropriately. This report investigated an outbreak of *S. aureus* in a football team and identified the transmission of a methicillin-resistant *S. aureus* ST5 strain amongst four players, which was closely linked to the fusidic acid-resistant clone from New Zealand. Two different strains of

methicillin-susceptible *S. aureus*, ST15 and ST291, were also found to have colonised and spread amongst a small population of players. In order to manage and arrest *S. aureus* outbreaks in professional athletes engaged in contact sports, it is essential to educate players and staff in addition to having good personal and environmental hygiene practices and policies in place. The overall environment and amenities specific to the needs of the football team would better promote an environment of wellbeing and mitigate risk of outbreaks like this.

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In conclusion, *Outbreak of community-acquired Staphylococcus aureus skin infections in an Australian professional football team* (Shaban, Li *et al* 2020) has demonstrated the utility of the mPCR/RLB based binary typing assay in a community setting, to screen for potential clusters of transmission which may be further examined using WGS. The simplicity of the mPCR/RLB assay enables rapid generation of actionable subtyping results. Using this approach, the study identified transmission of a methicillin-resistant *S. aureus* ST5 strain in addition to two circulating methicillin-sensitive *S. aureus* strains, ST15 and ST291. In addition to utility in the macro and micro epidemiology in the hospital context, this chapter has shown the utility of the genomic epidemiology in identifying community transmission of *Staphylococcus aureus.*

Conclusion

Conventional, generally species-specific typing methods have traditionally been the mainstay in bacterial epidemiology studies. Resolution of epidemiological typing has been improved by the development of typing methods employing molecular analysis of microbial DNA. However, new typing methods are often applied without critical evaluation of their performance characteristics. Struelens and colleagues have developed a schema for systematic evaluation of new subtyping assays. (Struelens, 1996).

An MRSA-centric focus on subtyping of *S. aureus* has limited our understanding of MSSA epidemiology in a variety of settings. This is because assays used are not optimised for MSSA specifically. Here, targets were selected as the first step in developing a binary typing assay for MSSA. A reference library of strains was established and interrogated for 80 potential targets for binary typing. These were identified from a review of the literature (Coldea *et al*., 2013; Feng *et al*., 2008; Ho *et al*., 2008; Kobayashi *et al*., 1995; Monecke *et al*., 2013; Song *et al*., 2016; Tong *et al*., 2015; Suzuki *et al*., 2006), the existing MRSA mPCR/RLB assay and whole genome sequencing analysis. Targets selected include a variety of potentially informative recognised virulence factors. Including these targets raises the intriguing possibility of using the assay as a tool for exploring bacterial phenotype/ disease phenotype relationships.

Using AuSeTTs, (O'Sullivan *et al*., 2013) within the reference set of 137 isolates, 121 unique binary types were distinguishable. The maximum Simpson's diversity index of 0.9977 (95%CI, 0.9960 to 0.9993) was achieved with a subset of just 24 of the 80 targets. Targets selected were varied and included toxin genes, antimicrobial resistance markers and phage derived open reading frames. The most common binary type accounted for three percent of the collection.

Changes were incorporated into the final 43 lane mPCR/RLB assay format to accommodate 20 informative and/or discriminatory targets. Controls, both positive and negative, were also included

(*nuc, fem*A and *mec*A). AuSeTTS was rerun with several targets of potentially clinical interest force included (TSST and *lukS*-PVL). In the final analysis for 20 discriminatory targets; ϕMu50B, SAV0858, *cad*B, *amp* and phage integrase are removed and *bla* was an addition. For the final format of the mPCR/RLB, Simpson's index of diversity is calculated at 0.991 (95% CI 0.987 – 0.996).

Oligonucleotide primers and probes were designed for the MSSA mPCR/RLB assay. Optimisation of the assay was performed for the mPCR and the reverse line blot and standard laboratory procedures were developed.

Assessment of prospective subtyping assays against standard performance criteria is an important critique and should be incorporated into subtyping assay development pipelines. The MSSA mPCR/RLB assay was assessed for against consensus guidelines for performance (Struelens, 1996). The assay performed well in rapidity, typeability, accessibility, ease of use, discriminatory power, and stability. Reproducibility was calculated as 0.93, slightly below the target of >0.95, and is likely attributable to single binary profile differences for a small number of isolates on repeat testing. A limitation of this assessment was that the lack of enhanced epidemiological data, including bed tracking, meant epidemiological concordance was difficult to thoroughly assess.

the utility of the mPCR/RLB binary typing assay in epidemiological investigations of methicillin-sensitive *Staphylococcus aureus* (MSSA) was investigated prospectively in the hospital setting in chapter three. Specifically, 213 MSSA strains isolated from blood cultures collected at ten hospitals in New South Wales in 2017, were typed using the assay. A diverse population of MSSA was responsible for bacteraemia. There was evidence in this dataset of limited temporospatial clustering of rare binary types. Of the 24 clusters of rare binary types detected, three contained two isolates each from the same facility with collection dates within 28 days. Targeted application of whole genome sequencing with single nucleotide polymorphism (SNP) analysis and collection of patient movement data at the ward level would help elucidate these potential clusters further.

In Chapter four, the utility of the mPCR/RLB binary typing assay in epidemiological investigations of MSSA is further investigated in the hospital setting. MSSA strains isolated from any source at one metropolitan hospital in western Sydney, New South Wales, in 2017 were typed using the assay. Again, the strains typed were diverse with 254 unique binary types were identified. Simpson's index of diversity was calculated using the AuSeTTS software package (O'Sullivan *et al*., 2013) as 0.993 (95%CI 0.991 – 0.994). The most prevalent binary profiles, designated as binary types 40, 130, and 152, each accounted for 3.13% of isolates.

There were 52 temporal clusters of two to seven isolates with collection dates separated by intervals less than or equal to 28 days. There were 79 clusters of uncommon binary types with less than or equal to five representative strains. Of these clusters, 24 were both of an uncommon binary type and temporally linked. There were no spatial clusters identified, with most isolates being from samples collected in the emergency department. Movement of patients withing the facility after sample collection was unknown, and limited cluster detection.

In chapter five, a published study *Outbreak of community-acquired Staphylococcus aureus skin infections in an Australian professional football team* (Shaban, Li *et al* 2020) demonstrates the utility of the assay in a community-based setting and its use in combination with the MRSA mPCR/RLB assay.

The study identified transmission of a methicillin-resistant *S. aureus* ST5 strain in addition to two circulating methicillin-sensitive *S. aureus* strains, ST15 and ST291. In addition to utility in the macro and micro epidemiology in the hospital context, this chapter has shown the utility of the genomic epidemiology in identifying community transmission of *Staphylococcus aureus.*

Given the significant burden of disease due to *Staphylococcus aureus*, such as skin and soft tissue infections, in the community and their associated morbidity, extending the use of the assay to this setting significantly increases the potential applicability of the mPCR/RLB binary typing assay.

In summary a MSSA mPCR/RLB assay is developed as useful epidemiological tool. The utility of the assay is demonstrated above in the healthcare and community settings (chapters 3, 4 and 5). Epidemiological data is required to further inform analysis of potential clusters identified by the assay. The criteria used in defining clusters, including the time between collection dates for which isolates are excluded as duplicate, and the number of isolates which are deemed to constitute a rare binary type may affect the findings of cluster analysis significantly. Considering combinations of cluster types, for example temporospatial clusters, increases the complexity of analysis involved.

Moving forward, it is planned that additional isolates will be studied prospectively using the assay. These studies will need to include in-depth epidemiological data, including but not limited to details of patient movements through the hospital. This will allow more informed and detailed analysis of clusters and transmission chains. WGS analysis will be performed on isolates identified as potential clusters in future investigations and potentially completed ones.

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Supplementary Material

Table 8: Binary profiles with count of instances

eta	etb	sea	sec	sed	see	PVL	tsst	bla	dfrk	ermC	coa	splE	N046	SAV0881	PV83-orf2	phi11-44	sltORF257	SA1801	SAV1974	MRSA Type	Count
							■					■			■		■	■		4254	43
												■			■					152	29
			■				■	■				■			■					71832	21
												■						■		130	20
■			■	■	■	■	■	■	■	■	■		■	■	■		■	■	■	655359	20
												■								128	19
														■						40	18
				■										■						32808	16
																		■		2	14
												■			■			■		146	14
						■		■							■		■			10268	13
						■		■							■		■		■	10269	12
					■													■		16386	10
		■						■				■			■			■		133266	9
								■										■		2050	9
						■		■				■		■	■		■			10420	8
			■					■				■								67712	8
		■				■						■			■		■	■		139414	8
												■						■		138	8
		■										■						■		131202	7
														■				■		42	7
		■										■			■			■		131218	7
																				0	6
								■										■		2058	6
			■					■												67584	6
								■						■						2088	6
■													■		■			■		524378	6
								■				■						■		2178	6
				■	■													■		49154	6
												■			■			■		154	5
						■						■			■		■	■		8342	5
		■					■					■			■			■		135322	5
		■					■					■			■		■	■		135318	5
												■		■	■		■			180	5
		■				■									■		■	■		139286	5
			■					■										■		67586	5
					■							■	■					■		16770	5
							■							■				■	■	4131	5
												■	■					■		386	5
												■		■	■		■	■		182	4
												■			■		■	■		150	4
		■				■		■				■			■		■	■		141462	4
						■		■	■			■						■		11394	4
			■				■	■										■		71682	4
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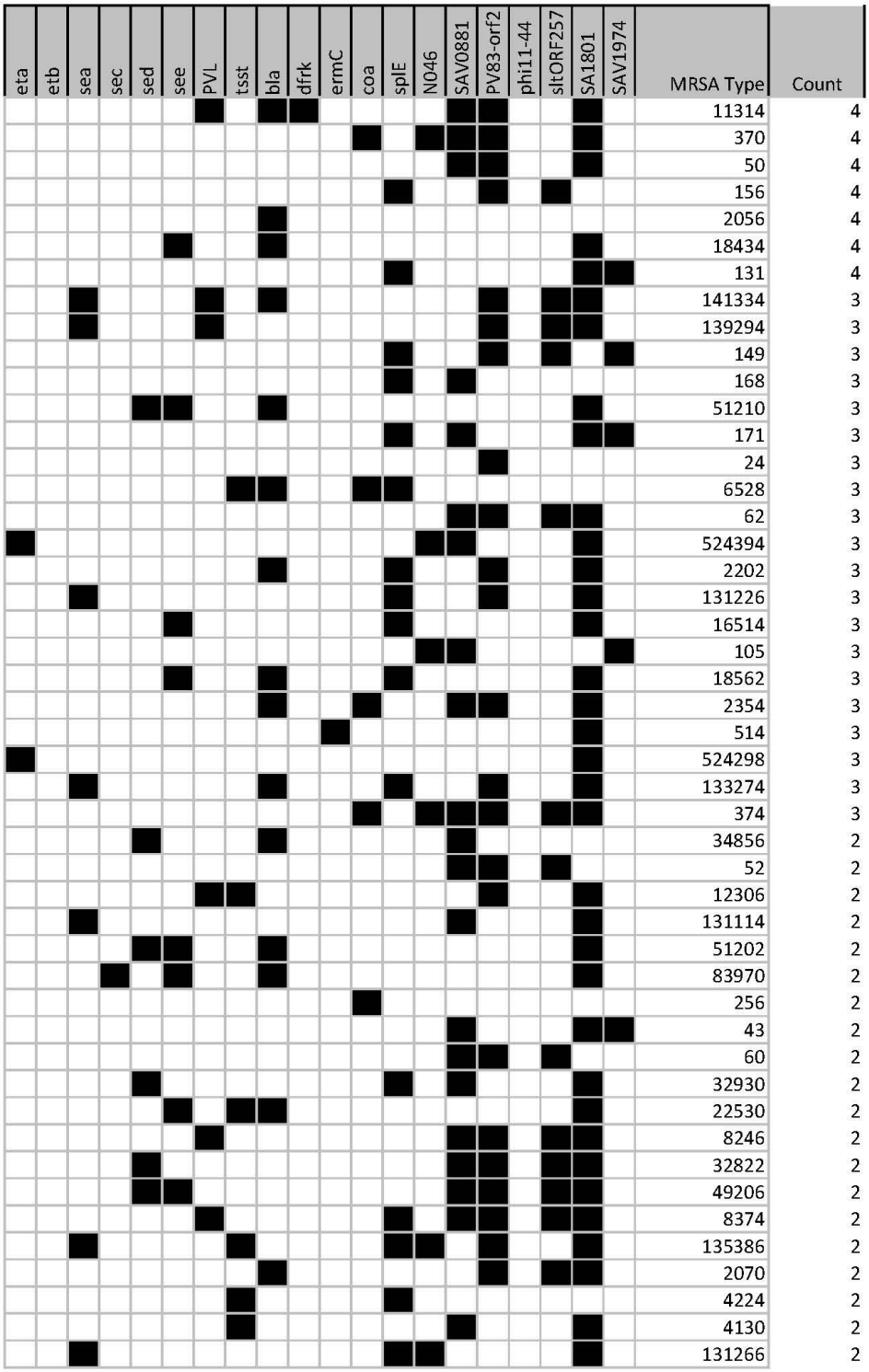
						■									■		■			8220	4



eta	etb	sea	sec	sed	see	PVL	tsst	bla	dfrk	ermC	coa	splE	N046	SAV0881	PV83-orf2	phi11-44	sltORF257	SA1801	SAV1974	MRSA Type	Count
						■		■	■					■	■			■		11314	4
											■		■	■	■			■		370	4
														■	■			■		50	4
												■			■		■			156	4
								■												2056	4
					■			■										■		18434	4
												■						■	■	131	4
		■				■		■							■		■	■		141334	3
		■				■									■		■	■		139294	3
												■			■		■		■	149	3
												■		■						168	3
				■	■			■										■		51210	3
												■		■				■	■	171	3
															■					24	3
							■	■			■	■								6528	3
														■	■		■	■		62	3
■													■	■				■		524394	3
								■				■			■			■		2202	3
		■										■			■			■		131226	3
					■							■						■		16514	3
													■	■					■	105	3
					■			■				■						■		18562	3
								■			■			■	■			■		2354	3
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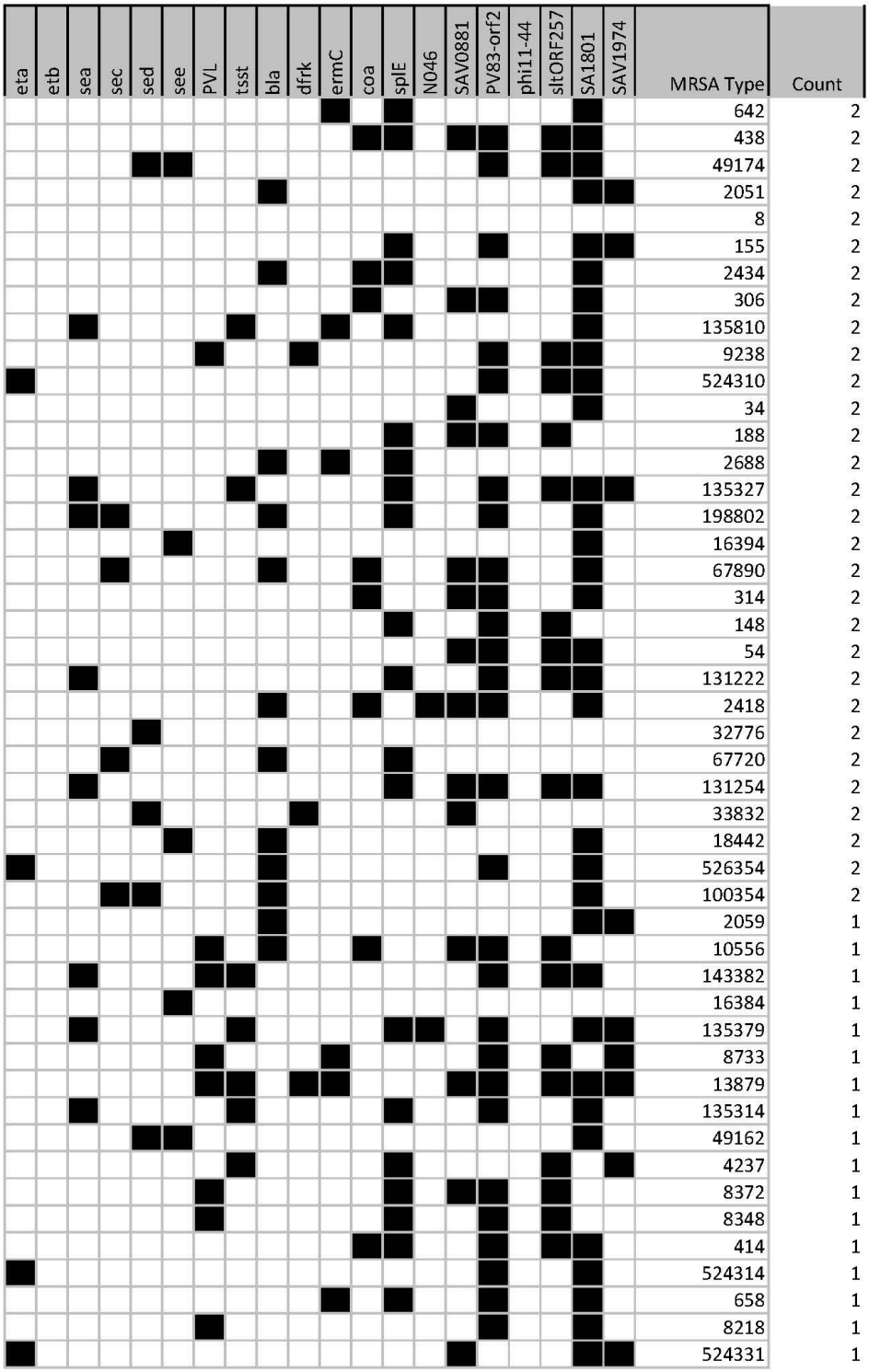
		■						■				■			■			■		133274	3
											■		■	■	■		■	■		374	3
				■				■						■						34856	2
														■	■		■			52	2
						■	■								■			■		12306	2
		■												■				■		131114	2
				■	■			■										■		51202	2
			■		■			■										■		83970	2
											■									256	2
														■				■	■	43	2
														■	■		■			60	2
				■								■		■				■		32930	2
					■		■	■										■		22530	2
						■								■	■		■	■		8246	2
				■										■	■		■	■		32822	2
				■	■									■	■		■	■		49206	2
						■						■		■	■		■	■		8374	2
		■					■					■	■		■			■		135386	2
								■							■		■	■		2070	2
							■					■								4224	2
							■							■				■		4130	2
		■										■	■					■		131266	2



eta	etb	sea	sec	sed	see	PVL	tsst	bla	dfrk	ermC	coa	splE	N046	SAV0881	PV83-orf2	phi11-44	sltORF257	SA1801	SAV1974	MRSA Type	Count
										■		■						■		642	2
											■	■		■	■			■		438	2
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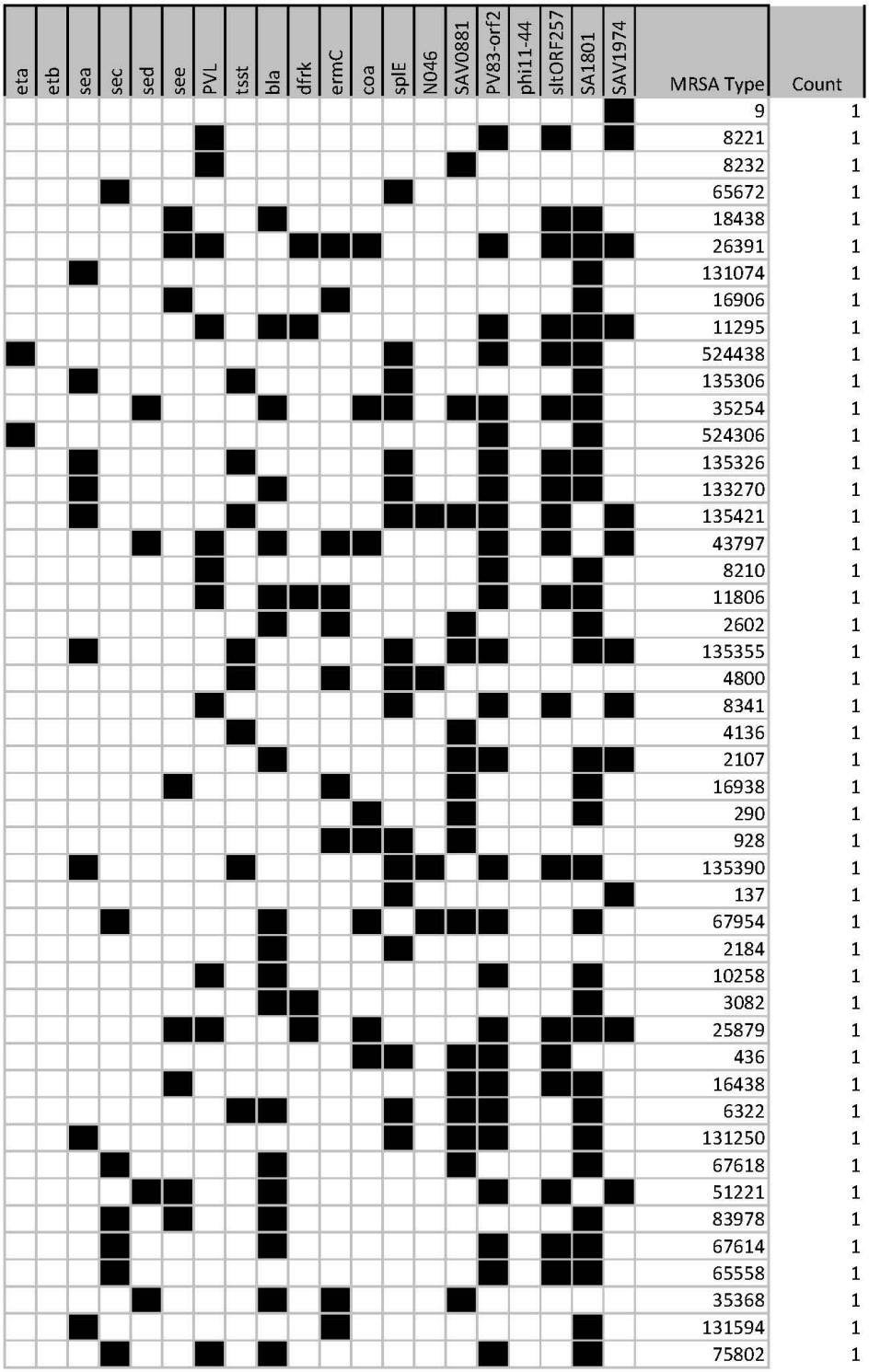
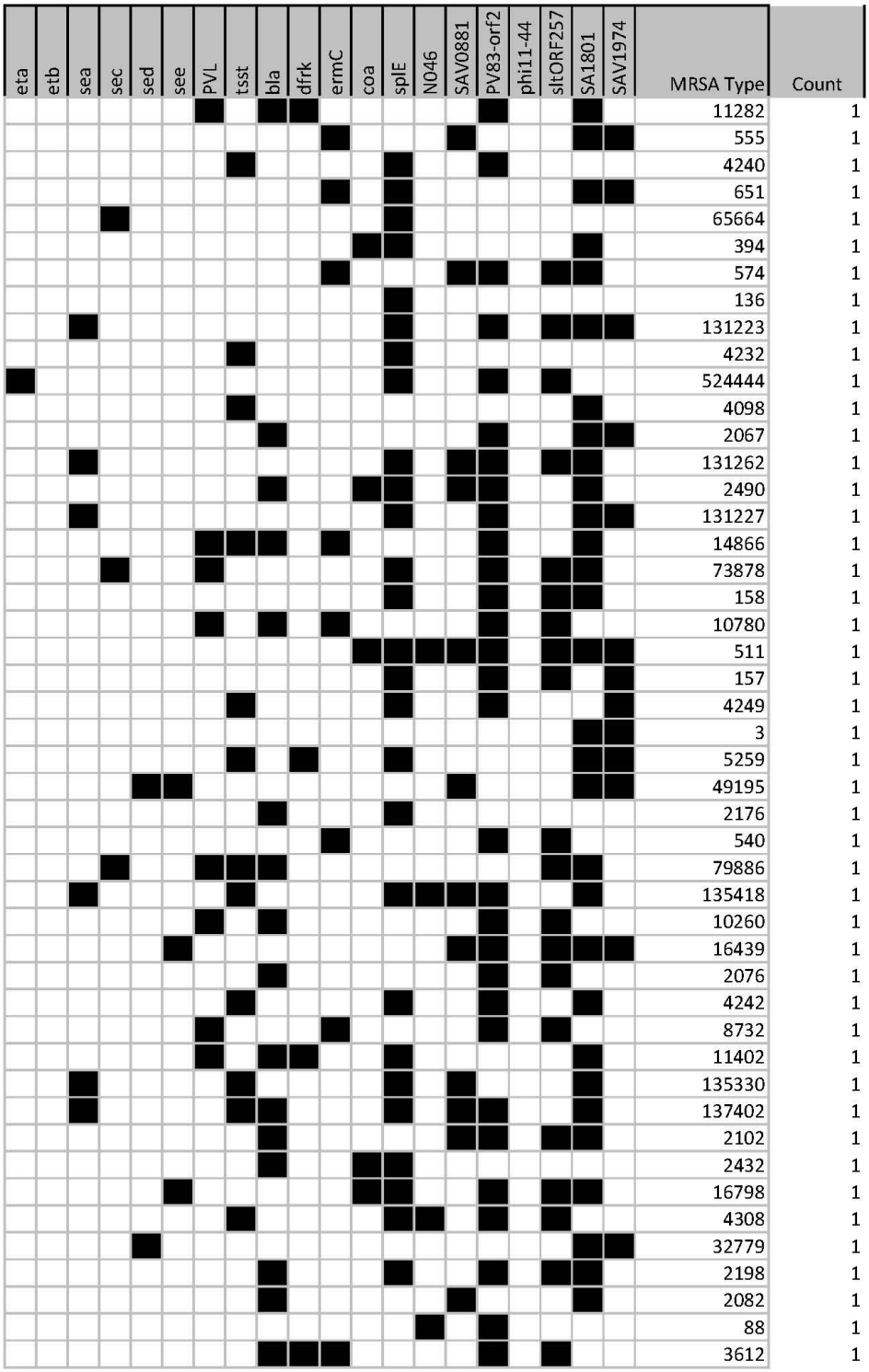
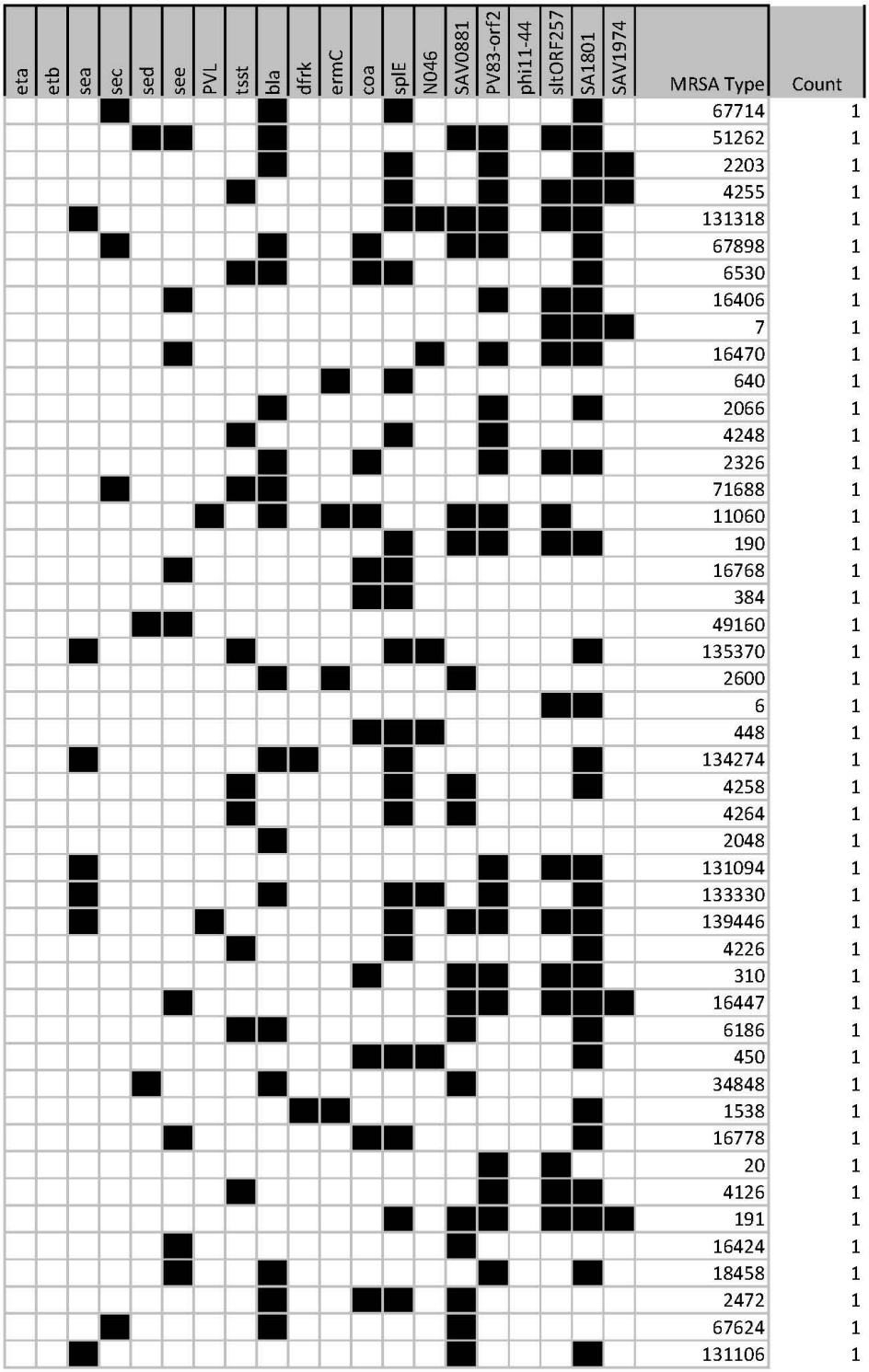
								■										■	■	2051	2
																				8	2
												■			■			■	■	155	2
								■			■	■						■		2434	2
											■			■	■			■		306	2
		■					■			■		■						■		135810	2
						■			■						■		■	■		9238	2
■															■		■	■		524310	2
														■				■		34	2
												■		■	■		■			188	2
								■		■		■								2688	2
		■					■					■			■		■	■	■	135327	2
		■	■					■				■			■			■		198802	2
					■													■		16394	2
			■					■			■			■	■			■		67890	2
											■			■	■			■		314	2
												■			■		■			148	2
														■	■		■	■		54	2
		■										■			■		■	■		131222	2
								■			■		■	■	■			■		2418	2
				■																32776	2
			■					■				■								67720	2
		■										■		■	■		■	■		131254	2
				■					■					■						33832	2
					■			■										■		18442	2
■								■							■			■		526354	2
			■	■				■										■		100354	2
								■										■	■	2059	1
						■		■			■			■	■		■			10556	1
		■				■	■								■		■	■		143382	1
					■															16384	1
		■					■					■	■		■			■	■	135379	1
						■				■					■		■		■	8733	1
						■	■		■	■				■	■		■	■	■	13879	1
		■					■					■			■			■		135314	1
				■	■													■		49162	1
							■					■					■		■	4237	1
						■						■		■	■		■			8372	1
						■						■			■		■			8348	1
											■	■			■		■	■		414	1
■															■			■		524314	1
										■		■			■			■		658	1
						■									■			■		8218	1
■														■				■	■	524331	1



eta	etb	sea	sec	sed	see	PVL	tsst	bla	dfrk	ermC	coa	splE	N046	SAV0881	PV83-orf2	phi11-44	sltORF257	SA1801	SAV1974	MRSA Type	Count
																			■	9	1
						■									■		■		■	8221	1
						■								■						8232	1
			■									■								65672	1
					■			■									■	■		18438	1
					■	■			■	■	■				■		■	■	■	26391	1
		■																■		131074	1
					■					■								■		16906	1
						■		■	■						■		■	■	■	11295	1
■												■			■		■	■		524438	1
		■					■					■						■		135306	1
				■				■			■	■		■	■		■	■		35254	1
■															■			■		524306	1
		■					■					■			■		■	■		135326	1
		■						■				■			■		■	■		133270	1
		■					■					■	■	■	■		■		■	135421	1
				■		■		■		■	■				■		■		■	43797	1
						■									■			■		8210	1
						■		■	■	■					■		■	■		11806	1
								■		■				■				■		2602	1
		■					■					■		■	■			■	■	135355	1
							■			■		■	■							4800	1
						■						■			■		■		■	8341	1
							■							■						4136	1
								■						■	■			■	■	2107	1
					■					■				■				■		16938	1
											■			■				■		290	1
										■	■	■		■						928	1
		■					■					■	■		■		■	■		135390	1
												■							■	137	1
			■					■			■		■	■	■			■		67954	1
								■				■								2184	1
						■		■							■			■		10258	1
								■	■									■		3082	1
					■	■			■		■				■		■	■	■	25879	1
											■	■		■	■		■			436	1
					■									■	■		■	■		16438	1
							■	■				■		■	■			■		6322	1
		■										■		■	■			■		131250	1
			■					■						■				■		67618	1
				■	■			■							■		■		■	51221	1
			■		■			■										■		83978	1
			■					■							■		■	■		67614	1
			■												■		■	■		65558	1
				■				■		■				■						35368	1
		■								■								■		131594	1
			■			■		■							■			■		75802	1



eta	etb	sea	sec	sed	see	PVL	tsst	bla	dfrk	ermC	coa	splE	N046	SAV0881	PV83-orf2	phi11-44	sltORF257	SA1801	SAV1974	MRSA Type	Count
						■		■	■						■			■		11282	1
										■				■				■	■	555	1
							■					■			■					4240	1
										■		■						■	■	651	1
			■									■								65664	1
											■	■						■		394	1
										■				■	■		■	■		574	1
												■								136	1
		■										■			■		■	■	■	131223	1
							■					■								4232	1
■												■			■		■			524444	1
							■											■		4098	1
								■							■			■	■	2067	1
		■										■		■	■		■	■		131262	1
								■			■	■		■	■			■		2490	1
		■										■			■			■	■	131227	1
						■	■	■		■					■			■		14866	1
			■			■						■			■		■	■		73878	1
												■			■		■	■		158	1
						■		■		■					■		■			10780	1
											■	■	■	■	■		■	■	■	511	1
												■			■		■		■	157	1
							■					■			■				■	4249	1
																		■	■	3	1
							■		■			■						■	■	5259	1
				■	■									■				■	■	49195	1
								■				■								2176	1
										■					■		■			540	1
			■			■	■	■									■	■		79886	1
		■					■					■	■	■	■			■		135418	1
						■		■							■		■			10260	1
					■									■	■		■	■	■	16439	1
								■							■		■			2076	1
							■					■			■			■		4242	1
						■				■					■		■			8732	1
						■		■	■			■						■		11402	1
		■					■					■		■				■		135330	1
		■					■	■				■		■	■			■		137402	1
								■						■	■		■	■		2102	1
								■			■	■								2432	1
					■						■	■			■		■	■		16798	1
							■					■	■		■		■			4308	1
				■														■	■	32779	1
								■				■			■		■	■		2198	1
								■						■				■		2082	1
													■		■					88	1
								■	■	■					■		■			3612	1



eta	etb	sea	sec	sed	see	PVL	tsst	bla	dfrk	ermC	coa	splE	N046	SAV0881	PV83-orf2	phi11-44	sltORF257	SA1801	SAV1974	MRSA Type	Count
			■					■				■						■		67714	1
				■	■			■						■	■		■	■		51262	1
								■				■			■			■	■	2203	1
							■					■			■		■	■	■	4255	1
		■										■	■	■	■		■	■		131318	1
			■					■			■			■	■			■		67898	1
							■	■			■	■						■		6530	1
					■										■		■	■		16406	1
																	■	■	■	7	1
					■								■		■		■	■		16470	1
										■		■								640	1
								■							■			■		2066	1
							■					■			■					4248	1
								■			■				■		■	■		2326	1
			■				■	■												71688	1
						■		■		■	■			■	■		■			11060	1
												■		■	■		■	■		190	1
					■						■	■								16768	1
											■	■								384	1
				■	■															49160	1
		■					■					■	■					■		135370	1
								■		■				■						2600	1
																	■	■		6	1
											■	■	■							448	1
		■						■	■			■						■		134274	1
							■					■		■				■		4258	1
							■					■		■						4264	1
								■												2048	1
		■													■		■	■		131094	1
		■						■				■	■		■			■		133330	1
		■				■						■		■	■		■	■		139446	1
							■					■						■		4226	1
											■			■	■		■	■		310	1
					■									■	■		■	■	■	16447	1
							■	■						■				■		6186	1
											■	■	■					■		450	1
				■				■						■						34848	1
									■	■								■		1538	1
					■						■	■						■		16778	1
															■		■			20	1
							■								■		■	■		4126	1
												■		■	■		■	■	■	191	1
					■									■						16424	1
					■			■							■			■		18458	1
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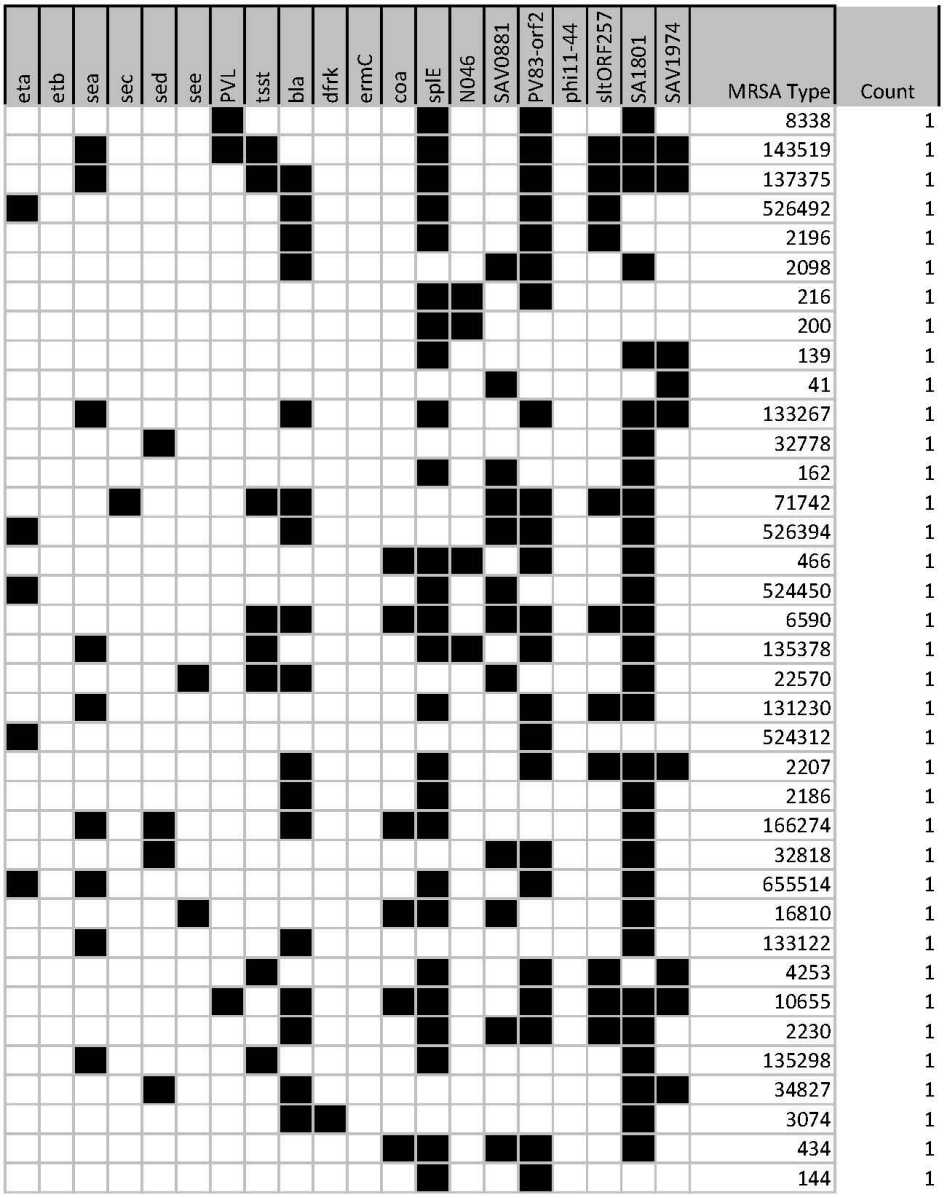

eta	etb	sea	sec	sed	see	PVL	tsst	bla	dfrk	ermC	coa	splE	N046	SAV0881	PV83-orf2	phi11-44	sltORF257	SA1801	SAV1974	MRSA Type	Count
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		■				■	■					■			■		■	■	■	143519	1
		■					■	■				■			■		■	■	■	137375	1
■								■				■			■		■			526492	1
								■				■			■		■			2196	1
								■						■	■			■		2098	1
												■	■		■					216	1
												■	■							200	1
												■						■	■	139	1
														■					■	41	1
		■						■				■			■			■	■	133267	1
				■														■		32778	1
												■		■				■		162	1
			■				■	■						■	■		■	■		71742	1
■								■						■	■			■		526394	1
											■	■	■		■			■		466	1
■												■		■				■		524450	1
							■	■			■	■		■	■		■	■		6590	1
		■					■					■	■		■			■		135378	1
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		■										■			■		■	■		131230	1
■															■					524312	1
								■				■			■		■	■	■	2207	1
								■				■						■		2186	1
		■		■				■			■	■						■		166274	1
				■										■	■			■		32818	1
■		■										■			■			■		655514	1
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		■						■										■		133122	1
							■					■			■		■		■	4253	1
						■		■			■	■			■		■	■	■	10655	1
								■				■		■	■		■	■		2230	1
		■					■					■						■		135298	1
				■				■										■	■	34827	1
								■	■									■		3074	1
											■	■		■	■			■		434	1
												■			■					144	1

Table 9: *In silico* binary profiles

Isolate	sea	sec	sed	lukS-PVL	Tn554tnpB	phiMu50B SAV0881	phiPV83 ORF2	phiMu50B SAV0858	phi11 nt4427-5251	phiSLT ORF257	phiN315 SA1801	phiMu50A SAV1974	phiSLT ORF182	Seq Type	Identity %
15969	+			+			+			+	+			ST1	100
15995	+			+			+			+	+			ST1	100
16000		+							+					ST1	100
16016	+										+			ST1	100
16028	+						+		+	+	+			ST1	100
16013	+				+	+			+	+ (WGS only)	+	+ (WGS only)		ST109	100
16006						+	+		+	+	+			ST1152	100
15968		+									+			ST12	100
16101	+										+			ST12	100
16102											+			ST12	100
15986				+			+		+	+		+		ST121	100
15998						+	+		+		+	+		ST121	99.97
16066				+			+		+	+				ST121	100
16100	+		+			+					+			ST1350	100
15962							+		+	+			+	ST15	100
15972									+					ST15	100
15996						+		+						ST15	100
15999						+	+		+	+				ST15	100
16003						+		+ (WGS only)			+			ST15	100
16004														ST15	100
16014														ST15	100
16033							+		+	+				ST15	100

16046			+								+			ST15	100
16061											+			ST15	99.97
16074						+	+	+	+	+	+			ST15	100
15979				+			+			+	+	- (RLB only)	+	ST1633	100
15970								+	+		+			ST188	99.97
15977			+ (WGS only)						+ (WGS only)		+			ST188	99.97
16011								+			+ (WGS only)			ST188	100
16023											+ (WGS only)			ST188	100
16036								+	+		+			ST188	100
16037								+	+		+			ST188	100
16042						+	+	+	+		+			ST188	100
16064					+				+		+			ST188	100
15963							+			+				ST188/ST1519	99.97
15984											+	+		ST20	100
16020											+			ST20	100
16051											+			ST20	100
16071							+			+	+			ST20	100
16093							+				+			ST20	100
15954						+					+	+		ST22	100
15994					+		+	+	+		+			ST2276	100
16041	+				+			+			+			ST239	100
15957							+			+ (WGS only)	+			ST25	100
16019							+		+					ST25	100
16027							+		+					ST25	100
16056						+	+				+			ST291	100
16085				+		+	+			+	+			ST291	100
16015	+				+		+				+	+		ST30	100
15958	+						+		+	+	+	+		ST34	100

15961	+						+		+	+	+	+		ST34	100
15980						+			+		+	+		ST34	100
16035						+	+		+	+	+	+		ST34	99.97
16063							+		+	+		+	+	ST34	100
16082							+		+		+	+		ST34	100
16089	+						+		+	+	+	+		ST34	100
15960		+						+	+		+	- (RLB only)		ST45	100
15966		+				+	+				+			ST45	100
16005		+									+			ST45	100
16040		+				+		+	+		+			ST45	100
16062						+					+			ST45	100
16075						+		+	+		+			ST45	100
16091			+				+		+		+	+		ST45	100
15955			+			+			+					ST5	100
15959							+			+	+			ST5	100
15964									+					ST5	100
15967			+			+			+					ST5	99.97
15973			+						+					ST5	100
15974							+			+	+			ST5	100
15976			+			+			+					ST5	100
15985			+						+		+			ST5	100
15991			+						+		+	+		ST5	100
16007						+			+					ST5	100
16008											+			ST5	100
16009			+								+			ST5	100
16010			+			+			+					ST5	100
16018			+			+			+					ST5	100
16021			+			+			+					ST5	100
16024	+ (WGS only)					+			+		+			ST5	100
16025						+	+		+	+	+			ST5	100
16044			+			+			+		+			ST5	100

16048			+			+	+				+			ST5	100
16053	+		+		+		+			+	+	+		ST5	100
16059						+		+	+		+			ST5	100
16067	+					+	+		+		+			ST5	100
16068	+					+	+			+	+			ST5	100
16077		+			+	+	+		+	+		+		ST5	100
16087			+			+	+			+	+			ST5	100
16095											+			ST5	100
16096	+		+								+			ST5	100
16098			+			+	+		+					ST5	100
16097											+			ST50	100
16052				+			+				+			ST72	100
15981						+			+			+		ST508	100
15997		+				+	+	+	+	+	+	+		ST508	100
16055				+					+		+			ST188	100
16088				+		+	+	+	+	+	+			ST508	100
16043									+					ST59	99.97
16058	+			+							+	+		ST59	100
16084							+		+		+	+		ST59	100
15965	+						+		+		+			ST6	100
15983	+						+				+			ST6	100
15989	+	+ (WGS only)				+	+		+		+			ST6	100
16012	+						+				+			ST6	100
16022	+						+		+		+			ST6	100
16054	+						+		+		+			ST6	100
16083	+						+				+			ST6	100
15987											+			ST672	99.97
15988											+			ST672	99.97
16001							+				+			ST672	100
16057						+	+		+		+			ST7	100
15978		+												ST72	100
16039		+												ST72	100

15956		+				+	+				+			ST78	100
15993					+	+	+			+	+			ST78	100
16034					+	+	+				+			ST78	100
16094		+			+	+	+				+			ST78	100
15992											+			ST8	100
16002					+		+				+			ST8	100
16086	+						+				+			ST30	100
16038											+			ST8	99.97
16047	+		+			+			+		+			ST8	100
16065				+			+		+	+	+	+		ST8	100
16090	+						+		+	+	+			ST1	100
16103							+			+		+		ST8	100
16092				+	+	+	+		+		+			ST109	100
15990	+						+			+	+	+		ST81	100
16029							+		+					ST833	99.97
16076						+	+				+			ST88	100
16099							+		+		+			ST89	100
15971							+		+					ST97	100
15975							+		+					ST97	100
15982							+		+					ST97	100
16026							+		+					ST97	100
16050							+		+		+			ST97	100
16060						+	+		+		+			ST97	100
16070							+		+		+			ST97	100