The clinical and molecular epidemiology of community-associated *Staphylococcus aureus* in northern Australia

by

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Declaration

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Charles Darwin University, is the result of my own investigations, and all references to ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any other degree.

Steven Ye Ching Tong

28 July 2010

Abstract

Staphylococcus aureus is a major human pathogen which has proved versatile in developing resistance to antimicrobials and acquiring virulence factors. Prior to 1990 methicillin-resistant *S. aureus* (MRSA) was principally a hospital-acquired pathogen, but in the past two decades there has been the global emergence of community-associated (CA) strains of MRSA. Early reports from the Northern Territory, Australia documented high rates of CA-MRSA in Indigenous people. It is unclear why CA-MRSA has emerged in this setting and what relationship CA-MRSA has with the circulating methicillin-susceptible *S. aureus* (MSSA) strains.

The epidemiology of *S. aureus* and CA-MRSA in northern Australia was described by determining population incidences for *S. aureus* infection and prospectively collecting *S. aureus* isolates at the Royal Darwin Hospital. The annual incidence of *S. aureus* bacteraemia was six times higher in the Indigenous compared to the non-Indigenous population. The disease spectra due to CA-MRSA and MSSA were similar. However, remote residence was associated with CA-MRSA.

Irrespective of methicillin-resistance, Panton-Valentine leukocidin (PVL) + isolates appeared more virulent, causing disease in younger, healthier hosts. However, isoforms of PVL conferred no differential clinical effect compared to each other. Diversity was found within the staphylococcal protein A (*spa*) locus in both CA-MRSA and MSSA clonal complex 93 isolates, suggesting multiple independent acquisitions of the methicillin-resistance mediating *mec*A gene.

Molecular typing assays were developed and utilised. These targeted the stable housekeeping genes of the multilocus sequence typing loci, the more rapidly evolving *spa* locus, and the gene encoding for the pore forming virulence factor PVL.

Together, the results support a hypothesis that CA-MRSA has arisen in *S. aureus* lineages in remote Indigenous communities where staphylococcal disease is highly prevalent. Standard treatment regimens for staphylococcal skin disease may no longer be effective in remote communities and investigations into alternative treatments were commenced.

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It is has been a great privilege to conduct my PhD studies at the Menzies School of Health Research (Menzies). My enjoyment of the last three years is due not only to the interesting topics of study but also the excellent people I have worked with. Menzies has been a wonderful environment to work and learn in.

Five people stand out as being integral to my PhD and my growth in learning about the research process. My supervisors Bart Currie, Allen Cheng and Deborah Holt have each generously provided their relevant expertise to various parts of the PhD. As my principal supervisor, Bart Currie's long term experience of conducting research in the Northern Territory has been invaluable in providing me with strategic direction and advice as to what projects to be involved with. He has also helped me to understand the importance of forming collaborative partnerships. Despite only being several years my senior, Allen Cheng already has an extraordinary research output and I continue to learn much from his productivity. I have borrowed heavily upon his biostatistical expertise. The laboratory based aspects of my research have been greatly facilitated by the practical know-how of Deborah Holt. Deb has always been there to answer my basic questions about running PCRs or gels.

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Finally, I want to thank my Lord Jesus Christ who created the universe and died and rose to bring me to God. It is a great privilege to peer into and better understand the biology of his universe and particularly the molecular evolutionary processes which drive the interactions between staphylococci and humans.

Declaration of author's contribution

This thesis is substantially my own work and was performed under the guidance of my supervisors Bart Currie, Deborah Holt and Allen Cheng, and also Philip Giffard. The papers and abstracts listed in the thesis in which I was sole first author were written by me. For the *Staphlylococcus aureus* typing paper (Chapter 8), on which I was joint first author, I wrote the paper with contributions from Deborah Holt and Philip Giffard. I collected the majority of the data for the case-control study (Chapter 3) and enrolled and followed-up all the patients in the pilot randomised controlled trial for skin sore treatments (Chapter 5). I designed and performed the laboratory work for the *spa* typing assay (Chapter 6) and the PVL variants assay (Chapter 7). I conceived, designed and wrote the bioinformatic software to facilitate the development of the high-resolution melting based typing assays for *S. aureus* (Chapter 8). All the statistical analysis presented in the thesis was performed by myself with advice from Allen Cheng.

I hereby specifically acknowledge the following contributions of others in collecting data and performing laboratory assays:

Chapters 3 and 4: Craig Boutlis initiated the case-control study and Emma Bishop commenced the recruitment of patients and collection of clinical data. Rachael Lilliebridge co-ordinated the storage of isolates for the case-control study and performed the molecular typing on these isolates. Paul Southwell, Gary Lum, Desmond Chih, and the Northern Territory Government Pathology Service Microbiology Laboratory identified and collected isolates. Elizabeth Canale, Marianne Martinello and Jonas Zehnder were medical students who assisted in collecting patient data.

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Publications

Papers directly arising from this work

Refereed journals

Tong SY, Anstey NM, Lum GD, Lilliebridge RA, Stephens DP, Currie BJ. Fatal community-associated methicillin-resistant *Staphylococcus aureus* pneumonia after influenza. Med J Aust. 2008;188:61. (Appendix V)

Lithgow AE, **Tong SY**. MRSA: The emerging problem of community-associated MRSA: Necrotising pneumonia in a 19-month-old Aboriginal boy. The Northern Territory Disease Control Bulletin. 2008;15:22–4. (Appendix VI)

Tong SY, McDonald MI, Holt DC, Currie BJ. Global implications of the emergence of community-associated methicillin-resistant *Staphylococcus aureus* in Indigenous populations. Clin Infect Dis. 2008;46:1871–8. (Appendix III)

Tong SY, Bishop EJ, Lilliebridge RA, Cheng AC, Spasova-Penkova Z, Holt DC, Giffard PM, McDonald MI, Currie BJ, Boutlis CS. Community-associated strains of methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *S. aureus* in Indigenous northern Australia: epidemiology and outcomes. J Infect Dis. 2009;199:1461–70. (Appendix IV)

Tong SY, Lilliebridge RA, Holt DC, McDonald MI, Currie BJ, Giffard PM. High-resolution melting analysis of the *spa* locus reveals significant diversity within sequence type 93 methicillin-resistant *Staphylococcus aureus* from northern Australia. Clin Microbiol Infect. 2009;15:1126–31. (Appendix X)

Tong SY, Giffard PM, Holt DC. CA-MRSA: Emerging remotely. Microbiology Australia. 2009;30:185–6. (Appendix XII)

Tong SY, Andrews RM, Kearns T, Gundjirryirr R, McDonald MI, Currie BJ, Carapetis JR. Trimethoprim-sulfamethoxazole compared to benzathine penicillin for treatment of impetigo in Aboriginal children: a pilot randomised controlled trial. J Paediatr Child Health 2010;46:131–3. (Appendix VIII)

Tong SY, Lilliebridge RA, Holt DC, Coombs GW, Currie BJ, Giffard PM. Rapid detection of H and R Panton-Valentine leukocidin isoforms in *Staphylococcus aureus* by high-resolution melt analysis. Diagn Microbiol Infect Dis 2010;67:399–401. (Appendix XI)

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<u>Manuscript in preparation</u>

Lilliebridge RA[#], **Tong SY**[#], Giffard PM, Holt DC. MLST based *Staphylococcus aureus* typing scheme using high-resolution melting analysis of SNP nucleated PCR fragments. Manuscript in preparation.

[#] indicates joint first author

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Tong SYC, Andrews RM, Kearns T, Gundjirryirr R, Currie BJ, McDonald MI, Carapetis JR. Trimethoprim-sulfamethoxazole compared to benzathine penicillin for impetigo in Aboriginal children – a pilot randomised controlled trial. Annual Scientific Meeting of the Northern Territory branch of the Royal Australian College of Physicians, November 2008, Darwin, Australia.

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Abbreviations used in the text

AFLP	Amplified fragment length polymorphism
aOR	Adjusted odds ratio
bp	Base pair
βHS	Beta haemolytic streptococcus
BPG	Benzathine penicillin G
CA-MRSA	Community-associated methicillin-resistant *Staphylococcus aureus*
CC	Clonal complex
CI	Confidence interval
C_T	Cycle threshold
D	Simpson's index of diversity
DNA	Deoxyribonucleic acid
GAS	Group A streptococcus
GBS	Group B streptococcus
GCS	Group C streptococcus
H	Histidine
HA-MRSA	Healthcare-associated methicillin-resistant *Staphylococcus aureus*
HCA	Healthcare-associated
HRM	High-resolution melting
ICU	Intensive care unit
IM	Intramuscular
LOS	Length of stay
MALDI-TOF	Matrix-assisted laser desorption ionisation - time of flight
MelT	Melting type
Minim	Mini-MLST
MLST	Multilocus sequence type
MLVA	Multilocus variable number tandem repeat analysis
mMRSA	Multidrug-resistant methicillin-resistant *Staphylococcus aureus*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
nmMRSA	Non-multidrug-resistant methicillin-resistant *Staphylococcus aureus*
NT	Northern Territory

OR	Odds ratio
PCR	Polymerase chain reaction
PCR/ESI-MS	PCR coupled to electrospray ionisation – mass spectrometry
PFGE	Pulsed-field gel electrophoresis
PVL	Panton-Valentine leukocidin
R	Arginine
RDH	Royal Darwin Hospital
SCC*mec*	Staphylococcal cassette chromosome *mec*
SD	Standard deviation
SNP	Single nucleotide polymorphism
spa	staphylococcal protein A
SSTI	Skin and soft tissue infection
ST	Sequence type
T_m	Melting temperature
TMP-SMZ	Trimethoprim-sulfamethoxazole
UK	United Kingdom
VNTR	Variable number tandem repeat
WA	Western Australia
WSPP	Western Samoan Phage Pattern

Chapter 1. Introduction

Staphylococcus aureus is a major human pathogen which has proved versatile in developing resistance to antimicrobials and acquiring virulence factors. Prior to 1990 methicillin-resistant *S. aureus* (MRSA) was principally a hospital-acquired pathogen, but in the past two decades there has been the global emergence of community-associated (CA) strains of MRSA. Early reports from the Northern Territory, Australia documented high rates of CA-MRSA in Indigenous people. It is unclear why CA-MRSA has emerged in this setting and what relationship CA-MRSA has with the circulating methicillin-susceptible *S. aureus* (MSSA) strains.

The first half of this thesis describes my efforts to define the epidemiology of CA-MRSA in the Top End of the Northern Territory. As my studies progressed it became clear that a focus on CA-MRSA alone was too narrow and hence the scope of the thesis more broadly encompasses the clinical and molecular epidemiology of *S. aureus* in general in the Top End. The thesis begins with a background about *S. aureus* in Indigenous populations, with a particular focus on the emergence of CA-MRSA in Australia (Chapter 2). This is followed by a comparison of CA-MRSA with MSSA infections from the Royal Darwin Hospital using a case-control study design (Chapter 3). Chapter 4 provides a more detailed analysis of the combined clinical and molecular epidemiology of *S. aureus* and concentrates on Panton-Valentine leukocidin (PVL) and specific *S. aureus* lineages.

The high prevalence of *S. aureus* and CA-MRSA as a cause of impetigo in remote communities led us to conduct a pilot randomised controlled trial of an alternative treatment to the current Northern Territory standard treatment regimen of benzathine penicillin. This pilot study is described in Chapter 5.

The second half of this thesis relates to molecular genotyping assays I developed and utilised. Chapters 6 and 7 focus on determining variation in the hypervariable *spa* locus and the gene encoding for PVL. Chapter 8 describes using high-resolution melting analysis to interrogate highly informative single nucleotide polymorphisms

in the multilocus sequence type loci of *S. aureus* and *Streptococcus pyogenes*. Chapter 9 provides the conclusions and recommendations for future research.

The thesis has been written so that the references for each chapter are placed at the end of the chapter. This has been done for the convenience of the reader and to also reflect the text of the actual publications that make up some of the chapters. However, it is recognised that some references are repeated in different chapters.

The actual portable document files of published papers, together with the grant application for a randomised controlled trial of alternative treatments to intramuscular penicillin for impetigo in Aboriginal children, are attached as appendices.

Chapter 2. The emergence of community-associated methicillin-resistant *Staphylococcus aureus* in Indigenous populations

This chapter provides a review of the literature concerning community-associated methicillin-resistant *S. aureus* (MRSA) and introduces the hypothesis that community-associated MRSA has emerged from Indigenous populations in Australia.

The following was published in Clinical Infectious Diseases. See Appendix III.

Tong SY, McDonald MI, Holt DC, Currie BJ. Global implications of the emergence of community-associated methicillin-resistant *Staphylococcus aureus* in Indigenous populations. Clin Infect Dis. 2008; 46: 1871-8.

2.1 Abstract

The emergence of community-associated methicillin-resistant *Staphylococcus aureus* (community-associated MRSA) in Australia may have been facilitated by conditions in socially disadvantaged populations – particularly, remote Australian Aboriginal communities. The appearance of community-associated MRSA was first noticed in Australia during the early 1980s; subsequently, several genetically diverse strains have independently emerged from geographically distinct regions. Molecular and epidemiological studies support the role of genetic transfer of resistance determinants (SCC*mec*IV) in this process. Conditions in Aboriginal communities – namely, domestic crowding, poor hygiene, and high rates of scabies, pyoderma and antibiotic use – have facilitated both the clonal expansion and *de novo* emergence of strains of community-associated MRSA. Combating the worldwide emergence and spread of community-associated MRSA may require novel community-level control strategies targeted at specific groups, such as remote Indigenous populations.

2.2 Introduction

Community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a leading cause of skin and soft-tissue infections in many parts of the world. One strain, USA300, now causes up to 70% of skin and soft-tissue infections that present to emergency departments across the United States[1]. Community-associated MRSA emerged in Australia during the early 1980s[2, 3]; in contrast with the United States, there continues to be an increasingly recognised diversity of strains in circulation in Australia[4]. Three previously recognised clonal groups were described from geographically distinct regions, as was a newly documented clonal group apparently unique to tropical northern Australia. The present review describes the emergence of these clonal groups and hypothesises that conditions in socially disadvantaged populations have facilitated emergence. We follow the story of community-associated MRSA in Australia and discuss the public health implications. **Table 2-1** defines some of the terms used in this review.

Australia has played a significant role in the history of *S. aureus* epidemiology. In 1952, a pandemic clone of penicillin-resistant *S. aureus*, termed "phage type 80/81," was first isolated from neonatal infections in Sydney[5]. Phage type 80/81 subsequently caused severe nosocomial and community infections throughout the developed world during the 1950s and 1960s[6] and more recent work has demonstrated phage type 80/81 to be a Panton-Valentine leukocidin (PVL) positive sequence type (ST) 30 strain[7]. This epidemic receded with the introduction of methicillin and related penicillinase-stable antibiotics during the 1960s, only to be replaced by the emergence of MRSA. After sporadic reports of MRSA during the 1960s, an MRSA strain emerged in Melbourne and Sydney hospitals during the late 1970s that subsequently became endemic in Australian and overseas hospitals[8].

In 1980, the appearance of MRSA infections in injection drug users in Detroit heralded the next wave of staphylococcal disease[9]. Unlike patients with healthcare-associated MRSA, the majority of these patients were young and otherwise healthy and had not recently been hospitalised. These new strains were labelled "community-acquired MRSA".

Table 2-1. Glossary of terms used in the introduction about community-associated methicillin-resistant *Staphylococcus aureus*

Clones / strains	Varieties of *S. aureus* which differ in their basic genetic background, determined by various typing techniques; in Australia, the most common community-associated MRSA clones are WA-MRSA-1 (ST1), Queensland clone (ST93), and the Western Samoan Phage Pattern (WSSP) (ST30); in the United States, USA300 is predominant
Multilocus sequence type	Strains can be differentiated according to the DNA sequence of 7 housekeeping genes; the sequence is assigned a unique "sequence type"; the genetic relatedness of strains can be inferred, and closely related strains can be grouped together as a clonal complex.
*mec*A	The gene encoding a modified cell wall component that leads to resistance to methicillin and other β-lactam antibiotics
Staphylococcal cassette chromosome (SCC)	The mobile genetic element on which *mec*A resides; there are different types of SCC*mec* (I to V), which vary in size, mobility and whether other resistance genes are carried; SCC*mec* can transfer from one staphylococcus to another
Community-associated MRSA	Strains of MRSA which are typically not multiresistant to antibiotics and harbour SCC*mec*IV as the resistance determinant; community-associated MRSA has arisen from the community but now causes infections in hospitals as well as in the community
Healthcare-associated MRSA	Strains of MRSA which are genetically distinct from community-associated MRSA, are multiresistant to antibiotics and usually harbour SCC*mec*II or SCC*mec*III as the resistance determinant; cause infections almost exclusively in patients with healthcare contact.

2.3 Community-associated MRSA in Australia

Community-associated MRSA was initially noticed in Australia during the early 1980s[3], and the first detailed description, in 1989, was of patients from remote Aboriginal communities of Western Australia[2]. After the Western Australian government decided to make MRSA colonisation and infection a notifiable disease in 1985[10], it became apparent that MRSA was increasingly isolated from patients without prior hospital contact. Most of these patients came from remote Aboriginal communities in the Kimberley region (**Figure 2-1**) in the tropical north of Western Australia. In contrast to previous MRSA strains, the isolates were resistant only to β-lactam antimicrobials, and genetic analysis by pulsed-field gel electrophoresis (PFGE) demonstrated a "new" type of MRSA[2]. WA-MRSA, as it became known, spread throughout Western Australia and into major metropolitan centres. Community studies found that 42% of inhabitants of one remote community and 24% of another were colonised with WA-MRSA[11].

Similar strains of non-multiresistant MRSA were also present in the Northern Territory, the jurisdiction adjacent to northern Western Australia. Between 1991 and 1995, infections caused by community-associated strains outnumbered those of healthcare-associated MRSA at the Royal Darwin Hospital in the tropical Top End of the NT[12, 13], which suggests that community-associated MRSA was already widespread. The majority of these infections occurred in people from rural and remote Indigenous communities.

Although the documentation of community-associated MRSA was more accurate and complete in Western Australia, the incidence of infection during the early 1990s was substantially higher in the Northern Territory. On the basis of population data from the 1996 national census, the incidence of community-associated MRSA isolation in Western Australia[14] from January 1991 through June 1995 was 43 isolations per 100,000 population. The incidence of community-associated MRSA infection in the Top End of the Northern Territory[12] over the same time period was 81 infections per 100,000 population. The true difference in incidence is probably much greater, because notification of infections and colonisations (including screening samples) was mandatory for all Western Australia laboratories, whereas clinical infections at only the Royal Darwin Hospital were included in notification from the Northern

Territory. This suggests a possibly earlier emergence of community-associated MRSA in the Northern Territory than in Western Australia, with widespread establishment in the Northern Territory population.

As molecular tools advanced, it became clear that community-associated MRSA was not a "feral descendant" of healthcare-associated MRSA strains that had escaped into the community[15]; rather, the emergence of community-associated MRSA was independent of healthcare-associated MRSA. Strains of different genetic backgrounds were arising from discrete regions of Australia[16], and all community-associated MRSA strains were different from the traditional healthcare-associated MRSA strains. Indeed, in contrast to community-associated MRSA, healthcare-associated MRSA has generally been restricted to a small number of clones that have spread globally with the movement of carrier patients and health staff[17]. Molecular analysis of Northern Territory isolates found them to be genetically distinct from Western Australia strains[16]. It also became evident that WA-MRSA was not simply one strain but was at least five unrelated strains[18]. In contrast to the clonal transfer of healthcare-associated MRSA from one colonised or infected patient to another within the hospital setting, often via the hands of healthcare workers, the picture in Australia indicated multiple independent emergences of community-associated MRSA. The highest rates of notification in Western Australia were from two widely separated remote regions – the Kimberley and the more southerly Goldfields[19]. In the Top End of the Northern Territory, Aboriginal people were 13 times more likely than non-Aboriginal people to be infected with community-associated MRSA[12].

In Queensland, on the opposite side of the continent from Western Australia, 2 additional clones were identified: the Western Samoan Phage Pattern (WSPP) clone (ST30-MRSA-IV), first described in the Pacific Islander population, and the Queensland MRSA clone (ST93-MRSA-IV), which appeared in both Caucasian and Aboriginal populations. The WSPP clone was first documented in Brisbane in 1997[20]. These isolates were related to a clone causing an epidemic of community-associated MRSA in New Zealand during the mid- to late 1990s[21, 22]. The New Zealand epidemic was centred in areas of Auckland densely populated by Pacific Islanders, and several studies confirmed that Samoans were at highest risk of colonisation, infection, and bacteraemia with WSPP[23]. Ten isolates from Australia, New Zealand, and Western Samoa were identical by PFGE[22] and supported the

postulate that WSPP arrived in Australia via New Zealand during the 1980s and 1990s[20]. Poor living conditions in Samoa were cited as factors leading to the emergence of WSPP, although supportive evidence is scant[22].

Queensland-MRSA (ST93) came to prominence when Caucasians were also noted to have community-associated MRSA infections[24]. Although the first reported case of fatal necrotising pneumonia caused by community-associated MRSA in Australia was in a young Aboriginal man, it was initially thought that ST93 was uncommon in Aboriginal populations[25]. Subsequent studies found that three of four cases of community-associated MRSA bacteraemia due to ST93 were in Aboriginal patients[26] and that ST93 was not carried in attendees to urban Brisbane general practices[27] but was carried by 7% of school children in Aboriginal communities[28]. Evidence suggests that the Queensland clone emerged from Aboriginal communities[29, 30].

The most recently described clonal group in Australia, NT-MRSA, was found to predominate in remote Aboriginal communities in the Top End of the Northern Territory. NT-MRSA comprises clonal complex 75 (cc75), which contains ST75. In a longitudinal, community-based study of pyoderma, 71% of community-associated MRSA isolates were cc75[31]. Although ST75 had been identified in previous Australian-wide surveys of community-associated MRSA[4, 18, 32] and indeed labelled as WA MRSA-8, it was determined that all the isolates were actually from the Northern Territory. Recent typing of the Northern Territory isolates causing infections in 1991[12] reveals that NT-MRSA was already present at that time (D.C.H., unpublished data). This clone almost certainly emerged from the local Northern Territory population.

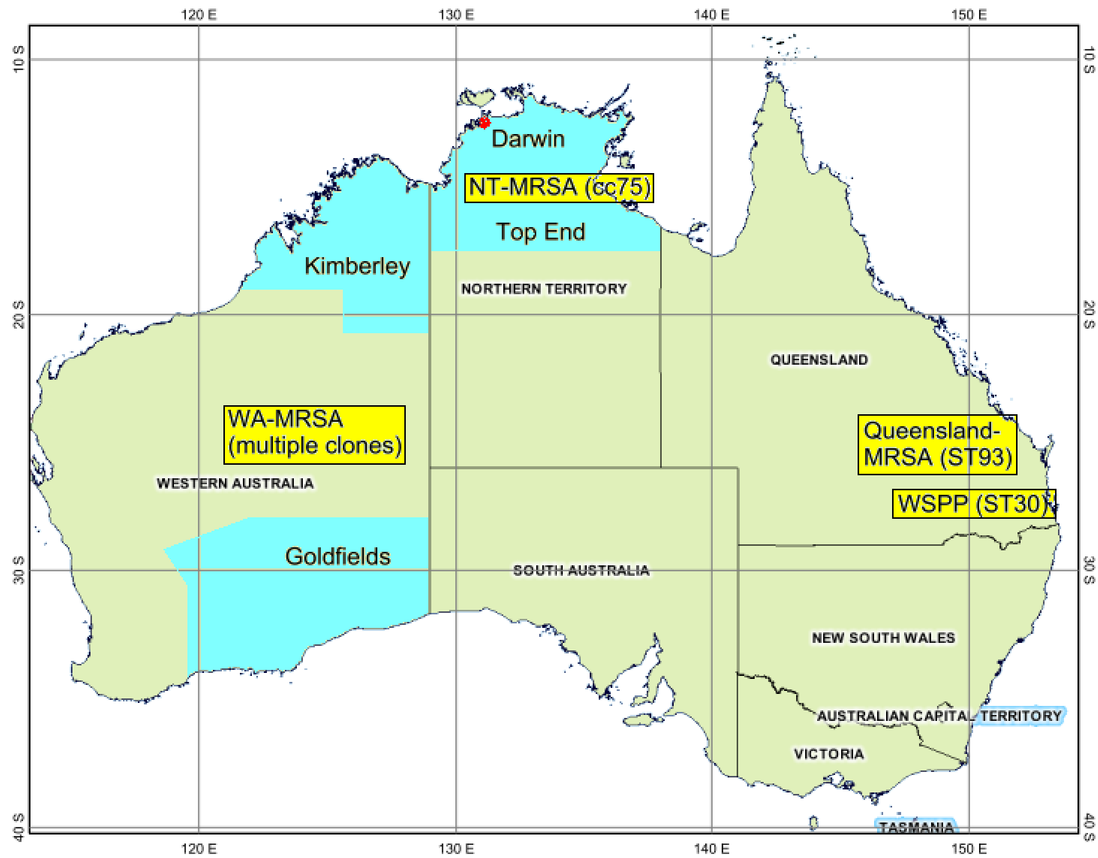

Figure 2-1. Community-associated methicillin-resistant *Staphylococcus aureus* strains in Australia and regions from which these strains have appeared.

cc, clonal complex; NT, Northern Territory; ST, sequence type; WA, Western Australia; WSPP, Western Samoan Phage Pattern.

2.4 Mechanisms for the emergence of community-associated MRSA

Methicillin resistance is mediated by the *mec*A gene, which is carried on one of several types of staphylococcal cassette chromosomes (SCCs), which are mobile genetic elements. Community-associated MRSA typically has type IV SCC*mec*, which is smaller and more mobile than the types I–III SCC*mec* seen in healthcare-associated MRSA[33]. We hypothesise that, in addition to clonal transmission of community-associated MRSA, there is local *de novo* emergence of new clones of community-associated MRSA when SCC*mec*IV, or more rarely SCC*mec*V, is transferred via site-specific integration into already prevalent community strains of methicillin-susceptible *S. aureus* (MSSA). That is, not only is one clone of community-associated MRSA spreading, but the gene mediating resistance is also repeatedly jumping from one staphylococcus to another – a "gene outbreak." In addition to a diversity of community-associated MRSA clones arising from geographically distinct regions, we would also expect to find the following: that SCC*mec*IV-positive and SCC*mec*IV-negative isolates of the same clone circulate together, that community-associated MRSA and MSSA infections are epidemiologically and clinically similar, that *in vivo* transfer of SCC*mec*IV between staphylococcal isolates does occur, and that environmental conditions favouring such transmission are present among populations from which community-associated MRSA has emerged.

Molecular studies that include both MRSA and MSSA strains from confined localities and time spans demonstrate the co-circulation of MSSA and MRSA strains of the same or a similar genetic backbone[31, 34, 35]. It appears that clones of MRSA that harbour SCC*mec*IV have only lately emerged, suggesting recent and possibly frequent acquisition of SCC*mec*IV by background MSSA strains[35]. In the Northern Territory community study, cc75 accounted for 25% of all isolates, and within cc75, the ratio of MRSA to MSSA was 2:1[31]. This clone has not been reported elsewhere to date, and what we have is a prevalent MSSA strain and its direct SCC*mec*IV-harbouring descendant coexisting in an isolated environment.

Early studies of the epidemiology of community-associated MRSA found striking similarities between community-associated MRSA and MSSA infections, leading authors to conclude that "the clinical syndromes associated with *S. aureus* isolation

are independent of methicillin susceptibility"[36, 37]. However, because one particular clone of community-associated MRSA – USA300 – has become dominant in the United States, slight differences between community-associated MRSA and MSSA infections have emerged[38]. This is likely related to various virulence factors carried by USA300 that have made it such a successful clone. For instance, the presence of arginine catabolic mobile element in USA300 is thought to confer a selective advantage for growth and survival in the host[39].

In contrast, in northern Australia, where no one clone has become dominant, we have found no difference with regards to the proportion of skin and soft tissue infections, need for admission, need for surgery and length of stay for patients with community-associated MRSA infections, compared with patients with MSSA infections, presenting to the Royal Darwin Hospital[40]. Although USA300, Queensland-MRSA, and the WSPP clone carry the Panton-Valentine leukocidin genes (*lukS-PV* and *lukF-PV*), NT-MRSA and the original WA-MRSA clones are *pvl* negative[41]. Most Australian reports of severe community-associated MRSA infections are associated with the Queensland clone[26], which supports the hypothesis that virulence is determined by factors other than the presence of SCC*mec*. However, *pvl* has been detected recently in some WA-MRSA strains[4], emphasising the importance of ongoing clinical and molecular surveillance for changing virulence in community-associated MRSA.

Evidence of the *in vivo* transfer of SCC*mec*IV to an MSSA strain was elegantly provided by Wielders et al.[42], when they described a patient in whom initially an MSSA and then an MRSA were isolated. The isolates were identical except for the presence of *mec*A, and the *mec*A was identical to that excised from a *Staphylococcus epidermidis* isolate from the same patient. It appeared that the MSSA had acquired *mec*A from the *S. epidermidis* to become MRSA.

2.5 Common factors in groups at high risk for community-associated MRSA

Populations at high risk for community-associated MRSA outbreaks include sporting teams[43], incarcerated persons[44], the military[45], children in day care facilities[46], men who have sex with men[47], and Indigenous communities across the world[37, 48, 49]. A

study of community-associated MRSA infections in an American football team found that infections developed at turf abrasion sites and in players who had more frequent skin contact with other players. Hygiene practices were suboptimal, and antibiotic use was much higher among team players than among the general population[43]. Investigations of outbreaks in jails in the United States identified poor hygiene practices, close contact with MRSA-infected inmates, and poor access to medical care as risk factors for community-associated MRSA infection[44].

Indigenous populations often live in remote and isolated settings, although there are crowded living conditions within communities. As has been the case in Australia, community-associated MRSA appears to have emerged from these settings. In remote Alaskan villages, outbreaks of community-associated MRSA skin and soft-tissue infections were associated with prior antibiotic use and use of communal saunas from which community-associated MRSA was recovered[48, 49]. An epidemic of community-associated MRSA occurred in an American Indian rural community with crowded housing conditions, poor access to health care and high rates of skin disease, leading the authors to comment that "community-associated MRSA may be found in ever-increasing numbers in other communities of low socioeconomic status", and that "rural communities are not sheltered"[37]. We contend that not only are such communities "not sheltered", but may in fact be where community-associated MRSA is emerging from and subsequently spreading to the wider population.

2.6 Factors contributing to the emergence of community-associated MRSA from Australian Aboriginal communities

Risk factors for emergence and transmission of community-associated MRSA – namely, crowding, poor hygiene, skin infections and antibiotic use – are highly prevalent in Australian Aboriginal communities[50]. Crowding is most severe in the NT, with reports of a mean of 3.4 persons per bedroom in one study[51] and up to 7.5 per bedroom in another[52]. Water supplies are deficient and unreliable[53], and >60% of households were found to have no or poorly functioning facilities for either washing children, washing clothes or removing faeces[51]. Although it is difficult to make causative links between independent factors, it is likely that domestic crowding, poor

hygiene, and associated socio-demographic factors contribute to extremely high rates of scabies and impetigo in Aboriginal communities[50, 51].

The prevalence of scabies is 25% in adults and 65% in children in some Northern Territory Aboriginal communities[54], and by the age of one year, 63% and 69% of children have respectively presented with scabies and skin sores to community clinics[55]. The prevalence of impetigo in children (<15 years of age) has been up to 70%[54], but we more recently observed an overall prevalence of 20%[52]. Group A streptococcus (GAS) has previously been the primary pathogen, found in over 80% of pyoderma lesions[50], but the pattern appears to be changing in the Northern Territory. In a more recent study, *S. aureus* was recovered from 57% of pyoderma lesions and group A streptococcus from 29% of lesions (usually with *S. aureus*)[52]. It is not clear why the microbiological epidemiology is changing, but with the emergence of community-associated MRSA, intramuscular benzathine penicillin may no longer be the most appropriate antibiotic for the treatment of skin sores in these communities. The ongoing heavy burden of staphylococcal skin infection in crowded settings is likely to be associated with high rates of antibiotic use and to facilitate person-to-person transmission of community-associated MRSA and the transfer of SCC*mec*IV into resident MSSA strains.

The zoonotic potential of MRSA has been noted with horses, pigs, cows, and domestic pets[56-59]. Poor dog health and dog overpopulation are major problems in many Aboriginal communities, and dogs are an intriguing potential contributor to community-associated MRSA emergence. One community study found a median of three (and up to 17) dogs per household, and it was common for dogs to have open wounds[60]. The probable transmission of community-associated MRSA between dogs and humans, in both directions, has been documented elsewhere[59] and would seem even more likely in Aboriginal communities. An additional consideration is that dogs are also colonised with other staphylococcal species, including *Staphylococcus sciuri*[61]. The *S. sciuri* genome contains a *mec*A homologue which is thought possibly to be the evolutionary precursor of *mec*A now found in MRSA[33]. Therefore, it is possible, not only that antibiotic pressure and overcrowding is amplifying the transmission of community-associated MRSA strains in both dogs and humans, but that community-associated MRSA origins may relate to non-*S. aureus* staphylococcal species in dogs.

2.7 What can we do?

There is a rising prevalence of community-associated MRSA infections worldwide, and evidence is accumulating that community-associated MRSA is not simply replacing MSSA but is adding to the overall burden of staphylococcal disease[62, 63]. Community-associated MRSA strains also cause healthcare-associated and nosocomial MRSA infections; people who are colonised on admission can serve as a source of transmission within the hospital environment[11, 64-68]. This was recognised early in the community-associated MRSA reports from the Northern Territory[13]. Of concern, clones in Australia are beginning to acquire more virulence and antibiotic resistance determinants[4]. Furthermore, if there is truly ongoing acquisition of SCC*mec*IV by local MSSA strains, we are likely to see increasing *de novo* emergence of community-associated MRSA in community settings. Combating the emergence and spread of community-associated MRSA may require novel infection control strategies targeted at specific groups at the community level.

The role of subpopulations acting as the foci for the emergence and amplification of infectious diseases has been long recognised with sexually transmitted and vector borne diseases[69]. "Core transmitters" with crusted scabies have also been identified as important in driving the ongoing scabies outbreaks in remote Aboriginal communities, which in turn underlie high rates of pyoderma[50]. There is growing appreciation of the importance of such "core groups" in the epidemiology of antimicrobial-resistant pathogens, including community-associated MRSA[70-72]. In the United States, large jails housing up to 20,000 inmates have been identified as likely foci for the amplification and subsequent spread of community-associated MRSA into the wider community[45, 71, 73]. Public health interventions directed at these "superspreader institutions" are predicted to have a disproportionate effect on controlling the epidemic of community-associated MRSA[70]. Authorities in the United States have produced guidelines aimed at reducing the transmission of community-associated MRSA within prisons, the military and in the general population[45, 74]. One correctional facility in Texas has demonstrated significant reductions in community-associated MRSA infections through improvements in screening for and care of skin infections, personal hygiene, and antibiotic therapy[75].

Although Australia's healthcare system ranks highly internationally, the health inequalities between Aboriginal and non-Aboriginal Australians are well documented. A considerable increase in resources is required to enable remote Aboriginal communities to meet published recommendations for control of community-associated MRSA. It could be that we are paying a heavy microbiological price for the neglect of Aboriginal housing and health hardware needs in remote communities over the past 2 decades. Improving living conditions for Aboriginal Australians should reduce rates of skin infection, and, if our hypothesis is correct, public health strategies targeted at screening for and appropriate treatment of skin infections should slow the emergence of new community-associated MRSA strains. Clinical trials are required to determine if the current recommendation of intramuscular benzathine penicillin is still the most appropriate antibiotic for treatment of impetigo in remote Aboriginal communities. Despite appropriate calls for accelerated development of new antibiotics to treat resistant organisms[76], we must bear in mind that the unfettered use of antimicrobial agents in disadvantaged communities without addressing underlying socioeconomic conditions is likely to further promote the emergence of microbial resistance.

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Chapter 3. Epidemiology and outcomes of community-associated methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *S. aureus*

This chapter describes the clinical epidemiology of community-associated MRSA in comparison to methicillin-susceptible *S. aureus* in northern Australia. Population level data on *S. aureus* bacteraemia were also examined. Analysis of the impact of Panton-Valentine leukocidin (PVL) was restricted to non-multidrug-resistant methicillin-resistant *S. aureus* isolates in this chapter. However, this analysis is extended to include methicillin-susceptible *S. aureus* in chapter 4 where PVL is the major focus. Two case reports of severe community-associated MRSA are found in appendices V and VI.

The following was published in the Journal of Infectious Diseases. See Appendix IV.

Tong SY, Bishop EJ, Lilliebridge RA, Cheng AC, Spasova-Penkova Z, Holt DC, Giffard PM, McDonald MI, Currie BJ, Boutlis CS. Community-associated strains of methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *S. aureus* in Indigenous northern Australia: epidemiology and outcomes. J Infect Dis. 2009; 199: 1461-70.

3.1 Abstract

Background. Some strains of non-multidrug-resistant methicillin-resistant *Staphylococcus aureus* (nmMRSA) in Australia are likely to have emerged from strains of methicillin-susceptible *S. aureus* (MSSA) in remote Aboriginal communities.

Objective. To describe the clinical epidemiology of infection due to community-associated MRSA strains in an Australian tropical hospital setting with a significant Aboriginal population and to compare infections caused by community-associated

strains of MRSA, healthcare-associated strains of MRSA, and MSSA strains with respect to demographic risk factors and clinical outcomes.

Methods. We queried the microbiology database for the Top End of the Northern Territory, Australia, to determine population incidences for *S. aureus* infections and conducted a prospective matched case-control study to compare infection due to nmMRSA, MSSA or multidrug-resistant MRSA at the Royal Darwin Hospital.

Results. The annual incidence of *S. aureus* bacteraemia was 65 cases per 100,000 population, but in the Aboriginal population was 172 cases per 100,000 population (odds ratio [OR] compared with non-Aboriginal population, 5.8 [95% confidence interval {CI}, 3.8–8.9]). Female sex (adjusted OR [aOR], 1.5 [95% CI, 1.1–2.0]) and remote residence (aOR, 1.8 [95% CI, 1.2–2.5]) were associated with the isolation of nmMRSA rather than MSSA, but disease spectrum and outcomes were similar. Among those from whom nmMRSA was isolated, Aboriginal patients were younger (aOR for each additional year, 0.94 [95% CI, 0.92–0.96]), more likely to be female (aOR, 3.8 [95% CI, 1.7–8.5]), and more likely to reside in a remote community (aOR, 29 [95% CI, 8.9–94]) than non-Aboriginal patients. The presence of Panton-Valentine leukocidin in nmMRSA was associated with double the odds of sepsis (aOR, 2.2 [95% CI, 1.1–4.6]).

Conclusions. The association of nmMRSA with female sex and remote residence supports the hypothesis that nmMRSA arose from MSSA strains in remote Aboriginal communities where staphylococcal disease is highly prevalent. The similar clinical spectrum and outcomes for nmMRSA and MSSA infections suggest that virulence is not correlated with resistance phenotype.

3.2 Introduction

Infection due to community-associated strains of methicillin-resistant *Staphylococcus aureus* (CA-MRSA strains) was first recognised in Australia in people from remote Aboriginal communities[1]. Reports of genetically distinct strains of CA-MRSA from other geographically removed regions followed[2-4]. The parallel emergence of infection due to CA-MRSA strains in North America also included outbreaks in rural Indigenous communities[5, 6]. Despite the possibility that CA-MRSA strains emerged from such communities and the impact of these strains on their inhabitants, there have been no detailed studies comparing the clinical epidemiology of infection due to CA-MRSA strains in Indigenous and non-Indigenous people.

Early studies noted clear differences between CA-MRSA strains and healthcare-associated strains of MRSA (HA-MRSA strains) with respect to the patients affected and types of infection caused. Individuals infected with CA-MRSA strains were younger and more likely to have skin and soft-tissue infections (SSTIs), and Indigenous people were overrepresented in this group[5, 6]. In contrast, there were notable similarities between infections due to CA-MRSA strains and infections due to methicillin-susceptible *S. aureus* (MSSA) strains with regard to risk factors and clinical illness[5, 7]. Molecular studies demonstrated that HA-MRSA and CA-MRSA strains were genetically distinct[5, 8]. However, CA-MRSA strains and MSSA strains with the same or similar genetic backgrounds co-circulated in the same environment[9, 10]. Accordingly, it has been proposed that CA-MRSA strains emerge locally from prevalent circulating MSSA strains via acquisition of the *mec*A gene[11-13].

We conducted a case-control study at the Royal Darwin Hospital (RDH) with the following 2 objectives: (1) to describe the clinical epidemiology of infection due to CA-MRSA strains in an Australian tropical hospital setting with a significant Aboriginal population, and (2) to compare infections caused by CA-MRSA, HA-MRSA, and MSSA strains with respect to demographic risk factors and clinical outcomes. We hypothesised that infection due to CA-MRSA strains and infection due to MSSA strains would be similar in most of these key aspects.

3.3 Methods

3.3.1 Study setting and design

The 330-bed RDH is located in the Top End of the Northern Territory of Australia at a tropical latitude of 12°23' S. It is the tertiary referral centre for the city of Darwin, two regional hospitals, and over 70 remote communities, serving a population of 176,000 and an area of 510,000 km^2. Aboriginal Australians comprise 27% of the regional population but make up >50% of the RDH inpatient and emergency department population. Approval for the study was granted by the Human Research Ethics Committee of the Northern Territory Department of Health and Familiess and Menzies School of Health Research.

We sought non-duplicate clinical isolates of *S. aureus* over a 12-month period from 18 April 2006 through 17 April 2007 by querying the microbiology database that links all three Top End hospitals. There was no routine hospital screening for MRSA colonisation during the study period. We considered non-multidrug-resistant MRSA (nmMRSA) isolates to represent CA-MRSA strains and considered multidrug-resistant MRSA (mMRSA) isolates to represent HA-MRSA strains[14]. nmMRSA isolates were defined phenotypically as those resistant to <3 non–β-lactam antibiotic classes, and mMRSA isolates were defined as those resistant to ≥3 non–β-lactam antibiotic classes. Antibiotic phenotype has previously been shown to accurately predict the genotype of CA-MRSA strains[15], and this result has been validated in recent studies from Queensland[14], Western Australia[16], and an Australia-wide survey[12] that included isolates from Darwin. After excluding non-resident patients, we calculated incidence rates for the isolation of *S. aureus* on the basis of published population data from 2006[17] and correlated this with measures of regional socioeconomic disadvantage[18] and residential remoteness[19]. For the former analysis, only regions that had ≥10 *S. aureus* isolates and an available score for the index of relative socioeconomic disadvantage were included.

We then conducted a case-control study of patients at the RDH in which we compared nmMRSA infections with infections caused by MSSA and mMRSA over the same 12-month period. All nmMRSA and mMRSA isolates newly identified in the microbiology laboratory were collected daily, as well as ≤4 consecutive MSSA isolates for each nmMRSA isolate. Laboratory staff did not include isolates from

patients known to have had *S. aureus* isolated in the past month. We used sequential laboratory receipt numbers to match eligible patients who had nmMRSA isolated to the next two eligible patients who had MSSA isolated.

3.3.2 Data collection

By prospectively reviewing the clinical records of the patients from whom each of the isolates were recovered, we collected information concerning demographic characteristics, healthcare-associated risk factors, comorbidities, clinical details of the infection, treatment details, and outcome at discharge. To restrict our analysis to incident staphylococcal infections, we excluded patients from further analysis if they had previously been enrolled in the study or if they had a prior existing unresolved *S. aureus* infection. We excluded patients who had only attended an outpatient clinic or dialysis unit and did not require treatment at the emergency department or hospital admission. We also excluded patients transferred to the RDH who had been admitted elsewhere for >24 hours, patients for whom the treatment intention was palliative, and patients for whom clinical notes were unavailable.

Healthcare-associated infection was defined in accordance with previously published criteria[20] and included nosocomial infection or the presence of any of the following risk factors during the year prior to collection of the sample that yielded the culture result of interest in the present study: (1) residence in a long-term care facility, (2) prior admission to an acute care facility, (3) use of central intravenous catheters or long-term venous access devices, (4) use of urinary catheters, (5) use of other long-term percutaneous devices, (6) prior surgical procedures, and/or (7) need for any form of dialysis. Nosocomial infection was defined by an isolate obtained from a sample collected >48 hours after hospital admission. Previously published criteria were used to define infection, sepsis, and the systemic inflammatory response syndrome[21]. We defined colonisation as a positive microbiological culture result in the absence of clinical features of infection.

3.3.3 Laboratory methods

The laboratory identified *S. aureus* isolates by use of standard methods and conducted susceptibility testing using an automated system (Vitek 2 V4.01; bioMérieux) and the Kirby-Bauer disk diffusion method in accordance with the guidelines of the Clinical and Laboratory Standards Institute[22]. A disk-approximation

test was used to detect inducible clindamycin resistance. Real-time polymerase chain reaction was used to verify the identity of nmMRSA isolates by confirming the presence of the *nuc*A and *mec*A genes; it was also used to determine the presence of *pvl* genes[10].

3.3.4 Statistical analysis

Statistical significance for crude analysis of dichotomous variables was determined using the χ^2 test or Fisher's exact test. Nonparametric data were compared using the Mann-Whitney U test. We performed multivariate conditional logistic regression analysis with stepwise backward elimination of variables to identify the risk factors associated with infection due to nmMRSA compared with those for infection due to MSSA, and we performed multivariate logistic regression analysis of risk factors associated with severity of illness. The likelihood ratio test was used to assess the statistical significance of candidate risk factors. We examined the association between incidence of *S. aureus* infection and measures of regional socioeconomic disadvantage and remoteness by linear regression. If obvious outliers were present we used robust regression methods. Two-sided P values of <.05 were considered significant. Statistical analysis was performed with Stata (version 9.2; StataCorp).

3.4 Results

From 18 April 2006 through 17 April 2007, there were 2227 *S. aureus* isolates recovered from distinct patients from the three Top End hospitals; nmMRSA accounted for 343 (15%) of the isolates, MSSA accounted for 1748 (79%), and mMRSA accounted for 136 (6%). There were 110 (5%) non-duplicate isolates recovered from blood culture, which were distributed as follows: 17 were nmMRSA, 83 were MSSA, and 10 were mMRSA. The annual incidence of *S. aureus* bacteraemia was 65 cases per 100,000 population, and for infection due to MRSA, the incidence was 16 cases per 100,000 population. The annual incidence of *S. aureus* bacteraemia in the Aboriginal population was 172 cases per 100,000 population, and for the non-Aboriginal population, the incidence was 30 cases per 100,000 population (odds ratio [OR], 5.8 [95% confidence interval [CI], 3.8–8.9]). The total annual incidence of isolation of *S. aureus* was 1248 isolations per 100,000 population, and the incidence of nmMRSA isolation was 193 isolations per 100,000 population. There was a strong correlation between incident isolation of *S. aureus* and measures of regional socioeconomic disadvantage and remoteness (**Figure 3-1**).

At the RDH during the same time period, there were 1693 non-duplicate *S. aureus* isolates recovered; 291 (17%) of these were nmMRSA, 1265 (75%) were MSSA, and 137 were (8%) mMRSA. As an estimate of community-onset cases, of 728 isolates recovered from patients presenting to the emergency department, 117 (16%) were nmMRSA, 582 (80%) were MSSA, and 29 (4%) were mMRSA. Prior to matching, there were 1007 eligible patients and isolates in the case-control study. **Figure 3-2** presents details about exclusion criteria and the number of participants and isolates at each stage of the study. After matching, there were 239 patients who had nmMRSA isolated, 478 who had MSSA isolated, and 90 who had mMRSA isolated available for further analysis. The demographic characteristics of the control patients who had MSSA isolated were representative of the overall population of patients infected with MSSA in the Top End (data not shown). Of the 807 isolates studied, 403 (50%) were considered to represent community-associated strains, and 404 (50%) were considered to represent healthcare-associated strains. There were 153 (19%) isolates recovered from patients who satisfied the definition of nosocomial infection. A similar proportion of nmMRSA and MSSA isolates were healthcare-associated strains (P=.15) or were nosocomially acquired (P=.12) (**Table**

3-1). In nmMRSA isolates, the presence of *pvl* genes was associated with community acquisition rather than nosocomial acquisition (OR, 4.4 [95% CI, 2.5–7.8]).

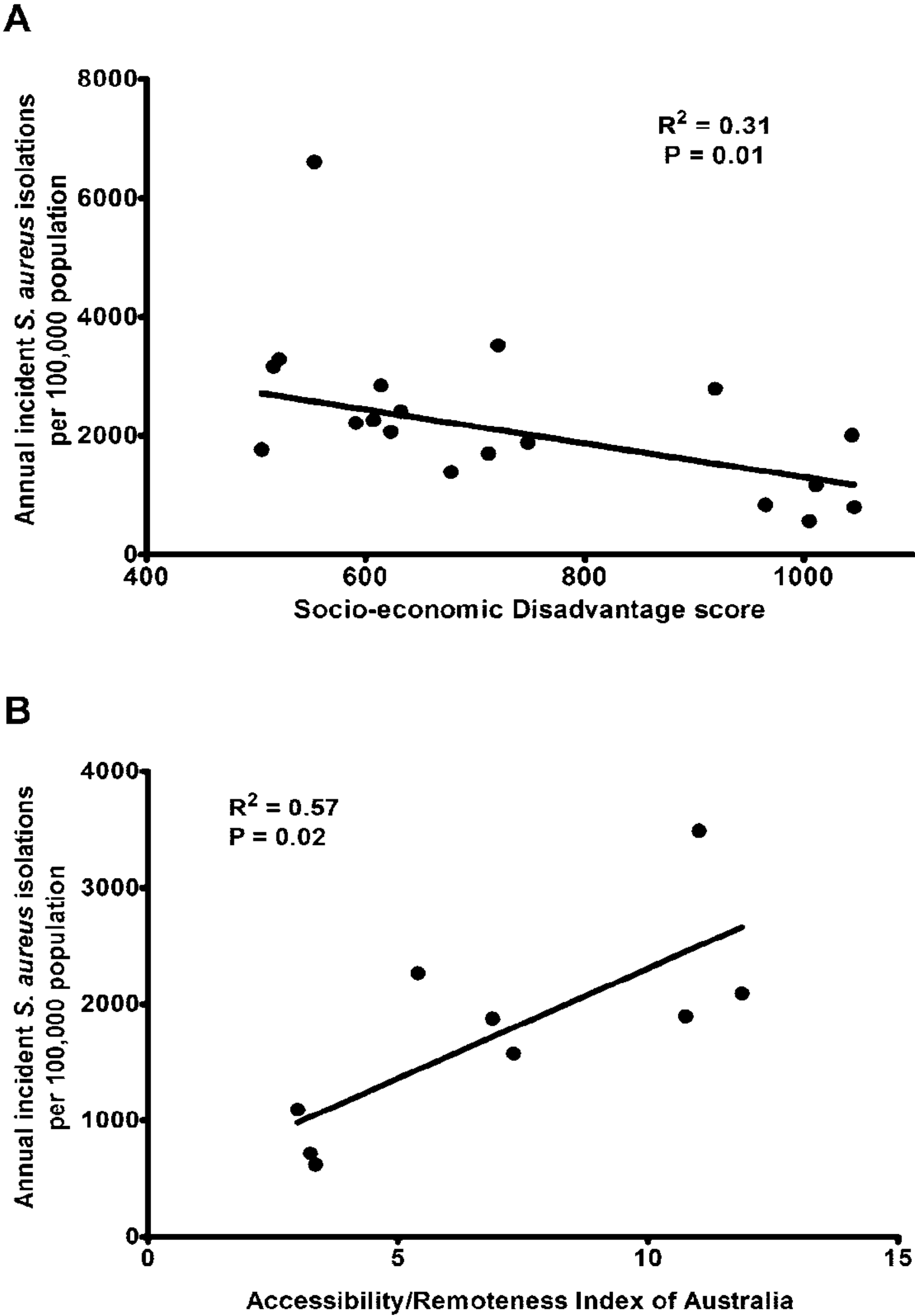

Figure 3-1. Incident isolation of *Staphylococcus aureus* in the Top End.

The annual incidence (18 April 2006 through 17 April 2007) is plotted against the regional index of socioeconomic disadvantage score (A) where a lower score indicates greater disadvantage and the accessibility / remoteness index (B) where a higher score indicates greater remoteness. Regression was performed using robust methods (A) and standard linear regression (B).

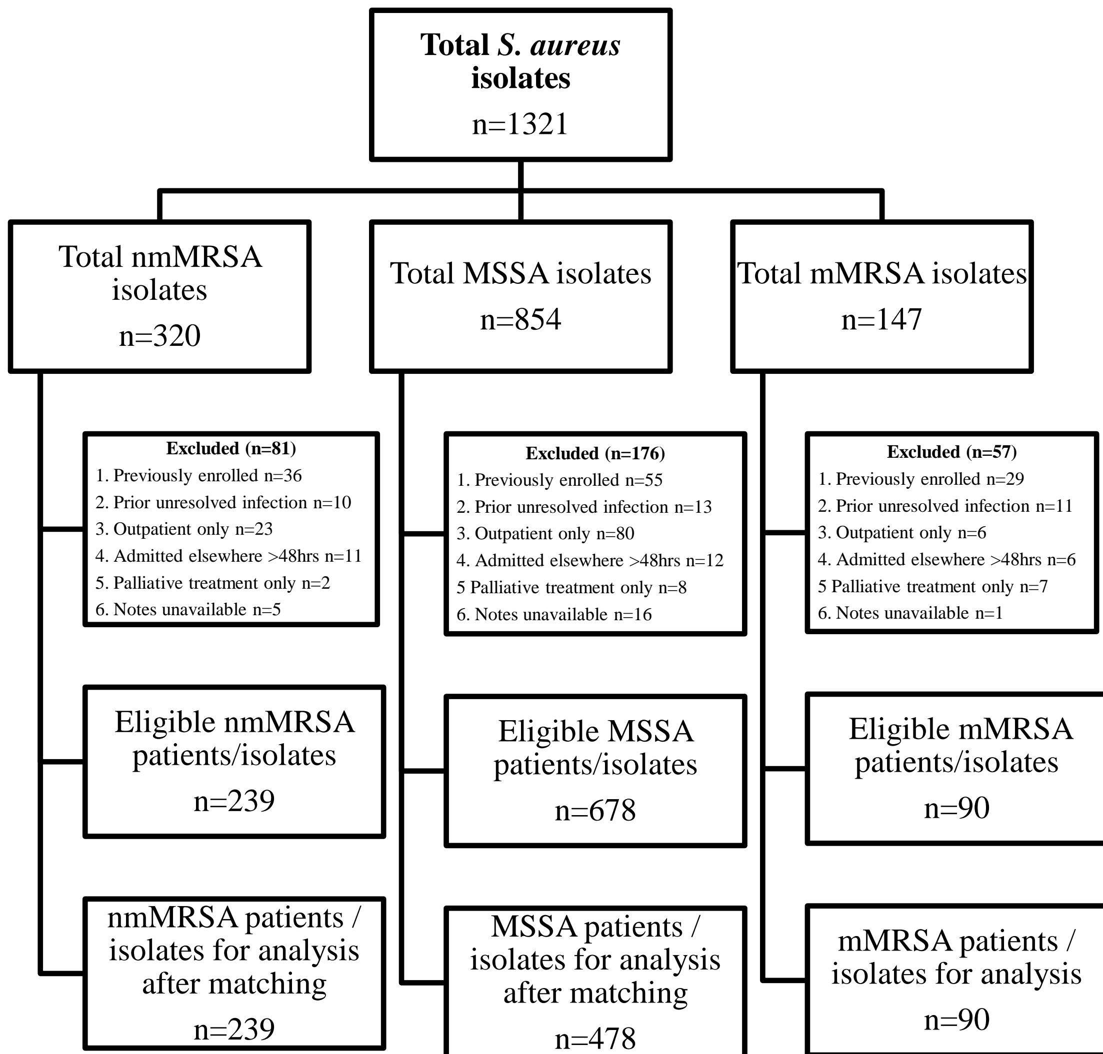

Figure 3-2. Flow chart detailing *Staphylococcus aureus* isolates collection.

Details of the number of *Staphylococcus aureus* isolates collected, the exclusion criteria applied, the number of eligible isolates, and the number of matched isolates used for the case-control study, 18 April 2006 – 17 April 2007. nmMRSA, non-multidrug-resistant, methicillin-resistant *S. aureus*; mMRSA, multidrug-resistant MRSA; MSSA, methicillin-susceptible *S. aureus*.

3.4.1 Epidemiological and clinical characteristics

Patients who had nmMRSA isolated and patients who had MSSA isolated were of a similar age (P = .10) but younger than those who had mMRSA isolated (P < .01) (**Table 3-1**). Patients who had nmMRSA isolated were less likely to be male than those who had MSSA or mMRSA isolated. A similar proportion of patients who had nmMRSA isolated and patients who had MSSA isolated were of Aboriginal ethnicity, but patients who had nmMRSA isolated were more likely to reside remotely. Otherwise, similarities between patients who had nmMRSA isolated and those who had MSSA isolated were evident with regard to the presence of comorbidities and laboratory indices. The patients who had mMRSA isolated were clearly a distinct group, being more likely to be hospitalised with comorbidities as well as having significantly higher serum creatinine levels and lower albumin and hematocrit levels (data not shown).

3.4.2 Severity and primary site of infections

The distribution of clinical syndromes and severity of illness is shown in **Table 3-1**. The vast majority of infections were SSTIs. Overall 629 (87%) of 720 infections (87 colonised patients were excluded) were SSTIs, accounting for ~90% of nmMRSA and MSSA infections (198 of 225 infections and 390 of 436 infections, respectively) and 41 (69%) of 59 mMRSA infections. Boils, abscesses, and nonsurgical wound infections accounted for ~60% of nmMRSA and MSSA SSTIs (115 of 198 infections and 242 of 390 infections, respectively), whereas surgical site infections made up 21 (51%) of 41 mMRSA SSTIs. When colonised individuals were excluded, sepsis, severe sepsis, and septic shock accounted for 52 (23%) of 225 nmMRSA infections, 108 (25%) of 436 MSSA infections, and 23 (39%) of 59 mMRSA infections. There was no statistically significant difference between nmMRSA and MSSA when comparing the severity of illness (P = .49), the distribution of overall clinical syndromes (P = .78), or the clinical syndromes in the SSTI group alone (P = .12).

3.4.3 Outcomes

The majority of patients were admitted to the hospital, and ~50% of patients who had nmMRSA or MSSA isolated required surgical intervention (120 of 239 patients and 251 of 478 patients, respectively). The median length of stay was short for patients who had nmMRSA isolated (four days) or MSSA isolated (three days) but prolonged

for patients who had mMRSA isolated (28 days) cases. The in-hospital mortality rate was higher for patients who had mMRSA isolated (**Table 3-1**) than for the other groups.

3.4.4 Antimicrobial susceptibility

Disk-approximation testing of erythromycin-resistant isolates revealed that inducible clindamycin resistance was present in 52 (22%) of 239 nmMRSA isolates and 77 (16%) of 478 MSSA isolates, respectively. All nmMRSA and MSSA isolates were susceptible to trimethoprim-sulfamethoxazole and rifampicin, and most were susceptible to tetracycline (233 nmMRSA isolates [97%] and 475 MSSA isolates [99%]) and fusidic acid (206 [86%] nmMRSA isolates and 456 [95%] MSSA isolates). Patients who had nmMRSA isolated were less likely than those who had MSSA or mMRSA isolated to receive an antibiotic that was active against the causative organism (i.e., active antibiotic). The active antibiotics most commonly prescribed as inpatient therapy for nmMRSA infection were vancomycin (prescribed for 43 [54%] of 79 patients) and trimethoprim-sulfamethoxazole (prescribed for 49 [62%] of 79 patients).

3.4.5 Comparison of Aboriginal to non-Aboriginal patients who had nmMRSA isolated

Aboriginal patients who had nmMRSA isolated were significantly younger, more likely to be from remote regions, and less likely to be male than non-Aboriginal patients who had this organism isolated (**Table 3-2**). Despite a younger age, diabetes mellitus and chronic renal disease were more common in Aboriginal patients and the length of stay was longer. Scabies was diagnosed 14 times more frequently in Aboriginal patients. Non–multidrug-resistant MRSA isolates from Aboriginal patients were less likely to be *pvl* positive.

Table 3-1. Characteristics of patients from whom nmMRSA, MSSA and mMRSA were isolated.

				Unadjusted OR (95% CI)	
Variable type, characteristic	nmMRSA (n=239)	MSSA (n=478)	mMRSA (n=90)	nmMRSA vs MSSA	nmMRSA vs mMRSA
Demographic characteristics					
Age (median)	39	34	59		
Female sex	107 (45)	166 (35)	28 (31)	1.5 (1.1–2.1)	1.8 (1.0–3.1)
Aboriginal	135 (56)	240 (50)	30 (33)	1.3 (0.9–1.8)	2.6 (1.5–4.5)
Remote residence	81 (34)	366 (23)	14 (16)	1.7 (1.2–2.4)	2.8 (1.4–5.7)
HCA risk present	114 (48)	201 (42)	89 (99)	1.3 (0.9–1.7)	0.0 (0.0–0.1)
Nosocomial acquisition	39 (16)	58 (12)	56 (62)	1.4 (0.9-2.2)	0.1 (0.1–0.2)
Comorbidities					
≥1 comorbidity	76 (32)	136 (28)	69 (77)	1.2 (0.8–1.7)	0.1 (0.1–0.3)
Diabetes	47 (20)	73 (15)	32 (36)	1.4 (0.9–2.1)	0.4 (0.3–0.8)
Chronic respiratory disease	17 (7)	27 (6)	22 (24)	1.3 (0.6–2.5)	0.2 (0.1–0.5)
Chronic renal disease	15 (6)	23 (5)	19 (21)	1.3 (0.6–2.7)	0.3 (0.1–0.6)
Coronary artery disease	27 (11)	29 (6)	26 (29)	2.0 (1.1–3.5)	0.3 (0.2–0.6)
Chronic liver disease	5 (2)	17 (4)	12 (13)	0.6 (0.2–1.7)	0.1 (0.0–0.4)
Injection drug user	2 (1)	3 (1)	1 (1)	1.3 (0.1–11)	0.8 (0.0–45)
Scabies	17 (7)	27 (6)	5 (6)	1.3 (0.6–2.5)	1.3 (0.4–4.7)
Severity of infection[a]					
Colonisation	14 (6)	42 (9)	31 (34)	0.6 (0.3–1.2)	0.1 (0.1–0.2)
Localised infection	173 (72)	328 (69)	36 (40)	1.2 (0.8–1.7)	3.9 (2.3–6.8)
Sepsis	47 (20)	101 (21)	20 (20)	0.9 (0.6–1.4)	0.9 (0.5–1.6)
Severe sepsis	1 (1)	3 (1)	2 (2)	0.7 (0.0–8.3)	0.2 (0.0–3.6)
Septic shock	4 (2)	4 (1)	1 (1)	2.0 (0.4–11)	1.5 (0.1–75)
Primary site of infection [b]					
Skin and soft tissue infection [c]	198 (88)	390 (89)	41 (69)	0.9 (0.5–1.5)	3.1 (1.4–6.5)
Cellulitis	32 (14)	58 (13)	5 (8)	1.1 (0.7–1.8)	1.8 (0.6–6.2)
Abscess and/or boil	90 (40)	163 (37)	2 (3)	1.1 (0.8–1.6)	19 (4.8–164)
Surgical site	24 (11)	29 (7)	21 (36)	1.7 (0.9–3.1)	0.2 (0.1–0.5)
Diabetic foot wound or ulcer	10 (4)	23 (5)	6 (10)	0.8 (0.3–1.9)	0.4 (0.1–1.4)

Pyoderma	12 (5)	33 (8)	1 (2)	0.7 (0.3–1.4)	3.3(0.5–142)
Non-surgical wound	25 (11)	79 (18)	6 (10)	0.6 (0.4–0.9)	1.1 (0.4–3.5)
Other	5 (2)	5 (1)	0 (0)	2.0 (0.5–7.4)	1.6 (0.2–74)
Bone and/or joint	6 (3)	9 (2)	1 (2)	1.3 (0.4–4.1)	1.6 (0.2–74)
Bloodstream [d]	5 (2)	14 (3)	3 (5)	0.7 (0.2–2.0)	0.4 (0.1–2.8)
Respiratory tract	9 (4)	11 (2)	7 (12)	1.6 (0.6–4.3)	0.3 (0.1–1.0)
Urinary tract	2 (1)	2 (1)	4 (7)	1.9 (0.1–27)	0.1 (0.0–0.9)
Other	5 (2)	10 (2)	3 (5)	1.0 (0.3–3.2)	0.4 (0.1–2.8)
Treatment received					
Surgery	120 (50)	251 (53)	25 (28)	1.1 (0.8–1.5)	2.6 (1.5–4.6)
Active antibiotic therapy					
As inpatient	79 (33)	388 (81)	46 (51)	0.1 (0.1–0.2)	0.5 (0.3–0.8)
As inpatient or at discharge	100 (42)	442 (92)	46 (51)	0.1 (0.0–0.1)	0.7 (0.4–1.1)
Outcomes					
Hospitalisation	197 (82)	388 (81)	83 (92)	1.1 (0.7–1.7)	0.4 (0.1–0.9)
ICU admission	12 (5)	25 (5)	12 (13)	1.0 (0.4–2.0)	0.3 (0.1–0.9)
Length of stay, median, days	4	3	28		
In-hospital mortality	6 (3)	9 (2)	8 (9)	1.3 (0.4–4.3)	0.3 (0.1–0.9)

Note. Data are no. (%) of subjects unless otherwise indicated. CI, confidence interval; HCA, health care-associated; ICU, intensive care unit; OR, odds ratio.

[a] P=0.49 by Fisher's exact analysis of severity of infection using 5x2 table of nmMRSA vs MSSA infection; P<0.01, for comparison of nmMRSA vs mMRSA infection.

[b] Patients with colonisation alone were excluded; for this part of the analysis, there were 225 nmMRSA infections, 436 MSSA infections, and 59 mMRSA infections. P=0.78 by Fisher's exact analysis for all infections using 6x2 table of nmMRSA vs MSSA infection; P<0.01, using 7x2 table of nmMRSA vs mMRSA infection.

[c] P=0.12 by Fisher's exact analysis for skin and soft-tissue infection using 7x2 table of nmMRSA vs MSSA infection; P<0.01, for comparison of nmMRSA and mMRSA infection.

[d] There were 49 cases of bacteraemia, the source of infection was unidentified for 22 cases. For the other 27 cases of bacteraemia, the primary source of infection was considered to be skin and soft tissue for 11, bone and/or joint for 7, respiratory for 3, urinary tract for 2, and other for 4.

Table 3-2. Characteristics of patients with nmMRSA, stratified by ethnicity.

Variable type, characteristic	Ethnicity: Aboriginal (n=135)	Ethnicity: Non-Aboriginal (n=104)	Unadjusted OR (95% CI)	*P*
Demographics				
Age (median)				
Median, years	30	43		<0.01
<18 years	46 (34)	8 (8)	6.2 (2.7–16)	<0.01
Remote residence	77 (57)	4 (4)	33 (11–129)	<0.01
Female sex	75 (56)	32 (31)	2.8 (1.6–5.0)	<0.01
HCA risk present	72 (53)	42 (40)	1.7 (1.0–2.9)	0.05
Nosocomial acquisition	26 (19)	13 (13)	1.7 (0.8–3.8)	0.16
Comorbidities				
≥1 Comorbidity	48 (36)	28 (27)	1.5 (0.8–2.7)	0.16
Diabetes	36 (27)	11 (11)	3.1 (1.4–7.1)	<0.01
Chronic renal disease	14 (10)	1 (1)	11 (1.7–508)	<0.01
Scabies	16 (12)	1 (1)	14 (2.1–586)	<0.01
Type of infection				
Abscess	41 (30)	49 (47)	0.5 (0.3–0.9)	<0.01
Bacteraemia	5 (4)	0 (0)	NA	0.07
Systemic infection	31 (25)	21 (21)	1.2 (0.6–2.5)	0.50
Outcomes				
Hospitalisation	115 (85)	82 (79)	1.5 (0.7–3.2)	0.20
Length of stay, median, days	5	2		0.02
Surgery	62 (46)	58 (56)	0.7 (0.4–1.2)	0.13
In-hospital mortality	4 (3)	2 (2)	1.6 (0.2–17)	0.61
Resistance and virulence features of isolate				
PVL	60 (48)	62 (66)	0.5 (0.3–0.9)	0.01
Erythromycin resistance	38 (28)	21 (20)	1.5 (0.8–3.0)	0.16
Clindamycin resistance	33 (24)	19 (18)	1.4 (0.7–2.9)	0.25

Note. Data are no. (%) of patients, unless otherwise indicated. CI, confidence interval; HCA, healthcare-associated; OR, odds ratio; PVL, Panton-Valentine leukocidin.

3.4.6 Multivariate analysis

Because the characteristics of patients who had mMRSA isolated were clearly different from those of patients who had nmMRSA or MSSA isolated, we concentrated on comparing predictors for the isolation of nmMRSA to predictors for the isolation of MSSA. After multivariate conditional logistic regression, older age (adjusted OR [aOR] for each additional year, 1.01 [95% CI, 1.00–1.02]), female sex (aOR, 1.5 [95% CI, 1.1–2.0]), and remote residence (aOR, 1.8 [95% CI, 1.2–2.5]) predicted isolation of nmMRSA. Among patients who had nmMRSA isolated, Aboriginal patients were younger (aOR for each additional year, 0.94 [95% CI, 0.92–0.96]), more likely to be female (aOR, 3.8 [95% CI, 1.7–8.5]), more likely to reside remotely (aOR, 29 [95% CI 8.9–94]), more likely to have chronic renal impairment (aOR, 42 [95% CI, 4.5–403]), and less likely to be infected with a *pvl*-positive strain (aOR, 0.2 [95% CI, 0.1–0.6]) than non-Aboriginal patients.

After multivariate logistic regression comparing sepsis (sepsis, severe sepsis, or septic shock) with localised infection only, the presence of comorbidities (aOR, 1.8 [95% CI, 1.1–2.8]) and remote residence (aOR, 1.6 [95% CI, 1.0–2.6]) predicted sepsis. SSTIs were associated with localised infection (aOR, 0.2 [95% CI, 0.1–0.3]). The isolate's methicillin-resistance phenotype was not associated with severity of disease (**Table 3-3**). For nmMRSA, the presence of *pvl* was the only factor significantly associated with sepsis (aOR, 2.2 [95% CI, 1.1–4.6]).

Table 3-3. Multivariate analysis of variables associated with localised infection versus sepsis

Variable	Unadjusted		Adjusted	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Resistance phenotype of isolate				
nmMRSA	0.8 (0.6–1.2)	0.45	1.0	
MSSA	0.9 (0.7–1.3)	0.62	1.2 (0.8–1.8)	0.34
mMRSA	2.0 (1.2–3.5)	0.01	1.7 (0.8–3.4)	0.14
Age				
Reference	1.0		1.0	
Each additional year	1.0(1.00–1.01)	0.41	1.0(0.98–1.01)	0.34
Sex				
Male	1.0		1.0	
Female	1.2 (0.9–1.7)	0.24	1.2 (0.8–1.7)	0.38
Ethnicity				
Non-Aboriginal	1.0		1.0	
Aboriginal	1.1 (0.8–1.5)	0.61	0.8 (0.5–1.2)	0.28
Residence location				
Urban	1.0		1.0	
Remote	1.5 (1.0–2.1)	**0.04**	1.6 (1.0–2.6)	**0.05**
Comorbidity				
None	1.0		1.0	
≥1	1.8 (1.3–2.6)	**<0.01**	1.8 (1.1–2.8)	**0.02**
HCA risk present				
No	1.0		1.0	
Yes	1.3 (0.9–1.8)	0.12	0.9 (0.6–1.3)	0.50
Type of infection				
Non-SSTI	1.0		1.0	
SSTI	0.2 (0.1–0.3)	**<0.01**	0.2 (0.1–0.3)	**<0.01**

Note. For this analysis, "sepsis" was defined as sepsis, severe sepsis, or septic shock. Bold type indicates significant P values (P < .05). CI, confidence interval; HCA, healthcare-associated; nmMRSA, non–multidrug-resistant, methicillin-resistant *Staphylococcus aureus*; mMRSA, multidrug-resistant MRSA; MSSA, methicillin-susceptible *S. aureus*; OR, odds ratio; SSTI, skin and soft-tissue infection.

3.5 Discussion

The present study has revealed a heavy burden of staphylococcal disease in Aboriginal populations and defined the epidemiological and clinical relationship between nmMRSA and MSSA in northern Australia. The findings that patients who had nmMRSA isolated were significantly less likely to be male than those who had MSSA isolated and that remote residence was a strong predictor of nmMRSA isolation provide important insights into the emergence of nmMRSA. Evidence that clinical disease presentation and outcomes were independent of resistance phenotype suggests that, in our setting, virulence is not convergent with resistance phenotype.

The incidences of both MSSA infection and nmMRSA infection are much higher in Aboriginal populations. These incidence rates strongly correlate with measures of remoteness and socioeconomic disadvantage across regions of the Top End. The annual incidence of *S. aureus* bacteraemia – 65 cases per 100,000 population – is almost double the rate estimated for the whole of Australia, which is 35 cases per 100,000 population[23] and more than double the rates recently reported from Canada[24] and Sweden[25]. When patients were stratified by ethnicity, the non-Aboriginal population of the Top End had an annual incidence of bacteraemia similar to that of the rest of Australia but the incidence among the Aboriginal population is six times higher, at 172 cases per 100,000 population.

Clinical and epidemiological risk factors do not appear to reliably distinguish between infection due to CA-MRSA strains and infection due to MSSA strains[26, 27], and outcomes following hospital discharge are comparable[28]. In Taiwan, there was no difference in the mortality rate for bacteraemia due to CA-MRSA strains and that of bacteraemia resulting from MSSA[29]. Our case-control study also demonstrated that nmMRSA and MSSA cause similar infections in a comparable group of patients. Similarities existed with respect to disease spectrum and outcomes, as well as most demographic and epidemiological features. This was in marked contrast to the results for mMRSA, which was isolated from a clearly distinct group of patients and resulted in different infections and outcomes.

Multivariate analysis revealed two striking differences between patients who had nmMRSA isolated and those who had MSSA isolated. Our finding that those who had nmMRSA isolated were more likely to be female, or rather, less likely to be

male, is surprising and intriguing. Previous studies with a comparable design[14, 26, 27, 30, 31] have consistently shown a predominance of males among patients with staphylococcal infections, which, to our knowledge, remains unexplained. After stratifying patients who had nmMRSA isolated according to ethnicity, we found that females comprised more than half of the Aboriginal patients but less than a third of non-Aboriginal patients; this latter figure mirrored that of patients who had MSSA isolated across the study population, with 166 (35%) of 478 being female. Of note, Aboriginal patients were significantly younger, and children (age, <18 years) accounted for 46 (34%) of 135 Aboriginal patients, compared with 8 (8%) of 104 non-Aboriginal patients. Referral bias is unlikely to explain the observed differences in the sex of people requiring treatment in a major tertiary hospital. In our community setting, men with mild infections are less likely than women and children to present to community health clinics, but this is unlikely to be the case for severe infections. It has been postulated that infection with nmMRSA may be more dependent on skin-to-skin or fomite-to-skin transmission, rather than resulting from endogenous colonisation[32]. It has also been demonstrated that infants and mothers often share strains of *S. aureus*[33]. In Aboriginal communities, domestic crowding – as many as 7.5 people per bedroom – facilitates the transmission of infectious agents within households[34], and women provide most of the child care. Thus women, through their closer household contact with children, may be more likely to develop nmMRSA infection than men.

Although both MSSA and nmMRSA are more common among Aboriginal people than non-Aboriginal people in the Top End, we have found that remote residence, rather than ethnicity itself, is most strongly associated with infection due to nmMRSA rather than MSSA. This contrasts with the results of studies from the United States, in which ethnicity was associated with nmMRSA infection[30, 31, 35, 36]. The remote areas of the Top End include Aboriginal communities characterised by socioeconomic disadvantage in relation to health, housing, education, and employment[18]. Because women and children were overrepresented among Aboriginal patients who had nmMRSA isolated, our findings support the postulate that factors in remote Aboriginal communities, such as domestic crowding and poor hygiene as well as high rates of scabies, skin sores, and antibiotic use, may contribute to the transmission and emergence of nmMRSA[13]. In addition, children in remote

communities repeatedly present to community clinics with respiratory tract and skin infections[37] and are typically treated with penicillin and cephalosporin antibiotics.

The methicillin-resistance phenotype did not predict whether an infection was localised or led to sepsis, and it was not predictive of the clinical spectrum of disease. Unlike the situation in the United States, where virulence and resistance have converged in the predominant USA300 clone[38], there continues to be a diversity of nmMRSA strains circulating in Australia[12]. Our current study and another from the Australian state of Queensland have demonstrated that virulence does not correlate with resistance phenotype in these settings[14]. We have previously established that nmMRSA and MSSA strains from the same clonal complexes circulate in remote Top End communities[10], suggesting that staphylococcal chromosome cassette (SCC) *mec*IV has independently been acquired by MSSA on multiple occasions in our environment. It has been demonstrated that the deletion of SCC*mec*IV in an isogenic mutant of a clinical USA300 isolate had no effect on competitive fitness or virulence[38] and that the presence of different SCC*mec* elements does not have a strong impact on bacterial fitness[39]. It is therefore unsurprising that we found the clinical spectrum and outcomes of disease due to nmMRSA and MSSA to be similar.

Interestingly, the presence of *pvl* genes in nmMRSA was associated with double the odds of sepsis. There continues to be debate over the importance of Panton-Valentine leukocidin (PVL), and mouse infection models have not consistently shown a major role in pathogenesis[40]. A recent clinical study found no significant difference between PVL-positive and PVL-negative USA400 isolates with respect to their propensity to cause infection or colonisation[41]. However, there were only ten PVL-positive isolates in that study, and there was a trend linking the presence of PVL with increasing severity of disease. Our findings support previous studies that demonstrated that patients with pneumonia or bone and joint infections caused by PVL-positive *S. aureus* were systemically more unwell that those with infections caused by PVL-negative *S. aureus*[42, 43].

nmMRSA has been present in northern Australia and the RDH since at least the early 1990s[4, 44]. Over the past 10 to 15 years, the percentage of nmMRSA and mMRSA isolates among the overall population of *S. aureus* isolates has increased from 4%[4] to 291 (17%) of 1693 for nmMRSA and from 3%[4] to 137 (8%) of 1693 for mMRSA. However, nmMRSA has not outstripped mMRSA as a nosocomial pathogen at the

RDH. In 1991–1995, nmMRSA constituted 40%[44] of nosocomial MRSA isolates, compared with 39 (41%) of 95 in this 2006–2007 case-control study. The finding that nmMRSA and MSSA infection had similar clinical profiles and outcomes calls into question current infection control strategies that target MRSA on the basis of methicillin-resistance phenotype alone. It is possible that the spread of a potentially more virulent, PVL-positive MSSA strain may have more significant consequences than the spread of a PVL-negative nmMRSA strain, and therefore, it should be targeted for more aggressive infection control interventions. Molecular diagnostic methods may facilitate a different approach. One Australian state, Western Australia, has recently attempted to implement a "search and destroy" approach to PVL-positive MRSA strains in the community[45]; this extends existing policies of public health agencies being notified of all MRSA isolations and all MRSA isolates being sent to a reference typing laboratory.

Our study has some limitations. We did not interview patients directly and were unable to assess the impact of factors such as domestic crowding, prior antibiotic use, and the mode of acquisition for SSTIs. Our incidence figures and case-control study pertain only to patients presenting to hospitals and do not reflect the overall community burden of staphylococcal infections. Nevertheless, these hospitals service an area of 510,000 km^2, and our study provides important insights into staphylococcal infections in this large tropical region. Additionally, our calculations are likely to result in an underestimation of the already high incidence of *S. aureus* infection. Among children in some remote communities, the prevalence of pyoderma is 20%, from which *S. aureus* can be cultured in 57% of cases[10]. Hence, in these communities the prevalence of staphylococcal infection among children is >11,000 cases per 100,000 population, and the annual incidence of all staphylococcal infections is even higher. We considered nmMRSA to represent CA-MRSA strains. However, ST22-MRSA-IV, an epidemic HA-MRSA strain, is typically resistant only to ciprofloxacin +/- erythromycin, and may thus be misclassified as CA-MRSA. Subsequent genotyping found that ST22 only represented 2.5% of the nmMRSA isolates at the RDH. Finally, although co-infections with other organisms are likely to be present we did not directly account for this in the analysis.

Staphylococcal disease, including that due to nmMRSA, imposes a disproportionate burden on remote Aboriginal communities in northern Australia. Our study

demonstrates that female sex and residence in a remote community is associated with nmMRSA infection, and remote residence is likely a marker of social disadvantage that encompasses overcrowding, poor housing conditions, and lack of access to the physical infrastructure needed to maintain skin hygiene. There are well-founded fears that nmMRSA will become a nosocomial pathogen, but primary prevention also requires consideration of public health issues, such as housing and community hygiene. In our situation, nmMRSA is still principally a community pathogen – arising and spreading from within the community setting.

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Chapter 4. Clinical correlates of Panton-Valentine leukocidin, PVL isoforms and clonal complex in the *Staphylococcus aureus* population of northern Australia

This chapter links the clinical and molecular epidemiology of *S. aureus* in northern Australia.

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Tong SY, Lilliebridge RA, Bishop EJ, Cheng AC, Holt DC, McDonald MI, Giffard PM, Currie BJ, Boutlis CS. Clinical correlates of Panton-Valentine leukocidin, PVL isoforms and clonal complex in the *Staphylococcus aureus* population of northern Australia. J Infect Dis 2010; 202: 760–769.

4.1 Abstract

Background. Regional differences in the prevalence of Panton-Valentine leukocidin (PVL) and PVL isoform harbouring strains and the local population structure of *Staphylococcus aureus* may influence the clinical spectrum of *S. aureus* infections.

Methods. Using a prospective collection of *S. aureus* isolates from northern Australia, we determined differences between infections caused by: (1) PVL+ and PVL- isolates; (2) PVL histidine (H) and arginine (R) isoform harbouring isolates; and (3) different lineages, including the genetically divergent clonal complex (CC) 75 and the PVL+ CC93.

Results. PVL+ isolates comprised 54% (128/239) of community-associated methicillin-resistant and 40% (95/239) of methicillin-susceptible *S. aureus* (MSSA) isolates. There were 113 H and 110 R isoform harbouring isolates. PVL was associated with truly community-acquired disease, younger age and presentations with sepsis. We found no differences in infections due to H compared to R isoform

harbouring isolates. CC93 was the most prevalent lineage. The genetically divergent CC75 caused similar clinical disease to other *S. aureus* clones.

Conclusions. PVL+ and PVL- infections are clearly distinct. MSSA contributes a large, but under-recognised, burden of PVL+ disease. Compared to elsewhere in the world, there is a relative abundance of the clade that contains CC93 and CC121 in both northern Australia and Asia.

4.2 Introduction

The emergence of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections beginning in the 1990s has been a global phenomenon. Among the earlier reports were those from the United States[1] and Indigenous Australian communities[2]. Recently, the CA-MRSA epidemic in the United States has been dominated by one clone, USA300, which appears to have undergone a rapid radiation and replaced other CA-MRSA clones[3]. In Australia, where USA300 is rare, there continues to be a diversity of circulating CA-MRSA clones[4]. In northern Australia, two lineages are of particular interest. Clonal complex (CC) 75 is phylogenetically diverged, but phenotypically similar to other *S. aureus*[5, 6]. The second lineage, CC93, is apparently unique to Australia. Initially described in 2001[7], CC93 expresses the pore-forming leukotoxin Panton-Valentine leukocidin (PVL) and has spread throughout the nation, becoming the predominant CA-MRSA clone in Australia[4, 8].

An association between the presence of PVL and severe staphylococcal infections such as necrotising pneumonia has been previously noted[9, 10]. The vast majority of CA-MRSA isolates in the US are PVL+ USA300[3], whereas only 50% of CA-MRSA isolates in Australia are PVL+[4]. Recent studies have demonstrated sequence variations within the PVL gene *lukSF-PV*[11-13]. Molecular modelling suggests that the amino acid substitution of His_{176} to Arg_{176} (H and R isoforms) may affect PVL function[11]; however, clinical studies are lacking. USA300 isolates carry the R isoform but most other clones isolated outside of the United States carry the H isoform[11, 12]. Fifty percent of our PVL+ CA-MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates from northern Australia carry the R isoform. The majority of these isolates are CC93.

Thus, the diverse molecular epidemiology of *S. aureus* in tropical northern Australia provides a unique opportunity to investigate whether clinical and epidemiological differences exist between infections caused by: (1) PVL+ and PVL- isolates; (2) PVL H and R isoforms harbouring isolates; and (3) different lineages, including CC75 and CC93. In addition, we placed the population structure of *S. aureus* in northern Australia within the context of recent findings regarding *S. aureus* in East Asia.

4.3 Methods

4.3.1 Study setting and design

The 330 bed Royal Darwin Hospital (RDH) is located in the tropical Top End of the Northern Territory. It is the tertiary referral center for the capital city Darwin, along with two regional hospitals and over 70 remote communities; all together encompassing a population of 190,000 and an area of 510,000 square kilometers. Both paediatric and adult patients are cared for at the RDH and are included in this study. Indigenous Australians comprise 27% of the total population, but account for over 50% of the RDH inpatient and emergency department population. Ethics approval for the study was granted by the Human Research Ethics Committee of the Northern Territory Department of Health and Families and the Menzies School of Health Research.

We initially conducted a case-control study that compared infections caused by non-multi-resistant methicillin-resistant *S. aureus* (nmMRSA) to those caused by MSSA at the RDH over a 12 month period from April 2006[14]. For this current study, we matched eligible nmMRSA patients to the next eligible MSSA patient using sequential laboratory specimen receipt numbers. We collected information concerning demographics, health-care associated risk factors, co-morbidities, clinical details of the infection, treatment details, and outcomes at discharge. Sepsis was defined in adults and children by the systemic inflammatory response syndrome criteria[15, 16].

4.3.2 Laboratory methods

The RDH laboratory identified *S. aureus* isolates by standard methods and conducted susceptibility testing using an automated Vitek 2 instrument (Biomerieux, V4.01) and Kirby-Bauer disc diffusion method in accordance with Clinical and Laboratory Standards Institute standards[17]. A disc approximation test ("D-test") was used to detect inducible clindamycin resistance. Real-time PCR verified the identity of nmMRSA isolates by confirming the presence of the *nuc*A and *mec*A genes[18]. We phenotypically defined nmMRSA as those resistant to <3 non-β-lactam antibiotic classes and considered these to represent CA-MRSA strains. Antibiotic phenotype

has previously been shown to accurately predict CA-MRSA genotypes[19] with validation in recent Australian studies[4, 20].

We genotyped isolates using a SNP genotyping system based on the multilocus sequence type (MLST) database as previously described[18, 21]. In short, a kinetic PCR method was used to interrogate 8 highly discriminatory SNPs, allowing isolates to be assigned into clonal complexes. To confirm isolates as CC93, we also performed a high-resolution melt (HRM) assay interrogating *aroE* position 252[22]. We determined the presence of the *lukF-PV* gene in all isolates[18, 21] and then used an HRM assay to determine the H and R isoform status of all PVL+ isolates. The primers for this HRM assay are: PVL527F (5'-CAACAAAACTATATCAGTGAAGTAGAAC-3'), PVL527R (5'-TCCCCATTGAACACTTTTTGAA-3'). The isoforms were clearly discriminated as the melting temperature of the amplified product for the H isoform was 1°C less than that for the R isoform[23] (and see Chapter 7).

4.3.3 Statistical analysis

Comparisons of infections due to PVL+ and PVL- isolates and between CCs were stratified by methicillin-resistance (as the study included all nmMRSA isolates but only a random subset of MSSA isolates). Statistical significance for crude analysis of dichotomous variables was determined using Chi-square or Fisher's exact test, and for continuous variables a Student's t-test or analysis of variance. Non-parametric data were compared using the Mann-Whitney U test. We conducted multivariate logistic regression analysis with stepwise backwards elimination of variables to identify associations with the outcomes of PVL status, PVL isoform status, sepsis and abscess formation. Variables with a univariate P-value of <0.20 were included in the initial models. The likelihood ratio test was used to assess the statistical significance of candidate risk factors. Two-sided P-values of <0.05 were considered significant. Statistical analysis was performed with Stata 10.1 (StataCorp, Texas, USA). Phylogenetic analysis was performed on concatenated MLST data using the neighbour-joining algorithm (Kimura two-parameter distance estimation) as implemented in MEGA v.4.1[24].

4.4 Results

During the study period, 1693 non-duplicate *S. aureus* isolates were recovered from patients at the RDH, with MSSA outnumbering nmMRSA at a ratio of 4.3:1. There were 1265 (75%) MSSA, 291 (17%) nmMRSA and 137 (8%) multi-resistant MRSA isolates. Patient characteristics, outcomes and corresponding molecular data from 239 eligible nmMRSA and 239 matched MSSA isolates were included in the case-control analysis. Twenty-two clonal complexes (CCs) were represented (**Figure 4-1**). Eight of the CCs were represented by both nmMRSA and MSSA isolates. Of the 478 isolates, 223 were PVL+, of which 113 and 110 harboured the H and R isoforms respectively. PVL+ isolates belonged to 8 CCs with 5 CCs harbouring the H isoform and 3 the R isoform. Seven of these 8 CCs had both PVL- and PVL+ isolates.

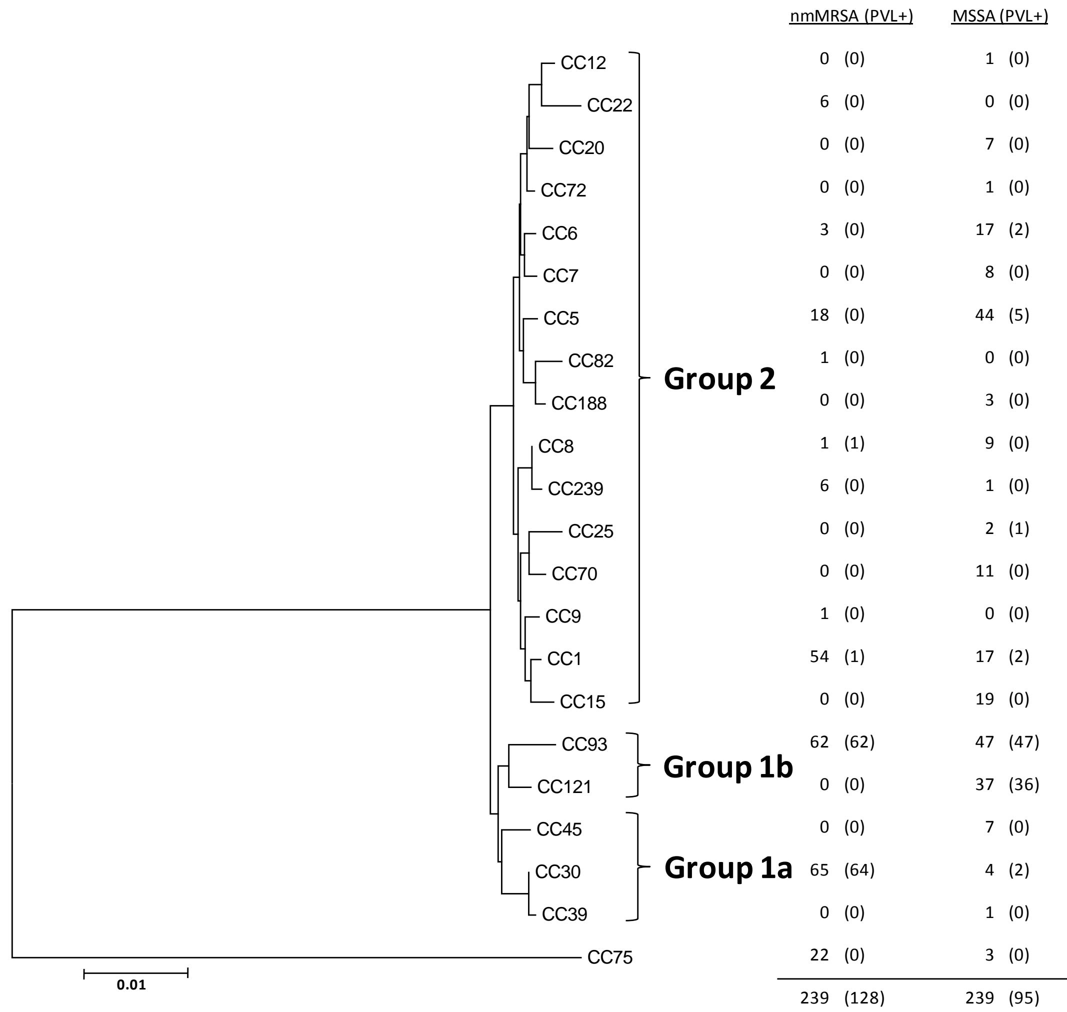


Figure 4-1. Phylogenetic relationship of *Staphylococcus aureus* clonal complexes

A neighbour-joining tree based on the concatenated sequences of the founder sequence types representing each of the clonal complexes (CC) in the collection. Three major clades are demonstrated (Groups 1a, 1b and 2) and CC75 is phylogenetically diverged from the rest of *Staphylococcus aureus*. The number of isolates in each clonal complex which are non-multidrug-resistant methicillin-resistant *S. aureus* (nmMRSA), methicillin-susceptible *S. aureus* (MSSA) and which harbour Panton Valentine leukocidin (PVL) are provided.

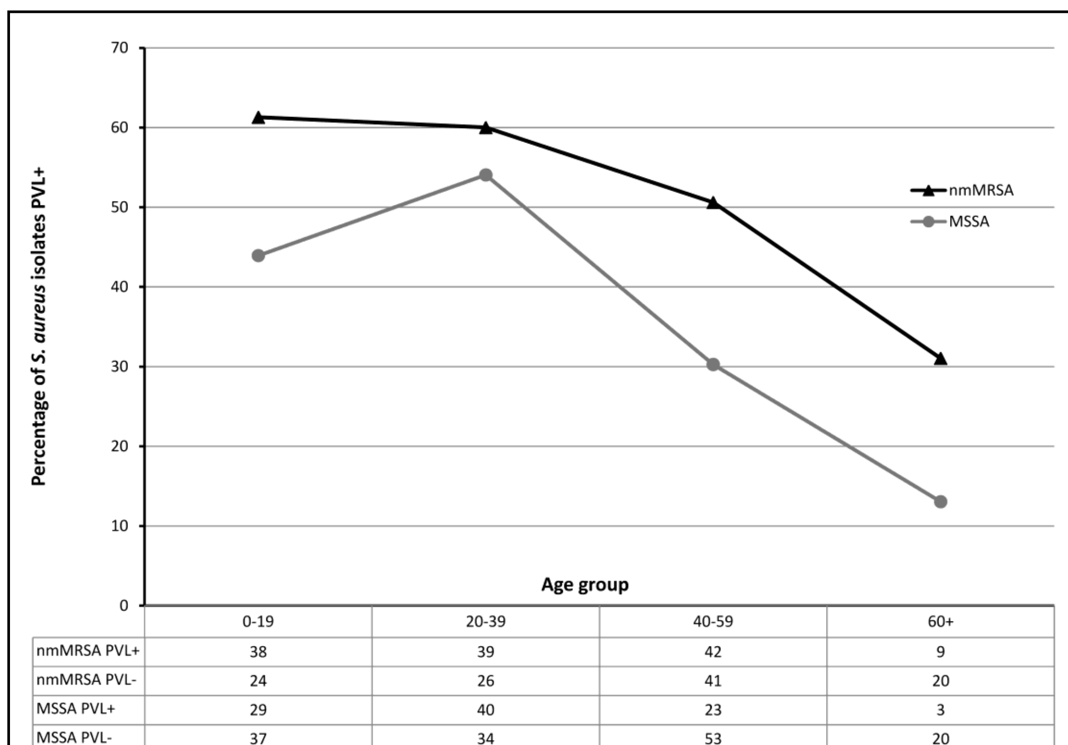
4.4.1 Comparison between PVL+ and PVL- isolates

The large numbers of both PVL+ and PVL- isolates and their distribution across clonal complexes allowed comparisons between infections caused by PVL+ and PVL- isolates. Univariate analysis for both nmMRSA and MSSA subgroups revealed PVL+ isolates to affect younger patients with fewer comorbidities and fewer health-care associated risk factors. Overall, 73% (163/223) of PVL+ isolates were community-acquired compared to only 41% (104/255) of PVL- isolates (P<0.001). In contrast, we have previously shown that only 1% of multidrug-resistant MRSA isolates were community-acquired in our setting[14]. The proportional incidence of isolates harbouring PVL stratified by age group showed steady decline with age for both nmMRSA and MSSA (**Figure 4-2**). Of patients <40 years, 55% (146/267) had infections caused by PVL+ isolates , compared to only 36% (77/211) of those >40 years (P<0.001). Skin and soft tissue infections and abscesses were seen more commonly in PVL+ infections but there was no difference in bacteraemia. Despite a greater proportion of patients requiring surgery, the length of stay was shorter for PVL+ infections. Multivariate logistical modelling revealed that increasing age (adjusted OR [aOR] 0.98 [95% CI, 0.97–0.99]), the presence of a health-care associated risk factor (aOR 0.32 [95% CI, 0.20–0.50]) and resistance to fusidic acid (aOR 0.10 [95% CI, 0.03–0.30]) reduced the odds of a PVL+ infection, while the presence of an abscess (aOR 9.44 [95% CI, 5.30–16.81]) and methicillin-resistance (aOR 2.50 [95% CI, 1.59–3.94]) increased the odds of a PVL+ infection.

To determine whether PVL was associated with sepsis, we built a further multivariate logistic regression model comparing an outcome of sepsis with localised infection. Significant predictors of sepsis were the presence of PVL (aOR 1.94 [95% CI, 1.21–3.11]), remote residence (aOR 1.73 [95% CI, 1.09–2.75]) and the presence of at least one comorbidity (aOR 1.66 [95% CI, 1.02–2.70]. A skin and soft tissue infection reduced the odds of sepsis (aOR 0.39 [95% CI, 0.21–0.73]). Notably, in contrast to the presence of PVL, methicillin-resistance and clonal complex were not associated with sepsis. The presence of PVL was associated with abscess formation on multivariate logistic regression modelling for both nmMRSA (OR 10.3 [95% CI 4.5–23.9]) and MSSA (OR 11.4 [95% CI 5.4–23.7]).

4.4.2 Comparison between PVL+ H and R isoform isolates

There was no difference across all characteristics when comparing infections due to isolates harbouring H and R isoforms on univariate analysis (**Table 4-2**). A multivariate logistic regression model also found no significant associations.



	0-19	20-39	40-59	60+
nmMRSA PVL+	38	39	42	9
nmMRSA PVL-	24	26	41	20
MSSA PVL+	29	40	23	3
MSSA PVL-	37	34	53	20

Figure 4-2. Percentage of *S. aureus* isolates harbouring Panton-Valentine leukocidin by age group.

nmMRSA, non-multidrug-resistant methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; PVL, Panton-Valentine leukocidin. Raw numbers are provided below the graph.

Table 4-1. Panton-Valentine leukocidin (PVL) + and PVL- infections stratified by methicillin-resistance

	nmMRSA (n=239)							MSSA (n=239)						
	PVL+ (n=128)		PVL- (n=111)		Unadjusted OR (95% CI)		P	PVL+ (n=95)		PVL- (n=144)		Unadjusted OR (95% CI)		P
Variable type, characteristic														
Demographic trait														
Age (median), years	34		42				0.007	30		40				0.004
Male sex	76	(59)	56	(50)	0.7	(0.4–1.2)	0.167	70	(74)	86	(60)	0.5	(0.3–0.9)	0.027
Indigenous	64	(50)	71	(64)	0.5	(0.3–0.9)	0.030	46	(48)	74	(51)	0.9	(0.5–1.5)	0.653
Remote residence	40	(31)	41	(37)	0.9	(0.5–1.3)	0.355	25	(26)	32	(22)	1.3	(0.7–2.8)	0.468
Clinical risk factors														
Health-care associated risk	40	(31)	74	(67)	0.2	(0.1–0.4)	<0.001	20	(21)	77	(53)	0.2	(0.1–0.4)	<0.001
Nosocomial acquistion	10	(8)	29	(26)	0.2	(0.1–0.5)	<0.001	3	(3)	25	(17)	0.2	(0.0–0.5)	0.003
≥1 Comorbidity	27	(21)	49	(44)	0.3	(0.2–0.6)	<0.001	12	(13)	52	(36)	0.3	(0.1–0.5)	<0.001
Diabetes	17	(13)	30	(27)	0.4	(0.2–0.8)	0.009	6	(6)	29	(20)	0.3	(0.1–0.7)	0.005
Chronic renal disease	6	(5)	9	(8)	0.6	(0.2–1.6)	0.282	0	(0)	13	(9)	NA	NA	0.003
Coronary artery disease	8	(6)	19	(17)	0.3	(0.1–0.8)	0.011	1	(1)	10	(7)	0.1	(0.0–1.1)	0.065
Injecting drug use	1	(1)	1	(1)	0.9	(0.1–14)	0.919	0	(0)	1	(1)	NA	NA	0.416
Type and severity of infection														
Skin and soft tissue	121	(95)	91	(82)	3.8	(1.5–9.4)	0.004	89	(94)	118	(82)	3.3	(1.3–8.3)	0.013
Abscess	64	(50)	8	(7)	13	(6–29)	<0.001	46	(48)	11	(8)	11	(5–24)	<0.001

Pneumonia	2	(2)	7	(6)	0.2	(0.5–1.2)	0.075	1	(1)	7	(5)	0.2	(0.0–1.7)	0.141
Bacteraemia	5	(4)	5	(5)	0.9	(0.2–3.1)	0.818	6	(6)	7	(5)	1.3	(0.4–4.1)	0.987
Sepsis	35	(27)	20	(18)	1.7	(0.9–3.2)	0.089	24	(25)	30	(21)	1.3	(0.7–2.4)	0.423
Outcome														
Length of stay (median), days	2.5		5				0.001	2		4				<0.001
Hospitalisation	105	(82)	92	(83)	0.9	(0.5–1.8)	0.863	71	(75)	115	(80)	0.7	(0.4–1.4)	0.352
Surgery received	87	(68)	33	(30)	5.0	(2.9–8.7)	<0.001	68	(72)	39	(27)	6.8	(3.8–12)	<0.001
In-hospital mortality	2	(2)	4	(4)	0.4	(0.1–2.4)	0.328	0	(0)	3	(2)	NA	NA	0.157
Antibiotic susceptibility														
Erythromycin resistant	9	(7)	50	(45)	0.1	(0.0–0.2)	<0.001	28	(29)	12	(8)	4.6	(2.2–9.6)	<0.001
Clindamycin resistant	9	(7)	43	(39)	0.1	(0.1–0.3)	<0.001	28	(29)	10	(7)	5.6	(2.6–12)	<0.001
Fusidic acid resistant	1	(1)	31	(28)	0.0	(0.0–0.1)	<0.001	3	(3)	10	(7)	0.4	(0.1–1.6)	0.218

Note: nmMRSA, non-multidrug-resistant methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*; PVL, Panton-Valentine leukocidin; HCA, health care-associated.

Table 4-2. Comparison of infections due to Panton-Valentine leukocidin H and R isoform harbouring isolates.

Variable type, characteristic	H isoform (n=113)		R isoform (n=110)		Unadjusted OR (95% CI)		P
Demographic trait							
Age, median, years	33		30				0.337
Male sex	71	(65)	75	(66)	0.9	(0.5–1.6)	0.774
Indigenous	56	(51)	54	(48)	0.9	(0.5–1.5)	0.641
Remote residence	37	(34)	28	(25)	0.6	(0.4–1.2)	0.147
Clinical risk factors							
HCA risk	30	(27)	30	(27)	1.0	(0.5–1.7)	0.903
Nosocomial acquisition	5	(5)	8	(7)	1.6	(0.5–5.1)	0.423
≥1 Comorbidity	17	(15)	22	(19)	1.3	(0.7–2.7)	0.431
Diabetes	11	(10)	12	(11)	1.1	(0.5–2.5)	0.879
Chronic renal disease	4	(4)	2	(2)	0.5	(0.1–2.7)	0.399
Coronary artery disease	6	(5)	3	(3)	0.5	(0.1–1.9)	0.298
Type and severity of infection							
Skin and soft tissue	103	(94)	107	(95)	1.2	(0.4–3.7)	0.737
Abscess	60	(55)	50	(44)	0.7	(0.4–1.1)	0.125
Sepsis	31	(28)	28	(25)	0.8	(0.5–1.5)	0.565
Outcome							
LOS, median, days	2		2				0.764
Hospitalisation	89	(81)	87	(77)	0.8	(0.4–1.5)	0.474
Surgery received	76	(69)	79	(70)	1.0	(0.6–1.8)	0.894
In-hosp mortality	0	(0)	2	(2)			0.161

Isolate characteristics

mecA +ve	64	(58)	64	(57)	1.0	(0.8–1.3)	0.816
Erythromycin resistant	21	(19)	16	(14)	0.7	(0.3–1.4)	0.324
Clindamycin resistant	21	(19)	16	(14)	0.7	(0.3–1.4)	0.325
Fusidic acid resistant	0	(0)	4	(4)			0.046

Note: nmMRSA, non-multidrug-resistant methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*; H, Panton-Valentine leukocidin (PVL) histidine isoform; R, PVL arginine isoform; HCA, healthcare-associated ; LOS, length of stay.

4.4.3 Population structure and clinical outcomes of different clones

Phylogenetic analysis (**Figure 4-1**) demonstrates the divergence of CC75 from all other clones and the clustering of PVL+ isolates within the Group 1 clade. Given that we have taken a random sample of MSSA isolates that is likely to be representative of the overall MSSA population and that MSSA outnumbers nmMRSA at a 4.3:1 ratio, we estimate that MSSA accounts for approximately 70% of PVL+ isolates. Almost all Group 1b clade (CC93 and CC121) isolates were PVL+ and represented 87% (83/95) of all PVL+ MSSA isolates.

We compared the clinical features and isolate characteristics of clonal complexes that were represented by more than 20 isolates when stratified by methicillin-resistance (**Table 4-3**). Together these lineages comprised 69% (331/478) of all isolates. Across group comparisons revealed significant differences for patients with regards to age, sex, ethnicity, remote residence, presence of comorbidities, health-care associated risk factors, nosocomial acquisition, presentation with abscess, length of stay and receipt of surgery. There were also significant differences in antibiotic resistance profile and presence of PVL. There was no difference in the outcomes of bactaeremia and sepsis.

The principally PVL+ clones, CC30, 93 and 121, affected patients with fewer comorbidities and health-care associated risk factors. Only 7% (14/211) of these infections were acquired in hospital, indicating a lack of penetration of these community clones into the nosocomial environment. In contrast, CC1, 5 and 75 affected patients with more comorbidities and health-care associated risk factors and had higher rates of nosocomial acquisition.

CC93 was numerically the predominant clone comprising 26% (62/239) of nmMRSA and 20% (47/239) of MSSA isolates. All harboured the PVL R isoform. There were no significant differences between CC93-nmMRSA and CC93-MSSA. CC75 was principally isolated from Indigenous patients (82% [18/22]; P=0.0062 when compared to non-CC75 isolates) and appeared less likely to cause presentations with sepsis. Erythromycin and inducible clindamycin resistance was present in over 50% of CC1 and CC121 isolates.

Table 4-3. Comparison of characteristics of infections due to different clonal complexes

	Clonal complex																	
	nmMRSA										MSSA							
Variable type, characteristic	CC1 (n=54)		CC30 (n=65)		CC75 (n=22)		CC93 (n=62)		Other (n=36)		CC93 (n=47)		CC5 (n=44)		CC121 (n=37)		Other (n=111)	
Demographic trait																		
Age (median), years[a]	40		33		39		35		48		31		36		30		40	
Male sex[b]	27	(50)	40	(62)	14	(64)	36	(58)	15	(42)	36	(77)	35	(80)	25	(68)	60	(54)
Indigenous[c]	39	(72)	33	(51)	18	(82)	31	(50)	14	(39)	23	(49)	20	(45)	22	(59)	55	(50)
Remote residence[d]	26	(48)	22	(34)	11	(50)	18	(29)	4	(11)	10	(21)	9	(20)	15	(41)	23	(21)
Clinical risk factors																		
≥1 Comorbidity[e]	21	(39)	13	(20)	6	(27)	15	(24)	21	(58)	6	(13)	16	(36)	5	(14)	37	(33)
HCA risk[f]	36	(67)	21	(32)	12	(55)	19	(31)	26	(72)	10	(21)	23	(52)	10	(27)	54	(49)
Nosocomial acquisition[g]	15	(28)	6	(9)	2	(9)	5	(8)	11	(31)	3	(6)	7	(16)	0	(0)	18	(16)
Type and severity of infection																		
Skin and soft tissue[h]	45	(83)	62	(95)	19	(86)	58	(94)	28	(78)	45	(96)	38	(86)	33	(90)	91	(82)
Abscess[i]	5	(9)	36	(55)	1	(5)	27	(44)	3	(8)	21	(45)	7	(16)	18	(49)	11	(10)
Bacteraemia	3	(6)	0	(0)	1	(5)	5	(8)	1	(3)	3	(6)	3	(7)	3	(8)	4	(4)
Pneumonia	3	(6)	1	(2)	2	(9)	1	(2)	2	(6)	0	(0)	1	(2)	1	(3)	6	(5)
Sepsis	13	(24)	15	(23)	3	(14)	19	(31)	5	(14)	8	(17)	12	(27)	13	(35)	21	(20)

Outcome																		
Length of stay (median)[j]	6.5		2		2		3.5		4.5		2		3		2		4	
Length of stay (mean)	32		7		9		6		18		9		15		5		20	
Hospitalisation	50	(93)	54	(83)	14	(64)	51	(82)	28	(78)	33	(70)	33	(75)	29	(78)	91	(82)
Surgery received[k]	19	(35)	43	(66)	5	(23)	42	(68)	11	(31)	34	(72)	14	(32)	26	(70)	33	(30)
In hospital mortality	2	(4)	0	(0)	1	(5)	2	(3)	1	(3)	0	(0)	0	(0)	0	(0)	3	(3)
Isolate characterisitics																		
PVL positive[l]	1	(2)	64	(98)	0	(0)	62	(100)	1	(3)	47	(100)	5	(11)	36	(97)	7	(6)
PVL R isoform[m]	1	(2)	0	(0)	0	(0)	62	(100)	1	(3)	47	(100)	0	(0)	0	(0)	2	(2)
Erythromycin resistant[n]	32	(59)	1	(2)	6	(26)	8	(13)	12	(33)	8	(17)	2	(5)	20	(54)	10	(9)
Clindamycin resistant[o]	29	(54)	1	(2)	5	(23)	8	(13)	9	(25)	8	(17)	2	(5)	20	(54)	8	(7)
Fusidic acid resistant[p]	29	(54)	0	(0)	0	(0)	0	(0)	0	(0)	1	(2)	0	(0)	0	(0)	12	(11)

Note: nmMRSA: non-multi-resistant methicillin-resistant *Staphylococcus aureus*; MSSA: methicillin-susceptible *S. aureus*; HCA: health-care associated; PVL: Panton Valentine leukocidin; R: arginine isoform of PVL. a Kruskal-Wallis, p=0.004; b $\chi 2$, p=0.004; c $\chi 2$, p=0.007; d $\chi 2$, p<0.001; e $\chi 2$, p<0.001; f $\chi 2$, p<0.001; g $\chi 2$, p<0.001; h $\chi 2$, p=0.041; i $\chi 2$, p<0.001; j Kruskal-Wallis, p=0.001; k $\chi 2$, p<0.001; l $\chi 2$, p<0.001; m $\chi 2$, p<0.001; n $\chi 2$, p<0.001; o $\chi 2$, p<0.001; p $\chi 2$, p<0.001..

4.5 Discussion

There are a number of parallels and contrasts between the community-associated MRSA epidemics described in north America and our experience in northern Australia[25, 26]. In north America, the USA400 clone appeared to be most prominent prior to 2001[27], but has since been displaced by USA300 across most of the United States[3]. Both USA300 and USA400 typically express PVL. In northern Australia, despite the presence of community-associated MRSA since at least the early 1990s[28], there continues to be a diversity of strains, with the three major clones together representing approximately 75% of isolates. One of these clones, CC1, is usually PVL-. However, our finding that a significant proportion of both community-associated MRSA and MSSA isolates are PVL+ CC93, reflects the growing emergence of this lineage across Australia since it was first described in 2000[8]. As with USA300 and USA400, cases of severe staphylococcal disease have been described with CC93[29, 30].

In this current study, we have defined epidemiological associations with PVL, and also PVL H and R isoforms in a large prospective sample of patients with infections caused by diverse community-associated *S. aureus* clones. Many previous studies have principally focused on community-associated MRSA; one of the key results in this study is the unanticipated high rate of PVL positivity in MSSA isolates.

Early epidemiological studies highlighted the association between PVL and necrotising pneumonia, furunculosis and severe bone and joint infections[9, 10, 31]. One of the initial papers described an association between PVL and lethal necrotising pneumonia in young adults but the overall numbers were small with only 16 cases of PVL+ pneumonia of which 8 were recruited retrospectively[10]. More recent studies have failed to find an association between the presence of PVL and poorer outcomes in complicated skin and soft tissue infections or invasive disease[32, 33]. Conflicting results from experimental studies have also led to a significant degree of controversy regarding the pathogenic role of PVL. In one study using a mouse model, it was reported that PVL was directly causative of necrotising pneumonia[34]. However, it was subsequently determined that an unintended point mutation in the *agr* promoter of the *S. aureus* isolate used resulted in defective virulence gene regulation and explained the observed phenotype[35]. Furthermore, mammalian neutrophils from

different species differ in their susceptibility to PVL. Mouse neutrophils are more resistant than human and rabbit neutrophils to PVL[36-39]. Therefore mouse models may not be appropriate for determining the role of PVL in human disease and could explain the negative findings from several studies[40, 41]. A recent study using a rabbit model of necrotising pneumonia compared the virulence of a USA300 wild-type strain with that of an isogenic PVL-deletion mutant and found that expression of PVL resulted in increased pathogenicity[42].

Our findings demonstrate a clear difference in the patient population affected and disease caused by PVL+ compared to PVL- isolates, irrespective of methicillin-resistance phenotype. Patients with PVL+ isolates were younger by close to 10 years on average and had fewer comorbidities. Despite PVL being significantly associated with sepsis, patients with PVL+ infections had a shorter length of hospital stay. Patients with PVL+ infections were less likely to have health-care associated risk factors, regardless of methicillin-resistance phenotype. In other words, even among community-associated strains of *S. aureus*, PVL+ strains were much more likely to be truly community-acquired. Only 6% (13/223) of PVL+ isolates were nosocomially-acquired compared to 21% (54/255) of PVL- isolates and 62% of multidrug-resistant MRSA isolates[14]. PVL+ strains are endemic in our population and cause discharging skin abscesses but have made minimal incursions into the hospital setting. There are rightly concerns that PVL+ community-associated MRSA strains such as USA300 will infiltrate and replace health-care associated genotypes of MRSA[43]. However, it is not clear whether traditional transmission models for MRSA can be applied to CA-MRSA[44]. Our findings would suggest that, in our setting, true nosocomial transmission of PVL+ strains (both nmMRSA and MSSA) is uncommon.

Another striking finding was that the age specific incidence of PVL+ compared to PVL- infections declined following a peak in the 20-39 year age group. Serological studies have demonstrated that PVL+ *S. aureus* infections elicit an anti-PVL IgG response[45, 46]. However, no consistent correlation was found between antibody levels and increasing age among individuals without evidence of current *S. aureus* infection[46]. Prospective longitudinal studies would help to determine whether such antibodies provide protective immunity or perhaps contribute to host susceptibility[47], and thus impact upon the age specific incidence of PVL+ infections.

Of PVL isoforms, the H and R isoforms appear to the most significant numerically and by geographic distribution. Molecular models suggest the R isoform may lead to increased pore formation and therefore greater leukotoxicity[11]. We found no difference in the epidemiological and clinical characteristics of infections caused by PVL H and R isoform harbouring isolates, suggesting to us that the R isoform confers no differential clinical effect. This accords with recent laboratory findings of no difference in leukotoxicity between strains bearing the H and R isoforms[48]. However, as the majority of R isoforms were CC93 isolates, we cannot exclude the lesser possibility that the genomic background or other virulence factors carried by CC93 neutralise a real clinical difference. Notably, the other clone of *S. aureus* that typically harbours the R isoform is USA300. While not as extensive, the prevalence and distribution of CC93 in Australia is also expanding[4, 8]. Whether the R isoform enhances transmissibility of *S. aureus* compared to the H isoform requires further exploration.

Previous work has demonstrated that all nasal colonising *S. aureus* genotypes can cause invasive disease[49, 50]. Our examination of the local population structure of *S. aureus* and associated detailed clinical features revealed several additional points. Both CC1 and CC5, common lineages locally and globally, typically caused disease in older, sicker patients. Carriage studies of *S. aureus* have demonstrated PVL-strains of CC1 and CC5 to be common nasal colonisers[6, 49, 51, 52]. Our findings would suggest that these strains are well-adapted human commensals that mainly cause opportunistic infections in vulnerable hosts.

In contrast, CC93 and the other PVL+ clones affect younger, healthier patients. CC93 was the most common clone identified in our collection, being significantly represented among both nmMRSA and MSSA strains. Previous typing of the staphylococcal protein A (*spa*) gene has revealed similar diversity of *spa* types in both the nmMRSA and MSSA CC93 isolates from northern Australia. Rather than a recent clonal expansion of a particular CC93-MRSA *spa* type following a single acquisition of SCC*mec*, the multiple CC93-MRSA *spa* types in a geographically and chronologically localised collection of isolates is better explained by either an earlier SCC*mec* acquisition with subsequent *spa* type diversification or multiple independent acquisitions of SCC*mec*[22].

An unexpected finding was the importance of CC121, an exclusively MSSA clone in our collection that comprised 15% (37/239) of all MSSA isolates, all but one of which was PVL+. Colonisation surveys in remote Western Australian Indigenous communities revealed CC121 to comprise 6.7% of MSSA isolates[52] but ours is the first report to attribute clinical disease to this clone in Australia. The impact of CC121 and CC93 in Australia has likely been previously underestimated due to a focus on nmMRSA.

Antibiotic resistance to non β-lactams differed according to clonal complex. Notably, of CC1 nmMRSA isolates, over 50% were resistant to fusidic acid and over 50% were resistant to erythromycin. CC121 also demonstrated over 50% resistance to erythromycin. In contrast, there was almost no resistance to fusidic acid in other clonal complexes. The association of fusidic acid resistance in CC1 has previously been noted in Western Australia[53].

The phylogenetically diverged CC75 lineage is one of emerging interest. Apart from impetigo[18], this is the first report that CC75 is capable of causing similar clinical disease, including bacteraemia, to other *S. aureus* clones. However, two observations may indicate a comparatively less virulent phenotype. First, CC75 is under-represented in clinical isolates where it represents only 9% of nmMRSA isolates compared to 71% of nmMRSA isolates recovered from impetigo lesions in remote Indigenous communities[18]. Second, the observed rates of sepsis due to CC75 are probably lower than with other lineages.

Intriguingly, the presence of CC75 has recently been reported in Cambodia and possibly Indonesia and Malaysia[6]. This may relate to well described direct population contacts between Macassan traders from Indonesia and Indigenous people of northern Australia that pre-dated the European colonisation of Australia[54]. Also, a relative abundance of Group 1b strains in Asia[6, 51] compared to elsewhere in the world has been noted and we have found a similar over-representation of this clade with CC121 and CC93 together comprising 26% and 35% of our nmMRSA and MSSA isolates respectively. In summary, the emerging picture of *S. aureus* in the Top End of Australia is of a mixture of globally prevalent lineages such as CC1, CC5 and CC30 co-existing with lineages that belong to either the highly diverged CC75 or the Group 1b clade. CC121 is very widespread in the Asia-Pacific region, while CC93 appears to have emerged in Australia.

Limitations of our study include not collecting and typing all MSSA isolates presenting to the laboratory during the study period. However, MSSA isolates were collected in a random manner by sequential matching of laboratory receipt codes throughout the study period and should be representative of all MSSA isolates during this period. Importantly, we have been careful to stratify the analysis by methicillin-resistance and have demonstrated that the associations with PVL were consistent for both nmMRSA and MSSA. However, it must be borne in mind that epidemiological associations with PVL should be distinguished from pathophysiological proof of causation. We did not perform SCC*mec* typing and the SNP based typing method does not provide the resolution of full MLST or PFGE. Thus, we cannot determine differences in clinical features of some sub-clones within clonal complexes.

In conclusion, we have determined the clinical disease spectrum caused by prevalent strains of *S. aureus* in northern Australia, including descriptions for CC93 and CC75. While differences in clinical disease existed based on the genetic background as assessed by clonal complex, these differences, including the outcome of sepsis, were principally explained by the presence of PVL. Even among community-associated lineages of *S. aureus*, PVL was a strong predictor of truly community-acquired disease. In our setting, the greater burden of PVL+ disease is carried by MSSA and focusing on nmMRSA alone will lead to under-recognition and under-estimations of the impact of PVL+ clones such as CC93 and CC121.

4.6 References

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Chapter 5. Alternative treatments for impetigo in remote communities

The high prevalence of community-associated methicillin-resistant *S. aureus* (CA-MRSA) recovered from impetigo lesions in Aboriginal children living in remote communities led us to question whether benzathine penicillin G continues to be the best treatment of impetigo in these settings. To ascertain the feasibility and acceptability of a large randomised controlled trial comparing BPG to alternative oral therapies in remote Aboriginal communities I conducted a pilot study in one community. Based on the results of this pilot study, we submitted an ultimately successful grant application to the National Health and Medical Research Council to conduct a more definitive randomised controlled trial. The grant application, of which I took a lead in writing, is attached as appendix IX. The other principal investigators are: Jonathan Carapetis, Bart Currie, Ross Andrews and Malcolm McDonald. The randomised controlled trial has begun recruiting patients as of November 2009.

The following report detailing the pilot study has been published in the Journal of Paediatrics and Child Health. See Appendix VIII.

Tong SY, Andrews RM, Kearns T, Gundjirryirr R, McDonald MI, Currie BJ, Carapetis JR. Trimethoprim-sulfamethoxazole compared with benzathine penicillin for treatment of impetigo in Aboriginal children: a pilot randomised controlled trial. J Paediatr Child Health 2010; 46: 131–133.

5.1 Introduction

Impetigo is highly prevalent in some remote Australian Aboriginal communities and serious complications include sepsis[1], acute post-streptococcal glomerulonephritis[2] and possibly rheumatic heart disease[3]. Benzathine penicillin G (BPG), to which Group A streptococcus (GAS) is susceptible but most strains of *Staphylococcus aureus* are not, is the currently recommended treatment in the remote Aboriginal setting[4]. However, *S. aureus*, including community-associated methicillin-resistant *S. aureus* (CA-MRSA), may be replacing GAS as the dominant cause of impetigo[5]. BPG may no longer be an effective treatment and the intramuscular (IM) injections are painful and poorly tolerated. We conducted a pilot randomised controlled trial comparing IM BPG to oral trimethoprim-sulfamethoxazole (TMP-SMZ) for treatment of impetigo in Aboriginal children. Our objective was to determine if such a study was feasible and acceptable in an Aboriginal community setting, and to perform a preliminary comparison of the efficacy of TMP-SMZ with IM BPG.

5.2 Methods

We visited households in a remote Aboriginal community in the Top End of the Northern Territory, Australia. Children aged between two months and 16 years with a diagnosis of purulent or crusted impetigo were eligible for inclusion, but were excluded if they had: 1) received any antibiotic in the previous seven days, or IM BPG in the previous 30 days; 2) a known allergy to study medications; or 3) evidence of sepsis, cellulitis, or abscesses. Patients or their care-givers provided written consent. The local ethics committee provided ethics approval, and the trial was registered (ACTRN12607000592448).

Participants were randomised to receive either a single dose of IM BPG (45mg/kg up to 900mg) or TMP-SMZ (4 + 20 mg/kg up to 160 + 800 mg) orally twice daily for 5 days. The allocation sequence utilised a permuted block design with blocks of four and six. Allocation was concealed by using sealed, sequentially marked, opaque envelopes. We did not stratify for age, gender or severity of impetigo.

The primary outcome was successful treatment of impetigo lesions at day 7 after the commencement of treatment. Treatment was deemed successful if sores were healed or improved in terms of drying of crusts and purulence and decrease in size of lesions, and unsuccessful if unchanged or worse. Assessment was by clinical examination and also by review of photographs by two investigators blinded to treatment allocation. Secondary outcomes were 1) the bacterial resolution of sores at day 4 and day 7; and 2) successful treatment at day 4. At baseline and on days 4 and 7, we documented the presence, size, appearance and number of skin sores, took digital photographs of affected areas and also swabs from sores if they were purulent or crusted. Swabs were placed in Amie's medium and transported back to Darwin for routine culture and antimicrobial susceptibility testing. Analysis was by intention-to-treat. For children whose sores were no longer purulent or crusted and thus did not have a swab taken, it was assumed that microbiological clearance had occurred.

5.3 Results

Between January 23 and 26 2008, we screened 111 children, with 18 potentially eligible for inclusion. Thirteen consented and were randomly assigned to receive TMP-SMZ (7 participants) or IM BPG (6 participants). There was no difference in age, gender or severity of sores between the two groups. *S. aureus* was recovered in skin sore swabs from all 13 participants (three CA-MRSA) and β-haemolytic streptococci from 6 (four GAS). All *S. aureus* isolates were susceptible to TMP-SMZ and for all except two children the *S. aureus* isolates were penicillin-resistant. These two children had penicillin-susceptible strains isolated at baseline concurrently with a MSSA in one case and a CA-MRSA in the other. Both of these children were randomised to IM BPG. Seven days after randomisation, in all 7 patients assigned to TMP-SMZ, and 5 of 6 patients assigned to IM BPG treatment was successful (relative risk (RR) of successful treatment in the TMP-SMZ group, 1.2; $p = 0.46$; 95% confidence interval (CI) 0.83, 1.72). See **Table 5-1**. Treatment was successful after 4 days in 6 of 7 treated with TMP-SMZ and 3 of 6 with IM BPG (RR 1.7; $p = 0.27$; 95% CI 0.73, 4.03). Microbiological clearance was documented by day 4, in 5 of 7 treated with TMP-SMZ and 2 of 6 with IM BPG (RR 2.1; $p=0.28$; 95% CI 0.63, 7.30); and by day 7, in all 7 treated with TMP-SMZ compared to 3 of 6 with IM BPG (RR 2.0; $p=0.07$; 95% CI 0.90, 4.45). Irrespective of treatment received, we did not recover β-haemolytic streptococci at day 4 from any patients from whom it was initially recovered. Of the three children with CA-MRSA, clearance was observed by day 7 in both children treated with TMP-SMZ, but not the child given IM BPG. No adverse effects, in particular rash or gastrointestinal upset, from either study medication were observed or reported. There was 78–83% agreement between the clinical assessment and that of blinded assessors.

Table 5-1. Treatment given, clinical assessments and microbiological swab results.

Age, sex	Treatment	Severity	Day 0	Day 4		Day 7	
			Culture	**Clinical**	**Culture**	**Clinical**	**Culture**
2.1, F	Penicillin	Mild	MSSA, GAS	Unchanged	MSSA	Unchanged	MSSA
3.1, M	Penicillin	Mild	MSSA, GAS	Cured	No swab taken	Cured	No *S.aureus* or βHS
4.9, F	Penicillin	Mild	CA-MRSA, GAS	Unchanged	CA-MRSA	Improved	CA-MRSA
3.6, M	Penicillin	Severe	CA-MRSA	Improved	No *S.aureus* or βHS	Lost to follow-up	Lost to follow-up
5.4, F	Penicillin	Severe	MSSA	Improved	MSSA	Improved	No swab taken
7.1, F	Penicillin	Severe	MSSA	Unchanged	MSSA	Improved	MSSA
7.3, F	TMP-SMZ	Mild	MSSA, GAS	Improved	MSSA	Improved	No *S.aureus* or βHS
7.1, F	TMP-SMZ	Mild	MSSA	Improved	No *S.aureus* or βHS	Cured	No swab taken
4.8, M	TMP-SMZ	Severe	MSSA, GCS	Improved	No *S.aureus* or βHS	Improved	No swab taken
4.0, M	TMP-SMZ	Severe	CA-MRSA	Improved	MSSA	Improved	No *S.aureus* or βHS
6.5, M	TMP-SMZ	Mild	MSSA	Unchanged	No *S.aureus* or βHS	Improved	No swab taken
4.1, M	TMP-SMZ	Severe	MSSA	Improved	No *S.aureus* or βHS	Improved	No swab taken
3.0, F	TMP-SMZ	Severe	MSSA, GBS	Improved	No *S.aureus* or βHS	Cured	No swab taken

Note. TMP-SMZ, trimethoprim-sulfamethoxazole; MSSA, methicillin-sensitive *S. aureus*; CA-MRSA, community-associated methicillin-resistant *S. aureus*; βHS, beta hemolytic streptococcus; GAS, Group A streptococcus; GBS, Group B streptococcus; GCS, Group C streptococcus.

5.4 Discussion

Both IM BPG and TMP-SMZ were efficacious in healing impetigo in this pilot study. The overall cure or improvement at seven days in 12 of 13 children, is better than the rate of 61% previously seen with placebo, and equates well with rates of 75-95% with topical or other systemic treatments[6]. Our secondary endpoints suggested the possibility of earlier clinical improvement and microbiological clearance, as well as more successful clearance by day 7 in the TMP-SMZ group. The detected prevalence of impetigo of 16% (18/118 screened) accords well with that found in recent surveys of 10–20% in other Aboriginal communities[5] and is still unacceptably high. Based on the results of this pilot study, we have obtained funding to proceed to a formal randomised controlled trial comparing IM BPG and TMP-SMZ. See the project synopsis in Appendix IX.

5.5 References

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Chapter 6. High-resolution melting analysis of the *spa* locus

This chapter begins the second main section of the thesis which concerns the laboratory and bioinformatic studies I undertook. This chapter describes the use of a high-resolution melting platform to interrogate the hypervariable *spa* locus of *S. aureus*.

The following was published in Clinical Microbiology and Infection. See Appendix X.

Tong SY, Lilliebridge RA, Holt DC, McDonald MI, Currie BJ, Giffard PM. High-resolution melting analysis of the *spa* locus reveals significant diversity within sequence type 93 methicillin-resistant *Staphylococcus aureus* from northern Australia. Clin Microbiol Infect. 2009; 15: 1126–31.

6.1 Abstract

High-resolution melting analysis is an inherently robust, easy and inexpensive approach to the examination of genomic regions containing single-nucleotide polymorphisms and hypervariable loci. *Staphylococcus aureus* sequence type (ST) 93 is a singleton, Panton-Valentine leukocidin-positive clone unique to Australia. A high-resolution melting-based method for the identification of ST93 was developed, and a similar approach was used to reveal diversity within the *spa* locus of this lineage. Statistical and graphical methods that account for instrumental and operator-dependent variation in high-resolution melting curves were developed, to allow greater confidence and reproducibility in deciding whether another curve is truly different from the baseline curve of an amplicon with known sequence. The data support a very early acquisition, or multiple independent acquisitions, of SCC*mec* by ST93 methicillin-susceptible *S. aureus* (MSSA), and the coexistence of MSSA and methicillin-resistant *S. aureus* versions of the same lineage within northern Australia.

6.2 Introduction

In the evolving Australian epidemics of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA), the most common multilocus sequence types (MLSTs) are 1, 93 and 30[1]. Sequence type (ST) 93 methicillin-resistant *S. aureus* (MRSA), first described in 2000[2], is an MLST singleton clone, is typically Panton-Valentine leukocidin (PVL)-positive, and is a major cause of severe CA-MRSA disease in Australia[2-4]. The other common PVL-positive clone, ST30-MRSA, probably arrived in Australia via large population migrations in the 1980s[5]. ST93-MRSA is the most rapidly expanding CA-MRSA clone throughout Australia[1], and now constitutes 33% of Australian CA-MRSA isolates[6], and has also been exported to the UK by travellers[7]. Previous investigators found little genetic diversity within ST93-MRSA, suggesting that it arose from one PVL-positive subtype of ST93 methicillin-susceptible *S. aureus* (MSSA)[8].

The polymorphic X region of the staphylococcal protein A (*spa*) gene rapidly evolves, and *spa* sequence typing can elucidate patterns of diversity within sequence types. High-resolution melting (HRM) analysis, a new approach to analysing genetic variation, is cost-effective, simple, and robust. It has been shown to be highly effective for resolving *spa* types[9]. Although empirical methods to define HRM curves as the same or different have been used, such methods have not been based upon statistical analysis[9]. Our aims were to: (i) develop an HRM-based method for the rapid identification of ST93; and (ii) determine the *spa* locus diversity of ST93-MRSA, ST93-MSSA and ST30-MRSA isolates from northern Australia. Of particular interest was whether ST93-MRSA represents a highly conserved clone with a very recent origin as compared with ST93-MSSA.

6.3 Methods

Isolates were collected in community-based studies of impetigo conducted in remote Aboriginal communities from 2003 to 2005[10], and in a prospective study of CA-MRSA infections at the Royal Darwin Hospital (RDH) from 2006 to 2007[11]; three ST93-MSSA isolates were collected in 1991 at the RDH (**Table 6-1**). The community studies had revealed the highly divergent clonal complex (CC) 75 to be the predominant *S. aureus* clone in remote communities in northern Australia[10].

A robust and efficient method for identifying ST93 was developed. An ST93-specific single-nucleotide polymorphism (SNP), *glpF* 165, was identified in the *S. aureus* MLST database using the software Minimum SNPs[12]. This is a three-state SNP, with the 'C' allele completely specific for ST93, the 'A' allele completely specific for CC75, and the 'T' allele possessed by all other known STs. To add redundancy, we also used the MLST database-derived SNP *aroE* 252, which has been determined to be diagnostic for ST93. This SNP also has three allelic states[13], with the 'G' allele being unique to ST93. Primers were designed to generate amplicons containing these SNPs.

The diversity of ST93 was assessed by performing HRM analysis of the *spa* locus of 30 isolates each, identified as ST93-MRSA and ST93-MSSA. Additionally, 30 isolates of PVL-positive ST30-MRSA from northern Australia, previously characterised using a kinetic PCR method to examine eight SNPs[13], were similarly analysed.

We used a Rotor-Gene 6000 instrument (Corbett Life Science) for the HRM analysis. DNA was extracted using the QIAamp DNA minikit protocol for Gram-positive bacteria with lysostaphin. Each reaction contained 5 µL of Platinum SYBR Green qPCRSuperMix-UDG (2x; Invitrogen Life Technologies), 0.5 µM each primer and 1 µL of a 1:5 dilution of the DNA template in a final volume of 10 µL. The real-time PCR thermocycling parameters were: 50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 5 s and 60°C for 30 s; 72°C for 2 min; and 50°C for 20 s; this was followed by HRM ramping with fluorescence data acquisition at 0.05°C increments. The primers and HRM ramping temperatures are shown in **Table 6-2**. Reactions were routinely carried out in duplicate. The exported HRM curves are available as an Excel file: http://www3.interscience.wiley.com/journal/122324935/suppinfo. The

spa locus was sequenced in representative isolates that yielded the different curves, and *spa* types were assigned according to the *spa* database[14].

We defined 95% CIs for the HRM curves corresponding to isolates of the same sequenced *spa* type by exporting the normalised fluorescence data of the HRM curves to calculate the mean and standard deviation (SD) at each temperature of the melting protocol. This was only performed when there were at least six isolates of the same *spa* type. At each temperature, the 95% CIs were calculated as the mean ± (1.96 x SD). We generated difference graphs by subtracting this mean normalised fluorescence from the normalised fluorescence of each curve at each temperature, and defined the 95% CIs for the difference graph as 0 ± (1.96 x SD).

Table 6-1. High-resolution melting (HRM) curves and corresponding *spa* types and repeats.

	HRM curve (no. of isolates)	*spa* genotype (no. sequenced)	*spa* repeats
ST93-MRSA[a] (Figure 6-1c)	HRM curve 1 (23)	t202 (8)	11–17–23–17–17–16–16–25
	HRM curve 3 (2)	t1819 (2)	11–17–23–17–16–16–25
	HRM curve 4 (1)	t4675 (1)	11–17–17–17–16–16–25
	HRM curve 5 (4)	t1811 (4)	11–17–17–16–16–25
ST93-MSSA (Figure 6-1d)	HRM curve 1 (26)[a, b]	t202 (5)	11–17–23–17–17–16–16–25
	HRM curve 2 (1)[c]	t4178 (1)	11–17–23–17–17–16–16–16–25
	HRM curve 6 (2)[c]	t4699 (2)	11–17–16–16–25
	HRM curve 10 (1)[b]	t4698 (1)	04–16–16–25
ST30-MRSA[a] (Figure 6-1e)	HRM curve 7 (27)	t019 (7)	08–16–02–16–02–25–17–24
	HRM curve 8 (2)	t138 (2)	08–16–02–25–17–24
	HRM curve 9 (1)	t4700 (1)	08–275–02–16–02–25–17–24

Note. MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*; ST, sequence type.
[a] All ST93-MRSA, ST30-MRSA and 21 ST93-MSSA isolates were from the 2006-2007 Royal Darwin Hospital collection.
[b] Six ST93-MSSA isolates were from the 2003–2005 community-based studies.
[c] Three ST93-MSSA isolates were from the 1991 Royal Darwin Hospital collection.

Table 6-2. Primers and ramping temperatures for high-resolution melting reactions.

Primer name	Primer sequence (5’ 3’)	Ramping temperatures
glpF 165 F	ACCCAGCGGTGTCTTTAGCTCTTGCAT	72–84°C
glpF 165 R	ATACAATCGTTGCTCCGACA	
aroE 252 F	GTGGATAGGGTATAATACAG	68–81°C
aroE 252 R	ACCTGCGCCCAAAATTAAAA	
spa 1095 F	AGACGATCCTTCGGTGAGC	75–87°C
spa 1517 R	GCTTTTGCAATGTCATTTACTG	

6.4 Results

We developed HRM-based methods for the examination of both SNP regions, SNP *glpF* 165 and SNP *aroE* 252, using isolates of known ST. For each SNP region, three easily discriminated curves were obtained (**Figure 6-1a, b**). During the development of the *glpF* 165 assay, some unexpected results were obtained. The initial primer set allowed amplification of a product that generated identical HRM curves for ST93 and CC75, even though CC75 is highly divergent from all other *S. aureus*, and the two PCR products differed at seven positions. Examination of the seven SNPs indicated a neutral balance of hydrogen bonds, with an identical GC content. Redesign of the primers (*glpF* 165F and *glpF* 165R) allowed clear discrimination of ST93 and CC75, with the amplified regions now having differing GC contents. Of the possible amplified *aroE* 252 regions, ST93, among all the STs, has a unique GC content as well as the highest melting temperature, and therefore generates a unique HRM curve. We were unable to consistently generate an *aroE* amplicon for CC75, and further work to better characterise this locus for CC75 is proceeding. It was concluded that examining the *glpF* 165 and *aroE* 252 SNP regions by HRM analysis is a rapid and robust method for identifying ST93. It has the added benefits of incorporating redundancy and allowing the identification of CC75, which is another major northern Australian clonal complex.

HRM analysis of the *spa* locus revealed four and three clearly distinct curves for ST93-MRSA and ST93-MSSA isolates, respectively (**Figure 6-1c, d** and **Table 6-1**). Of 14 sequenced ST93 isolates associated with HRM curve 1, 13 had the same *spa* type of t202, and one contained an additional 24-bp repeat, t4178. Closer examination of the t4178 HRM difference curve, with the mean t202 curve as the baseline, revealed part of the curve sitting outside the 95% CI curve, suggesting that the t4178 and t202 HRM curves are indeed different (**Figure 6-2a**). The five other curves correlated with different *spa* types. Comparison of the raw data of HRM curves of two ST93-t202 isolates produced in another laboratory[9] showed that they were consistent with our t202 curves, although a part of the curve at 72°C, well away from the melting temperature, was outside the 95% CI (**Figure 6-2a**). Within ST30-MRSA, three slightly different curves could be distinguished on both the normalised fluorescence and the difference curves (**Figure 6-1e** and **Figure 6-2b**). The *spa* loci of seven isolates with HRM curve 8 were sequenced; all had the same *spa* type of

t019. The two other curves correlated with different *spa* types (**Table 6-1**). **Figure 6-1f** shows curves from all three groups of isolates.

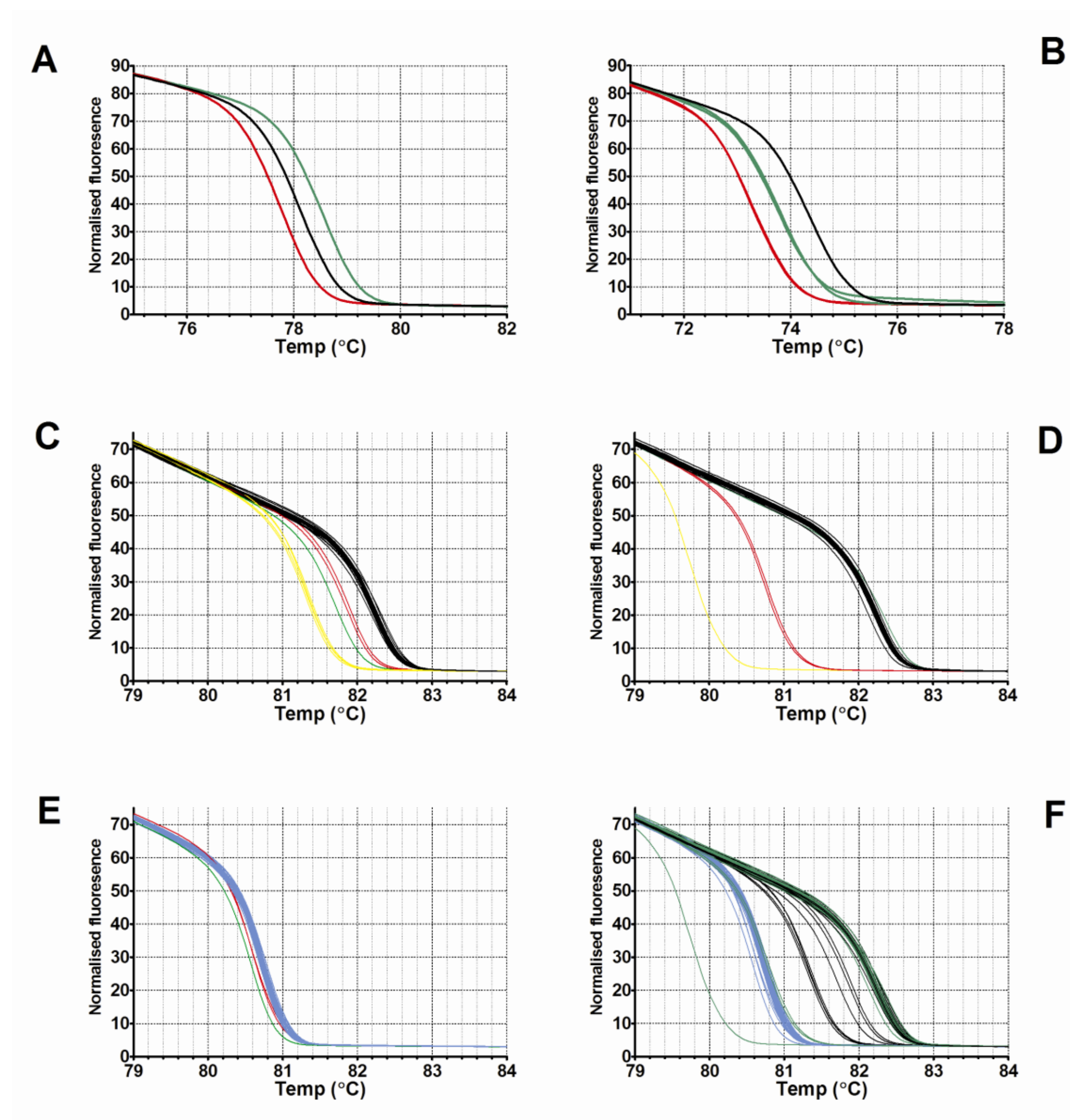

Figure 6-1. High-resolution melting curves of the *glpF* 165, *aroE* 252 and *spa* loci (c-f).

(a) Representative curves corresponding to each allele at *glpF* 165: C (black) is specific for ST93, and A (green) is specific for CC75. (b) Representative curves for the *aroE* 252 region: the black curve is specific for sequence type (ST) 93. (c) ST93 methicillin-resistant *Staphylococcus aureus* (ST93-MRSA) showing four curves: curve 1 (black), curve 3 (red), curve 4 (green), and curve 5 (yellow). (d) ST93 methicillin-susceptible *S. aureus* (ST93-MSSA) showing four curves: curve 1 (black), curve 2 (green), curve 6 (red), and curve 10 (yellow). (e) ST30-MRSA showing three curves: curve 7 (blue), curve 8 (red), and curve 9 (green). (f) ST93-MRSA (black), ST93-MSSA (green) and ST30-MRSA (blue) combined.

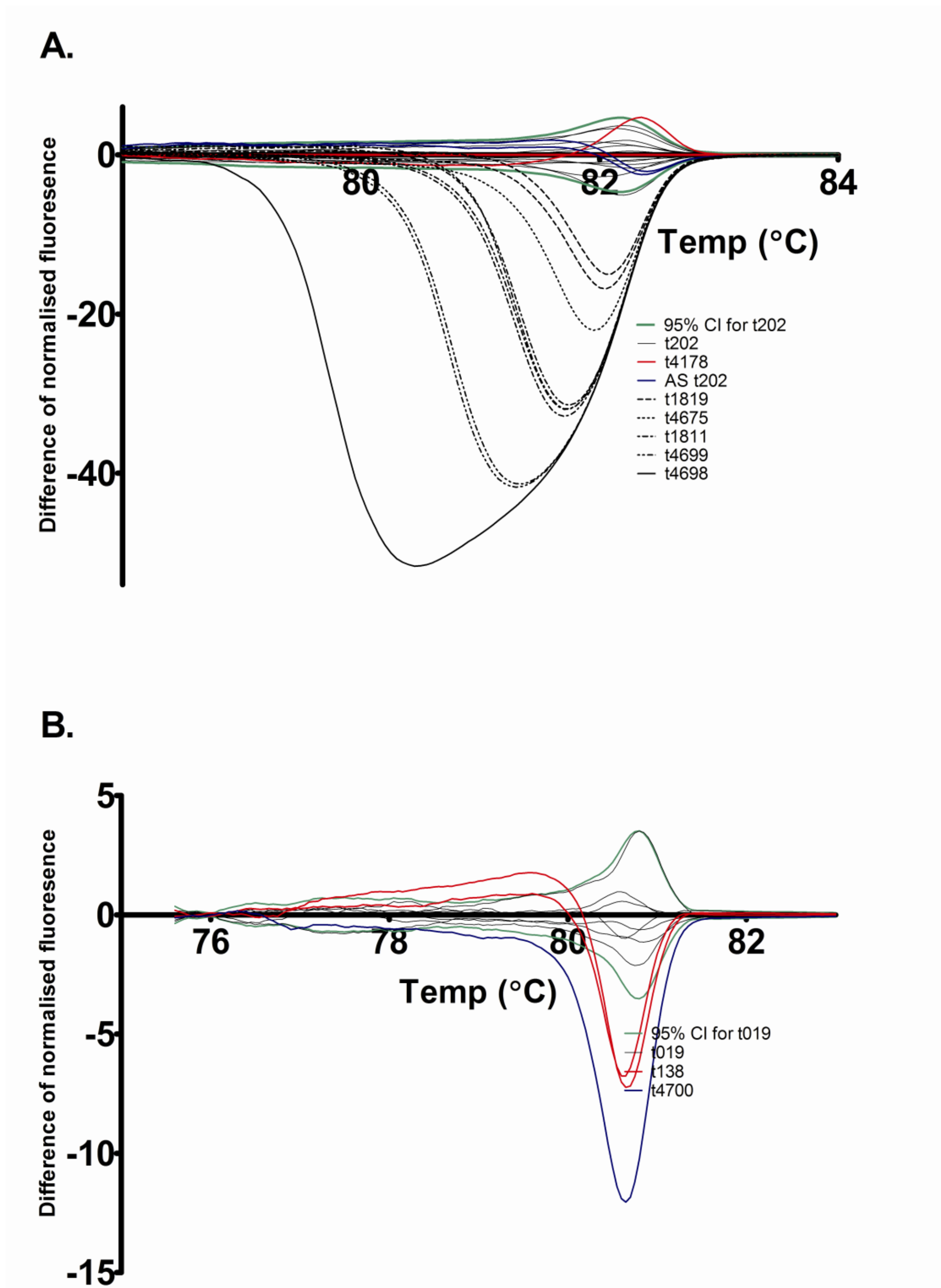

Figure 6-2. Difference graph of high-resolution melting curves of *spa* loci.

(a) Curves for sequence type (ST) 93 isolates. The mean of the known t202 curves was used as the baseline. The green lines indicate the 95% CIs for the 13 t202 curves, and the thin black lines indicate each t202 curve. The blue lines (AS t202) refer to t202 curves derived from the raw data of the study by Stephens et al[9], and sit within the 95% CI, whereas part of the t4178 (red) curve sits outside the 95% CI. (b) Curves for ST30 isolates. The mean of the known t019 curves was used as the baseline. The green lines indicate the 95% CIs for the seven t019 curves, and the thin black lines indicate each t019 curve.

6.5 Discussion

All of the ST93-MRSA isolates were from the 2006–2007 hospital-based study, and the finding of four *spa* types within these 30 isolates revealed surprising *spa* type diversity for a group of chronologically and geographically localised isolates from a hospital serving a population of only 176 000. Similarly localised[15-17], and some even larger[18, 19], studies of CA-MRSA revealed no *spa* type diversity among ST30, ST80 and USA300 isolates. The diversity in ST93-MRSA more closely mirrors that seen in studies across broader geographical regions and time periods, where single STs contained up to five *spa* types[20-23]. Larsen et al.[22, 23] linked this diversity within ST80 and USA300 to multiple international importations of these strains into Denmark. Such importation could explain the diversity that we demonstrated in the widely distributed ST30-MRSA, but not that in ST93-MRSA.

The diversity in both ST93-MRSA and ST93-MSSA does not support the hypothesis that ST93-MRSA is undergoing an explosive clonal expansion derived from a recent single instance of SCC*mec* acquisition. Rather, the data support an early acquisition of SCC*mec*, with subsequent rearrangements of the *spa* sequence or multiple independent acquisitions of SCC*mec*, and coexistence of MSSA and MRSA versions of the same lineage. A recent study of ST5 revealed that SCC*mec* has probably been acquired repeatedly in geographical regions within ST5, and predicted that MRSA haplotypes within a geographical region should also be present in MSSA from that region[24]. Our findings support this prediction; for instance, the commonest *spa* type, t202, is present in ST93-MRSA from RDH and in ST93-MSSA from both RDH and community study collections. Interestingly, all 21 ST93-MSSA isolates from the 2006–2007 hospital collection were *spa* type t202, raising the possibility of a current epidemic of ST93-MSSA-t202, as compared with endemic ST93-MRSA. However, these 21 isolates were not linked epidemiologically in terms of time or patient residence, and only one was nosocomially acquired.

HRM analysis is a single-step closed-tube reaction. It is inherently robust, easy, and inexpensive to optimise and perform. It can also be used to examine hypervariable loci[9]. The derivation of SNP sets from sequence alignments using the software Minimum SNPs, and the development of HRM-based assays for the examination of these SNPs, is a straightforward and efficient approach for assembling genotyping

procedures for specific purposes. We have been able to accurately discriminate two major clones in northern Australia from all other clones and also from each other.

HRM-mediated *spa* examination can differentiate closely related *spa* types, and provides further resolving power for genotyping. It is less expensive than full *spa* sequencing; each sequencing reaction in our institution costs US $20, as compared with US $0.50 for reagents for each HRM run. The Rotor-Gene 6000 software and previous publications have used a single HRM curve of an amplicon of known sequence as the baseline to generate difference curves. However, despite the robust methods, there is inevitably instrumental and operator error, resulting in slight variations in HRM curves generated from identical amplicons. We have developed a simple technique that incorporates this variation and allows greater confidence and reproducibility in deciding whether another curve is truly different from the baseline curve of known sequence. With appropriate calibration of instruments, it should also allow portability of the HRM curves and inter-laboratory comparison, as demonstrated in this article.

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Chapter 7. High-resolution melting detection of Panton-Valentine leukocidin variants

This chapter describes the development of an assay to detect sequence variants of the gene encoding for Panton-Valentine leukocidin.

The following has been published in Diagnostic Microbiology and Infectious Disease. See Appendix XI.

Tong SY, Lilliebridge RA, Holt DC, Coombs GW, Currie BJ, Giffard PM. Rapid detection of H and R Panton-Valentine leukocidin variants in *Staphylococcus aureus* by high-resolution melt analysis. Diagn Microbiol Infect Dis 2010; 67: 399–401.

7.1 Abstract

We designed a real-time PCR and high-resolution melting assay to simultaneously detect the presence of the Panton-Valentine leukocidin gene and discriminate histidine and arginine variants. Of 223 *Staphylococcus aureus* isolates from northern Australia, variants clustered by clonal complex. All clonal complex 93 isolates harboured the arginine variant. This is a rapid, single-step, closed-tube assay.

7.2 Introduction

The majority of community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains produce Panton-Valentine leukocidin (PVL) which is encoded by the co-transcribed genes *lukS-PV* and *lukF-PV*[1]. Recent studies have demonstrated sequence variations within *lukSF-PV*[2-4]. In particular, a single nucleotide polymorphism (SNP) at nucleotide site 527 leads to an A → G His_{176} to Arg_{176} substitution; the H and R variants. USA300 (clonal complex [CC] 8) and USA400 (CC1) isolates have been found to carry the R variant but most clones isolated outside of the United States carry the H variant[2, 3]. To date, the only isolate with the R variant not from CC1 or CC8 is an Australian isolate of CC93[3]. CC93 is the most rapidly expanding CA-MRSA clone in Australia and a major cause of severe CA-MRSA disease[5]. Molecular modelling suggests these variants may affect PVL function[2] and a rapid means of discriminating these variants would facilitate clinical studies.

High-resolution melt (HRM) analysis is a newly emerging technology that employs the ability to monitor with fluorescence the separation of two strands of DNA with increasing temperatures[6]. The combination of real-time PCR and HRM can be used to determine the presence or absence and also sequence variants of a gene. We designed a high-throughput HRM-based assay to simultaneously detect the presence of *lukSF-PV* and discriminate the H and R variants. We determined the distribution of H and R variants in a collection of hospital based isolates from the tropical Top End of northern Australia.

7.3 Methods

Isolates were collected as part of a case-control study, comparing CA-MRSA to MSSA infections at the Royal Darwin Hospital. Full details of the study design have been previously published[7]. For this study, isolates from eligible CA-MRSA patients were matched 1:1 to the next eligible MSSA patient using sequential laboratory numbers. DNA was extracted using the QIAamp® DNA minikit (QIAGEN, Melbourne, Australia) protocol for Gram-positive bacteria with lysostaphin. We typed isolates into clonal complexes using a SNP genotyping system based on the multilocus sequence type (MLST) database as previously described[8]. To confirm isolates as CC93, we also performed an HRM assay interrogating *aroE* position 252[9]. Pulsed-field gel electrophoresis, as described elsewhere, was performed on selected isolates[10].

Previously, our laboratory detected *lukSF-PV* by amplifying a region of the *lukF-PV* gene[8, 11]. For this HRM assay, we designed primers to amplify a 57 bp fragment of *lukS-PV* spanning nucleotide position 527 with primers: PVL527F (5'-CAACAAAACTATATCAGTGAAGTAGAAC-3'), PVL527R (5'-TCCCCATTGAACACTTTTTGAA-3'). PCR fragments containing the 57bp HRM-assay amplicon were obtained using flanking primers PVLlukS1F (5'-TGGTCCATCAACAGGAGGTA), PVLlukS1R (5'-TGGGGGTAATTCATTGTCTG-3') and these were sequenced on an ABI3730XL (Applied Biosystems) machine at a commercial centre (Macrogen, Seoul, Korea).

HRM reactions were performed on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) system. Each HRM reaction contained 5 µL Platinum® SYBR® Green qPCRSuperMix-UDG (2x, Invitrogen Life Technologies), 0.5 µM of each primer and 1 µL of a 1:5 dilution of the DNA template in a final volume of 10 µL. The real-time PCR thermocycling parameters were: 50°C for 2 min, 95°C for 2 min, 1 cycle each of 95°C for 2s and decreasing annealing temperatures from 65°C to 61°C in 1°C decrements for 10s, 35 cycles of 95°C for 2s and 60°C for 10s, 72°C for 2 min, and 50°C for 20 s, followed by HRM ramping from 64°C to 74°C with fluorescence data acquisition at 0.3°C increments. Raw HRM curves were normalised by the Rotor-Gene 6000 software, using normalisation regions of 64.5°C to 65.0°C and 73.2°C to 73.7°C.

7.4 Results

There were 239 CA-MRSA and 239 MSSA isolates collected for analysis. Initial testing for *lukF-PV* on real-time PCR revealed 223 positive and 255 negative isolates. All 223 *lukF-PV* positive isolates were analysed with the HRM assay, together with 40 negative isolates that were chosen for representatives across different clonal complexes. All 223 *lukF-PV* positive isolates amplified with the HRM primers, and the 40 *lukF-PV* negative isolates either amplified late or not at all (**Figure 7-1**). At a normalised fluorescence threshold of 0.2, all positive isolates amplified before cycle 23 and all negative isolates amplified after cycle 27. Late amplifying isolates are likely to harbour *lukD* which has some sequence homology with *lukS* at the primer sites. Using a cycle threshold cut-off of 25 provided 100% sensitivity and 100% specificity for detecting *lukSF-PV*.

The HRM assay discriminated between the H and R variants with melting temperatures differing by 1°C. These differences are clear on both the HRM normalised curves and the standard dF/dT based melting profile (**Figure 7-2**). The results for 20 isolates were confirmed by DNA sequencing.

Overall, there were 110 H variants and 113 R variants. All 109 CC93 isolates, regardless of *mecA* status, were R variants. There were 4 other R variant isolates: three CC1s and one CC8. Using pulsed-field gel electrophoresis the CC8 and CC1 isolates were characterised as USA300 and USA400 respectively. The majority of H variants were CC30 (66 isolates) and CC121 (36 isolates).

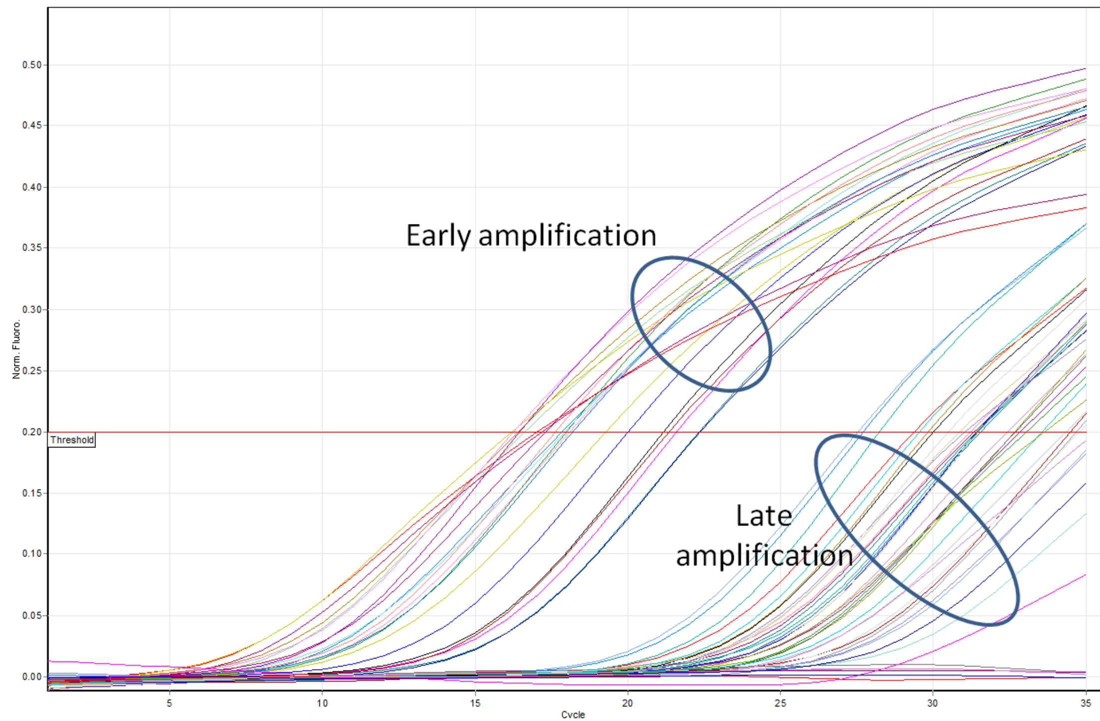
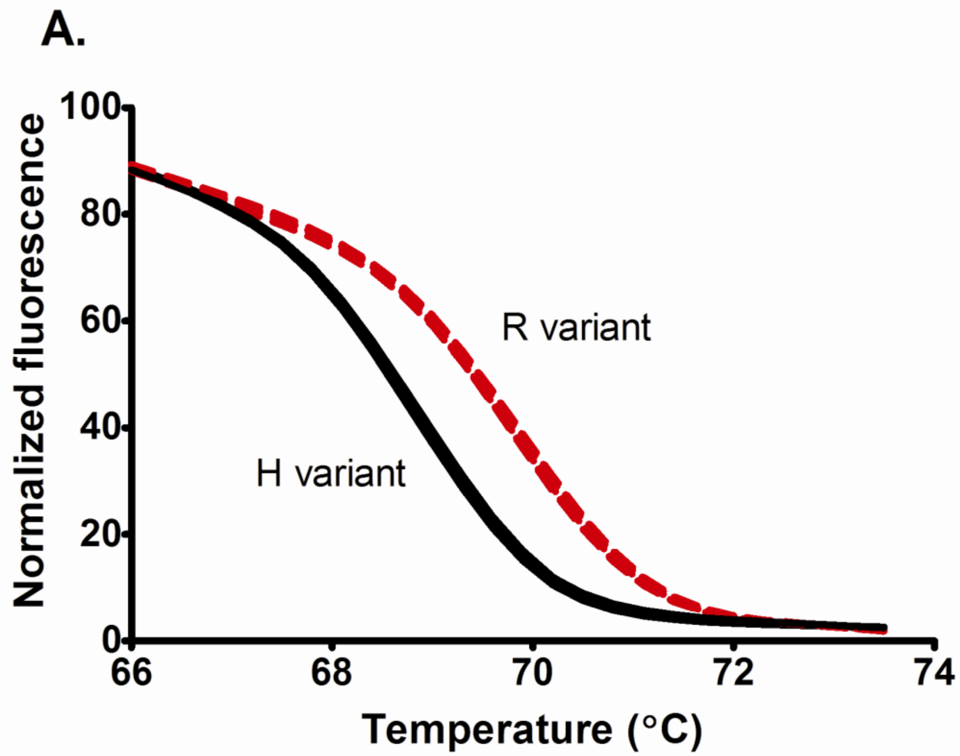


Figure 7-1. Real time PCR amplification curves of Panton-Valentine leukocidin (PVL) positive and negative isolates.

The PVL+ isolates all have a cycle threshold of <23, and PVL- isolates have a cycle threshold of >27. PVL- isolates which amplify late are likely to harbour *lukDE*; others do not amplify at all, most likely indicating the absence of both *lukSF-PV* and *lukDE*.



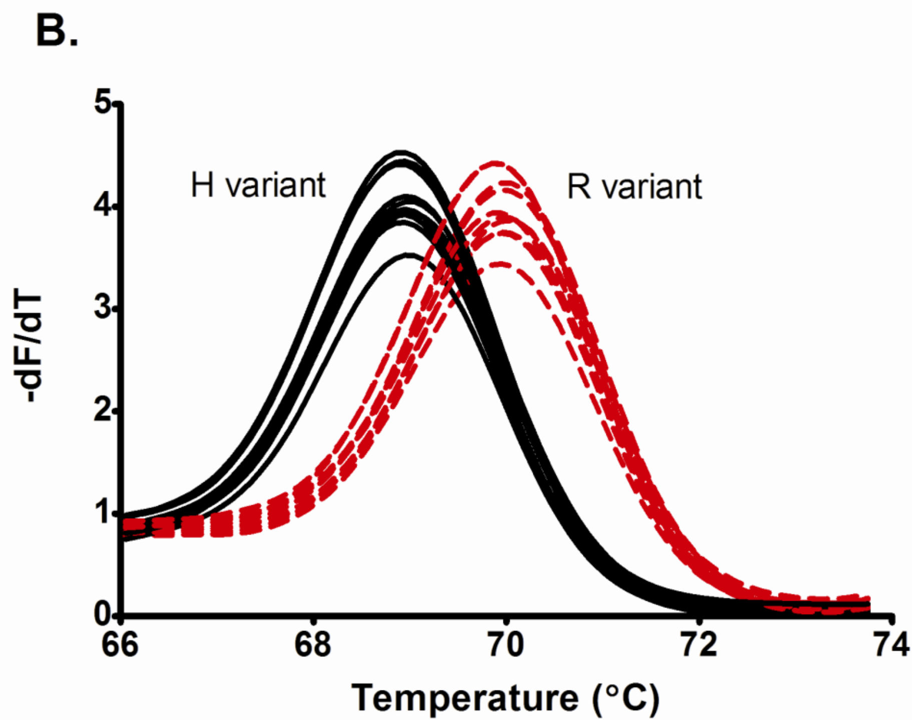


Figure 7-2. High-resolution melt (A) and standard melting temperature (B) curves for isolates of known sequence.

Histidine (H) variants (black, unbroken lines, n=10) and arginine (R) variants (red, broken lines, n=10).

7.5 Discussion

In addition to the first description of the presence of USA300 in the Northern Territory, we have found a major epidemic Australian clone, CC93, to universally harbour the R variant. Unlike USA300, CC93 does not contain the arginine catabolic mobile element which may be central to the transmissibility of USA300[12]. Whether the R variant of PVL confers epidemic properties to these respective clones is yet to be determined. In contrast to O'Hara *et al.*[2], but in agreement with the findings of Dumitrescu *et al.*[3], we found no correlation between PVL variant and the presence of *mecA* (P=0.82). This is well illustrated by CC93, where both CC93-MRSA and CC93-MSSA harbour the R variant. The most likely explanation is that the presence of the R variant pre-dates the acquisition of *mecA*.

In summary, we have developed and utilised a high-throughput, single step, closed tube HRM assay that simultaneously detects for the presence of PVL and also discriminates its two major variants without any additional cost in terms of time or money compared to the standard real-time PCR assays currently used for the detection of *pvl* genes. Although we have found no difference in the epidemiological and clinical characteristics of infections caused by PVL H and R isoform harbouring isolates (see Chapter 4), this finding should be validated in other studies.

7.6 References

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Chapter 8. MLST based *Staphylococcus aureus* typing scheme using high-resolution melting analysis of SNP nucleated PCR fragments

This chapter describes the development of a multilocus sequence type based method for genotyping bacterial species using high-resolution melting analysis. The practical demonstration of its utility is provided by using *S. aureus* as a model organism.

The title and paper content will be modified prior to submission. The authors will be the following:

Lilliebridge RA[#], Tong SY[#], Giffard PM, Holt DC.

[#] Joint first authorship.

8.1 Abstract

Microbial genotyping techniques are widely used to track epidemics and to understand the population structure of microbial species. Multilocus sequence typing (MLST) has proved extremely valuable in elucidating the population biology of bacterial pathogens. However, MLST requires sequence analysis of seven DNA fragments from housekeeping genes. Mass spectrometry based methods to interrogate MLST loci fragments have been developed to improve the throughput of genotyping while maintaining consistency with MLST derived population structures. We describe methods to determine highly informative SNPs in the MLST concatenated sequences of bacterial species and the subsequent application of high-resolution melting (HRM) technology to interrogate regions which contain these SNPs. *Staphylococcus aureus* was used as a model organism and we generated a predicted key which facilitates the translation from multilocus sequence types to HRM types. The methods were validated using a sample of diverse *S. aureus* isolates from northern Australia and we obtained a Simpson's index of diversity of 0.980 in comparison to MLST. HRM analysis is a single-step, closed tube process that is

relatively low in cost and this typing scheme accurately resolved isolates into MLST clonal complexes. The tools described allow for an efficient pipeline to develop similar genotyping schemes for other bacterial species.

8.2 Introduction

Staphylococcus aureus is a global pathogen which causes a range of infections from simple skin and soft tissue infections to life threatening invasive infections. Treatment is complicated by the widespread prevalence of resistance to methicillin in both the hospital and community setting. The importance of *S. aureus* to human health is reflected by the many typing schemes available. These include pulsed-field gel electrophoresis (PFGE), multi-locus variable number tandem repeat analysis (MLVA), and sequencing of regions of the genome. The practical choice for these techniques is guided by their simplicity, convenience, resolving power, cost and availability of resources. The image based typing methods such as PFGE and amplified fragment length polymorphism (AFLP) are highly discriminatory but comparison between laboratories can be problematic[1]. Sequence based techniques including multilocus sequence typing (MLST)[2] and single locus approaches such as *spa* typing[3] have a key advantage of portability and the development of online databases. There are now over 1500 unique *S. aureus* sequence types on the MLST database (http://saureus.mlst.net/) and studies using MLST analysis have provided a rich understanding of the population structure of *S. aureus*[4]. However, MLST requires sequencing of the internal fragments of 7 genes which is comparatively slow and expensive and less amenable to high-throughput applications.

Typing techniques have been developed which are based on MLST, but without the need for sequencing. Two such techniques use PCR coupled to electrospray ionisation – mass spectrometry (PCR/ESI-MS) and matrix-assisted laser desorption ionisation – time of flight mass spectrometry (MALDI-TOF)[5, 6]. These make use of the MLST data and provide genotypes which are consistent with the population structure and clonal complexes determined from MLST. However, it is unclear how widely these techniques will be utilised given the still limited availability and expense of the required mass spectrometry instruments.

Highly informative single nucleotide polymorphisms (SNPs) can be determined from the MLST databases using a program such as Minimum SNPs[7, 8]. These are SNPs which have been calculated to maximise the Simpson's index of diversity (*D*) and interrogation of several of these SNPs can provide a high degree of resolution and infer genotypes that are consistent with the MLST population structure[9, 10]. To date,

the SNP interrogation platforms have included differential primer extension (kinetic PCR)[10, 11] and padlock probes with tag microarrays[12].

We describe here a MLST based typing scheme for *S. aureus* using the emerging technology of high-resolution melting (HRM) to analyse PCR fragments containing informative SNPs. HRM employs the ability to monitor with fluorescence the separation of two strands of DNA with increasing temperatures[13]. The dynamics of the melting curves produced can vary according to sequence variations in the DNA fragments. As these fragments may contain more than one SNP, HRM analysis may provide significantly more information than platforms that only interrogate one SNP. We also demonstrate the application of the software Minimum SNPs and HRMType to generate MLST based typing schemes.

8.3 Methods

8.3.1 Concatenated MLST sequences

The concatenated MLST sequences for 1508 STs were downloaded from the *S. aureus* MLST site (http://saureus.mlst.net/) in November 2009. Four STs (STs 753, 957, 1166 and 1471) with an incorrect concatenated sequence length and one ST (ST1110) with a likely frame shift at position 81 of the *glpF* locus were subsequently removed from the alignment. Ten STs (STs 102, 202, 204, 208, 215, 220, 663, 773, 1079, 1407) were removed on the basis that their *tpi* alleles and in some cases *pta* alleles were >10% diverged from the other *S. aureus* alleles. A further nine STs which are likely members of clonal complex 75 (STs 75, 258, 850, 883, 1223, 1248, 1284, 1304, and 1306) were also removed as this clonal complex shows significant divergence from other *S. aureus* and may represent a distinct species or sub-species[14]. The concatenated sequences were analysed using MEGA 4.02[15].

8.3.2 Identification of *D* optimised SNPs useful for high-resolution melting analysis

We had previously identified a set of eight SNPs with a Simpson's index of diversity (*D*) of 0.95 for the MLST dataset[10]. We now aimed to generate a set of *D* optimised SNPs which were likely to contribute to a change in the melting temperature of the DNA (i.e., principally transitions) for interrogation by HRM. From the original SNP set, we used SNPs which were transitions for most STs (concatenated sequence positions 210, 2100, 2316 and the 2521/2523 combination) as forced inclusions and SNPs which were transversions (positions 162, 1695 and 2189) as forced exclusions in Minimum SNPs[7]. Any additional SNPs identified by Minimum SNPs which were transversions for most STs were also excluded. Transitions which were in close proximity to each other were favoured as they could be included in a single small PCR product for HRM analysis. The final SNPs chosen were 78 and 210 (*arcC* locus), 543 and 610 (*aroE* locus), 1663 (*gmk* locus), 2100 (*pta* locus), 2316 (*tpi* locus) and 2521/2523 (*tpi* locus).

8.3.3 Melting typing by real time PCR and high-resolution melting analysis

Primers were manually designed in the regions flanking the identified SNPs in each locus. We wrote in-house programs as "do" files in Stata 10.9 (StataCorp, Texas) to analyse regions internal to the primers. We have called the program HRMType and it generates a prediction of the number and order of HRM curves for each region based on the G+C content from the MLST sequence data. Each predicted curve was given a number based on its G+C content and a HRM profile was generated for each ST based on the expected curve number for each region. This profile was designated the "Melting Type" (MelT) for the ST and a key was generated which facilitated the translation between MelT profile and ST.

8.3.4 Isolates used for validation of methodology

Isolates used for this study were clinical *S. aureus* isolates collected as part of a case-control study at the Royal Darwin Hospital from April 2006 to April 2007[16]. A single colony on Horse Blood agar was inoculated into 5mL of Todd-Hewitt broth (Oxoid) and grown overnight at 37°C with agitation. DNA was extracted from the pelleted cells using a QIAamp DNA mini kit (Qiagen) using the protocol for Gram positive bacteria with lysostaphin. Purified DNA was eluted in 200ul 10mM Tris HCl, 0.5mM EDTA, pH9, and diluted 1:10 in H_2O prior to use in PCR.

Real time PCR reactions contained 5 µL Platinum® SYBR® Green qPCRSuperMix-UDG (2x, Invitrogen Life Technologies), 0.4µM of each primer and 1µl of the diluted DNA extraction in a total volume of 10ul. The reactions were performed on a Rotorgene 6000 device (Corbett Life Science). Cycling conditions were 50°C for 2 minutes, 95°C for 2 minutes followed by 40 cycles of 95°C for 3 seconds, 56°C for 18 seconds and 72°C for 12 seconds. HRM was carried out from 66°C to 84°C in 0.1^{o}C increments for 2 seconds each. For each fragment, primer sequences, fragment lengths and the predicted number of HRM curves are shown in **Table 8-1**.

Raw HRM curves were normalised by the Rotorgene 6000 software v 1.7 (Corbett) and the normalisation temperatures for the different fragments are provided in **Table 8-1**. Difference graphs of the normalised curves were obtained using a ST239 isolate as the baseline curve.

Table 8-1. Details of the six fragments used in the typing scheme.

Fragment name	SNP(s) position(s) in concatentated MLST sequence	Primers (5' – 3')	Fragment size (bp)	HRM normalisation regions (°C)	Number of predicted curves	Predicted ΔTm (°C)	Actual ΔTm (°C)
arcC78/210	78, 210	TGGATACTTGTGGTGCAATG CGTATAAAAAGGACCAATTGGTTT	181	74–75 81–82	6	0.23	0.19-0.25
aroE88/155	543, 610	TAAATATTCCAATTGAAGATTTTC CTGCATTAATCGCTTGTTCA	140	67–68 76–77	6	0.29	0.49-0.67
gmk286	1663	GAAGTAGAAGGTGCAAAGC CAAGTGATCTAAACTTGGAGG	83	69–70 78–79	4	0.49	0.32-0.73
pta294	2100	CCTTGTGAATCAAGTTCTGGATTG TGCAGCACATTCAACAGG	158	75–76 82–83	5	0.26	0.29-0.42
tpi36	2316	TTGATGATTTACCAGTTCCGATTG GATGAAGAAATTAACAAAAAAGCGCA	219	75–76 81–82	6	0.19	0.14-0.26
tpi241/243	2521, 2523	GCCCCATCAATATCAGTTTGTG GTAAATCATCAACATCTGAAGAT	168	74–75 80–81	4	0.24	0.42

The normalisation regions refer to the temperatures we selected to normalise the fluorescence curves using the Corbett Rotorgene software. The actual ΔT_m was calculated by finding the difference in the mean T_m of successive curves (each with multiple reactions) and is presented as the range of these differences. The ΔT_m for *aroE88/155* was calculated for its 2nd melting domain.

8.3.5 MLST of specific isolates and loci

We carried out sequencing of selected MLST loci of some isolates to clarify any discrepancies between the MelT results and the original kinetic PCR typing method, using standard MLST primers and methodology for *S. aureus*[2].

8.3.6 Determination of the concordance between HRM typing and the MLST-defined *S. aureus* population structure

Determination of the concordance between the predicted MelT and the MLST-defined population structure of *S. aureus* was carried out *in silico*. An eBURST analysis[17] was performed on the entire *S. aureus* MLST database. The group definition was set to "six alleles in common", and each group was defined as a clonal complex (CC). "Sub-CCs" within CCs were identified on the basis of the presence of a progenitor with at least five single locus variants (SLVs). In the case of large CCs with several sub-CCs and chains of STs with SLV relationships, the boundary between sub-CCs was placed at the half way point between sub-CC progenitors on the eBURST diagram. For each CC/subCC, the analysis was performed as follows:

1. The STs that belong to the CC/sub-CC were compiled.
2. The MelT(s) that correspond to all of these STs were compiled.
3. For each of these MelTs, the following steps were carried out:
    a. The complete list of STs that correspond to that MelT was compiled.
    b. Each of these STs within the MelT was classified according to whether or not it belonged to the CC/sub-CC of interest.
    c. The MelT was classified as to whether or not it was diagnostic for the CC/sub-CC of interest. The criterion was that at least 50% of the STs within a MelT must belong to the CC/sub-CC of interest, for that MelT to be diagnostic for that CC/sub-CC.
    d. All the STs in the MLST database were classified as being true positives, false positives, true negatives or false negatives. If the MelT was diagnostic for the CC/sub-CC, then STs that corresponded to this MelT, and were members of the CC/sub-CC of interest, were classified as true positives. STs that correspond to this MelT and did not belong to the CC/sub-CC of interest were classified as false positives. All other STs were classified as true negatives. If the MelT was not diagnostic for the

CC/sub-CC, then STs that correspond to this MelT, and were members of the CC/sub-CC of interest, were classified as false negatives. All other STs were classified as true negatives.

4. The true and false positives and true and false negatives from all the MelTs were summed.
5. The summed values were used to calculate the overall sensitivity, specificity and positive predictive value of the Mini-MLST (Minim) method for the CCs and sub-CCs.

8.3.7 Comparison with randomly generated fragments

We adapted HRMType to calculate the D value for randomly generated fragments from the concatenated sequence. Individual fragments were not allowed to span more than one MLST locus in the concatenated sequence. We used fragment sizes with the same size as our six chosen fragments, and also with sizes ranging from 20 to 150bp. Multiple iterations allowed us to determine the distributions of D and the transformation $-log_{10}(1\text{-}D)$ for these randomly generated fragments. Additionally, for each position in the MLST concatenated sequence we performed a multivariate linear regression analysis with the outcome variable $-log_{10}(1\text{-}D)$ and the independent variable was the absence or presence of that position within the selected fragments. Adjustment was made for fragment size. This provided a measure of association between the presence of that position and the $-log_{10}(1\text{-}D)$ and this coefficient of association was plotted against the concatenated position to generate a SNP association map. This analysis was repeated for eight other bacterial species using MLST data from the relevant websites linked to http://www.mlst.net/. The bacterial species were *Burkholderia pseudomallei*, *Campylobacter spp.*, *Enterococcus faecium*, *Haemophilus influenza*, *Streptococcus agalactiae*, *Streptococcus pneumonia*, *Streptococcus pyogenes*, and *Streptococcus suis*.

8.4 Results

8.4.1 Majority of SNPs are informative for high-resolution melting analysis in the MLST sequence data

A total of 1484 STs from the *S. aureus* MLST database were included in the final alignment of concatenated MLST sequences. Unidirectional nucleotide pair frequency calculations revealed that each ST differed from any other ST at an average of 27 nucleotides and that 22 of these involved a change in G+C content, indicating that overall 81% of SNPs would be informative on the HRM platform.

8.4.2 HRM curve prediction

The melting temperature is dependent on the length and G+C content of DNA fragments and salt concentration of the mixture. Where the salt concentration and length of fragments are constant the difference in melting temperatures (ΔT_m) can be expressed as $\Delta T_m = 41((GC_2 - GC_1)/N)$, where GC is the number of G+C residues and N the total length of the fragments[18, 19]. The predicted ΔT_m for a change of one GC for each of the fragments is provided in **Table 8-1**. It would be expected that the resolving capability of HRM is likely to decrease as fragment size increases and that once fragments are greater than 200bp the ΔT_m will be less than 0.2°C.

Each ST was assigned a MelT profile based on the HRM curves predicted for the 6 regions from their allele sequence data using HRMType. The 6 regions are highly discriminatory with a predicted 269 MelT profiles and a *D* of 0.979 compared to full MLST for the 1484 STs used

8.4.3 Validation on previously typed isolates

We used a collection of clinical isolates[16] which had previously been assigned to clonal complexes by a SNP-based kinetic PCR technique and a limited number had full MLST data for validation. We chose over 100 isolates that represented the spectrum of the clonal complexes identified and that would be predicted to provide the greatest representation of curves for each amplified region. Within our collection we had 4 of the 6 predicted curves for *arcC78/210*, 4 of 6 for *aroE88/155*, 3 of 4 for *gmk286*, 3 of 5 for *pta294*, 5 of 6 for *tpi36* and 2 of 4 for *tpi241/243* (**Figure 8-1**).

The HRM curves were reproducible and we could consistently discriminate between curves. As an example, for *arcC78/210* the mean difference in T_m between replicates of the same isolate was 0.03°C (SD 0.02) and there was no overlap between curves with the ΔT_m ranging from 0.19 to 0.25°C (**Figure 8-1A**). The actual ΔT_m values for each fragment were approximately as predicted except for the curves for *aroE88/155* where two melting domains present in the fragment resulted in inaccurate ΔT_m predictions.

For *aroE88/155* and *tpi*36 extra curves which had not been predicted were also observed (**Figure 8-1**). For the *aroE88/155* fragment isolates with 24 G+C residues were separated into two groups. These curves (designated curve 24 and 24.5) showed very different melt curves with curve 24.5 melting earlier in the first melting domain than both curves 24 and 25 but being indistinguishable in the later melting domain from curve 25 (**Figure 8-1B**). Analysis of the G+C content over the length of the *aroE* fragment using a window size of 5 and an increment of 1 clearly showed the presence of two regions of differing G+C content. The order of curves 24, 24.5 and 25 could be predicted by analysing the G+C content of the two domains separately (data not shown). An additional curve was also seen for *tpi*36 (**Figure 8-1E**). ST93 isolates produced a curve which melted later than the curve 66 predicted for this fragment and sequencing of *tpi* in these isolates confirmed that there were no unexpected SNPs. Analysis of the G+C content along the length of the *tpi* fragment sequence showed that the ST 93 allele had a slightly higher G+C content in the central region of the fragment possibly accounting for its later melting curve. We assigned ST93 as curve 67 for *tpi*36 as its T_m was approximately that of the predicted curve 67, although we had no representatives of curve 67 in our collection. We refined the MelT key by assigning STs with the identical sequence in the interrogated regions for *aroE88/155* and *tpi36* to these additional curves which added further resolving power to the method. With this refinement, there were 300 MelT profiles and a *D* value of 0.980. An example of the key for the first 30 STs is provided in **Table 8-2**.

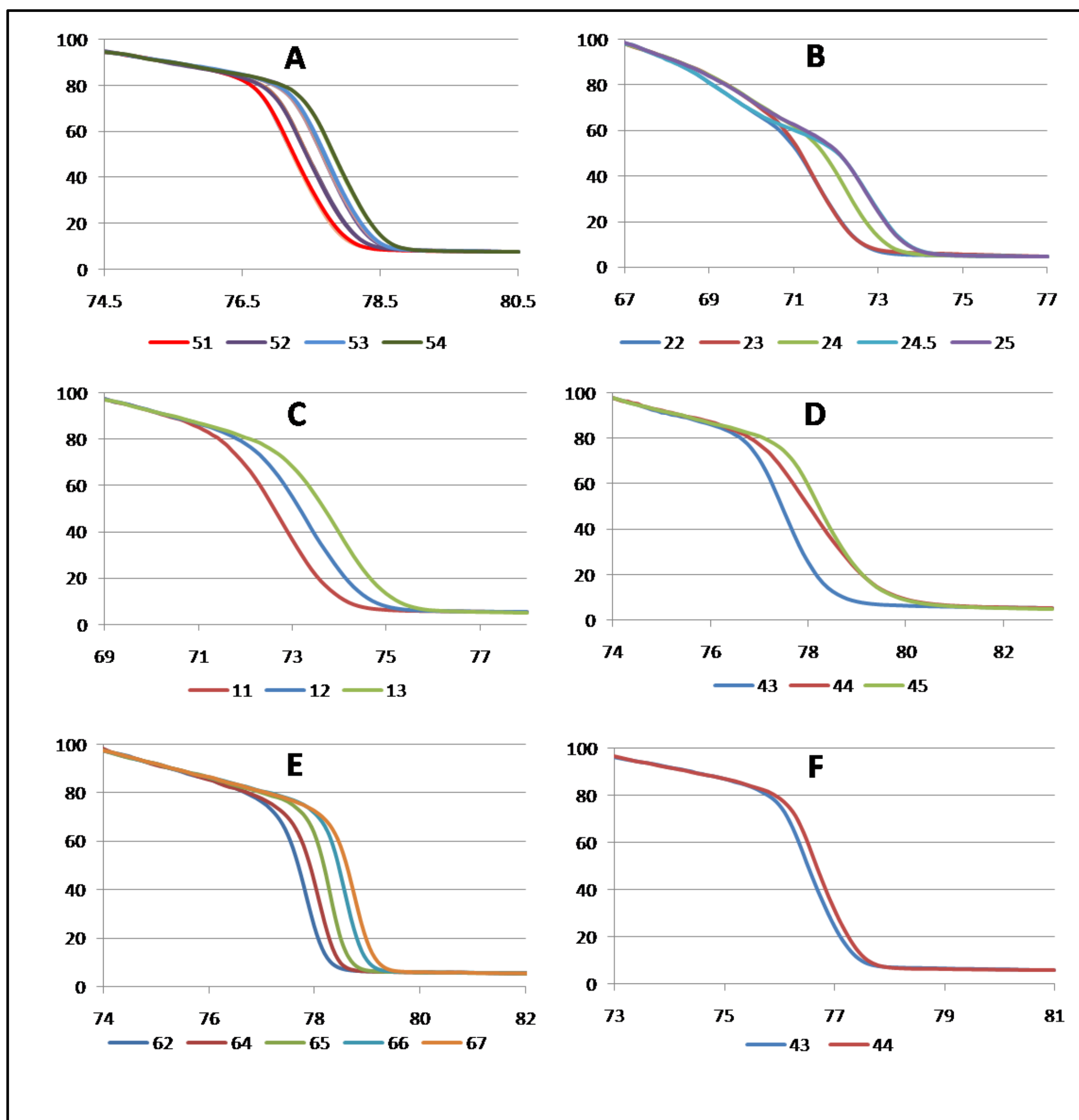

Figure 8-1. High-resolution melting curves for the six fragments.

The six fragments are *arcC78/210* (A), *aroE88/155* (B), *gmk286* (C), *pta294* (D), *tpi36* (E) and *tpi241/243* (F). The x-axis refers to the temperature in °C and the y-axis the normalised fluorescence. The curves are labelled by the number of G+C residues contained in the corresponding fragment. For *arcC78/210* there are 24 curves presented to demonstrate the reproducibility and ability to discriminate multiple curves. All other regions are presented with one representative curve. The only unexpected order of curves was for *tpi36* where the sequence for ST93 was predicted to result in curve 66 but instead resulted in curve 67.

The HRM curves can also be represented as difference graphs by using one of the curves as a baseline comparator. We chose to always use a ST239 isolate as the comparator because it is a commonly found ST and its curves are generally one of the middle curves for each fragment. For the *aroE88/155* fragment in particular, the difference graph better demonstrated the separation between curves (**Figure 8-2**).

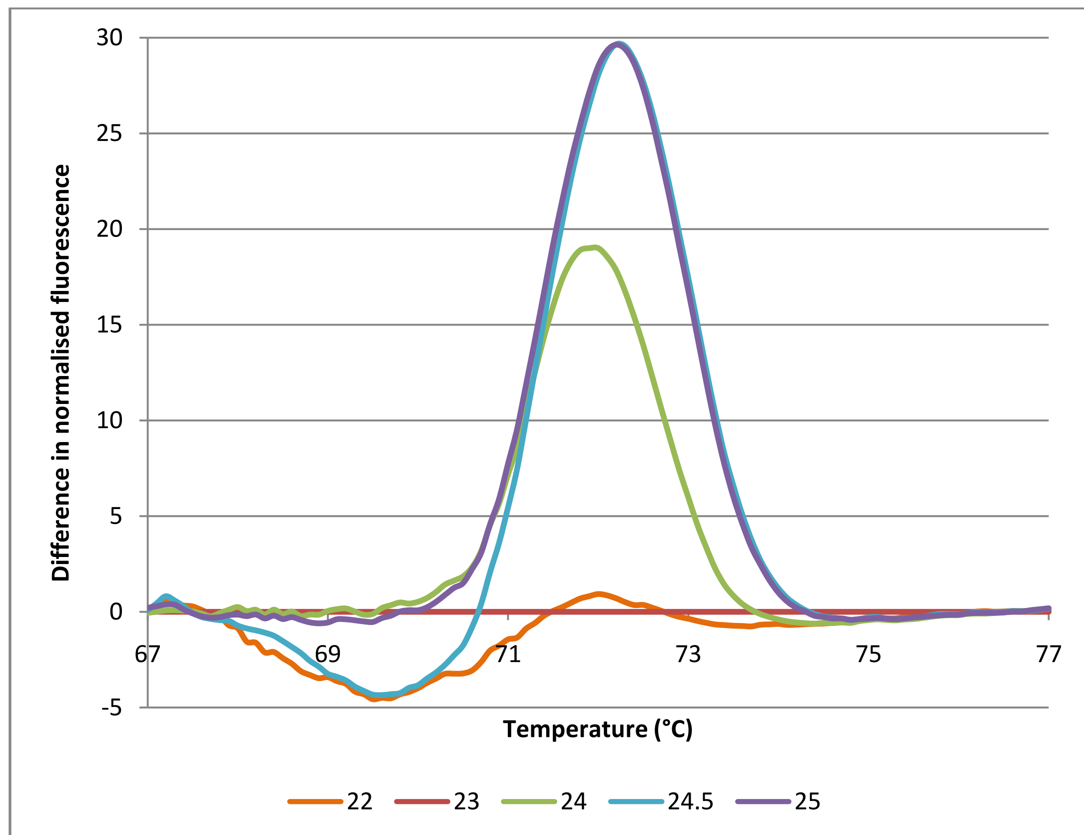

Figure 8-2. Difference graph for *aroE88/155.*

The high-resolution melting curves are compared with curve 23 (which represents ST239) as the baseline. The two melting domains are evident with the first between 68 and 71°C and the second between 71 and 74°C.

The results obtained with the HRM typing method largely correlated with the results previously obtained using the kinetic PCR SNP typing method. For those isolates where a discrepancy occurred between the methods, the relevant MLST allele of the isolate was sequenced. In all cases the HRM typing result was correct, with the kinetic PCR SNP result either failing to resolve a single locus variant within a clonal complex or being incorrectly recorded in a small number of cases. The HRM was able to discriminate the common local CA-MRSA ST93 from all other STs, which the SNP typing method did not. A table of isolates and corresponding results based on kinetic PCR SNP typing, HRM typing and sequencing is provided in **Appendix I**.

Table 8-2. The key for sequence types, HRM curve numbers and melting types.

ST	arcC	aroE	gmk	pta	tpi36	tpi241	MelT
1	53	24	13	43	66	43	**225**
2	51	24.5	12	45	65	43	**69**
3	53	24	13	43	66	43	**225**
4	53	24.5	12	44	65	44	**239**
5	53	23	12	43	66	43	**193**
6	52	23	12	43	66	43	**95**
7	52	23	12	44	65	43	**103**
8	52	23	13	44	65	43	**119**
9	52	23	13	43	66	43	**114**
10	52	23	12	43	64	44	**92**
11	53	23	12	43	66	43	**193**
12	53	23	12	44	64	44	**196**
13	53	23	12	44	64	44	**196**
14	53	22	13	43	65	43	**182**
15	54	22	13	43	65	43	**267**
16	53	22	13	43	65	43	**182**
17	51	23	12	44	64	43	**23**
18	54	22	13	43	65	43	**267**
19	51	24.5	12	44	62	43	**60**
20	54	23	12	43	65	43	**271**
21	53	25	11	43	64	44	**248**
22	53	25	11	43	64	44	**248**
23	53	25	11	43	64	44	**248**
24	51	24.5	12	45	65	43	**69**
25	54	24	13	43	64	44	**290**
26	54	24	13	43	64	44	**290**
27	52	23	13	43	66	42	**113**
28	54	24	13	43	64	44	**290**
29	51	24.5	12	44	62	43	**60**
30	51	24.5	12	45	65	44	**70**

Note. Only the first 30 of 1484 sequence types are provided here. The full key is provided as **Appendix II**. ST, sequence type; MelT, melting type.

8.4.4 HRM genotypes are concordant with the *S. aureus* population structure

It is desirable that the results of the Minim typing method for *S. aureus* be concordant with the population structure defined by MLST. This would allow the inference of the CC from the MelT data. This was tested against 40 CCs and "sub-CC's", which are CCs within larger CCs (**Table 8-3**). This demonstrates that Minim typing is very effective at assigning isolates to CCs. The concordance is least accurate with the closely related CC8, CC5 and CC7. Sub-CC6 (regarded here as part of CC5) is discriminated from ST5, but not from several ST5 SLVs. CC7 is not discriminated from sub-CC247 which is within CC8 (**Table 8-3**).

Table 8-3. Concordance between HRM types and MLST sequence type

CC	Sub-CC	Specificity	Sensitivity	PPV
8	All	97%	97%	83%
8	8	99%	92%	89%
8	239	99%	91%	79%
8	72	99.6%	71%	63%
8	707	99.8%	83%	63%
5	All	99%	93%	91%
5	5	99%	86%	85%
5	9	99.9%	96%	93%
5	6	99%	89%	30%
5	228	99%	46%	43%
30	All	99%	96%	93%
30	30	99%	85%	87%
30	34	99.5%	79%	68%
30	39	99%	100%	43%
30	36	Not discriminated from CC30, sub-CC30		
1	All	98%	94%	87%
1	1	99%	90%	81%
1	97	99%	95%	72%
1	188	99.8%	77%	85%
1	71	Not discriminated from CC1, sub-CC1		
1	573	Not discriminated from CC1, sub-CC1		
45	All	99.6%	89%	94%
15	All	99.8%	96%	94%
121	All	99%	91%	83%
22	All	100%	100%	100%

59	All	99.8%	97%	91%
88	All	99.9%	92%	92%
88	88	100%	80%	100%
88	78	99.9%	100%	90%
133	All	100%	100%	100%
398	All	99%	95%	66%
12	All	99%	89%	68%
25	All	99.9%	95%	95%
151	All	99.8%	100%	80%
89	All	100%	81%	100%
20	All	99,7%	93%	78%
80	All	100%	93%	100%
7	All	99%	75%	33%
101	All	99.8%	90%	75%

8.4.5 Comparison with randomly generated fragments

To determine if using Minimum SNPs to nucleate fragments is more efficient than randomly choosing fragments we ran a simulation to randomly select 6 fragments, each fragment equal in size to our tested fragments. For simplicity we assumed that curves would be generated as predicted by the G+C content of the fragments. After 1000 simulations, the mean number of MelT profiles and mean $-log_{10}(1\text{-}D)$ values were 131 (SD 28) and 1.19 (SD 0.17) respectively, with none of the randomly selected combinations providing better resolution than our originally chosen set which provided 269 MelT profiles and a $-log_{10}(1\text{-}D)$ of 1.68 (**Figure 8-3**).

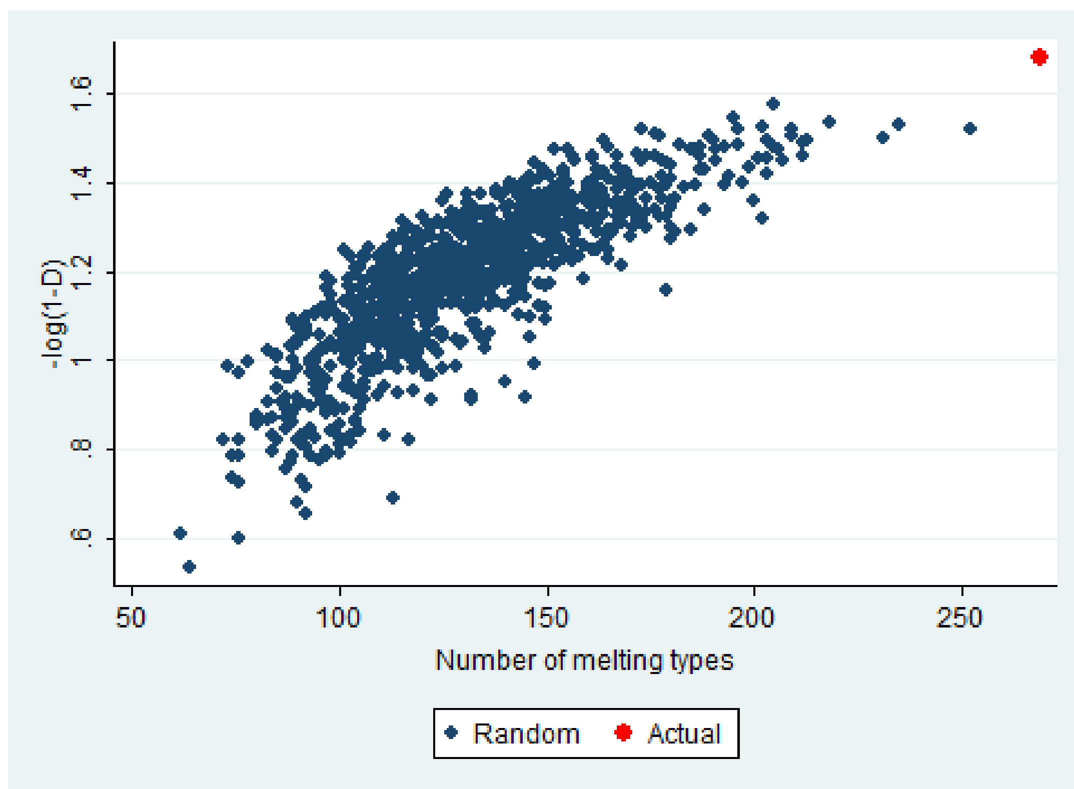

Figure 8-3. Comparison of resolution obtained with the actual selected fragments and 1000 randomly chosen fragments of the same size.

The actual fragments outperforms randomly selected fragments with the generation of more melting types and a larger $-log_{10}(1\text{-}D)$ value.

However we noted a wide range in resolution and to investigate the underlying reasons we ran the simulation to randomly select six fragments, with all fragments being of the same length in each simulation. This was performed 500 times each for fragment sizes of 20 to 150bp at increments of 10bp. Thus the dataset comprised 7000 randomly selected combinations of six fragments. Both the number of MelTs and $-log_{10}(1\text{-}D)$ value increased as the proportional coverage of the MLST concatenated sequence increased with fragments of increasing size (**Figure 8-4**). However there was a wide variation in the genotyping resolution. Using *S. aureus* as an example, with fragments of 20bp, there was a large range of *D* (0.03 to 0.88), $-log_{10}(1\text{-}D)$ (0.01 to 0.92) and number of MelTs (9 to 44). This suggested that certain regions were more important than others in obtaining greater resolution. The most likely explanation is that these regions contain highly informative SNPs.

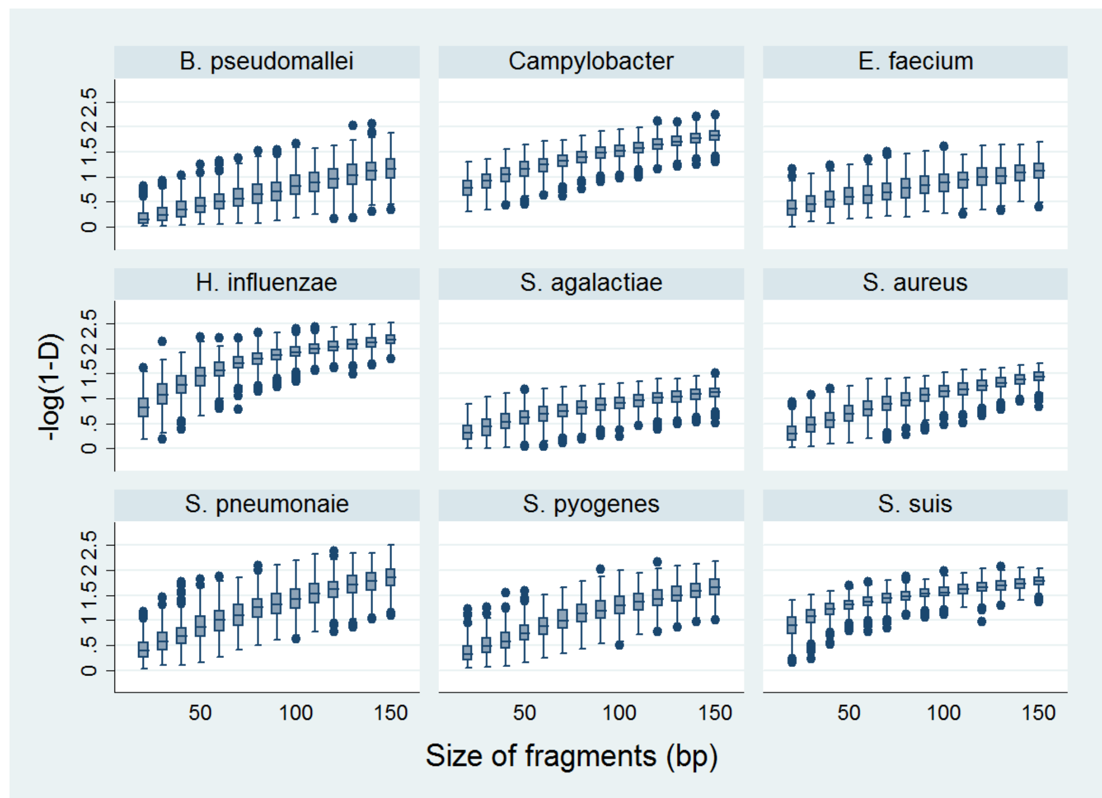

Figure 8-4. The resolution obtained with increasing coverage of the concatenated MLST sequence for 9 bacterial species.

The graphs were generated by performing 500 iterations with fragments of sizes 20 bp incrementing to 150 bp. The resolution obtained as measured by $-log_{10}(1\text{-}D)$ is shown to vary between bacterial species.

8.4.6 SNP association maps

Therefore, we investigated the association between increasing resolution and incorporation of individual SNPs in the randomly selected fragments by generating a fine mapping SNP association map. This demonstrated that there are eight regions of the concatenated sequence that, if included in the selected fragments, independently result in high *D* values (**Figure 8-5**). These regions correlate with the *D* optimised SNPs chosen by Minimum SNPs and the reason our originally selected six fragments performed so well is that the majority of these regions are included. Thus this SNP association map confirms the utility of Minimum SNPs but also provides a graphical means of representing the number and relative importance of D optimised SNPs across the whole concatenated sequence.

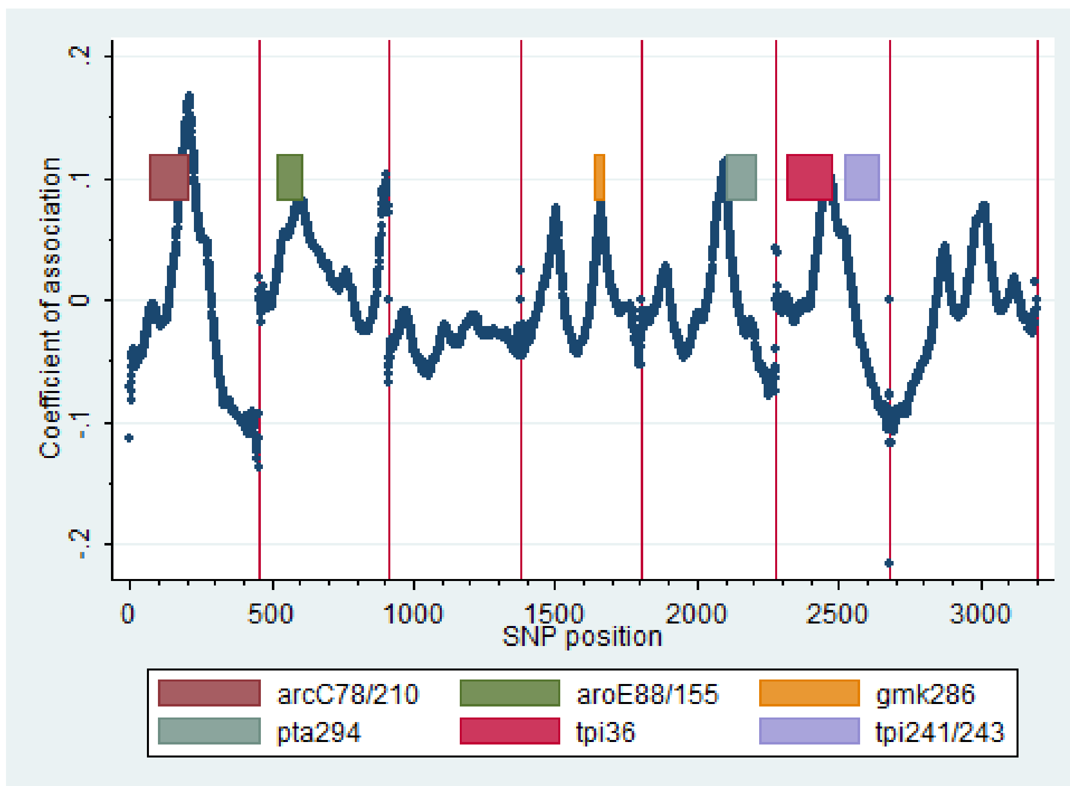

Figure 8-5. SNP association map for *S. aureus* with the positions of the 6 selected fragments.

Peaks in the coefficient of association demonstrate regions which, if included in random fragments, are associated with a high $-log_{10}(1\text{-}D)$ value (i.e., contain informative SNPs). The selected fragments were nucleated around informative SNPs determined by Minimum SNPs and this figure confirms that the SNPs and regions covered were well chosen.

We repeated the simulation of randomly choosing six fragments of sizes from 20bp to 150bp for eight other bacterial species and compared the SNP association maps with that of each other and of *S. aureus*. This revealed that there are informative SNPs in all these species, but the number and degree of association of the SNPs varies (**Figure 8-6**). These SNP association maps can be used to guide the selection of fragments to optimise the *D* value. We also demonstrated that the resolution able to be achieved is not uniform between bacterial species (**Figure 8-4**). For example, it requires less coverage of the concatenated sequence to obtain a median *–log(1-D)* >1.0 for *H. influenzae* (6 fragments of size 30bp, or 180bp/3057bp (6%)) compared to *S. aureus* (6 fragments of 90bp, or 540bp/3198bp (17%)) and *B. pseudomallei* (6 fragments of 130bp, or 780bp/3399bp (23%)). It is apparent that bacterial species with large clusters of related sequence types as determined by eBurst analysis[17] (e.g., *B, pseudomallei*, *S. aureus* and *E. faecium*) are more difficult to resolve using SNP based methods than species with fewer and smaller clusters of related sequence types (e.g., *H. influenzae*, *S. pneumoniae*, and *S. suis*). For example, *B. pseudomallei* has two populations; an Australian population that has greater allelic diversity, and a Southeast Asian population that has a high frequency of recombination resulting in a large cluster of highly connected sequence types[20].

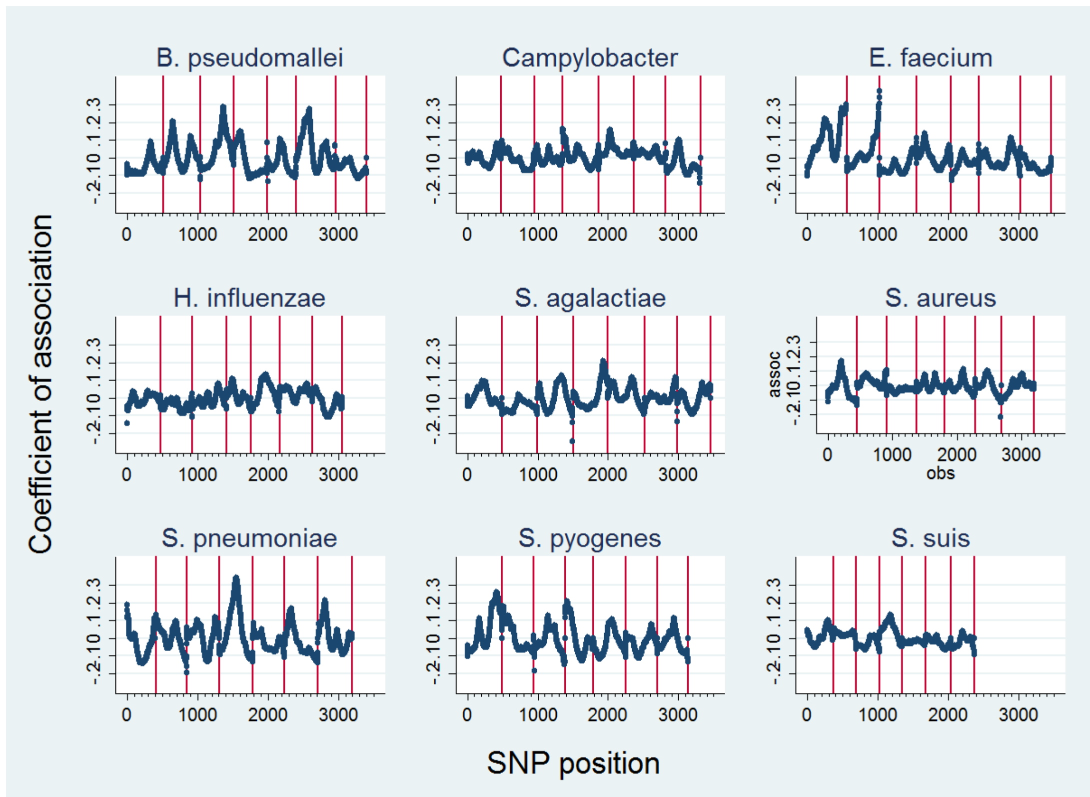

Figure 8-6. SNP association maps for 9 bacterial species.

There is variation in the number and degree of association of informative SNPs when bacterial species are compared. The selection of informative SNPs and regions for typing schemes can be guided by these graphs.

8.5 Discussion

Despite the increasing capability to rapidly generate sequencing data at multilocus and genome wide levels, laboratories of smaller size and in resource-poor regions are likely to continue to find the cost and timeliness of such technologies prohibitive. However, the availability of well curated databases of these sequences on the internet allows mining of this data to find highly informative subsets of the data. We demonstrate the practical application of such an approach to genotyping *S. aureus* by determining highly informative SNPs in multilocus sequence typing databases and interrogating these SNPs with the emerging but relatively low cost high-resolution melting platform. Six single-step PCR and HRM reactions are shown to provide a highly informative degree of genotyping resolution.

The predicted order of melting temperatures based on total G+C content was essentially accurate with the exception of *aroE88/155* which melted in two domains. While this was not predicted, incorporating information from the melting curve shape actually adds to the resolving power from that fragment. In addition, the *tpi36* fragment produced an extra melt curve for ST93 which had not been predicted based on the total G+C content alone. The ST93 allele had a slightly higher G+C content in the central region of the fragment which may account for its later melting curve. The key for converting MelTs to MLSTs was easily adjusted to incorporate this additional information. Our experience suggests that targeting smaller fragments will make the predicted keys more accurate and that HRM curves from fragments of >200bp may be more difficult to resolve as the difference in melting temperatures between curves will be <0.2°C.

The concordance between *S. aureus* MLST and Minim typing is very good. The experiment carried out was a very stringent test of concordance. The results in general and positive predictive values in particular represent an underestimate of the utility of Minim typing for inferring an isolate's CC. Several factors contribute to this underestimation. Firstly, all STs were weighted equally in the calculations. However, eBURST analysis of the *S .aureus* MLST database reveals that the isolates that possess the MLST of the CC progenitor are over-represented in actual collections of isolates, and the CC progenitors are very effectively discriminated by Minim typing. Secondly, "false positives" have a great impact on the positive

predictive value when, as is the case in this analysis, the true positives are a small proportion of the total. The false positives were STs that are singletons or members of the "wrong" CC that possess a MelT characteristic of a particular CC. The great majority of examples arise from instances in which the singleton or the "wrong" CC is closely related to the correct CC, or the ST is intermediate between two different CCs. In other words, the numbers of false positives are overstated, because of, for example, STs that are double locus variants (DLVs) of CC progenitors but simply happen not to be linked to the CC progenitor by a chain of SLV relationships, and are in consequence classed as singletons in this analysis. And thirdly the MelTs that are ambiguous with respect to the CC defined are known. If unambiguous assignment to a CC is necessary, then the "ambiguous" MelTs may trigger further analysis using e.g. the interrogation of additional CC specific SNPs or full MLST analysis. However, for many purposes, defining the isolate simply on the basis of its MelT, and accepting some ambiguity in the assignment to CCs, would be sufficient.

A limitation of the method is that new sequence types may not be detected. This could occur if SNPs are found outside of the amplified fragments or, if within the amplified fragments, SNPs in new alleles result in the same G+C content as previously known alleles. However, the use of six fragments and the accompanying key means that isolates with combinations of curve alleles that do not fit into the existing key are likely to represent new STs and should be fully sequence typed to confirm this. We identified two isolates that are single-locus variants of ST93 by such a means.

Our methods can be applied to other important bacterial species and we provide the theoretical basis for choosing SNPs and indications as to the resolving power achievable for these different species. The use of HRMType allows the accurate *in-silico* generation and optimisation of the typing scheme. This software could be adapted to select fragments and generate a key for other platforms such as with base composition analysis using PCR/ESI-MS[5]. It is also flexible in that the number, size and position of the fragments can be varied and therefore a typing scheme can be tailored according to the needs of the user, balancing convenience, resolving power and cost.

HRM based typing has several attractive features including that the amplification and HRM analysis is a single-step, closed tube process. It is rapid, taking a matter of

hours from DNA extraction to HRM, and relatively low cost, at approximately one tenth the cost of full MLST. It is equally cost effective as a high-throughput method or for small numbers of samples and with appropriate calibration can allow for inter-laboratory comparison of results. Our typing scheme accurately resolves isolates into MLST clonal complexes and could usefully be incorporated into a progressive hierarchical typing scheme[21]. For *S. aureus* this could be used in conjunction with interrogation of hypervariable regions (e.g. *spa* typing[3, 22]) and virulence and resistance genes.

8.6 References

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Chapter 9. Conclusions

9.1 Findings and implications of the epidemiological studies

Since initial reports of the presence of community-associated MRSA in the Northern Territory from the early 1990s[1, 2], there has been little documented research to further elucidate the epidemiology of *S. aureus*, and particularly community-associated MRSA, in our region. Interest in this area was re-invigorated when community-associated MRSA was found to represent 23% of *S. aureus* isolates recovered from impetigo lesions in children from remote Aboriginal communities in 2004–2005[3]. However, it was unclear what the burden and pattern of disease was in patients in the hospitals for the region.

The major findings and implications of the epidemiological studies conducted were:

- An annual incidence of *S. aureus* bacteraemia of 172 cases per 100,000 in the Aboriginal population of the Top End, which was 5.8 times that of the non-Aboriginal population.
- Community-associated MRSA accounts for 15% of *S. aureus* isolates recovered from patients in the three Top End hospitals. This needs to be considered by clinicians instituting empirical treatment for patients with suspected staphylococcal infections.
- The disease spectrum and outcomes of infections due to community-associated MRSA and MSSA were similar. This suggests that in our setting, virulence is not convergent with resistance phenotype.
- Community-associated MRSA infections, in comparison to those caused by MSSA, were associated with female sex and residence in a remote area. This finding supports the hypothesis that community-associated MRSA has arisen from remote communities where factors such as domestic crowding and poor hygiene as well as high rates of scabies, skin sores, and antibiotic use may contribute to the transmission and emergence of community-associated MRSA. Therefore, without improvements in the housing and health hardware of remote communities and implementation of programs to ensure better skin hygiene of

children, it is likely that community-associated MRSA will continue to represent a significant proportion of circulating *S. aureus* in northern Australia.

- Panton-Valentine leukocidin was harboured by 54% of community-associated MRSA and 40% of MSSA isolates. PVL was associated with truly community-acquired disease, younger age and presentations with sepsis. These findings provide confirmation of the epidemiological link between PVL and more severe disease, particularly in younger, healthier hosts.
- The greater burden of PVL+ disease is caused by MSSA. Focusing on community-associated MRSA will lead to under-recognition and under-estimations of the impact of PVL+ clones.
- There is a diversity of *S. aureus* lineages circulating in northern Australia. These lineages include the PVL+ CC93 and CC121, and the phylogentically diverged clonal complex 75. The relative abundance of CC93, CC121 and presence of CC75 in both northern Australia and Asia may relate to past human population movements.
- A pilot randomised controlled study comparing trimethoprim-sulfamethoxazole to benzathine penicillin for treatment of impetigo in Aboriginal children demonstrated the feasibility and acceptability of such a study in remote Aboriginal communities. These results were instrumental in obtaining funding to conduct a larger and more definitive randomised controlled trial.

9.2 Findings and advances from the laboratory studies

High-resolution melting analysis is an emerging technology that can be used to interrogate DNA fragments for variations in sequence and size. The advantages of the HRM platform are its rapid, single-step, closed tube nature and its relative low cost compared to full sequence analysis. HRM analysis was utilised for a number of applications.

The major findings and advances from the laboratory aspects of this thesis were:

- The development and application of bioinformatic tools to facilitate multilocus sequence typing (MLST) derived genotyping schemes using HRM analysis. The tools produced will allow an efficient pipeline to developing similar genotyping schemes for other bacterial species.
- Statistical and graphical methods that account for instrumental and operator-dependent variation in HRM curves were developed to allow greater confidence and reproducibility in deciding whether another curve is truly different from the baseline curve of an amplicon with known sequence.
- High-resolution melting analysis was used to interrogate the hypervariable *spa* locus and discriminate sequence variants of the Panton-Valentine leukocidin gene. This revealed surprising diversity in the *spa* locus of sequence type 93 MRSA and MSSA and supports the hypothesis that there is ongoing independent acquisitions of SCC*mec* by ST93-MSSA. The clinical outcomes of infections due to isolates harbouring the histidine and arginine PVL isoforms were compared and no significant differences were identified.

9.3 Future directions

There is a need for ongoing monitoring of the burden of community-associated MRSA compared to MSSA to guide empirical therapy of staphylococcal infections, particularly for patients with sepsis due to suspected *S. aureus*. Together with rapid diagnostics targeting resistance and virulence determinants of *S. aureus*, interventional studies comparing different treatment regimens would help determine optimal management approaches to such patients. However, to obtain sufficient power for such studies national and international collaborations will be required.

The epidemiology of *S. aureus* infections in hospital patients and also as a cause of impetigo in remote communities in northern Australia is now clearer. Studies to determine the diversity and transmission of colonising strains of *S. aureus* in both hospitalised patients and in remote communities would deepen our understanding of the population biology of *S. aureus* as it interacts with the human host. This would provide indications as to whether strains differ in their tropism for skin or nasal colonisation and whether they vary in their predilection to circulate within the hospital or community environments and to cause invasive disease.

The role of Panton-Valentine leukocidin in contributing to the pathogenesis of *S. aureus* continues to generate controversy. The predilection of PVL+ isolates to cause infections in younger rather than older hosts raises the possibility that exposure to PVL can generate protective immunity. This hypothesis could be pursued by conducting a serological survey across different age groups in our setting.

Two *S. aureus* lineages stand out as deserving further investigation. CC75 is significantly diverged from the rest of *S. aureus*[4] and found to probably be less virulent than other *S. aureus*. Further genomic studies are underway to elucidate its exact phylogenetic and taxonomic position as well as to seek explanations for possible differences in virulence potential compared to other *S. aureus*.

ST93 is a rapidly expanding lineage in Australia[5]. However, its origins are unclear and understanding the phylogeography of its emergence in the past decade in Australia would provide insights into the nature of public health interventions required to limit its expansion.

Several of the above studies will be facilitated by the application of the described MLST based HRM genotyping scheme. It is likely that such genotyping schemes will be useful for molecular epidemiological studies of other bacterial species.

9.4 References

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Chapter 10. Appendices

Appendix I: Isolates used for high resolution melting MLST based typing

Isolate	arcC78/210	aroE88/155	gmk286	pta294	tpi36	tpi241/3	Minim (CC/ST)	Kinetic PCR (CC)	MLST/sequencing	Likely MLST
1263	53	25	11	43	64	44	ST22	CC22		ST22
1283	52	23	13	43	66	43	CC9 inc. ST9	CC9		ST9
1284	53	25	11	43	64	44	ST22	CC22	aroE sequenced correct for ST22	ST22
1293	51	22	12	45	67	44	ST93	CC93	MLST = ST93	ST93
1299	51	22	12	45	67	44	ST93	CC93 "ST93"	MLST = ST93	ST93
1322	51	22	12	45	67	44	ST93	CC93	MLST = ST93	ST93
3004	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3035	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3049	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3054	52	23	12	44	66	43	ST779 (cc779?) or ST1088 (cc154)	CC7		ST779 or ST1088
3056	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3062	52	23	12	44	66	43	ST779 (cc779?) or ST1088 (cc154)	CC7	tpi allele 1, correct for ST779 & ST1088	ST779 or ST1088
3064	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3065	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3083	51	22	12	45	67	44	ST93	CC93 "ST93"	tpi correct for ST93	ST93
3084	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3090	51	22	12	After 45	67	44	SLV of ST93	CC93 "ST93"	tpi correct for ST93; pta has SLV of ST93	ST93 SLV
3093	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3094	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3095	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3103	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3106	51	24.5	12	44	62	44	CC121	CC93 "other"	tpi allele 14, correct for ST121	CC121
3110	53	23	12	44	65	43	CC8/ST72	CC72 (CC8 - ST72?)		ST72/CC8
3113	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3115	51	24.5	12	44	62	44	CC121	CC93 "other"	tpi allele 14, correct for ST121	CC121
3132	51	24.5	12	44	62	44	CC121	CC93 "other"	tpi allele 14, correct for ST121	CC121
3134	54	24	13	43	64	44	CC25	CC25		CC25
3138	51	22	12	45	67	After 44	SLV of ST93	CC93 "ST93"	tpi SLV for ST93	ST93 SLV
3156	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3157	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3161	52	24	12	43	66	43	CC1 inc. ST188	CC188 (CC1 - ST188?)	MLST = ST188	ST188
3172	51	24.5	12	45	65	43	CC30 inc. ST39	CC39 (includes ST39)	aroE & gmk have been sequenced, correct for ST39	ST39
3177	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3179	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3193	51	24	12	43	65	43	CC88 inc. ST88	CC6 (includes ST88)	tpi sequenced correct for ST88	ST88
3194	53	23	12	44	64	44	CC12 inc. ST12	CC12	pta & tpi have been sequenced results don't match any known ST	New ST
3217	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3222	54	23	12	43	65	43	CC20 inc. ST20	CC20		CC20 inc. ST20
3239	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3247	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3260	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3285	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3296	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3299	51	24.5	12	44	62	44	CC121	CC93 "other"	aroE correct for ST121, 51, 29, 120, 95, 577 (all CC121)	CC121
3304	52	24	13	43	64	44	CC97 inc. ST70	CC70 (CC97 - ST70?)		CC97 inc. ST70
3307	51	22	12	45	67	44	ST93	CC93 "ST93"	tpi correct for ST93	ST93
3307	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3308	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3316	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3333	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3334	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3342	51	24.5	12	44	62	44	CC121	CC93 "other"		CC121
3370	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3404	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3406	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93

3417	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3420	54	23	12	43	65	43	CC20 inc. ST20	CC20		CC20 inc. ST20
3422	51	24.5	12	44	62	44	CC121 inc. ST121	CC93 "other"	aroE sequenced correct for ST121	ST121
3428	51	22	12	?	67	44	ST93	CC93 "ST93"		ST93
3434	51	24.5	between 11 & 12	44	62	44	looks to be CC121	CC93 "other"		
3437	52	24	12	44	65	43	CC101 inc. ST101	CC7 (includes ST101)	aroE sequenced to give allele 1. this is a ST101 allele	ST101
3455	51	22	12	45	67	44	ST93	CC93 "ST93"	tpi correct for ST93	ST93
3455	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3458	51	22	12	45	67	44	ST93	CC93 "ST93"		ST93
3477	52	24.5	12	44	65	44	CC45/ST617; OR ST454 (singleton)	CC93 "other"		ST617 (CC45) or ST454
3488	52	24.5	12	45	65	44	CC30, inc. ST34, ST189, but not ST30	CC30 (includes ST34)	aroE correct for ST30, 34 and 189; arcC correct for ST34, not 189 or 30	ST34
3508	52	24	13	43	64	44	CC97 inc. ST70	CC70 (CC97 - ST70?)		CC97 inc. ST70
3522	51	23	13	44	65	43	CC8 inc. ST239	CC239 (CC8 - ST239?)		ST239
3538	54	22	13	43	65	43	CC15 inc. ST15 (but not ST14)	CC14 (CC15 - ST14?)	tpi sequnced correct for ST14 & ST15	ST15
3600	53	24	13	43	66	43	CC1	CC1		ST1
3601	54	22	13	43	65	43	CC15 inc. ST15 (but not ST14)	CC14 (CC15 - ST14?)	tpi allele 11, correct for ST14 & ST15	ST15
3608	51	22	12	45	67	44	ST93	CC93 "ST93"	tpi correct for ST93	ST93
3617	54	22	13	43	65	43	CC15 inc. ST15 (but not ST14)	CC14		ST15
3619	53	23	12	43	66	43	ST5	CC5	arcC, aroE and tpi sequenced, so far correct for ST5	ST5
3624	52	23	13	44	65	43	CC8	CC8	arcC, aroE, gmk, tpi have been sequenced, correct for ST8	ST8
3640	54	24	13	43	64	44	CC25	CC25	arcC has been sequenced, correct for ST25	ST25
3653	54	22	13	43	65	43	CC15 inc. ST15 (but not ST14)	CC14 (CC15 - ST14?)		ST15
3655	53	24.5	12	44	65	44	CC45 inc. ST45	CC45		CC45 inc. ST45
3660	51	23	13	44	65	43	CC8 inc. ST239	CC239 (CC8 - ST239?)	tpi sequenced correct for ST239	ST2239
3667	51	24	12	43	65	43	CC88 inc. ST88	CC6 (includes ST88)	arcC, aroE and tpi sequenced correct for ST88 (cc88).	ST88
3669	52	23	12	44	66	43	ST779 (cc779?) or ST1088 (cc154)	CC7	tpi allele 1, correct for ST779 & ST1088	ST779 or ST1088
3677	54	23	12	43	65	43	CC20 inc. ST20	CC20		CC20 inc. ST20
3694	51	23	12	43	66	43	CC6/ST1018	CC6		
3702	54	22	13	43	65	43	CC15 inc. ST15 (but not ST14)	CC14 (CC15 - ST14?)	tpi allele 11, correct for ST14 & ST15	ST15
3704	53	23	12	43	66	43	ST5	CC5	aroE and glpFsequenced correct for ST5	ST5
3710	53	24	13	43	66	43	CC1	CC1	arcC, aroE, pta, tpi have been sequenced, correct for ST1	ST1
3713	53	24	13	43	66	43	ST1	CC20	tpi allele 1; correct for ST1, incorrect for CC20	ST1
3716	52	24	12	43	66	43	CC1 inc. ST188	CC188 (CC1 - ST188?)		ST188
3732	52	23	13	44	65	43	CC8	CC8		ST8
3746	53	23	12	43	66	43	ST5	CC5	MLST = ST5	ST5
3782	51	24.5	12	45	65	44	CC30 inc. ST30	CC30	MLST = ST30	ST30
3807	51	24.5	12	44	62	44	CC121 inc. ST121	CC93 "ST93"		CC121
3816	54	22	13	43	65	43	CC15 inc. ST15 (but not ST14)	CC14 (CC15 - ST14?)		ST15
staph123	53	23	12	43	66	43	CC5 inc. ST5	CC5		ST5
		Refers to where there is discrepency between Minim and kinetic PCR results. Most of these relate to isolates that were called as CC93 on kinetic PCR, but are in fact CC121.								
		Refers to isolates revealed to be new sequence types.								

Appendix II: Key for Minim typing scheme

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1	53	24	13	43	66	43	**225**	1	1
2	51	24.5	12	45	65	43	**69**	30	39
3	53	24	13	43	66	43	**225**	1	1
4	53	24.5	12	44	65	44	**239**	45	
5	53	23	12	43	66	43	**193**	5	5
6	52	23	12	43	66	43	**95**	5	6
7	52	23	12	44	65	43	**103**	7	
8	52	23	13	44	65	43	**119**	8	8
9	52	23	13	43	66	43	**114**	5	9
10	52	23	12	43	64	44	**92**	10	
11	53	23	12	43	66	43	**193**	5	5
12	53	23	12	44	64	44	**196**	12	
13	53	23	12	44	64	44	**196**	12	
14	53	22	13	43	65	43	**182**	15	
15	54	22	13	43	65	43	**267**	15	
16	53	22	13	43	65	43	**182**	15	
17	51	23	12	44	64	43	**23**	singleton	
18	54	22	13	43	65	43	**267**	15	
19	51	24.5	12	44	62	43	**60**	singleton	
20	54	23	12	43	65	43	**271**	20	
21	53	25	11	43	64	44	**248**	22	
22	53	25	11	43	64	44	**248**	22	
23	53	25	11	43	64	44	**248**	22	
24	51	24.5	12	45	65	43	**69**	30	39
25	54	24	13	43	64	44	**290**	25	
26	54	24	13	43	64	44	**290**	25	
27	52	23	13	43	66	42	**113**	5	9
28	54	24	13	43	64	44	**290**	25	
29	51	24.5	12	44	62	43	**60**	121	
30	51	24.5	12	45	65	44	**70**	30	30
31	51	24.5	12	45	65	44	**70**	30	30
32	51	23	12	45	65	44	**30**	30	30
33	51	24.5	12	45	65	44	**70**	30	30
34	52	24.5	12	45	65	44	**163**	30	34
35	54	22	13	43	64	43	**265**	15	
36	51	24.5	12	45	65	44	**70**	30	36
37	51	24.5	12	45	65	44	**70**	30	30
38	51	24.5	12	45	65	44	**70**	30	36
39	51	24.5	12	45	65	43	**69**	30	39
40	51	24.5	12	45	65	43	**69**	30	30
41	51	24.5	12	45	65	43	**69**	30	39
42	52	24.5	12	45	65	43	**162**	42	
43	51	24.5	12	45	65	43	**69**	30	39
44	53	25	11	43	64	44	**248**	22	
45	53	24.5	12	44	65	44	**239**	45	
46	53	24.5	12	45	65	44	**242**	45	
47	53	24.5	12	44	65	44	**239**	45	
48	53	24.5	12	44	65	44	**239**	45	
49	52	24.5	12	44	64	44	**156**	49	
50	51	24.5	12	44	65	45	**66**	50	
51	51	24.5	12	44	62	43	**60**	121	
52	52	24.5	12	44	64	44	**156**	singleton	
53	53	24.5	12	44	65	44	**239**	45	
54	53	24.5	12	44	65	44	**239**	45	
55	52	23	13	44	63	44	**115**	singleton	
56	54	22	13	43	65	43	**267**	15	
57	51	24.5	12	45	65	44	**70**	30	30

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
58	54	23	13	43	65	43	**279**	15	
59	52	23	12	44	64	43	**100**	59	
60	53	25	11	43	64	44	**248**	22	
61	54	22	13	43	65	43	**267**	15	
62	51	23	12	44	64	44	**24**	5	6
63	53	23	13	43	66	43	**205**	5	9
64	51	23	12	44	64	44	**24**	singleton	
65	51	23	12	44	64	44	**24**	65	
66	53	23	12	44	64	44	**196**	singleton	
67	53	23	12	44	64	44	**196**	67	
68	53	23	12	44	64	44	**196**	67	
69	53	24	13	43	66	43	**225**	1	1
70	52	24	13	43	64	44	**141**	1	71
71	52	24	13	43	64	44	**141**	1	71
72	53	23	12	44	65	43	**197**	8	72
73	53	23	12	43	66	43	**193**	5	5
74	51	24.5	12	45	65	44	**70**	30	30
76	53	24	13	43	66	43	**225**	1	1
77	51	24.5	12	44	65	44	**65**	30	30
78	51	24	12	43	64	43	**41**	88	78
79	53	25	12	43	64	44	**253**	22	
80	53	23	13	44	65	44	**208**	80	
81	53	24	13	43	66	43	**225**	1	1
82	51	24	12	43	62	44	**40**	88	88
83	53	23	12	43	66	43	**193**	5	5
84	53	24.5	12	43	65	44	**234**	89	
85	52	23	12	43	66	43	**95**	5	5
86	51	23	12	44	65	43	**26**	8	72
87	52	23	12	45	64	43	**107**	59	
88	51	24	12	43	65	43	**42**	88	88
89	53	24.5	12	43	65	44	**234**	89	
90	53	23	12	43	66	43	**193**	5	5
91	53	24.5	12	43	65	44	**234**	89	
92	53	24.5	12	43	65	44	**234**	89	
93	51	22	12	45	67	44	**13**	singleton	
94	52	23	13	44	65	43	**119**	8	8
95	51	24.5	12	44	62	44	**61**	121	
96	52	24	12	44	66	43	**136**	154	
97	52	24	13	43	64	44	**141**	1	97
98	53	23	12	43	66	43	**193**	5	5
99	53	23	12	43	65	43	**190**	5	5
100	53	23	12	43	66	43	**193**	5	5
101	52	24	12	44	65	43	**134**	101	
103	51	24	12	43	65	44	**43**	88	88
104	52	23	12	45	65	44	**109**	singleton	
105	53	23	12	43	66	43	**193**	5	105
106	52	24	12	44	65	43	**134**	101	
107	53	23	12	44	65	43	**197**	8	72
108	53	24.5	12	44	65	44	**239**	45	
109	52	22	13	43	66	43	**89**	5	9
110	51	23	14	44	65	43	**38**	8	239
111	53	23	12	43	65	43	**190**	5	228
112	52	23	12	44	65	43	**103**	8	247
113	52	23	13	44	65	43	**119**	8	8
114	53	23	12	43	66	43	**193**	5	5
115	52	24	13	43	64	44	**141**	1	97
116	52	24	13	43	65	44	**144**	1	97

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
117	52	24	13	43	65	44	**144**	1	97
118	52	24	13	43	65	45	**145**	1	97
119	52	24	13	43	65	45	**145**	singleton	
120	51	24.5	12	44	62	44	**61**	121	
121	51	24.5	12	44	62	44	**61**	121	
122	54	24.5	12	44	65	44	**294**	45	
123	51	24.5	12	44	62	43	**60**	121	
124	52	24	13	43	64	44	**141**	1	97
125	53	23	12	43	66	43	**193**	5	5
126	52	23	12	43	64	44	**92**	126	
127	54	24	13	43	64	44	**290**	singleton	
128	51	23	13	44	65	43	**35**	8	239
129	51	24	12	43	64	43	**41**	88	78
130	51	24.5	12	44	65	44	**65**	130	
131	51	24	12	44	63	44	**46**	133	
132	51	24	12	44	63	44	**46**	133	
133	51	24	12	44	63	44	**46**	133	
134	53	25	11	43	64	44	**248**	22	
135	52	23	12	43	66	43	**95**	5	5
136	51	23	12	43	62	44	**16**	singleton	
137	53	25	11	43	64	44	**248**	22	
138	52	24.5	12	44	64	44	**156**	49	
139	51	24	12	45	63	44	**50**	133	
140	51	24.5	12	45	64	44	**68**	140	
141	51	23	13	44	65	43	**35**	8	8
142	53	22	12	43	66	43	**178**	5	5
143	53	23	12	43	66	43	**193**	5	5
144	52	23	13	43	66	43	**114**	5	9
145	52	23	12	43	64	44	**92**	10	
146	53	23	12	43	66	43	**193**	5	5
147	53	24	13	43	66	43	**225**	1	1
148	53	23	12	43	66	43	**193**	5	5
149	53	23	12	43	66	43	**193**	5	5
150	51	24	12	43	64	43	**41**	88	78
151	51	22	12	45	65	43	**11**	151	
152	53	24	12	44	63	44	**215**	377	
153	53	22	13	44	65	44	**185**	80	
154	52	24	12	44	66	43	**136**	154	
155	52	23	13	44	65	43	**119**	8	8
156	52	23	12	44	64	43	**100**	59	
157	51	23	13	44	65	43	**35**	singleton	
158	51	23	13	44	65	43	**35**	8	239
169	54	22	13	43	65	43	**267**	15	
178	54	21	13	43	65	43	**263**	15	
182	52	21	12	44	65	44	**83**	182	
188	52	24	12	43	66	43	**128**	1	188
189	52	24.5	12	45	65	44	**163**	30	30
190	54	24.5	13	44	65	44	**297**	singleton	
192	53	23	12	44	66	43	**199**	5	5
193	51	24.5	12	44	62	44	**61**	121	
194	53	23	12	43	66	43	**193**	5	5
195	52	24.5	13	44	65	43	**167**	8	8
196	52	24.5	12	45	65	44	**163**	30	30
197	53	24.5	12	44	64	44	**237**	singleton	
198	53	24.5	12	44	64	44	**237**	45	
199	54	22	13	43	66	43	**268**	15	
200	51	24.5	12	45	65	44	**70**	30	36

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
201	53	24.5	12	43	64	44	**233**	singleton	
203	53	24	12	45	66	43	**220**	1	188
205	52	24	13	43	64	44	**141**	1	97
207	53	24.5	12	43	65	44	**234**	89	
210	51	24.5	12	45	65	44	**70**	30	30
211	53	24.5	12	44	65	44	**239**	singleton	
212	53	24	12	43	66	43	**213**	1	188
213	53	24	13	45	65	44	**231**	singleton	
214	53	25	12	43	65	44	**254**	singleton	
217	53	25	11	43	64	44	**248**	22	
221	53	23	13	43	66	43	**205**	5	5
222	53	23	13	44	65	43	**207**	singleton	
225	53	23	12	43	67	43	**195**	5	5
228	53	23	12	43	65	43	**190**	5	228
231	53	23	12	43	66	43	**193**	5	105
235	53	23	12	43	65	43	**190**	singleton	
238	51	23	13	44	65	43	**35**	8	239
239	51	23	13	44	65	43	**35**	8	239
240	51	23	13	44	65	43	**35**	8	239
241	51	23	13	44	65	43	**35**	8	239
243	51	24.5	12	45	65	44	**70**	30	30
246	51	23	12	44	65	43	**26**	8	239
247	52	23	12	44	65	43	**103**	8	247
250	52	23	13	44	65	43	**119**	8	8
254	52	23	13	44	65	43	**119**	8	8
255	51	23	12	43	64	43	**17**	88	78
256	53	24.5	12	44	65	45	**240**	45	
257	51	24	12	43	64	43	**41**	88	78
259	53	24	13	43	64	44	**222**	1	573
260	53	24	13	43	65	43	**223**	15	
261	53	24	13	43	65	43	**223**	15	
262	53	23	13	44	65	43	**207**	singleton	
263	53	22	13	44	64	44	**184**	singleton	
264	53	22	13	43	66	43	**183**	1	573
265	53	24.5	12	44	65	43	**238**	singleton	
266	53	23	12	43	65	43	**190**	5	5
267	53	23	12	43	66	43	**193**	5	5
268	53	23	12	43	65	43	**190**	5	5
269	53	23	12	43	65	43	**190**	5	5
270	53	23	12	44	64	44	**196**	5	228
271	53	23	12	44	65	43	**197**	5	228
272	53	23	12	44	64	44	**196**	5	228
273	53	23	12	43	66	43	**193**	5	5
274	53	23	12	44	64	44	**196**	5	228
275	53	23	12	44	65	43	**197**	5	228
276	53	23	12	44	65	43	**197**	5	228
277	53	23	12	44	65	43	**197**	5	228
278	53	24.5	12	44	65	44	**239**	45	
279	53	24.5	12	44	65	43	**238**	45	
280	53	21	12	44	65	44	**176**	45	
281	51	23	12	45	65	44	**30**	30	30
282	51	23	12	45	65	44	**30**	30	30
283	51	24.5	12	45	65	44	**70**	30	30
284	51	24.5	12	45	65	44	**70**	30	30
285	51	24.5	12	45	65	44	**70**	30	30
286	52	24	13	43	65	43	**143**	1	97
287	52	23	13	43	65	43	**111**	8	8

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
288	52	23	13	43	65	43	**111**	8	8
289	52	24	12	44	65	43	**134**	singleton	
290	52	24.5	12	45	65	43	**162**	singleton	
291	52	24.5	12	44	64	44	**156**	398	
292	52	24	12	43	65	43	**127**	1	188
293	52	24	12	43	65	43	**127**	1	188
294	52	24	12	43	66	43	**128**	1	188
295	52	23	12	43	66	43	**95**	1	188
296	52	24	12	43	66	43	**128**	1	188
297	54	24	13	43	65	43	**291**	25	
298	54	24.5	12	44	65	44	**294**	45	
299	54	21	12	44	65	44	**262**	45	
300	54	23	12	43	65	43	**271**	singleton	
301	52	23	12	44	64	44	**101**	301	
302	52	23	12	44	65	43	**103**	singleton	
303	52	24	12	45	65	44	**137**	singleton	
304	52	24.5	12	45	65	43	**162**	305	
305	52	23	12	45	65	43	**108**	305	
306	52	23	12	43	65	43	**93**	7	
307	52	23	12	43	65	43	**93**	singleton	
308	52	23	12	43	66	43	**95**	singleton	
309	52	23	12	43	65	43	**93**	7	
310	52	24	12	43	64	44	**126**	singleton	
311	52	23	12	45	65	44	**109**	30	30
312	52	21	12	43	64	44	**81**	singleton	
313	52	24.5	12	44	65	44	**158**	30	34
314	52	24.5	12	45	65	43	**162**	30	34
315	52	23	12	45	65	43	**108**	305	
316	52	24.5	12	45	65	44	**163**	30	34
317	52	22	12	43	65	43	**85**	singleton	
318	52	24	12	43	66	43	**128**	1	573
319	53	24.5	12	44	65	44	**239**	45	
320	53	22	12	44	65	44	**179**	45	
321	53	24.5	12	44	64	44	**237**	45	
322	53	24.5	12	44	65	44	**239**	45	
323	53	23	12	44	65	44	**198**	323	
324	52	23	12	44	65	43	**103**	singleton	
325	52	23	12	43	66	43	**95**	5	5
326	52	23	12	43	66	43	**95**	5	6
327	54	22	13	43	65	43	**267**	15	
328	54	23	12	43	66	43	**273**	5	5
329	51	24.5	12	44	65	45	**66**	singleton	
330	51	23	12	44	64	44	**24**	301	
331	51	24	12	43	65	43	**42**	singleton	
332	54	24.5	13	43	65	43	**296**	singleton	
333	54	22	13	43	65	43	**267**	15	
334	53	23	12	44	65	44	**198**	304	
335	53	23	12	43	66	43	**193**	5	5
336	52	23	12	44	65	43	**103**	8	247
338	52	23	12	44	64	43	**100**	59	
339	53	24	13	43	66	43	**225**	singleton	
340	52	23	13	44	65	43	**119**	8	8
341	53	25	12	44	65	44	**258**	45	
342	53	25	12	44	65	44	**258**	45	
343	51	23	13	44	65	43	**35**	8	239
344	51	23	13	44	65	43	**35**	8	8
345	52	23	13	44	65	43	**119**	8	8

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
346	51	24.5	12	45	65	44	**70**	30	36
347	52	24	13	43	64	44	**141**	1	97
348	51	24.5	12	44	65	45	**66**	50	
349	53	24	13	43	64	44	**222**	1	97
350	51	23	12	44	64	45	**25**	350	
351	51	22	12	45	65	43	**11**	151	
352	52	24	13	43	64	44	**141**	1	97
353	52	24.5	12	45	65	43	**162**	42	
354	51	23	12	44	64	45	**25**	350	
355	52	24	13	43	64	44	**141**	1	97
356	50	22	12	45	64	43	**1**	singleton	
357	52	24	13	43	64	44	**141**	singleton	
358	52	23	13	43	64	44	**110**	1	97
359	52	23	12	44	64	43	**100**	59	
360	51	24.5	12	45	65	44	**70**	30	30
361	54	23	13	44	66	43	**283**	672	
362	51	23	13	44	64	43	**33**	8	239
363	53	24	13	43	65	43	**223**	1	573
364	53	23	12	43	66	43	**193**	5	5
365	52	23	12	44	65	43	**103**	singleton	
366	52	23	12	43	66	43	**95**	singleton	
367	53	23	12	43	66	43	**193**	5	5
368	51	23	13	44	65	42	**34**	8	239
369	54	24	13	43	63	44	**288**	25	
370	51	23	13	44	65	43	**35**	singleton	
371	53	23	12	43	66	43	**193**	5	5
372	51	23	13	44	65	42	**34**	8	239
373	53	23	12	43	66	43	**193**	5	5
374	53	23	12	43	66	43	**193**	5	5
375	52	23	12	44	64	43	**100**	59	
376	53	23	12	43	65	44	**191**	89	
377	53	24	12	44	63	44	**215**	377	
378	51	24.5	13	45	65	44	**77**	30	30
379	53	24.5	12	43	65	44	**234**	89	
380	52	23	12	44	65	43	**103**	singleton	
381	53	23	12	43	66	43	**193**	5	5
382	51	24.5	12	45	65	44	**70**	30	30
383	52	23	13	44	65	43	**119**	8	8
384	52	24	12	43	64	44	**126**	singleton	
385	53	22	13	44	64	44	**184**	1343	
386	51	22	12	44	65	43	**8**	singleton	
387	52	24	13	43	64	44	**141**	1	97
388	51	24	13	44	63	44	**54**	133	
389	54	23	12	43	64	43	**270**	20	
390	53	24	13	43	66	43	**225**	1	1
391	53	24.5	12	44	65	44	**239**	45	
392	51	24.5	12	45	65	44	**70**	30	30
393	53	23	12	43	66	43	**193**	5	5
394	52	23	12	45	64	43	**107**	59	
395	53	24	12	44	64	44	**216**	395	
396	53	24.5	12	44	65	44	**239**	45	
397	53	23	13	44	65	44	**208**	80	
398	52	24.5	12	44	64	44	**156**	398	
399	51	24.5	12	44	62	44	**61**	121	
400	53	24	12	43	66	43	**213**	1	573
401	53	24	12	43	66	43	**213**	1	1
402	53	23	13	43	66	43	**205**	5	5

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
403	53	23	12	43	66	43	**193**	5	5
404	53	23	12	43	66	43	**193**	5	5
405	52	24	12	43	64	44	**126**	1	97
406	52	23	12	43	66	43	**95**	5	9
407	52	23	13	44	65	43	**119**	8	239
408	51	24	13	43	64	44	**52**	singleton	
409	51	24.5	13	44	62	44	**74**	121	
410	51	24	12	44	62	44	**45**	singleton	
411	53	24.5	13	43	65	44	**243**	singleton	
412	52	24	12	43	64	44	**126**	1	71
413	52	23	13	43	64	44	**110**	singleton	
414	51	24	13	44	62	44	**53**	121	
415	51	24.5	12	43	62	44	**58**	singleton	
416	51	24.5	13	44	62	44	**74**	45	
417	51	24.5	12	45	62	44	**67**	singleton	
418	51	24	13	44	63	44	**54**	133	
419	51	24	12	44	63	44	**46**	133	
420	51	24	12	44	63	44	**46**	133	
421	52	24	12	44	66	43	**136**	96	
422	52	24	12	44	64	44	**133**	singleton	
423	52	24	12	43	64	44	**126**	1	71
424	52	24	12	43	64	44	**126**	1	71
425	52	23	12	44	63	44	**98**	singleton	
426	53	24	12	44	64	44	**216**	395	
427	51	24.5	12	44	62	44	**61**	121	
429	51	24	12	44	64	44	**47**	singleton	
431	51	24.5	12	45	65	44	**70**	30	30
432	53	24	12	43	66	43	**213**	1	1
433	51	24.5	12	45	65	44	**70**	30	30
434	51	24.5	12	45	66	43	**71**	30	30
435	51	24.5	12	43	65	44	**59**	30	30
436	51	24.5	12	45	65	44	**70**	30	30
437	52	24	13	43	64	44	**141**	25	
438	52	23	12	43	64	44	**92**	singleton	
440	52	24.5	12	44	64	43	**155**	singleton	
441	51	24.5	12	45	65	44	**70**	30	30
442	51	24.5	12	45	65	44	**70**	30	30
443	51	24.5	12	45	65	44	**70**	30	30
444	50	23	13	44	65	43	**4**	8	8
445	53	22	12	43	65	43	**177**	445	
446	52	23	12	43	63	44	**91**	1021	
447	54	23	12	43	65	43	**271**	singleton	
448	52	23	13	43	66	43	**114**	5	9
449	52	23	13	43	65	43	**111**	8	8
450	52	23	13	44	65	43	**119**	8	8
451	52	23	12	44	65	43	**103**	8	8
452	51	24.5	11	45	65	44	**57**	30	30
453	54	24.5	13	43	65	43	**296**	15	
454	52	24.5	12	44	65	44	**158**	singleton	
455	53	24.5	12	44	65	44	**239**	45	
456	53	23	12	42	66	43	**188**	5	5
457	52	24.5	12	45	65	44	**163**	30	34
458	52	24	13	43	64	44	**141**	1	97
459	52	24	13	43	66	43	**146**	singleton	
460	54	24	13	43	66	43	**293**	460	
461	53	23	12	43	66	43	**193**	5	5
462	53	23	12	43	66	43	**193**	5	5

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
463	52	24	12	44	65	43	**134**	7	
464	52	24	13	44	64	44	**147**	1	97
465	54	22	13	43	65	43	**267**	15	
466	53	24.5	12	44	65	44	**239**	89	
467	53	23	12	44	66	43	**199**	singleton	
468	52	23	13	43	66	43	**114**	5	9
469	54	22	12	43	65	43	**264**	15	
470	53	24.5	12	44	65	44	**239**	45	
471	53	23	12	43	66	43	**193**	5	5
472	52	24.5	12	44	64	44	**156**	49	
473	51	23	12	43	66	43	**19**	singleton	
474	53	24	13	43	66	43	**225**	1	1
475	53	25	13	43	66	43	**259**	1	1
476	52	23	13	44	65	43	**119**	8	8
477	53	24	13	43	63	44	**221**	1	1
478	51	24	12	44	63	44	**46**	133	
479	54	23	12	44	64	44	**275**	479	
480	51	24.5	12	44	65	44	**65**	130	
481	51	24	12	44	63	44	**46**	133	
482	51	23	12	44	64	44	**24**	singleton	
483	51	24.5	12	44	65	44	**65**	130	
484	51	24.5	12	43	65	44	**59**	30	30
485	54	22	13	43	65	43	**267**	15	
486	53	24	12	44	64	44	**216**	15	
487	53	23	12	43	66	43	**193**	5	5
488	51	24.5	12	44	62	44	**61**	121	
489	51	24.5	12	44	62	44	**61**	121	
490	51	24	12	43	65	43	**42**	88	88
491	51	24.5	12	45	65	44	**70**	30	30
492	53	23	12	43	66	43	**193**	5	5
493	53	24	13	43	66	43	**225**	1	1
494	52	23	12	43	65	43	**93**	8	247
495	53	25	11	43	64	44	**248**	22	
496	53	23	12	43	66	43	**193**	5	105
497	53	24.5	12	44	65	44	**239**	45	
498	53	25	11	43	63	44	**247**	22	
499	52	23	12	44	65	43	**103**	8	247
500	51	24.5	12	45	65	44	**70**	30	30
501	51	23	13	44	65	43	**35**	8	8
502	52	24	13	43	64	44	**141**	1	97
503	51	24.5	12	44	65	44	**65**	30	30
504	51	22	12	45	65	43	**11**	151	
505	52	24	12	44	65	43	**134**	101	
506	53	24	13	43	66	43	**225**	1	573
507	52	23	13	44	65	43	**119**	8	8
508	53	24.5	12	44	65	44	**239**	45	
509	53	24.5	12	43	65	44	**234**	89	
510	52	23	13	44	65	43	**119**	8	8
511	52	23	13	44	66	43	**121**	8	8
512	53	23	12	43	66	43	**193**	5	5
513	54	23	12	43	65	43	**271**	20	
514	53	23	13	43	66	43	**205**	1	1
515	53	23	12	43	66	43	**193**	5	863
516	52	23	12	43	66	43	**95**	1	188
517	51	23	13	43	65	43	**31**	8	8
518	53	23	12	43	66	43	**193**	5	5
519	51	23	13	44	65	43	**35**	8	239

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
520	54	23	12	44	64	44	**275**	479	
521	51	24.5	12	44	62	44	**61**	singleton	
522	52	23	12	44	65	44	**104**	703	
523	53	24.5	13	43	66	43	**244**	1	1
524	53	25	11	45	64	44	**252**	22	
525	52	23	12	44	65	43	**103**	8	247
526	53	23	12	43	66	44	**194**	5	5
527	51	23	13	44	65	43	**35**	singleton	
528	52	23	13	44	65	43	**119**	8	8
529	53	23	12	43	66	43	**193**	singleton	
530	53	23	12	44	64	44	**196**	12	
531	52	21	12	44	65	44	**83**	singleton	
532	52	23	13	44	65	43	**119**	8	8
533	52	24.5	12	45	65	44	**163**	30	30
534	53	24.5	12	44	65	44	**239**	45	
535	51	24.5	12	45	65	44	**70**	30	30
536	53	24.5	12	44	65	44	**239**	45	
537	52	23	12	44	64	43	**100**	59	
538	52	24	13	43	64	44	**141**	1	97
539	51	24.5	12	45	65	44	**70**	30	30
540	52	22	13	43	66	43	**89**	5	9
541	52	24.5	12	44	64	44	**156**	398	
542	51	24.5	12	45	65	44	**70**	30	30
543	51	24.5	12	45	65	44	**70**	30	30
544	53	23	12	44	65	43	**197**	8	72
545	52	23	12	44	64	43	**100**	59	
546	53	24.5	12	44	65	44	**239**	45	
547	53	23	12	43	66	43	**193**	5	5
548	52	23	13	45	65	43	**122**	8	8
549	53	23	12	43	66	43	**193**	5	863
550	53	23	13	43	66	43	**205**	1	1
551	54	22	13	43	65	43	**267**	15	
552	52	23	13	44	65	43	**119**	8	8
553	53	23	12	43	65	43	**190**	5	5
554	53	24.5	12	43	66	43	**235**	5	5
555	53	24	12	44	64	44	**216**	395	
556	51	23	12	45	65	44	**30**	30	34
557	51	24.5	12	45	65	44	**70**	30	30
558	51	24.5	12	45	65	44	**70**	30	30
559	52	23	12	44	64	43	**100**	singleton	
560	51	24.5	12	45	65	44	**70**	30	30
561	53	24	12	44	65	43	**217**	101	
562	53	25	12	44	65	44	**258**	45	
563	52	23	13	44	65	43	**119**	8	8
564	54	24	13	43	64	44	**290**	25	
565	53	24.5	12	44	65	44	**239**	45	
566	54	23	13	43	64	44	**278**	singleton	
567	53	24	13	43	66	43	**225**	1	1
568	52	24	12	44	66	43	**136**	96	
569	52	23	13	44	65	43	**119**	8	8
570	53	23	12	43	66	43	**193**	5	5
571	53	23	13	44	65	43	**207**	singleton	
572	52	23	12	44	65	43	**103**	8	247
573	53	24	13	43	66	43	**225**	1	573
574	52	24.5	12	44	65	44	**158**	singleton	
575	53	23	12	43	66	43	**193**	5	5
576	52	23	13	44	65	43	**119**	8	8

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
577	51	24.5	12	44	62	44	**61**	121	
578	53	23	13	44	65	44	**208**	80	
579	51	24.5	12	45	65	43	**69**	30	36
580	52	24.5	12	44	64	44	**156**	singleton	
581	51	24.5	12	44	62	44	**61**	singleton	
582	54	22	13	43	65	43	**267**	15	
583	52	23	13	44	65	44	**120**	80	
584	52	23	13	43	66	43	**114**	5	9
585	51	24	13	44	65	43	**55**	8	239
586	52	23	13	44	65	43	**119**	8	8
587	52	23	12	43	66	43	**95**	5	5
588	53	24.5	12	44	65	44	**239**	singleton	
589	51	23	12	44	62	43	**20**	singleton	
590	52	24.5	12	43	64	44	**151**	singleton	
591	52	24.5	12	45	65	44	**163**	30	34
592	51	23	13	44	65	43	**35**	8	239
593	52	23	13	44	64	43	**116**	singleton	
594	52	23	13	44	65	43	**119**	8	8
595	53	24.5	12	44	65	44	**239**	45	
596	51	24.5	12	45	66	44	**72**	30	30
597	53	23	12	43	66	43	**193**	5	5
598	51	25	12	45	65	44	**80**	30	30
599	51	24.5	12	44	64	44	**63**	singleton	
600	52	23	13	44	65	43	**119**	singleton	
601	52	24.5	12	45	64	44	**161**	140	
602	53	23	13	44	65	44	**208**	80	
603	51	24.5	12	45	65	43	**69**	singleton	
604	53	23	12	44	64	44	**196**	12	
605	51	24.5	12	45	65	44	**70**	30	30
606	53	25	11	43	64	44	**248**	22	
607	51	24.5	12	45	65	44	**70**	30	30
608	53	24.5	12	44	65	44	**239**	45	
609	52	23	13	44	65	43	**119**	8	8
610	53	24	13	44	66	43	**230**	1	1
611	52	21	12	44	65	44	**83**	182	
612	52	23	13	44	65	42	**118**	8	8
613	52	23	13	44	65	43	**119**	8	8
614	51	23	13	44	65	43	**35**	8	239
615	53	23	12	44	65	43	**197**	8	72
616	53	25	11	44	64	44	**250**	singleton	
617	52	24.5	12	44	65	44	**158**	45	
618	51	24	12	43	64	43	**41**	88	78
619	52	23	13	44	65	43	**119**	singleton	
620	54	22	13	43	65	43	**267**	15	
621	52	24.5	12	44	64	44	**156**	398	
622	53	25	11	43	64	44	**248**	22	
623	53	23	13	44	65	43	**207**	8	8
624	51	23	12	44	65	43	**26**	8	247
625	51	24.5	12	45	65	44	**70**	singleton	
626	51	23	12	44	65	43	**26**	8	247
627	53	23	12	43	66	43	**193**	5	5
628	53	23	12	45	66	43	**202**	5	5
629	51	24	12	44	65	43	**48**	88	88
630	52	23	13	44	65	43	**119**	8	8
631	51	23	13	44	65	43	**35**	8	8
632	53	23	12	43	66	43	**193**	5	5
633	53	23	12	44	66	43	**199**	singleton	

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
634	53	23	12	43	66	43	**193**	5	5
635	53	25	13	44	65	44	**260**	80	
636	53	23	11	43	64	44	**186**	22	
637	51	23	13	44	65	43	**35**	8	239
638	53	22	13	43	65	43	**182**	15	
639	51	23	13	44	65	43	**35**	8	239
640	53	24.5	12	44	65	45	**240**	45	
641	53	23	12	43	66	43	**193**	5	5
642	51	23	12	45	65	44	**30**	30	30
643	52	24.5	12	44	63	45	**154**	643	
644	51	23	12	44	65	43	**26**	8	239
645	53	24	13	43	65	43	**223**	15	
646	52	23	13	44	65	43	**119**	8	8
647	51	24.5	12	45	65	44	**70**	30	30
648	51	24.5	12	45	65	44	**70**	30	30
649	51	24.5	12	44	62	44	**61**	121	
650	53	23	12	43	66	43	**193**	5	5
651	51	24.5	12	44	62	44	**61**	121	
652	51	23	12	44	62	44	**21**	121	
653	52	25	13	44	65	43	**174**	singleton	
654	53	23	12	43	66	43	**193**	5	5
655	51	25	12	45	65	44	**80**	singleton	
656	51	23	12	45	64	45	**29**	singleton	
657	52	25	12	44	65	44	**171**	657	
658	52	25	12	44	65	44	**171**	657	
659	52	23	13	44	64	44	**117**	singleton	
660	53	23	13	44	65	43	**207**	8	770
661	53	23	12	44	67	44	**200**	singleton	
662	52	23	13	44	65	43	**119**	singleton	
664	52	23	13	44	65	43	**119**	8	8
665	54	23	13	44	65	43	**281**	singleton	
666	54	23	13	44	65	43	**281**	singleton	
667	51	24.5	12	45	65	44	**70**	30	30
668	52	24	13	43	66	43	**146**	5	9
669	52	24	13	44	64	44	**147**	1	97
670	52	23	13	44	65	42	**118**	8	8
671	52	24	13	43	66	43	**146**	5	9
672	54	23	13	44	66	43	**283**	672	
673	53	23	13	44	65	43	**207**	8	770
674	52	23	13	44	65	43	**119**	8	8
675	53	23	12	43	66	43	**193**	5	5
676	53	23	12	43	66	43	**193**	5	5
677	51	24.5	12	45	65	44	**70**	30	30
678	53	23	12	43	66	43	**193**	5	5
679	53	23	12	43	66	43	**193**	5	5
680	51	23	12	44	65	45	**27**	singleton	
681	51	24.5	12	44	62	44	**61**	121	
683	52	23	13	43	65	43	**111**	8	8
684	51	23	13	44	65	43	**35**	8	239
685	52	23	13	44	65	44	**120**	8	8
686	51	24.5	12	44	62	44	**61**	121	
687	51	24.5	12	44	65	45	**66**	121	
688	51	24.5	12	44	62	44	**61**	121	
689	51	24.5	12	44	65	45	**66**	121	
690	51	24.5	12	44	62	44	**61**	121	
691	54	22	13	43	65	43	**267**	15	
692	52	22	13	44	64	44	**90**	692	

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
693	52	25	13	43	64	44	**173**	1	97
694	52	23	12	43	64	44	**92**	126	
695	51	24	12	44	63	44	**46**	133	
696	51	24	12	44	63	44	**46**	133	
697	52	24	13	43	64	44	**141**	1	97
698	51	23	12	44	65	45	**27**	50	
699	53	22	12	43	65	43	**177**	singleton	
700	51	24.5	12	44	65	44	**65**	130	
701	51	24	12	44	63	44	**46**	133	
702	52	23	12	44	65	44	**104**	703	
703	52	23	12	44	65	44	**104**	703	
704	51	24.5	12	45	65	44	**70**	30	30
705	51	22	12	45	65	43	**11**	151	
706	52	23	13	43	66	43	**114**	5	9
707	52	23	12	44	65	44	**104**	singleton	
708	53	23	12	43	66	43	**193**	5	5
709	52	24	13	43	64	44	**141**	1	97
710	53	23	12	43	67	43	**195**	5	5
711	51	24	12	44	63	44	**46**	133	
712	51	24	12	44	63	44	**46**	133	
713	51	24.5	13	45	65	44	**77**	30	36
714	53	24.5	12	45	65	44	**242**	30	30
715	52	24.5	12	45	65	44	**163**	30	34
716	52	23	13	44	65	44	**120**	8	8
717	53	23	13	44	64	44	**206**	12	
718	52	24.5	12	44	63	45	**154**	718	
719	52	23	13	44	65	43	**119**	8	8
720	51	24.5	12	44	62	43	**60**	121	
721	51	24	12	43	66	43	**44**	1	188
722	51	24.5	12	45	65	44	**70**	30	30
724	51	23	13	44	66	43	**37**	8	239
725	51	24.5	12	44	65	43	**64**	121	
726	53	24	13	43	66	43	**225**	1	1
727	53	24.5	12	45	64	44	**241**	singleton	
728	53	23	13	44	65	44	**208**	80	
729	52	23	13	44	65	43	**119**	8	8
730	53	23	12	43	66	43	**193**	5	5
731	51	24.5	12	45	65	43	**69**	30	30
732	52	23	13	44	66	43	**121**	singleton	one 20,
733	52	24	13	43	64	43	**140**	singleton	
734	51	23	13	44	65	43	**35**	8	239
735	51	24.5	12	44	65	44	**65**	45	
736	53	23	12	43	65	43	**190**	5	228
737	53	25	11	43	64	44	**248**	22	
738	52	23	13	44	65	43	**119**	8	8
739	52	24	12	44	65	43	**134**	101	
740	52	24.5	12	45	64	44	**161**	singleton	
741	51	23	12	43	64	44	**18**	126	
742	52	24	13	43	64	44	**141**	1	97
743	53	24	13	43	66	43	**225**	1	1
744	53	23	12	43	66	43	**193**	5	5
745	51	24	12	44	63	44	**46**	133	
746	52	24	13	43	64	44	**141**	1	97
747	52	24	13	43	64	44	**141**	1	97
748	53	23	13	43	66	43	**205**	1	1
749	52	23	12	44	64	43	**100**	59	
750	52	24	12	44	63	44	**131**	133	

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
751	53	24	13	43	66	43	**225**	1	1
752	52	24.5	12	44	64	44	**156**	398	
754	52	23	12	44	64	43	**100**	59	
755	53	24.5	12	44	65	44	**239**	45	
756	51	24.5	12	45	65	44	**70**	30	30
758	52	24	12	43	66	43	**128**	1	188
759	52	23	12	44	65	43	**103**	8	247
760	53	24	13	43	66	43	**225**	1	573
761	53	24	13	43	67	43	**226**	1	1
762	53	24	13	43	66	43	**225**	1	1
763	52	23	12	43	66	43	**95**	5	5
764	53	24	12	43	66	43	**213**	5	5
765	51	24.5	12	45	65	44	**70**	30	30
766	53	25	11	42	64	44	**246**	22	
767	52	22	12	44	64	44	**87**	398	
768	52	23	13	44	65	43	**119**	8	8
769	53	24	13	43	66	42	**224**	1	1
770	53	23	13	44	65	43	**207**	8	770
771	51	24	12	44	63	44	**46**	133	
772	53	24	13	44	66	43	**230**	1	1
773	51	24.5	13	44	65	45	**75**	50	
774	54	24	13	43	66	43	**293**	singleton	
775	54	23	12	43	65	43	**271**	20	
776	50	24.5	12	45	65	43	**7**	30	39
777	52	23	12	44	64	43	**100**	59	
778	53	23	13	44	64	44	**206**	singleton	
779	52	23	12	44	66	43	**105**	779	
780	51	24.5	12	45	65	44	**70**	30	30
781	54	24	13	43	64	44	**290**	25	
782	51	23	13	44	65	43	**35**	8	239
783	53	23	12	43	65	43	**190**	5	228
784	52	23	13	44	65	42	**118**	8	8
785	53	23	12	44	64	44	**196**	12	
786	52	23	12	44	64	43	**100**	59	
787	53	23	12	43	66	43	**193**	5	5
788	52	23	12	43	66	43	**95**	5	5
789	52	23	12	44	65	43	**103**	12	
790	53	23	12	43	66	43	**193**	5	5
791	53	24.5	12	44	65	44	**239**	45	
792	53	24.5	12	44	65	44	**239**	45	
793	52	24	12	43	66	43	**128**	1	188
794	53	24.5	12	44	65	44	**239**	45	
795	54	22	13	43	65	43	**267**	15	
796	54	22	13	43	65	43	**267**	15	
797	52	23	12	44	65	43	**103**	8	247
798	53	23	12	44	64	44	**196**	12	
799	54	23	12	43	65	43	**271**	20	
800	51	24.5	12	44	62	44	**61**	121	
801	53	23	12	43	66	43	**193**	5	5
802	54	22	13	43	65	43	**267**	15	
803	53	24.5	12	44	65	44	**239**	45	
804	52	24.5	12	44	64	44	**156**	398	
805	52	23	13	43	66	43	**114**	5	9
806	51	24.5	12	46	65	44	**73**	30	30
807	52	24.5	12	45	65	44	**163**	30	34
808	51	24.5	12	45	65	44	**70**	30	30
809	52	21	12	44	62	44	**82**	singleton	

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
810	53	24.5	12	43	64	44	**233**	810	
811	51	24.5	12	45	65	44	**70**	30	30
812	52	24	12	44	65	43	**134**	101	
813	52	24.5	12	44	64	44	**156**	398	
814	52	22	13	44	64	44	**90**	693	
815	52	24	13	43	64	44	**141**	1	97
816	52	22	12	44	62	44	**86**	singleton	
817	51	24.5	12	45	65	43	**69**	30	39
818	54	22	13	43	65	43	**267**	15	
819	51	24.5	12	45	65	44	**70**	30	30
820	53	24.5	12	44	65	44	**239**	45	
822	53	23	13	44	65	44	**208**	80	
823	53	23	12	43	66	43	**193**	5	5
824	51	23	12	44	65	43	**26**	8	247
825	51	24	12	43	65	43	**42**	88	88
826	53	23	12	43	66	43	**193**	5	5
827	51	24.5	12	45	64	44	**68**	30	30
828	52	23	13	44	65	43	**119**	8	8
829	53	24	13	43	66	43	**225**	1	1
830	52	23	13	44	64	43	**116**	8	8
831	52	23	12	44	65	43	**103**	8	0
832	54	22	13	43	65	43	**267**	15	
833	55	24	12	43	66	43	**300**	1	573
834	52	24	13	43	66	43	**146**	5	9
835	53	23	12	43	66	42	**192**	5	5
836	52	23	13	44	66	43	**121**	8	8
837	51	24.5	12	44	62	44	**61**	121	
838	53	23	12	43	66	42	**192**	5	5
839	53	25	11	43	64	44	**248**	22	
840	53	23	12	43	66	43	**193**	5	5
841	53	24	13	43	66	43	**225**	1	1
842	52	24	13	43	64	44	**141**	1	97
843	52	24	13	44	64	44	**147**	1	97
844	52	23	13	44	65	43	**119**	8	8
845	54	22	13	43	65	43	**267**	15	
846	53	25	12	44	65	43	**257**	singleton	
847	51	24.5	12	45	65	43	**69**	singleton	
848	53	24	13	43	66	43	**225**	1	573
849	52	23	12	44	65	43	**103**	7	
851	53	24.5	13	44	66	43	**245**	1	1
852	53	24	13	43	66	43	**225**	1	1
853	52	23	13	44	65	43	**119**	singleton	
854	53	25	11	43	64	44	**248**	22	
855	51	24.5	12	45	65	43	**69**	30	39
856	52	23	12	44	66	45	**106**	singleton	
857	53	24.5	12	43	66	44	**236**	89	
858	52	23	13	44	65	43	**119**	8	8
859	51	24	12	43	65	43	**42**	88	88
860	53	23	12	43	66	43	**193**	5	863
861	53	23	12	44	65	43	**197**	8	770
862	53	23	12	43	66	43	**193**	5	863
863	53	23	12	43	66	43	**193**	5	863
864	52	23	12	43	66	43	**95**	5	6
865	50	23	13	44	65	43	**4**	8	8
866	53	23	12	43	66	43	**193**	5	5
867	53	25	11	43	64	44	**248**	22	
868	53	23	12	43	65	44	**191**	5	5

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
869	54	22	13	43	65	42	**266**	15	
870	52	23	13	44	65	43	**119**	8	8
871	54	22	13	43	66	43	**268**	15	
872	53	24	13	43	66	42	**224**	1	1
873	51	23	12	44	66	44	**28**	singleton	
874	53	23	12	43	66	43	**193**	5	5
875	53	23	12	44	64	44	**196**	12	
876	51	24	12	43	65	43	**42**	88	88
877	51	24.5	12	45	65	44	**70**	30	30
878	51	24.5	12	45	65	44	**70**	30	30
879	52	23	13	44	65	43	**119**	8	8
880	54	24	13	43	64	43	**289**	25	
881	52	23	13	45	65	43	**122**	8	8
882	53	24.5	12	44	65	44	**239**	45	
884	53	24	13	43	66	43	**225**	1	1
885	52	23	13	44	65	43	**119**	8	8
886	51	24.5	12	45	65	44	**70**	30	30
887	53	24.5	12	44	65	44	**239**	45	
888	52	23	13	43	66	43	**114**	5	9
889	51	23	12	44	65	43	**26**	8	239
890	52	23	12	44	62	44	**96**	singleton	
891	53	24	13	43	66	43	**225**	1	1
892	53	23	12	43	66	43	**193**	5	5
893	54	21	13	43	65	43	**263**	15	
894	52	21	12	44	65	44	**83**	182	
895	51	24.5	12	45	65	44	**70**	30	30
896	51	25	11	43	64	44	**78**	22	
897	51	25	12	43	64	44	**79**	22	
898	54	22	13	43	64	43	**265**	15	
899	51	24.5	13	45	65	44	**77**	30	30
900	51	23	13	44	65	43	**35**	8	8
901	53	23	12	44	64	44	**196**	12	
902	53	25	11	43	64	44	**248**	22	
903	52	23	13	44	66	43	**121**	5	9
904	51	24.5	12	44	65	45	**66**	50	
905	54	23	12	43	65	43	**271**	20	
906	54	22	13	43	65	43	**267**	15	
907	52	24.5	12	45	65	44	**163**	30	34
908	54	22	13	43	65	43	**267**	15	
909	52	23	12	43	66	43	**95**	5	5
910	52	24.5	12	45	65	44	**163**	30	30
911	53	25	12	43	66	43	**255**	singleton	
912	54	23	13	43	66	43	**280**	913	
913	54	23	13	43	66	43	**280**	913	
914	54	24	13	43	66	43	**293**	913	
915	53	23	12	43	66	43	**193**	5	5
916	53	24.5	12	43	64	44	**233**	89	
917	53	23	12	44	66	43	**199**	8	72
918	52	23	12	43	63	44	**91**	1021	
919	51	23	13	44	65	44	**36**	singleton	
920	53	23	12	43	66	43	**193**	5	5
921	53	23	12	43	66	43	**193**	5	5
922	52	23	12	44	64	43	**100**	singleton	
923	52	23	13	44	65	43	**119**	8	8
924	53	24.5	12	44	65	44	**239**	45	
925	52	23	12	44	64	43	**100**	59	
926	53	23	12	43	66	43	**193**	5	5

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
927	53	25	11	43	64	44	**248**	22	
928	53	24	11	43	64	44	**211**	22	
929	53	24.5	12	44	65	44	**239**	45	
930	53	23	12	43	66	43	**193**	5	5
931	52	23	13	44	65	43	**119**	8	8
932	52	23	12	43	66	43	**95**	5	6
933	51	24.5	13	45	65	43	**76**	30	39
934	52	21	13	44	65	44	**84**	singleton	
935	53	23	12	43	66	43	**193**	5	5
936	51	24	12	45	65	43	**51**	singleton	
937	53	23	13	44	64	44	**206**	1343	
938	51	24.5	12	45	65	44	**70**	30	30
939	53	23	12	44	65	43	**197**	8	72
940	52	23	12	44	64	43	**100**	59	
941	54	22	13	43	65	43	**267**	15	
942	52	23	12	44	65	44	**104**	942	
943	52	23	12	44	65	43	**103**	7	
944	52	21	12	44	65	44	**83**	182	
945	52	21	12	44	65	44	**83**	182	
946	51	24.5	12	44	62	44	**61**	121	
947	50	24.5	12	44	64	44	**6**	singleton	
948	52	23	12	43	66	43	**95**	5	6
949	52	23	12	43	66	43	**95**	singleton	
950	53	23	12	43	66	43	**193**	5	5
951	52	23	12	44	64	43	**100**	59	
952	52	23	12	44	64	43	**100**	59	
953	52	24	13	43	64	44	**141**	1	97
954	53	25	12	43	64	44	**253**	22	
955	52	24.5	12	43	63	44	**150**	singleton	
956	51	24.5	12	43	65	44	**59**	singleton	
958	51	24.5	12	45	65	44	**70**	singleton	
959	54	22	13	43	65	43	**267**	15	
960	52	23	13	44	65	43	**119**	8	8
961	52	24.5	12	44	66	44	**159**	45	
962	51	24.5	12	45	65	44	**70**	30	30
963	55	22	13	43	65	43	**299**	15	
964	54	24	13	43	62	44	**287**	singleton	
965	53	23	12	42	66	43	**188**	5	5
966	52	23	12	44	64	43	**100**	59	
967	52	23	12	44	64	43	**100**	59	
968	52	23	13	43	66	43	**114**	5	9
969	52	23	12	44	64	43	**100**	59	
970	53	25	11	43	64	44	**248**	22	
971	53	23	12	44	64	44	**196**	12	
972	53	24.5	12	44	65	44	**239**	45	
973	51	24	12	44	63	44	**46**	133	
974	52	24.5	12	44	64	44	**156**	398	
975	51	24.5	12	45	65	44	**70**	30	30
976	52	23	13	44	65	43	**119**	8	8
977	51	24.5	12	45	65	44	**70**	30	30
978	53	23	12	44	64	44	**196**	8	72
979	54	23	12	44	66	43	**277**	singleton	
980	52	23	13	44	65	43	**119**	8	8
981	51	23	13	43	66	43	**32**	981	
982	51	24.5	12	44	65	43	**64**	singleton	
983	51	23	13	44	65	43	**35**	8	239
984	53	25	11	44	65	43	**251**	singleton	

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
985	52	23	13	44	65	43	**119**	8	8/247
986	53	23	12	43	66	43	**193**	5	5
987	52	24	13	43	64	44	**141**	1	97
988	52	24.5	12	44	62	43	**153**	121	
989	52	24.5	12	45	65	44	**163**	30	34
990	53	23	12	44	65	44	**198**	8	72
991	52	24.5	12	45	65	44	**163**	30	34
992	52	24.5	12	45	65	44	**163**	30	34
993	53	24	13	43	66	43	**225**	1	1
994	54	23	13	44	65	44	**282**	80	
995	52	23	13	44	65	42	**118**	8	8
996	51	23	13	43	65	43	**31**	8	239
997	53	23	13	44	65	44	**208**	80	
998	53	23	12	43	66	43	**193**	5	5
999	52	23	13	44	65	43	**119**	8	8
1000	53	24.5	12	43	65	44	**234**	89	
1001	51	24.5	12	45	65	44	**70**	30	30
1002	54	22	13	43	65	43	**267**	15	
1003	54	22	13	43	65	43	**267**	15	
1004	52	22	12	45	65	43	**88**	singleton	
1005	53	24	13	44	66	43	**230**	1	1
1006	52	24.5	12	44	64	44	**156**	singleton	
1007	51	24	12	44	63	44	**46**	133	
1008	52	24	12	44	63	44	**131**	133	
1009	53	23	13	43	64	44	**203**	1153	
1010	51	24.5	12	44	62	44	**61**	121	
1011	53	23	12	43	66	43	**193**	5	5
1012	53	24	11	44	64	44	**212**	395	
1013	52	23	13	44	65	43	**119**	8	8
1014	52	23	13	44	65	43	**119**	8	8
1015	53	24	13	43	66	43	**225**	1	1
1016	53	24.5	12	44	65	44	**239**	45	
1017	53	24.5	12	44	65	44	**239**	singleton	
1018	51	23	12	43	66	43	**19**	5	6
1019	53	23	12	43	65	43	**190**	20	
1020	51	24.5	12	44	62	44	**61**	121	
1021	52	23	12	43	63	44	**91**	1021	
1022	53	23	12	44	65	43	**197**	8	72
1023	53	24.5	12	44	65	44	**239**	45	
1024	51	24.5	12	45	65	44	**70**	30	30
1025	53	24	12	44	64	44	**216**	singleton	
1026	51	24.5	12	45	65	44	**70**	30	30
1027	52	24.5	12	44	66	45	**160**	singleton	
1028	54	22	12	43	65	43	**264**	20	
1029	54	24	13	43	64	44	**290**	25	
1030	54	24	13	43	64	44	**290**	25	
1031	51	22	13	43	65	43	**14**	15	15, two 45, four
1032	53	24.5	12	45	65	44	**242**	45	
1033	53	25	12	44	64	44	**256**	singleton	
1034	51	24.5	12	44	62	44	**61**	121	
1035	54	23	12	44	65	43	**276**	singleton	
1036	54	22	13	43	65	43	**267**	15	
1037	53	25	11	43	64	44	**248**	22	
1038	54	22	13	43	65	43	**267**	15	
1039	53	24	13	43	66	43	**225**	singleton	
1040	53	24	12	44	65	44	**218**	45	
1041	52	24.5	12	45	65	44	**163**	30	34

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1042	54	24	13	43	64	44	**290**	25	
1043	52	24	12	44	64	43	**132**	singleton	
1044	51	23	13	43	66	43	**32**	981	
1045	53	24.5	12	44	65	44	**239**	45	
1046	52	23	12	44	64	44	**101**	singleton	
1047	53	24	12	43	66	43	**213**	1	188
1048	53	23	12	44	65	43	**197**	7	
1049	52	24.5	12	45	65	43	**162**	30	34
1050	54	24	13	43	64	44	**290**	25	
1051	53	25	11	43	64	44	**248**	22	
1052	53	24.5	12	44	65	45	**240**	45	
1053	51	24.5	12	45	65	44	**70**	30	30
1054	53	24	13	43	66	43	**225**	1	1
1055	51	24.5	12	45	65	44	**70**	30	30
1056	54	24	13	43	65	44	**292**	25	
1057	51	24.5	12	45	65	44	**70**	30	30
1058	53	24.5	12	44	65	44	**239**	45	
1059	51	24.5	12	45	65	44	**70**	30	30
1060	52	24	13	43	64	44	**141**	1	97
1061	54	22	13	43	65	43	**267**	15	
1062	52	24.5	12	45	65	44	**163**	30	30
1063	53	24.5	12	44	65	44	**239**	45	
1064	54	24	12	43	65	43	**285**	20	
1065	52	23	13	44	65	43	**119**	8	8
1066	52	24.5	13	44	64	44	**166**	398	
1067	52	24.5	13	44	64	44	**166**	398	
1068	51	24	12	43	65	43	**42**	88	88
1069	52	24.5	12	44	64	44	**156**	398	
1070	53	24	13	43	66	43	**225**	1	1
1071	51	24.5	12	45	65	44	**70**	30	36
1072	52	24	13	43	64	44	**141**	1	97
1073	52	25	12	45	66	45	**172**	singleton	
1074	51	22	12	45	65	43	**11**	151	
1075	51	24	12	44	63	44	**46**	133	
1076	51	22	12	45	65	43	**11**	151	
1077	52	24	13	43	65	44	**144**	1	97
1078	51	22	12	45	65	43	**11**	151	
1080	53	25	11	43	64	44	**248**	22	
1081	53	24.5	12	44	65	44	**239**	45	
1082	53	25	11	43	64	44	**248**	22	
1083	51	24.5	12	45	65	44	**70**	30	30
1084	51	24.5	12	45	65	44	**70**	30	30
1085	50	23	12	44	65	45	**3**	singleton	
1086	52	24	12	44	65	43	**134**	101	
1087	53	24.5	12	44	65	45	**240**	45	
1088	52	23	12	44	66	43	**105**	779	
1089	52	24	13	44	66	43	**149**	154	
1090	51	24.5	12	44	62	44	**61**	121	
1091	53	24.5	12	44	65	44	**239**	45	
1092	52	23	13	43	66	43	**114**	5	9
1093	53	24	12	44	64	44	**216**	singleton	
1094	53	24.5	12	45	64	44	**241**	810	
1095	52	25	11	43	64	44	**169**	22	
1096	53	24.5	12	44	65	43	**238**	45	
1097	51	23	13	44	65	43	**35**	8	239
1098	53	24	13	43	66	43	**225**	1	1
1099	52	23	12	43	66	43	**95**	5	5

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1100	52	23	12	43	66	43	**95**	5	5
1101	51	24.5	12	45	65	43	**69**	30	30
1102	52	24.5	12	45	65	44	**163**	30	34
1103	51	24.5	12	45	65	44	**70**	30	30
1104	51	24.5	12	45	65	44	**70**	30	30
1105	52	23	13	44	66	43	**121**	8	8
1106	52	24	12	43	67	43	**130**	1	188
1107	51	24.5	12	45	65	44	**70**	30	30
1108	52	23	12	44	64	43	**100**	59	
1109	52	23	12	44	64	43	**100**	59	
1111	53	23	12	43	66	43	**193**	59	
1112	52	24.5	12	44	64	44	**156**	398	
1113	52	23	12	43	66	43	**95**	singleton	
1114	51	24.5	12	45	65	44	**70**	singleton	
1115	53	24	13	44	65	43	**228**	singleton	
1116	51	23	13	44	65	43	**35**	8	239
1117	53	25	11	42	64	44	**246**	22	
1118	54	23	12	44	64	44	**275**	479	
1119	52	24	13	43	65	43	**143**	1	71
1120	53	24.5	12	44	65	44	**239**	45	
1121	53	24	13	43	66	43	**225**	1	1
1122	51	22	12	45	66	43	**12**	151	
1123	51	22	12	45	65	43	**11**	151	
1124	51	22	12	45	65	43	**11**	151	
1125	52	24	13	43	64	44	**141**	1	71
1126	52	24	13	43	63	44	**139**	1	71
1127	52	24	13	43	64	44	**141**	1	71
1128	52	24	13	43	64	44	**141**	1	71
1129	53	24	13	43	64	44	**222**	1	97
1130	51	24.5	12	44	62	44	**61**	singleton	
1131	51	24.5	12	45	65	44	**70**	30	30
1132	53	25	11	43	64	44	**248**	22	
1133	53	24.5	12	45	65	44	**242**	45	
1134	54	23	12	43	65	43	**271**	20	
1135	53	24	13	43	66	43	**225**	1	1
1137	53	25	11	43	64	44	**248**	22	
1138	53	25	11	43	64	45	**249**	22	
1139	53	25	11	43	64	44	**248**	22	
1140	51	24	12	43	64	43	**41**	88	78
1141	52	22	13	43	66	43	**89**	5	9
1142	53	23	12	44	64	44	**196**	12	
1143	54	22	13	43	65	43	**267**	15	
1144	54	24	13	43	64	44	**290**	25	
1145	54	24	13	43	64	44	**290**	25	
1146	54	24	13	43	64	44	**290**	25	
1147	51	24.5	12	45	65	44	**70**	30	30
1148	53	22	12	43	65	43	**177**	445	
1149	52	23	12	44	64	43	**100**	59	
1150	52	23	13	44	65	43	**119**	8	8
1151	52	23	12	43	63	44	**91**	1021	
1152	52	23	13	44	65	43	**119**	8	8
1153	53	22	13	43	64	44	**181**	1153	
1154	52	24.5	13	43	66	43	**165**	5	9
1155	53	24	12	44	65	43	**217**	101	
1156	53	23	12	43	64	44	**189**	12	
1158	51	24.5	12	45	65	43	**69**	30	30
1159	52	23	12	44	65	44	**104**	7	

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1160	54	22	13	43	65	43	**267**	15	
1161	52	24.5	12	45	65	44	**163**	30	34
1162	52	23	12	43	64	44	**92**	10	
1163	51	24.5	12	45	65	44	**70**	30	30
1164	53	23	12	43	66	43	**193**	5	5
1165	53	24.5	12	44	65	44	**239**	45	
1167	53	24.5	12	44	65	44	**239**	45	
1168	51	24.5	12	45	65	44	**70**	30	30
1169	53	24.5	12	44	65	44	**239**	45	
1170	51	24.5	12	44	62	43	**60**	121	
1171	51	24.5	12	45	65	43	**69**	30	30
1172	51	24.5	12	44	62	43	**60**	121	
1173	52	23	13	44	65	43	**119**	8	8
1174	52	24	13	43	64	44	**141**	1	97
1175	52	24.5	12	43	65	44	**152**	89	
1176	53	23	12	43	66	43	**193**	5	5
1178	53	22	12	43	66	43	**178**	5	5
1179	52	24	13	43	64	44	**141**	1	97
1181	52	23	13	44	65	43	**119**	8	8
1182	52	22	13	43	66	43	**89**	5	9
1183	52	23	13	44	66	43	**121**	8	8
1184	53	23	12	44	66	43	**199**	5	5
1185	55	22	13	43	65	43	**299**	15	
1186	53	23	12	43	66	43	**193**	5	5
1187	53	23	12	43	66	43	**193**	5	105
1188	52	23	13	44	65	43	**119**	8	8
1189	52	24	13	44	65	43	**148**	8	8
1191	53	24	13	45	66	43	**232**	singleton	
1192	52	24	11	44	65	43	**124**	singleton	
1193	53	24	13	45	66	43	**232**	singleton	
1194	53	24.5	12	43	66	43	**235**	singleton	
1195	53	25	13	45	66	43	**261**	singleton	
1196	53	24	12	44	66	43	**219**	singleton	
1197	52	24	11	44	66	43	**125**	singleton	
1198	52	24	12	45	66	43	**138**	singleton	
1199	52	22	12	43	65	43	**85**	singleton	
1200	51	24	13	45	66	43	**56**	singleton	
1201	54	24	12	44	66	43	**286**	singleton	
1202	53	24	12	44	62	43	**214**	singleton	
1203	51	24.5	12	45	65	43	**69**	singleton	
1204	53	23	12	45	66	43	**202**	5	5
1205	52	23	12	44	63	43	**97**	singleton	
1206	53	24	13	43	66	43	**225**	1	1
1207	53	23	13	43	66	43	**205**	singleton	
1208	52	23	13	44	65	43	**119**	8	8
1209	52	23	12	44	65	44	**104**	942	
1210	51	24.5	12	44	62	44	**61**	121	
1211	53	24.5	12	44	65	44	**239**	45	
1212	51	24.5	12	44	65	44	**65**	45	
1213	51	24.5	12	44	62	44	**61**	121	
1214	53	24.5	13	43	66	43	**244**	1	1
1215	51	24	12	43	65	43	**42**	88	88
1216	53	22	13	43	64	43	**180**	1153	
1217	53	23	12	43	65	44	**191**	singleton	
1218	52	24	12	43	66	43	**128**	1	188
1219	51	24	12	43	65	43	**42**	88	88
1220	53	24	13	43	66	43	**225**	1	1

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1221	52	23	13	43	65	43	**111**	8	8
1222	52	23	12	43	65	44	**94**	singleton	
1224	52	23	12	44	64	43	**100**	59	
1225	51	23	13	44	65	43	**35**	8	239
1226	54	24	13	43	64	44	**290**	25	
1227	51	23	13	44	65	43	**35**	8	8
1228	51	23	13	44	65	43	**35**	8	239
1229	52	24	13	44	65	43	**148**	singleton	
1230	51	22	12	45	65	43	**11**	151	
1231	54	23	13	43	64	44	**278**	singleton	
1232	52	24.5	12	44	64	44	**156**	398	
1233	51	24.5	12	44	62	44	**61**	singleton	
1234	52	23	12	44	65	43	**103**	7	
1235	52	24	12	43	66	43	**128**	1	188
1236	52	24	12	43	66	43	**128**	1	188
1237	52	23	13	44	65	43	**119**	8	8
1238	52	23	13	44	65	43	**119**	8	8
1239	52	23	14	44	65	43	**123**	8	8
1240	53	23	13	44	65	43	**207**	8	770/72
1241	52	23	12	44	64	43	**100**	59	
1242	53	24.5	12	45	65	44	**242**	45	
1243	53	24	12	44	65	43	**217**	singleton	
1244	51	24.5	12	44	62	43	**60**	121	
1245	51	24.5	12	44	65	44	**65**	130	
1246	51	24	12	44	65	43	**48**	singleton	
1247	51	24	12	44	63	44	**46**	133	
1248	51	22	12	45	65	43	**11**	151	
1249	51	22	12	45	66	43	**12**	singleton	
1250	51	23	12	44	66	44	**28**	singleton	
1251	51	23	12	44	64	44	**24**	singleton	
1252	52	23	13	44	65	43	**119**	8	8
1253	52	23	13	44	65	43	**119**	8	8
1254	52	24	12	44	65	44	**135**	singleton	
1255	52	23	13	44	65	43	**119**	8	8
1256	53	24	13	43	66	43	**225**	1	1
1257	52	23	13	44	65	43	**119**	8	8
1258	52	22	13	43	66	43	**89**	5	9
1259	53	23	12	43	66	43	**193**	5	5
1260	53	23	13	44	65	44	**208**	80	
1261	52	24	12	43	66	44	**129**	1	188
1262	51	24.5	12	45	65	44	**70**	30	30
1263	51	24.5	12	45	65	43	**69**	30	39
1264	51	24	12	43	64	43	**41**	88	78
1265	52	24	12	43	66	43	**128**	1	188
1266	53	23	12	44	65	44	**198**	45	
1267	52	22	13	43	66	43	**89**	5	9
1268	53	23	12	44	64	44	**196**	12	
1269	52	23	13	44	65	43	**119**	8	8
1270	51	24	12	43	64	43	**41**	88	78
1271	50	24.5	12	44	62	44	**5**	121	
1272	51	24.5	12	45	66	44	**72**	30	39
1273	53	23	12	44	64	44	**196**	singleton	
1274	51	22	12	45	65	43	**11**	705	
1275	54	23	12	44	66	43	**277**	singleton	
1276	52	25	12	43	65	43	**170**	singleton	
1277	52	24.5	13	44	64	44	**166**	398	
1278	52	24	13	43	66	43	**146**	1	1

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1279	53	22	13	43	66	43	**183**	1	573
1280	52	24	12	44	66	43	**136**	singleton	
1281	54	22	12	43	65	43	**264**	20	
1282	51	23	13	44	65	43	**35**	8	239
1283	51	23	13	44	65	43	**35**	8	239
1285	51	23	13	44	65	43	**35**	8	239
1286	51	23	12	44	65	43	**26**	singleton	
1287	51	24	12	44	65	43	**48**	singleton	
1289	51	24	12	43	65	43	**42**	88	88
1290	53	23	13	44	66	43	**209**	singleton	
1291	52	23	12	44	64	42	**99**	59	
1292	52	24	13	43	66	43	**146**	5	9
1293	54	23	12	43	67	43	**274**	singleton	
1294	51	23	13	44	65	43	**35**	8	239
1295	52	24	12	43	65	43	**127**	singleton	
1296	51	23	13	44	65	43	**35**	8	239
1297	52	23	13	43	66	43	**114**	5	9
1298	51	24.5	12	44	65	44	**65**	30	30
1299	52	23	12	44	66	43	**105**	779	
1300	51	24.5	12	44	62	44	**61**	121	
1301	51	24.5	12	44	62	44	**61**	121	
1302	51	24.5	12	44	64	44	**63**	singleton	
1305	52	23	13	44	65	43	**119**	8	8
1306	51	22	12	45	65	42	**10**	singleton	
1307	53	23	11	43	66	43	**187**	5	5
1308	52	23	13	44	65	43	**119**	8	8
1309	51	23	13	44	65	43	**35**	8	239
1310	51	23	13	44	65	43	**35**	8	239
1311	51	23	12	44	64	43	**23**	59	
1312	51	23	13	44	65	43	**35**	8	239
1313	54	22	13	43	65	43	**267**	15	
1314	52	24.5	12	44	65	43	**157**	singleton	
1315	53	24	12	44	64	44	**216**	395	
1316	52	23	12	44	64	43	**100**	59	
1317	53	23	12	43	66	43	**193**	5	5
1318	52	24	12	44	65	43	**134**	101	
1319	53	24.5	12	44	65	45	**240**	45	
1320	51	24.5	12	45	65	43	**69**	30	30
1321	51	23	12	45	65	44	**30**	singleton	
1322	53	24.5	12	44	65	44	**239**	45	
1323	54	23	13	44	66	43	**283**	672	
1324	53	23	12	44	65	43	**197**	8	72
1325	53	23	12	43	66	43	**193**	5	5
1326	53	25	11	43	64	44	**248**	22	
1327	53	25	11	43	64	44	**248**	22	
1328	53	24	13	43	66	43	**225**	1	1
1329	51	23	13	44	65	43	**35**	8	8
1330	53	24.5	12	44	65	44	**239**	45	
1331	53	23	12	44	65	43	**197**	8	72
1332	52	24	12	43	66	43	**128**	1	188
1333	51	24.5	12	45	65	44	**70**	30	30
1334	52	23	13	44	65	42	**118**	8	8
1335	51	24.5	12	45	64	44	**68**	30	30
1336	53	24	13	43	66	43	**225**	1	1
1337	53	23	13	45	65	43	**210**	8	770
1338	52	23	13	44	65	43	**119**	8	8
1339	53	24	13	43	66	43	**225**	singleton	

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1340	53	22	12	43	66	43	**178**	5	5
1341	51	23	13	44	65	43	**35**	8	239
1342	53	23	12	43	66	43	**193**	5	5
1343	53	22	13	44	64	44	**184**	1343	
1344	52	22	13	44	64	44	**90**	692	
1345	53	24	13	43	66	43	**225**	singleton	
1346	53	23	12	43	66	43	**193**	5	5
1347	52	22	13	44	64	44	**90**	692	
1348	53	23	12	43	66	43	**193**	5	5
1349	52	23	12	44	66	45	**106**	singleton	
1350	53	23	12	43	66	43	**193**	5	5
1351	52	23	13	44	65	43	**119**	8	8
1353	52	23	13	44	65	42	**118**	8	8
1354	52	23	12	44	64	43	**100**	59	
1355	52	24.5	12	45	65	44	**163**	30	34
1356	52	24	13	43	64	44	**141**	1	97
1357	52	23	13	44	65	43	**119**	singleton	
1358	51	24.5	12	44	65	43	**64**	singleton	
1359	51	23	12	44	64	44	**24**	singleton	
1360	51	24	12	43	65	43	**42**	88	88
1361	51	23	12	44	63	44	**22**	singleton	
1362	52	23	12	43	66	43	**95**	5	6
1363	50	22	12	45	65	43	**2**	705	
1364	51	22	12	45	65	43	**11**	705	
1365	51	22	12	45	64	43	**9**	705	
1366	52	24	13	43	64	45	**142**	1	97
1367	52	24	13	43	64	44	**141**	1	97
1368	54	23	12	43	65	43	**271**	20	
1369	53	23	12	44	64	44	**196**	12	
1370	54	23	12	43	65	43	**271**	20	
1371	51	23	12	44	63	44	**22**	singleton	
1372	54	24	13	43	64	44	**290**	25	
1373	53	24.5	12	43	65	44	**234**	89	
1374	51	23	13	44	65	43	**35**	8	239
1375	52	24.5	12	44	64	44	**156**	398	
1376	52	23	13	43	66	43	**114**	5	9
1377	53	24.5	12	44	65	44	**239**	45	
1378	54	22	13	45	65	44	**269**	singleton	
1379	52	24	13	43	63	44	**139**	1	97
1380	54	23	12	44	64	44	**275**	479	
1381	52	23	13	44	65	43	**119**	8	8
1382	52	23	13	44	65	43	**119**	8	8
1383	51	24.5	12	44	65	44	**65**	singleton	
1384	51	24.5	12	44	62	43	**60**	121	
1385	53	23	13	44	65	44	**208**	80	
1386	51	24	12	44	65	44	**49**	singleton	
1387	52	24.5	12	45	66	44	**164**	30	34
1388	53	24.5	12	44	65	44	**239**	45	
1389	52	23	13	43	66	42	**113**	5	9
1390	51	24.5	12	45	65	44	**70**	30	30
1391	52	24.5	12	44	64	44	**156**	398	
1392	51	24.5	12	45	65	43	**69**	30	30
1393	53	24.5	12	44	65	44	**239**	45	
1394	53	24.5	12	44	65	44	**239**	45	
1395	53	23	12	44	64	44	**196**	12	
1396	53	24	13	43	66	43	**225**	1	1
1397	52	23	13	44	65	43	**119**	8	8

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1398	53	23	12	43	65	43	**190**	5	5
1399	52	24	11	44	65	43	**124**	101	
1400	52	24	12	44	65	43	**134**	101	
1401	51	23	13	43	65	43	**31**	8	239
1402	53	24	13	43	66	43	**225**	1	1
1403	54	23	13	43	65	43	**279**	15	
1404	53	24	13	44	65	43	**228**	singleton	
1405	52	23	13	44	65	43	**119**	8	8
1406	52	24.5	12	45	65	44	**163**	30	30
1408	51	24.5	12	45	65	44	**70**	30	30
1409	53	23	12	44	66	43	**199**	5	5
1410	53	23	12	43	65	43	**190**	5	228
1411	52	23	12	44	65	43	**103**	7	
1412	53	24.5	12	44	65	44	**239**	45	
1413	51	24.5	12	43	62	44	**58**	121	
1414	53	25	11	43	63	44	**247**	22	
1415	51	24.5	12	45	65	43	**69**	30	30
1416	53	23	12	43	66	43	**193**	5	5
1417	53	24.5	12	45	65	44	**242**	45	
1418	52	23	12	44	64	43	**100**	59	
1419	52	24	13	43	64	44	**141**	1	97
1420	52	23	13	43	65	44	**112**	singleton	
1421	51	24.5	12	44	62	44	**61**	121	
1422	51	24.5	12	45	64	44	**68**	30	30
1423	54	23	12	43	65	44	**272**	20	
1424	52	23	12	44	65	42	**102**	8	247
1425	51	23	13	44	65	42	**34**	8	239
1426	52	23	13	44	65	42	**118**	8	8
1427	53	23	12	43	66	43	**193**	5	5
1428	52	23	13	44	65	43	**119**	8	8
1429	53	23	12	43	65	43	**190**	5	863
1430	52	23	13	44	65	43	**119**	8	8
1431	52	21	12	44	65	44	**83**	singleton	
1432	53	24.5	12	44	65	44	**239**	45	
1433	51	24.5	12	44	62	45	**62**	singleton	
1434	53	23	12	44	65	43	**197**	8	72
1435	53	23	12	42	66	43	**188**	5	5
1436	51	24.5	12	45	65	44	**70**	30	30
1437	51	24.5	12	45	65	44	**70**	30	30
1438	51	24.5	12	45	65	44	**70**	30	30
1439	51	24.5	12	45	65	44	**70**	30	30
1440	53	23	13	44	65	44	**208**	80	
1441	54	22	13	43	65	43	**267**	15	
1442	53	24.5	12	43	65	44	**234**	89	
1443	53	24.5	12	43	65	44	**234**	89	
1444	53	24.5	12	44	65	44	**239**	45	
1445	54	22	13	43	65	43	**267**	15	
1446	54	25	11	43	64	44	**298**	22	
1447	53	23	12	43	66	43	**193**	5	5
1448	51	24.5	12	45	65	44	**70**	30	30
1449	51	24	12	42	65	43	**39**	88	88
1450	52	24.5	12	44	64	44	**156**	398	
1451	53	24	13	43	66	43	**225**	1	1
1452	51	24	12	44	63	44	**46**	133	
1453	52	24.5	12	44	65	44	**158**	398	
1454	53	23	12	43	66	43	**193**	5	5
1455	51	24	12	43	66	43	**44**	88	88

st	arcC	aroE	gmk	pta	tpi36	tpi241	MelT	CC	SubCC
1456	51	24.5	12	45	65	44	**70**	30	30
1457	53	23	12	43	66	43	**193**	5	5
1458	52	23	12	44	65	43	**103**	7	
1459	52	24.5	12	44	64	44	**156**	398	
1460	53	23	12	44	64	44	**196**	12	
1461	53	23	12	44	64	44	**196**	12	
1462	52	24	12	43	66	43	**128**	1	188
1463	53	24	13	43	66	43	**225**	1	1
1464	52	26	12	44	64	44	**175**	singleton	
1465	51	22	13	44	65	43	**15**	8	239
1466	51	24	12	43	64	43	**41**	88	88
1467	53	23	13	44	64	44	**206**	singleton	
1468	52	24.5	12	44	65	44	**158**	singleton	
1469	53	22	13	43	64	44	**181**	1153	
1470	54	23	12	43	65	43	**271**	20	
1472	51	24.5	12	45	65	44	**70**	30	30
1473	53	25	11	43	64	44	**248**	22	
1474	52	23	12	43	66	43	**95**	1	188
1475	51	23	12	45	65	44	**30**	singleton	
1476	52	24	13	43	64	44	**141**	1	97
1477	54	22	13	43	65	43	**267**	15	
1478	51	24.5	12	45	65	44	**70**	30	30
1479	54	22	13	43	65	43	**267**	15	
1480	53	23	12	43	66	43	**193**	5	5
1481	53	23	12	43	65	43	**190**	5	228
1482	51	24.5	12	45	65	44	**70**	30	30
1483	53	22	12	43	66	43	**178**	5	5
1484	51	23	12	45	65	44	**30**	30	30
1485	52	23	12	44	65	43	**103**	7	
1486	51	22	13	43	65	43	**14**	15	
1487	54	24.5	12	45	65	44	**295**	30	30
1488	53	23	12	44	64	44	**196**	12	
1489	53	23	12	43	66	43	**193**	5	105
1490	52	23	12	44	65	43	**103**	8	8
1491	54	22	12	43	65	43	**264**	15	
1492	54	24	12	43	64	44	**284**	25	
1493	51	23	13	44	65	43	**35**	8	239
1494	51	22	13	44	65	43	**15**	8	239
1495	53	23	13	43	66	43	**205**	5	5
1496	53	24	13	43	66	43	**225**	1	573
1497	52	23	13	44	65	43	**119**	8	8
1498	52	23	13	43	65	43	**111**	8	8
1499	52	24	13	43	65	43	**143**	8	8
1500	52	24.5	13	44	66	43	**168**	singleton	
1501	53	23	12	45	64	43	**201**	singleton	
1502	53	23	13	43	65	44	**204**	80	
1503	53	24	13	44	64	44	**227**	1	573
1504	53	24	13	43	66	43	**225**	1	573
1505	53	24	12	43	66	43	**213**	1	573
1506	53	24	13	44	65	44	**229**	singleton	
1507	53	23	12	44	65	43	**197**	8	72
1508	52	23	13	44	65	43	**119**	8	8

Appendix III. Global implications of the emergence of community-associated methicillin-resistant *Staphylococcus aureus* in Indigenous populations

REVIEW ARTICLE

Global Implications of the Emergence of Community-Associated Methicillin-Resistant *Staphylococcus aureus* in Indigenous Populations

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The emergence of community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) in Australia may have been facilitated by conditions in socially disadvantaged populations—particularly, remote Australian Aboriginal communities. The appearance of community-associated MRSA was first noticed in Australia during the early 1980s; subsequently, several genetically diverse strains have independently emerged from geographically distinct regions. Molecular and epidemiological studies support the role of genetic transfer of resistance determinants (SCC*mec*IV) in this process. Conditions in Aboriginal communities—namely, domestic crowding, poor hygiene, and high rates of scabies, pyoderma, and antibiotic use—have facilitated both the clonal expansion and de novo emergence of strains of community-associated MRSA. Combating the worldwide emergence and spread of community-associated MRSA may require novel community-level control strategies targeted at specific groups, such as remote Indigenous populations.

Community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a leading cause of skin and soft-tissue infections in many parts of the world. One strain, USA300, now causes up to 70% of skin and soft-tissue infections that present to emergency departments across the United States [1]. Community-associated MRSA emerged in Australia during the early 1980s [2, 3]; in contrast with the United States, there continues to be an increasingly recognized diversity of strains in circulation in Australia [4]. Three previously recognized clonal groups were described from geographically distinct regions, as was a newly documented clonal group apparently unique to tropical northern Australia. The present review describes the emergence of these clonal groups and hypothesizes that conditions in socially disadvantaged populations have facilitated emergence. We follow the story of community-associated MRSA in Australia and discuss the public health implications. Table 1 defines some of the terms used in this review.

Australia has played a significant role in the history of *S. aureus* epidemiology. In 1952, a pandemic clone of penicillin-resistant *S. aureus*, termed "phage type 80/81," was first isolated from neonatal infections in Sydney [5]. Phage type 80/81 subsequently caused severe nosocomial and community infections throughout the developed world during the 1950s and 1960s [6]. This epidemic receded with the introduction of methicillin and related penicillinase-stable antibiotics during the 1960s, only to be replaced by the emergence of MRSA. After sporadic reports of MRSA during the 1960s, an MRSA strain emerged in Melbourne and Sydney hospitals during the late 1970s that subsequently became endemic in Australian and overseas hospitals [7].

In 1980, the appearance of MRSA infections in injection drug users in Detroit heralded the next wave of staphylococcal disease [8]. Unlike patients with health care–associated MRSA, the majority of these patients were young and otherwise healthy and had not been recently hospitalized. These new strains were labeled "community-acquired MRSA."

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Table 1. Glossary of terms used in this review of community-associated methicillin-resistant *Staphylococcus aureus* (MRSA).

Term	Explanation
Clones and/or strains	Varieties of *S. aureus* that differ in their basic genetic background, determined by various typing techniques; in Australia, the most common community-associated MRSA clones are WA-MRSA-1 (ST1), Queensland clone (ST93), and the Western Samoan Phage Pattern clone (ST30); in the United States, USA300 is predominant
Multilocus sequence type	Strains can be differentiated according to the DNA sequence of 7 housekeeping genes; the sequence is assigned a unique "sequence type"; the genetic relatedness of strains can be inferred, and closely related strains can be grouped together as a clonal complex
*mec*A	The gene encoding a modified cell wall component that leads to resistance to methicillin and other β-lactam antibiotics
Staphylococcal cassette chromosome (SCC)	The mobile genetic element on which *mec*A resides; there are different types of SCC*mec* genes (I–V), which vary in size, mobility, and whether other resistance genes are carried; SCC*mec* can transfer from one staphylococcus to another
Community-associated MRSA	Strains of MRSA that are typically not multiresistant to antibiotics and harbor SCC*mec*IV as the resistance determinant; community-associated MRSA has arisen from the community but now causes infections in hospitals as well as in the community
Health care–associated MRSA	Strains of MRSA that are genetically distinct from community-associated MRSA, are multiresistant to antibiotics and usually harbor SCC*mec*II as the resistance determinant; cause infections almost exclusively in patients with health care contact

COMMUNITY-ASSOCIATED MRSA IN AUSTRALIA

Community-associated MRSA was initially noticed in Australia during the early 1980s [3, 9], and the first detailed description, in 1989, was of patients from remote Aboriginal communities of Western Australia [2]. After the Western Australian government decided to make MRSA colonization and infection a notifiable disease in 1985 [3], it became apparent that MRSA was increasingly isolated from patients without prior hospital contact. Most of these patients came from remote Aboriginal communities in the Kimberley region (figure 1) in the tropical north of Western Australia. In contrast to previous MRSA strains, the isolates were resistant only to β-lactam antimicrobials, and genetic analysis by PFGE demonstrated a "new" type of MRSA [2]. WA-MRSA, as it became known, spread throughout Western Australia and into major metropolitan centers. Community studies found that 42% of inhabitants of one remote community and 24% of another were colonized with WA-MRSA [10].

Similar strains of non–multiresistant MRSA were also present in the Northern Territory, the jurisdiction adjacent to northern Western Australia. Between 1991 and 1995, infections caused by community-associated strains outnumbered those of health care–associated MRSA at the Royal Darwin Hospital in the tropical Top End of the Northern Territory [11, 12], which suggests that community-associated MRSA was already widespread. The majority of these infections occurred in people from rural and remote indigenous communities.

Although the documentation of community-associated MRSA was more accurate and complete in Western Australia, the incidence of infection during the early 1990s was substantially higher in the Northern Territory. On the basis of population data from the 1996 national census, the incidence of community-associated MRSA isolation in Western Australia [13] from January 1991 through June 1995 was 43 isolations per 100,000 population. The incidence of community-associated MRSA infection in the Top End of the Northern Territory [11] over the same time period was 81 infections per 100,000 population. The true difference in incidence is probably much greater, because notification of infections and colonizations (including screening samples) was mandatory for all Western Australia laboratories, whereas clinical infections at only the Royal Darwin Hospital were included in notification from the Northern Territory. This suggests a possibly earlier emergence of community-associated MRSA in the Northern Territory than in Western Australia, with widespread establishment in the Northern Territory population.

As molecular tools advanced, it became clear that community-associated MRSA was not a "feral descendant" of health care–associated MRSA strains that had escaped into the com-

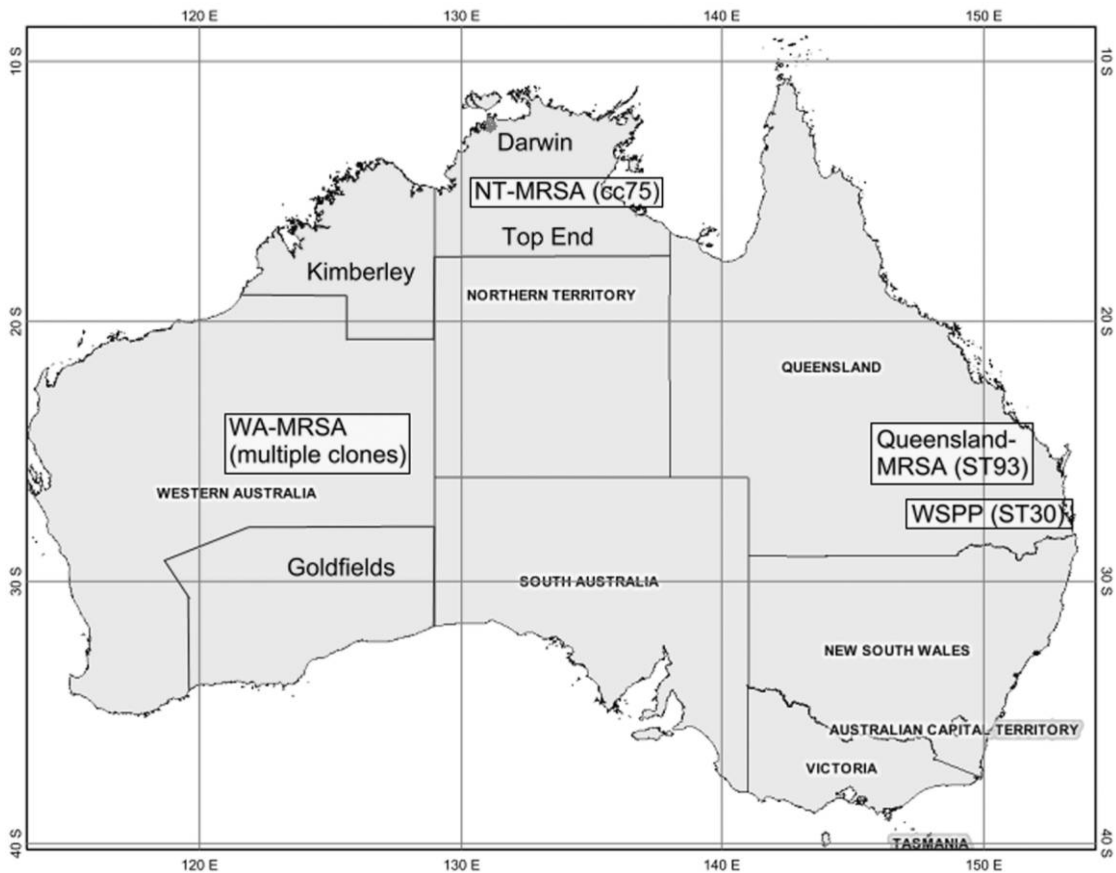

Figure 1. Community-associated methicillin-resistant *Staphylococcus aureus* strains in Australia and regions from which these strains have appeared. cc, clonal complex; NT, Northern Territory; ST, sequence type; WA, Western Australia; WSPP, Western Samoan Phage Pattern.

munity [14]; rather, the emergence of community-associated MRSA was independent of health care–associated MRSA. Strains of different genetic backgrounds were arising from discrete regions of Australia [15], and all community-associated MRSA strains were different from the traditional health care–associated MRSA strains. Indeed, in contrast to community-associated MRSA, health care–associated MRSA has generally been restricted to a small number of clones that have spread globally with the movement of carrier patients and health staff [16]. Molecular analysis of Northern Territory isolates found them to be genetically distinct from Western Australia strains [15]. It also became evident that WA-MRSA was not simply 1 strain but was at least 5 unrelated strains [17]. In contrast to the clonal transfer of health care–associated MRSA from one colonized or infected patient to another within the hospital setting, often via the hands of health care workers, the picture in Australia indicated multiple independent emergences of community-associated MRSA. The highest rates of notification in Western Australia were from 2 widely separated remote regions—the Kimberley and the more southerly Goldfields [18]. In the Top End of the Northern Territory, Aboriginal people were 13 times more likely than non-Aboriginal people to be infected with community-associated MRSA [11].

In Queensland, on the opposite side of the continent from Western Australia, 2 additional clones were identified: the Western Samoan Phage Pattern (WSPP) clone (ST30), first described in the Pacific Islander population, and the Queensland MRSA clone (ST93), which appeared in both white and Aboriginal populations. The WSPP clone was first documented in Brisbane in 1997 [19]. These isolates were related to a clone causing an epidemic of community-associated MRSA in New Zealand during the mid- to late 1990s [20, 21]. The New Zealand epidemic was centered in areas of Auckland densely populated by Pacific Islanders, and several studies confirmed that Samoans were at highest risk of colonization, infection, and bacteremia with WSPP [22]. Ten isolates from Australia, New Zealand, and Western Samoa were identical by PFGE [21] and supported the postulate that WSPP arrived in Australia via New Zealand during the 1980s and 1990s [19]. Poor living conditions in Samoa were cited as factors leading to the emergence of WSPP, although supportive evidence is scant [21].

Queensland MRSA (ST93) came to prominence when whites

were also noted to have community-associated MRSA infections [23]. Although the first reported case of fatal necrotizing pneumonia caused by community-associated MRSA in Australia was in a young Aboriginal man, it was initially thought that ST93 was uncommon in Aboriginal populations [24]. Subsequent studies found that 3 of 4 cases of community-associated MRSA bacteremia due to ST93 were in Aboriginal patients [25] and that ST93 was not carried in attendees to urban Brisbane general practices [26] but was carried by 7% of school children in Aboriginal communities [27]. Evidence suggests that the Queensland clone emerged from Aboriginal communities [28, 29].

The most recently described clonal group in Australia, NT-MRSA, was found to predominate in remote Aboriginal communities in the Top End of the Northern Territory. NT-MRSA comprises clonal complex 75 (cc75), which contains ST75. In a longitudinal, community-based study of pyoderma, 71% of community-associated MRSA isolates were cc75 [30]. Although ST75 had been identified in previous Australia-wide surveys of community-associated MRSA [4, 17, 31] and indeed had been labeled as WA-MRSA-8, it was determined that all the isolates were actually from the Northern Territory. Recent typing of the Northern Territory isolates causing infections during 1991 [11] reveals that NT-MRSA was already present at that time (D.C.H., unpublished data). This clone almost certainly emerged from the local Northern Territory population.

MECHANISMS FOR THE EMERGENCE OF COMMUNITY-ASSOCIATED MRSA

Methicillin resistance is mediated by the *mec*A gene, which is carried on 1 of several types of staphylococcal cassette chromosomes (SCCs), which are mobile genetic elements. Community-associated MRSA typically has type IV SCC*mec*, which is smaller and more mobile than the types I–III SCC*mec* seen in health care–associated MRSA [32]. We hypothesize that, in addition to clonal transmission of community-associated MRSA, there is local de novo emergence of new clones of community-associated MRSA when SCC*mec*IV, or more rarely SCC*mec*V, is transferred via site-specific integration into already prevalent community strains of methicillin-susceptible *S. aureus* (MSSA). That is, not only is one clone of community-associated MRSA spreading, but the gene mediating resistance is also repeatedly jumping from one staphylococcus to another—a "gene outbreak." In addition to a diversity of community-associated MRSA clones arising from geographically distinct regions, we would also expect to find the following: that SCC*mec*IV-positive and SCC*mec*IV-negative isolates of the same clone circulate together, that community-associated MRSA and MSSA infections are epidemiologically and clinically similar, that in vivo transfer of SCC*mec*IV between staphylococcal isolates does occur, and that environmental conditions favoring such transmission are present among populations from which community-associated MRSA has emerged.

Molecular studies that include both MRSA and MSSA strains from confined localities and time spans demonstrate the co-circulation of MSSA and MRSA strains of the same or a similar genetic backbone [30, 33, 34]. It appears that clones of MRSA that harbor SCC*mec*IV have only lately emerged, suggesting recent and possibly frequent acquisition of SCC*mec*IV by background MSSA strains [34]. In the Northern Territory community study, cc75 accounted for 25% of all isolates, and within cc75, the ratio of MRSA to MSSA was 2:1 [30]. This clone has not been reported elsewhere to date, and what we have is a prevalent MSSA strain and its direct SCC*mec*IV-harboring descendant coexisting in an isolated environment.

Early studies of the epidemiology of community-associated MRSA found striking similarities between community-associated MRSA and MSSA infections, leading authors to conclude that "the clinical syndromes associated with *S. aureus* isolation are independent of methicillin susceptibility" [35 (p. 595), 36]. However, because 1 particular clone of community-associated MRSA—USA300—has become dominant in the United States, slight differences between community-associated MRSA and MSSA infections have emerged [37]. This is likely related to various virulence factors carried by USA300 that have made it such a successful clone. For instance, the presence of arginine catabolic mobile element in USA300 is thought to confer a selective advantage for growth and survival in the host [38]. In contrast, in northern Australia, where no 1 clone has become dominant, we have found no difference with regard to the proportion of skin and soft-tissue infections, need for hospital admission, need for surgery, and length of hospital stay for patients with community-associated MRSA infections, compared with patients with MSSA infections, presenting to the Royal Darwin Hospital [39]. Although USA300, Queensland-MRSA, and the WSPP clone carry the Panton-Valentine leukocidin gene *(pvl)*, NT-MRSA and the original WA-MRSA clones are *pvl* negative [40]. Most Australian reports of severe community-associated MRSA infections are associated with the Queensland clone [25], which supports the hypothesis that virulence is determined by factors other than the presence of SCC*mec*. However, *pvl* has been detected recently in some WA-MRSA strains [4], emphasizing the importance of ongoing clinical and molecular surveillance for changing virulence in community-associated MRSA.

Evidence of the in vivo transfer of SCC*mec*IV to an MSSA strain was elegantly provided by Wielders et al. [41], when they described a patient in whom initially an MSSA and then an MRSA were isolated. The isolates were identical except for the presence of *mec*A, and the *mec*A was identical to that excised from a *Staphylococcus epidermidis* isolate from the same patient.

It appeared that the MSSA had acquired *mec*A from the *S. epidermidis* to become MRSA.

COMMON FACTORS IN GROUPS AT HIGH RISK FOR COMMUNITY-ASSOCIATED MRSA

Populations at high risk for community-associated MRSA outbreaks include sporting teams [42], incarcerated persons [43], the military [44], children in day care facilities [45], men who have sex with men [46], and indigenous communities across the world [36, 47, 48]. A study of community-associated MRSA infections in an American football team found that infections developed at turf abrasion sites and in players who had more frequent skin contact with other players. Hygiene practices were suboptimal, and antibiotic use was much higher among team players than among the general population [42]. Investigations of outbreaks in jails in the United States identified poor hygiene practices, close contact with MRSA-infected inmates, and poor access to medical care as risk factors for community-associated MRSA infection [43].

Indigenous populations often live in remote and isolated settings, although there are crowded living conditions within communities. As has been the case in Australia, community-associated MRSA appears to have emerged from these settings. In remote Alaskan villages, outbreaks of community-associated MRSA skin and soft-tissue infections were associated with prior antibiotic use and use of communal saunas from which community-associated MRSA was recovered [47, 48]. An epidemic of community-associated MRSA occurred in an American Indian rural community with crowded housing conditions, poor access to health care, and high rates of skin disease, which led the authors to comment that "community-associated MRSA may be found in ever-increasing numbers in other communities of low socioeconomic status" [36, p. 1204] and that "rural communities are not sheltered" [36, p. 1205]. We contend that not only are such communities "not sheltered," but that in fact they may be the milieu from which community-associated MRSA is emerging and subsequently spreading to the wider population.

FACTORS CONTRIBUTING TO THE EMERGENCE OF COMMUNITY-ASSOCIATED MRSA FROM AUSTRALIAN ABORIGINAL COMMUNITIES

Risk factors for emergence and transmission of community-associated MRSA—namely, crowding, poor hygiene, skin infections, and antibiotic use—are highly prevalent in Australian Aboriginal communities [49]. Crowding is most severe in the Northern Territory, with reports of a mean of 3.4 persons per bedroom in one study [50] and up to 7.5 per bedroom in another [51]. Water supplies are deficient and unreliable [52], and >60% of households were found to have no or poorly functioning facilities for either washing children, washing clothes, or removing feces [50]. Although it is difficult to make causative links between independent factors, it is likely that domestic crowding, poor hygiene, and associated sociodemographic factors contribute to extremely high rates of scabies and impetigo in Aboriginal communities [49, 50].

The prevalence of scabies is 25% in adults and 65% in children in some Northern Territory Aboriginal communities [53], and by age 1 year, 63% and 69% of all children in the community have presented with scabies and skin sores, respectively, to community clinics [54]. The prevalence of impetigo among children (<15 years of age) has been up to 70% [53], but we more recently observed an overall prevalence of 20% [51]. Group A streptococcus has previously been the primary pathogen, found in >80% of pyoderma lesions [49], but the pattern appears to be changing in the Northern Territory. In a more recent study, *S. aureus* was recovered from 57% of pyoderma lesions, and group A streptococcus was recovered from 29% of lesions (usually with *S. aureus*) [51]. It is not clear why the microbiological epidemiology is changing, but with the emergence of community-associated MRSA, intramuscular benzathine penicillin may no longer be the most appropriate antibiotic for the treatment of skin sores in these communities. The ongoing heavy burden of staphylococcal skin infection in crowded settings is likely to be associated with high rates of antibiotic use and to facilitate person-to-person transmission of community-associated MRSA and the transfer of SCC*mec*IV into resident MSSA strains.

The zoonotic potential of MRSA has been noted for horses, pigs, cows, and domestic pets [55–58]. Poor dog health and dog overpopulation are major problems in many Aboriginal communities, and dogs are an intriguing potential contributor to community-associated MRSA emergence. One community study found a median of 3 (and up to 17) dogs per household, and it was common for dogs to have open wounds [59]. The probable transmission of community-associated MRSA between dogs and humans, in both directions, has been documented elsewhere [58] and would seem to be even more likely in Aboriginal communities. An additional consideration is that dogs are also colonized with other staphylococcal species, including *Staphylococcus sciuri* [60]. The *S. sciuri* genome contains an *mec*A homologue that is thought to possibly be the evolutionary precursor of *mec*A now found in MRSA [32]. Therefore, it is possible, not only that antibiotic pressure and overcrowding is amplifying the transmission of community-associated MRSA strains in both dogs and humans, but that community-associated MRSA origins may relate to non–*S. aureus* staphylococcal species in dogs.

WHAT CAN WE DO?

There is a rising prevalence of community-associated MRSA infections worldwide, and evidence is accumulating that com-

munity-associated MRSA is not simply replacing MSSA but is adding to the overall burden of staphylococcal disease [61, 62]. Community-associated MRSA strains also cause health care–associated and nosocomial MRSA infections; people who are colonized on hospital admission can serve as a source of transmission within the hospital environment [10, 63–67]. This was recognized in early community-associated MRSA reports from the Northern Territory [12]. Of concern, clones in Australia are beginning to acquire more virulence and antibiotic resistance determinants [4]. Furthermore, if there is truly ongoing acquisition of SCC*mec*IV by local MSSA strains, we are likely to see increasing de novo emergence of community-associated MRSA in community settings. Combating the emergence and spread of community-associated MRSA may require novel infection-control strategies targeted at specific groups at the community level.

The role of subpopulations acting as the foci for the emergence and amplification of infectious diseases has been long recognized with sexually transmitted and vector-borne diseases [68]. "Core transmitters" with crusted scabies have also been identified as important in driving the ongoing scabies outbreaks in remote Aboriginal communities, which in turn underlie high rates of pyoderma [49]. There is growing appreciation of the importance of such "core groups" in the epidemiology of antimicrobial-resistant pathogens, including community-associated MRSA [69–71]. In the United States, large jails housing up to 20,000 inmates have been identified as likely foci for the amplification and subsequent spread of community-associated MRSA into the wider community [44, 70, 72]. Public health interventions directed at these "superspreader institutions" are predicted to have a disproportionate effect on controlling the epidemic of community-associated MRSA [69]. Authorities in the United States have produced guidelines aimed at reducing the transmission of community-associated MRSA within prisons, the military, and the general population [44, 73]. One correctional facility in Texas has demonstrated significant reductions in community-associated MRSA infections through improvements in screening for and care of skin infections, personal hygiene, and antibiotic therapy [74].

Although Australia's health care system ranks high internationally, the health inequalities between Aboriginal and non-Aboriginal Australians are well documented. A considerable increase in resources is required to enable remote Aboriginal communities to meet published recommendations for control of community-associated MRSA. It could be that we are paying a heavy microbiological price for the neglect of Aboriginal housing and health hardware needs in remote communities over the past 2 decades. Improving living conditions for Aboriginal Australians should reduce rates of skin infection, and, if our hypothesis is correct, public health strategies targeted at screening for and appropriate treatment of skin infections should slow the emergence of new community-associated MRSA strains. Clinical trials are required to determine whether the current recommendation of intramuscular benzathine penicillin is still the most appropriate antibiotic for treatment of impetigo in remote Aboriginal communities. Despite appropriate calls for accelerated development of new antibiotics to treat resistant organisms [75], we must bear in mind that the unfettered use of antimicrobial agents in disadvantaged communities without addressing underlying socioeconomic conditions is likely to further promote the emergence of microbial resistance.

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Appendix IV. Community-associated strains of methicillin-resistant *Staphylococcus aureus* and methicillin-susceptible *S. aureus* in Indigenous northern Australia: Epidemiology and outcomes

Community-Associated Strains of Methicillin-Resistant *Staphylococcus aureus* and Methicillin-Susceptible *S. aureus* in Indigenous Northern Australia: Epidemiology and Outcomes

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(See the editorial commentary by Turnidge, on pages 1416–8.)

Background. Some strains of non–multidrug-resistant, methicillin-resistant *Staphylococcus aureus* (nmMRSA) in Australia are likely to have emerged from strains of methicillin-susceptible *S. aureus* (MSSA) in remote Aboriginal communities.

Objective. To describe the clinical epidemiology of infection due to community-associated MRSA strains in an Australian tropical hospital setting with a significant Aboriginal population and to compare infections caused by community-associated strains of MRSA, health-care–associated strains of MRSA, and MSSA strains with respect to demographic risk factors and clinical outcomes.

Methods. We queried the microbiology database for the Top End of the Northern Territory, Australia, to determine population incidences for *S. aureus* infection and conducted a prospective matched case-control study to compare infection due to nmMRSA, MSSA, or multidrug-resistant MRSA at the Royal Darwin Hospital.

Results. The annual incidence of *S. aureus* bacteremia was 65 cases per 100,000 population, but in the Aboriginal population the incidence was 172 cases per 100,000 population (odds ratio [OR] compared with non-Aboriginal population, 5.8 [95% confidence interval {CI}, 3.8–8.9). Female sex (adjusted OR [aOR], 1.5 [95% CI, 1.1–2.0) and remote residence (aOR, 1.8 [95% CI, 1.2–2.5]) were associated with the isolation of nmMRSA rather than MSSA, but disease spectrum and outcomes were similar. Among those from whom nmMRSA was isolated, Aboriginal patients were younger (aOR for each additional year, 0.94 [95% CI, 0.92–0.96]), more likely to be female (aOR, 3.8 [95% CI, 1.7–8.5]), and more likely to reside in a remote community (aOR, 29 [95% CI, 8.9–94]) than non-Aboriginal patients. The presence of Panton-Valentine leukocidin in nmMRSA was associated with double the odds of sepsis (aOR, 2.2 [95% CI, 1.1–4.6]).

Conclusions. The association of nmMRSA infection with female sex and remote residence supports the hypothesis that nmMRSA arose from MSSA strains in remote Aboriginal communities where staphylococcal disease is highly prevalent. The similar clinical spectrum and outcomes for nmMRSA infection and MSSA infection suggest that virulence is not correlated with resistance phenotype.

Infection due to community-associated strains of methicillin-resistant *Staphylococcus aureus* (CA-MRSA strains) was first recognized in Australia in people from remote Aboriginal communities [1]. Reports of genetically distinct CA-MRSA strains from other geographically removed regions followed [2–4]. The parallel emergence of infection due to CA-MRSA strains in North America also included outbreaks in rural Indigenous communities [5, 6]. Despite the possibility that CA-MRSA strains emerged from such communities and

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the impact of these strains on their inhabitants, there have been no detailed studies comparing the clinical epidemiology of infection due to CA-MRSA strains in Indigenous and nonindigenous people.

Early studies noted clear differences between CA-MRSA strains and hospital-associated strains of MRSA (HA-MRSA strains) with respect to the patients affected and types of infection caused. Individuals infected with CA-MRSA strains were younger and more likely to have skin and soft-tissue infections (SSTIs), and Indigenous people were overrepresented in this group [5, 6]. In contrast, there were notable similarities between infections due to CA-MRSA strains and infections due to methicillin-susceptible *S. aureus* (MSSA) strains with regard to risk factors and clinical illness [5, 7]. Molecular studies demonstrated that HA-MRSA and CA-MRSA strains were genetically distinct [5, 8]. However, CA-MRSA strains and MSSA strains with the same or similar genetic backgrounds cocirculated in the same environment [9, 10]. Accordingly, it has been proposed that CA-MRSA strains emerge locally from prevalent circulating MSSA strains via acquisition of the *mecA* gene [11–13].

We conducted a case-control study at the Royal Darwin Hospital (RDH) with the following 2 objectives: (1) to describe the clinical epidemiology of infection due to CA-MRSA strains in an Australian tropical hospital setting with a significant Aboriginal population, and (2) to compare infections caused by CA-MRSA, HA-MRSA, and MSSA strains with respect to demographic risk factors and clinical outcomes. We hypothesized that infection due to CA-MRSA strains and infection due to MSSA strains would be similar in most of these key aspects.

METHODS

Study setting and design. The 330-bed RDH is located in the Top End of the Northern Territory of Australia at a tropical latitude of 12°23′ S. It is the tertiary referral center for the city of Darwin, 2 regional hospitals, and over 70 remote communities, serving a population of 176,000 and an area of 510,000 km^2. Aboriginal Australians comprise 27% of the regional population but make up >50% of the RDH inpatient and emergency department population. Approval for the study was granted by the Northern Territory Department of Health and Community Services Human Research Ethics Committee and the Menzies School of Health Research.

We sought nonduplicate clinical isolates of *S. aureus* over a 12-month period from 18 April 2006 through 17 April 2007 by querying the microbiology database that links all 3 Top End hospitals. There was no routine hospital screening for MRSA colonization during the study period. We considered non–multidrug-resistant MRSA (nmMRSA) isolates to represent CA-MRSA strains and considered multidrug-resistant MRSA (mMRSA) isolates to represent HA-MRSA strains [14]. nmMRSA isolates were defined phenotypically as those resistant to <3 non–β-lactam antibiotic classes, and mMRSA isolates were defines as those resistant to $\geqslant$3 non–β-lactam antibiotic classes. Antibiotic phenotype has previously been shown to accurately predict the genotype of CA-MRSA strains [15], and this result has been validated in recent studies from Queensland [14], Western Australia [16], and an Australia-wide survey [12] that included isolates from Darwin. After excluding nonresident patients, we calculated incidence rates for the isolation of *S. aureus* on the basis of published population data from 2006 [17] and correlated this with measures of regional socioeconomic disadvantage [18] and residential remoteness [19]. For the former analysis, only regions that had $\geqslant$10 *S. aureus* isolates and an available score for the index of relative socioeconomic disadvantage were included.

We then conducted a case-control study of patients at the RDH in which we compared nmMRSA infections with infections caused by MSSA and mMRSA over the same 12-month period. All nmMRSA and mMRSA isolates newly identified in the microbiology laboratory were collected daily, as well as $\leqslant$4 consecutive MSSA isolates for each nmMRSA isolate. Laboratory staff did not include isolates from patients known to have had *S. aureus* isolated in the past month. We used sequential laboratory receipt numbers to match eligible patients who had nmMRSA isolated to the next 2 eligible patients who had MSSA isolated.

Data collection. By prospectively reviewing the clinical records of the patients from whom each of the isolates were recovered, we collected information concerning demographic characteristics, health care–associated risk factors, comorbidities, clinical details of the infection, treatment details, and outcome at discharge. To restrict our analysis to incident staphylococcal infections, we excluded patients from further analysis if they had previously been enrolled in the study or if they had a prior existing unresolved *S. aureus* infection. We excluded patients who had only attended an outpatient clinic or dialysis unit and did not require treatment at the emergency department or hospital admission. We also excluded patients transferred to the RDH who had been admitted elsewhere for >24 h, patients for whom the treatment intention was palliative, and patients for whom clinical notes were unavailable.

Health-care associated infection was defined in accordance with previously published criteria [20] and included nosocomial infection or the presence of any of the following risk factors during the year prior to collection of the sample that yielded the culture result of interest in the present study: (1) residence in a long-term care facility, (2) prior admission to an acute care facility, (3) use of central intravenous catheters or long-term venous access devices, (4) use of urinary catheters, (5) use of other long-term percutaneous devices, (6) prior surgical procedures, and/or (7) need for any form of dialysis. Nosocomial infection was defined by an isolate obtained from a sample collected >48 h after hospital admission. Previously published criteria were used to define infection, sepsis, and the systemic inflammatory

response syndrome [21]. We defined colonization as a positive microbiological culture result in the absence of clinical features of infection.

Laboratory methods. The laboratory identified *S. aureus* isolates by use of standard methods and conducted susceptibility testing using an automated system (Vitek 2 V4.01; bioMérieux) and the Kirby-Bauer disk diffusion method in accordance with the guidelines of the Clinical and Laboratory Standards Institute [22]. A disk-approximation test was used to detect inducible clindamycin resistance. Real-time polymerase chain reaction was used to verify the identity of nmMRSA isolates by confirming the presence of the *nucA* and *mecA* genes; it was also used to determine the presence of *pvl* genes [10].

Statistical analysis. Statistical significance for crude analysis of dichotomous variables was determined using the χ^2 test or Fisher's exact test. Nonparametric data were compared using the Mann-Whitney *U* test. We performed multivariate conditional logistic regression analysis with stepwise backward elimination of variables to identify the risk factors associated with infection due to nmMRSA compared with those for infection due to MSSA, and we performed multivariate logistic regression analysis of risk factors associated with severity of illness. The likelihood ratio test was used to assess the statistical significance of candidate risk factors. We examined the association between incidence of *S. aureus* infection and measures of regional socioeconomic disadvantage and remoteness by linear regression. If obvious outliers were present we used robust regression methods. Two-sided *P* values of $<.05$ were considered significant. Statistical analysis was performed with Stata (version 9.2; StataCorp).

RESULTS

From 18 April 2006 through 17 April 2007, there were 2227 *S. aureus* isolates recovered from distinct patients from the 3 Top End hospitals; nmMRSA accounted for 343 (15%) of the isolates, MSSA accounted for 1748 (79%), and mMRSA accounted for 136 (6%). There were 110 (5%) nonduplicate isolates recovered from blood culture, which were distributed as follows: 17 were nmMRSA, 83 were MSSA, and 10 were mMRSA. The annual incidence of *S. aureus* bacteremia was 65 cases per 100,000 population, and for infection due to MRSA, the incidence was 16 cases per 100,000 population. The annual incidence of *S. aureus* bacteremia in the Aboriginal population was 172 cases per 100,000 population, and for the non-Aboriginal population, the incidence was 30 cases per 100,000 population (odds ratio [OR], 5.8 [95% confidence interval {CI}, 3.8–8.9]). The total annual incidence of isolation of *S. aureus* was 1248 isolations per 100,000 population, and the incidence of nmMRSA isolation was 193 isolations per 100,000 population. There was a strong correlation between incident isolation of *S. aureus* and measures of regional socioeconomic disadvantage and remoteness (figure 1).

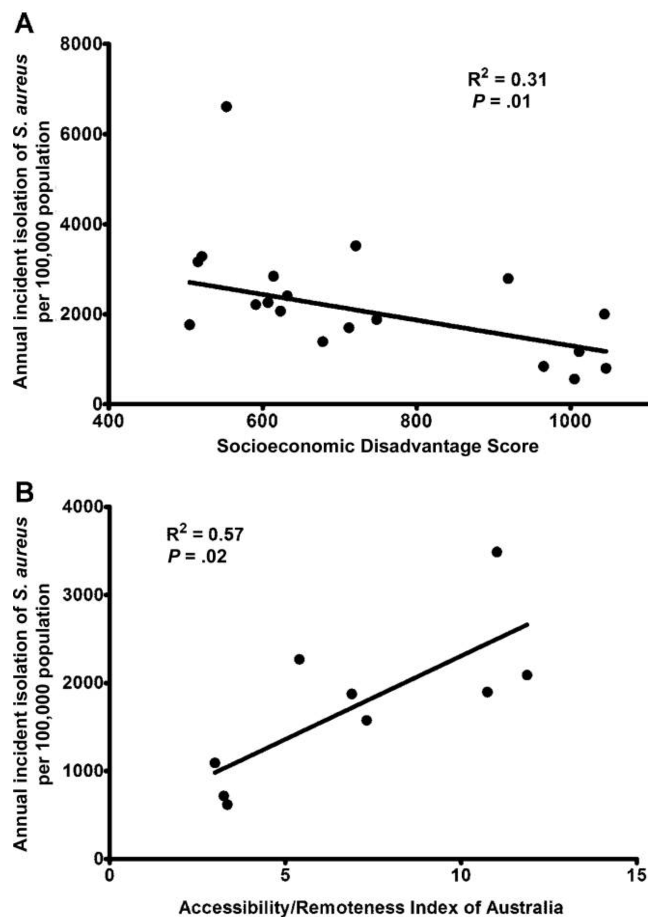

Figure 1. Incident isolation of *Staphylococcus aureus* from 18 April 2006 through 17 April 2007 in the Top End of the Northern Territory, Australia, plotted against the regional index of relative socioeconomic disadvantage score *(A)* and the accessibility/remoteness index of Australia *(B)*. For the socioeconomic score, a lower score indicates greater disadvantage; for the accessibility/remoteness index, a higher score indicates greater remoteness. Regression was performed using robust methods *(A)* and standard linear regression *(B)*.

At the RDH during the same time period, there were 1693 nonduplicate *S. aureus* isolates recovered; 291 (17%) of these were nmMRSA, 1265 (75%) were MSSA, and 137 were (8%) mMRSA. As an estimate of community-onset cases, of 728 isolates recovered from patients presenting to the emergency department, 117 (16%) were nmMRSA, 582 (80%) were MSSA, and 29 (4%) were mMRSA. Prior to matching, there were 1007 eligible patients and isolates in the case-control study. Figure 2 presents details about exclusion criteria and the number of participants and isolates at each stage of the study. After matching, there were 239 patients who had nmMRSA isolated, 478 who had MSSA isolated, and 90 who had mMRSA isolated available for further analysis. The demographic characteristics of the control patients who had MSSA isolated were representative of the overall population of patients infected with MSSA in the Top End (data not

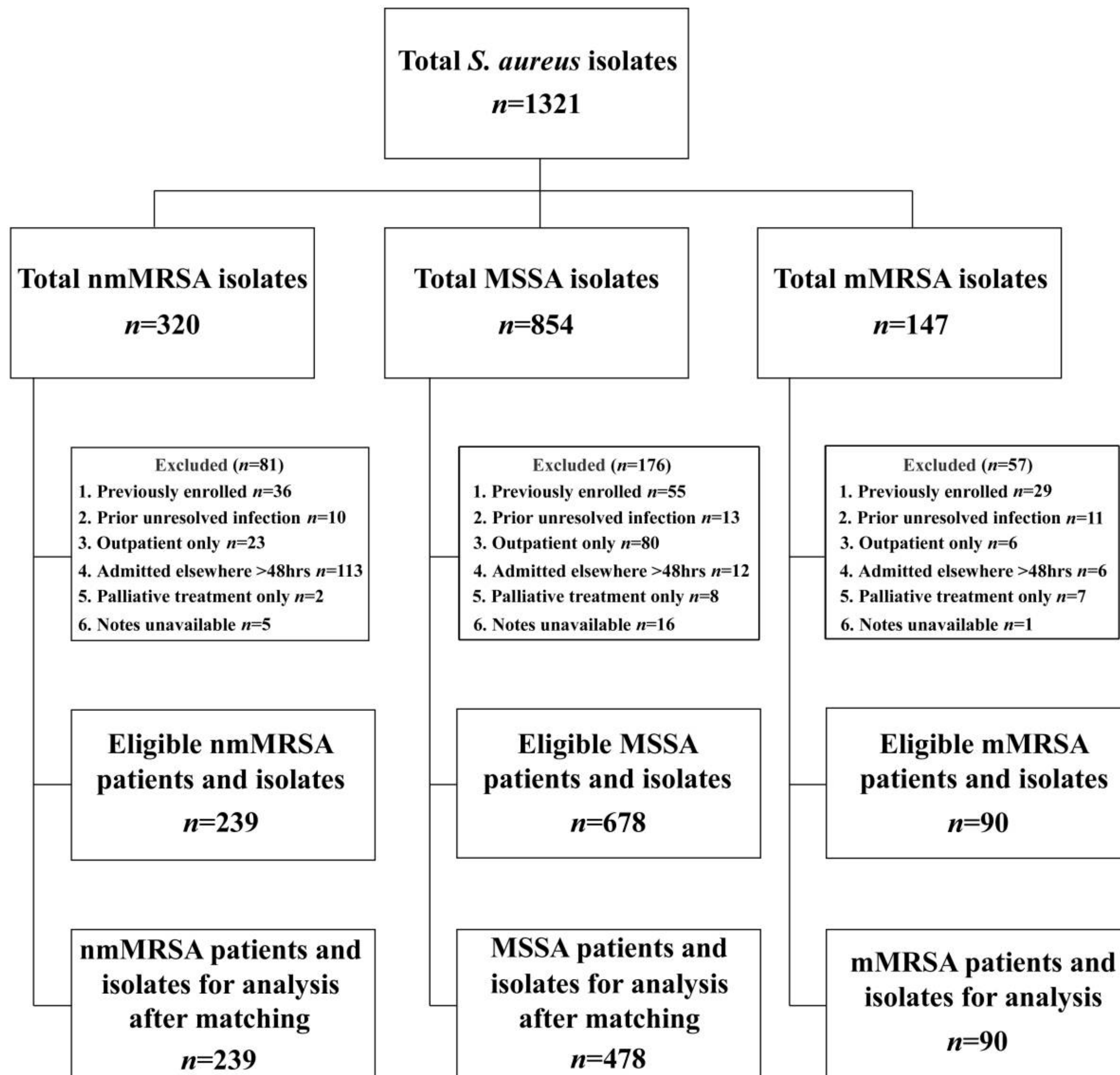

Figure 2. Flow chart detailing the no. of *Staphylococcus aureus* isolates collected, the exclusion criteria applied, the no. of eligible isolates, and the no. of matched isolates used for the Royal Darwin Hospital case-control study, 18 April 2006–17 April 2007. nmMRSA, non–multidrug-resistant, methicillin-resistant *Staphylococcus aureus;* mMRSA, multidrug-resistant MRSA; MSSA, methicillin-susceptible *S. aureus*.

shown). Of the 807 isolates studied, 403 (50%) were considered to represent community-associated strains, and 404 (50%) were considered to represent health care–associated strains. There were 153 (19%) isolates recovered from patients who satisfied the definition of nosocomial infection. A similar proportion of nmMRSA and MSSA isolates were health-care associated strains ($P = .15$) or were nosocomially acquired ($P = .12$) (table 1). In nmMRSA isolates, the presence of *pvl* genes was associated with community acquisition rather than nosocomial acquisition (OR, 4.4 [95% CI, 2.5–7.8]).

Epidemiological and clinical characteristics. Patients who had nmMRSA isolated and patients who had MSSA isolated were of a similar age ($P = .10$) but younger than those who had mMRSA isolated ($P < .01$) (table 1). Patients who had nmMRSA isolated were less likely to be male than those who had MSSA or mMRSA isolated. A similar proportion of patients who had nmMRSA isolated and patients who had MSSA isolated were of Aboriginal ethnicity, but patients who had nmMRSA isolated were more likely to reside remotely. Otherwise, similarities between patients who had nmMRSA isolated and those who had MSSA isolated were evident with regard to the presence of comorbidities and laboratory indices. The patients who had mMRSA isolated were clearly a distinct group, being more likely to be hospitalized with comorbidities as well as having significantly higher serum creatinine levels and lower albumin and hematocrit levels (data not shown).

Severity and primary site of infection. The distribution of clinical syndromes and severity of illness is shown in table 1. The vast majority of infections were SSTIs. Overall 629 (87%) of 720 infections (87 colonized patients were excluded) were SSTIs, ac-

Table 1. Characteristics of patients from whom non–multidrug-resistant, methicillin-resistant *Staphylococcus aureus* (nmMRSA), methicillin-susceptible *S. aureus* (MSSA), or multidrug-resistant MRSA (mMRSA) was isolated.

Variable type, characteristic	nmMRSA (n = 239)	MSSA (n = 478)	mMRSA (n = 90)	Unadjusted OR (95% CI): nmMRSA vs. MSSA infection	Unadjusted OR (95% CI): nmMRSA vs. mMRSA infection
Demographic trait					
Age, median, years	39	34	59		
Female sex	107 (45)	166 (35)	28 (31)	1.5 (1.1–2.1)	1.8 (1.0–3.1)
Aboriginal	135 (56)	240 (50)	30 (33)	1.3 (0.9–1.8)	2.6 (1.5–4.5)
Remote residence	81 (34)	366 (23)	14 (16)	1.7 (1.2–2.4)	2.8 (1.4–5.7)
HCA risk present	114 (48)	201 (42)	89 (99)	1.3 (0.9–1.7)	0.0 (0.0–0.1)
Nosocomial acquisition	39 (16)	58 (12)	56 (62)	1.4 (0.9–2.2)	0.1 (0.1–0.2)
Comorbidity					
⩾1 Comorbidity	76 (32)	136 (28)	69 (77)	1.2 (0.8–1.7)	0.1 (0.1–0.3)
Diabetes	47 (20)	73 (15)	32 (36)	1.4 (0.9–2.1)	0.4 (0.3–0.8)
Chronic respiratory disease	17 (7)	27 (6)	22 (24)	1.3 (0.6–2.5)	0.2 (0.1–0.5)
Chronic renal disease	15 (6)	23 (5)	19 (21)	1.3 (0.6–2.7)	0.3 (0.1–0.6)
Coronary artery disease	27 (11)	29 (6)	26 (29)	2.0 (1.1–3.5)	0.3 (0.2–0.6)
Chronic liver disease	5 (2)	17 (4)	12 (13)	0.6 (0.2–1.7)	0.1 (0.0–0.4)
Injection drug user	2 (1)	3 (1)	1 (1)	1.3 (0.1–11)	0.8 (0.0–45)
Scabies	17 (7)	27 (6)	5 (6)	1.3 (0.6–2.5)	1.3 (0.4–4.7)
Severity of infection[a]					
Colonization	14 (6)	42 (9)	31 (34)	0.6 (0.3–1.2)	0.1 (0.1–0.2)
Localized infection	173 (72)	328 (69)	36 (40)	1.2 (0.8–1.7)	3.9 (2.3–6.8)
Sepsis	47 (20)	101 (21)	20 (20)	0.9 (0.6–1.4)	0.9 (0.5–1.6)
Severe sepsis	1 (1)	3 (1)	2 (2)	0.7 (0.0–8.3)	0.2 (0.0–3.6)
Septic shock	4 (2)	4 (1)	1 (1)	2.0 (0.4–11)	1.5 (0.1–75)
Primary site or type of infection[b]					
Skin and soft tissue[c]	198 (88)	390 (89)	41 (69)	0.9 (0.5–1.5)	3.1 (1.4–6.5)
Cellulitis	32 (14)	58 (13)	5 (8)	1.1 (0.7–1.8)	1.8 (0.6–6.2)
Abscess and/or boil	90 (40)	163 (37)	2 (3)	1.1 (0.8–1.6)	19 (4.8–164)
Surgical site	24 (11)	29 (7)	21 (36)	1.7 (0.9–3.1)	0.2 (0.1–0.5)
Foot wounds or ulcers in a diabetic patient	10 (4)	23 (5)	6 (10)	0.8 (0.3–1.9)	0.4 (0.1–1.4)
Pyoderma	12 (5)	33 (8)	1 (2)	0.7 (0.3–1.4)	3.3 (0.5–142)
Nonsurgical wound	25 (11)	79 (18)	6 (10)	0.6 (0.4–0.9)	1.1 (0.4–3.5)
Other	5 (2)	5 (1)	0 (0)	2.0 (0.5–7.4)	1.6 (0.2–74)
Bone and/or joint	6 (3)	9 (2)	1 (2)	1.3 (0.4–4.1)	1.6 (0.2–74)
Bloodstream[d]	5 (2)	14 (3)	3 (5)	0.7 (0.2–2.0)	0.4 (0.1–2.8)
Respiratory tract	9 (4)	11 (2)	7 (12)	1.6 (0.6–4.3)	0.3 (0.1–1.0)
Urinary tract	2 (1)	2 (1)	4 (7)	1.9 (0.1–27)	0.1 (0.0–0.9)
Other	5 (2)	10 (2)	3 (5)	1.0 (0.3–3.2)	0.4 (0.1–2.8)
Treatment received					
Surgery	120 (50)	251 (53)	25 (28)	1.1 (0.8–1.5)	2.6 (1.5–4.6)
Active antibiotic therapy					
As inpatient	79 (33)	388 (81)	46 (51)	0.1 (0.1–0.2)	0.5 (0.3–0.8)
As inpatient or at discharge	100 (42)	442 (92)	46 (51)	0.1 (0.0–0.1)	0.7 (0.4–1.1)
Outcome					
Hospitalization	197 (82)	388 (81)	83 (92)	1.1 (0.7–1.7)	0.4 (0.1–0.9)
ICU admission	12 (5)	25 (5)	12 (13)	1.0 (0.4–2.0)	0.3 (0.1–0.9)
Length of stay, median, days	4	3	28		
In-hospital mortality	6 (3)	9 (2)	8 (9)	1.3 (0.4–4.3)	0.3 (0.1–0.9)

NOTE. Data are no. (%) of subjects unless otherwise indicated. CI, confidence interval; HCA, health-care associated; ICU, intensive care unit; OR, odds ratio.

[a] $P = .49$, by Fisher's exact analysis of severity of infection using 5×2 table of nmMRSA vs. MSSA infection; $P < .01$, for comparison of nmMRSA and mMRSA infection.

[b] Patients with colonization alone were excluded; for this part of the analysis, there were 225 nmMRSA infections, 436 MSSA infections, and 59 mMRSA infections. $P = .78$, by Fisher's exact analysis for all infections using 6×2 table of nmMRSA vs. MSSA infection; $P < .01$, using 7×2 table of nmMRSA vs. mMRSA infection.

[c] $P = .12$, by Fisher's exact analysis for skin and soft-tissue infection using 7×2 table of nmMRSA vs. MSSA infection; $P < .01$, for comparison of nmMRSA and mMRSA infection.

[d] There were 49 cases of bacteremia, but the bloodstream was considered the primary site of infection for only 22 cases. For the other 27 cases of bacteremia, the primary site of infection was considered to be skin and soft tissue for 11, bone and/or joint for 7, respiratory for 3, urinary tract for 2, and other for 4.

counting for ~90% of nmMRSA and MSSA infections (198 of 225 infections and 390 of 436 infections, respectively) and 41 (69%) of 59 mMRSA infections. Boils, abscesses, and nonsurgical wound infections accounted for ~60% of nmMRSA and MSSA SSTIs (115 of 198 infections and 242 of 390 infections, respectively), whereas surgical site infections made up 21 (51%) of 41 mMRSA SSTIs. When colonized individuals were excluded, sepsis, severe sepsis, and septic shock combined accounted for 52 (23%) of 225 nmMRSA infections, 108 (25%) of 436 MSSA infections, and 23 (39%) of 59 mMRSA infections. There was no statistically significant difference between nmMRSA and MSSA when comparing the severity of illness ($P = .49$), the distribution of overall clinical syndromes ($P = .78$), or the clinical syndromes in the SSTI group alone ($P = .12$).

Outcomes. The majority of patients were admitted to the hospital, and ~50% of patients who had nmMRSA or MSSA isolated required surgical intervention (120 of 239 patients and 251 of 478 patients, respectively). The median length of stay was short for patients who had nmMRSA isolated (4 days) or MSSA isolated (3 days) but prolonged for patients who had mMRSA isolated (28 days) cases. The in-hospital mortality rate was higher for patients who had mMRSA isolated (table 1) than for the other groups.

Antimicrobial susceptibility. Disk-approximation testing of erythromycin-resistant isolates revealed that inducible clindamycin resistance was present in 52 (22%) of 239 nmMRSA isolates and 77 (16%) of 478 MSSA isolates, respectively. All nmMRSA and MSSA isolates were susceptible to trimethoprim-sulfamethoxazole and rifampin, and most were susceptible to tetracycline (233 nmMRSA isolates [97%] and 475 MSSA isolates [99%]) and fusidic acid (206 [86%] nmMRSA isolates and 456 [95%] MSSA isolates). Patients who had nmMRSA isolated were less likely than those who had MSSA or mMRSA isolated to receive an antibiotic that was active against the causative organism (i.e., active antibiotic). The active antibiotics most commonly prescribed as inpatient therapy for nmMRSA infection were vancomycin (prescribed for 43 [54%] of 79 patients) and trimethoprim-sulfamethoxazole (prescribed for 49 [62%] of 79 patients).

Comparison of Aboriginal and non-Aboriginal patients who had nmMRSA isolated. Aboriginal patients who had nmMRSA isolated were significantly younger, more likely to be from remote regions, and less likely to be male than non-Aboriginal patients who had this organism isolated (table 2). Despite a younger age, diabetes mellitus and chronic renal disease were more common in Aboriginal patients and the length of stay was longer. Scabies was diagnosed 14 times more frequently in Aboriginal patients. Non–multidrug-resistant MRSA isolates from Aboriginal patients were less likely to be *pvl* positive.

Multivariate analysis. Because the characteristics of patients who had mMRSA isolated were clearly different from those of patients who had nmMRSA or MSSA isolated, we concentrated on comparing predictors for the isolation of nmMRSA to predictors for the isolation of MSSA. After multivariate conditional logistic regression, older age (adjusted OR [aOR] for each additional year, 1.01 [95% CI, 1.00–1.02]), female sex (aOR, 1.5 [95% CI, 1.1–2.0]), and remote residence (aOR, 1.8 [95% CI, 1.2–2.5]) predicted isolation of nmMRSA. Among patients who had nmMRSA isolated, Aboriginal patients were younger (aOR for each additional year, 0.94 [95% CI, 0.92–0.96]), more likely to be female (aOR, 3.8 [95% CI, 1.7–8.5]), more likely to reside remotely (aOR, 29 [95% CI 8.9–94]), more likely to have chronic renal impairment (aOR, 42 [95% CI, 4.5–403]), and less likely to be infected with a *pvl*-positive strain (aOR, 0.2 [95% CI, 0.1–0.6]) than non-Aboriginal patients.

After multivariate logistic regression comparing sepsis (sepsis, severe sepsis, or septic shock) with localized infection only, the presence of comorbidities (aOR, 1.8 [95% CI, 1.1–2.8]) and remote residence (aOR, 1.6 [95% CI, 1.0–2.6]) predicted sepsis. SSTIs were associated with localized infection (aOR, 0.2 [95% CI, 0.1–0.3]). The isolate's methicillin-resistance phenotype was not associated with severity of disease (table 3). For nmMRSA, the presence of *pvl* was the only factor significantly associated with sepsis (aOR, 2.2 [95% CI, 1.1–4.6]).

DISCUSSION

The present study has revealed a heavy burden of staphylococcal disease in Aboriginal populations and defined the epidemiological and clinical relationship between nmMRSA and MSSA in northern Australia. The findings that patients who had nmMRSA isolated were significantly less likely to be male than those who had MSSA isolated and that remote residence was a strong predictor of nmMRSA isolation provide important insights into the emergence of nmMRSA. Evidence that clinical disease presentation and outcomes were independent of resistance phenotype suggests that, in our setting, virulence is not convergent with resistance phenotype.

The incidences of both MSSA infection and nmMRSA infection are much higher in Aboriginal populations. These incidence rates strongly correlate with measures of remoteness and socioeconomic disadvantage across regions of the Top End. The annual incidence of *S. aureus* bacteremia—65 cases per 100,000 population—is almost double the rate estimated for the whole of Australia, which is 35 cases per 100,000 population [23] and more than double the rates recently reported from Canada [24] and Sweden [25]. When patients were stratified by ethnicity, the non-Aboriginal population of the Top End had an annual incidence of bacteremia similar to that of the rest of Australia but the incidence among the Aboriginal population is 6 times higher, at 172 cases per 100,000 population.

Clinical and epidemiological risk factors do not appear to reliably distinguish between infection due to CA-MRSA strains and infection due to MSSA strains [26, 27], and outcomes fol-

Table 2. Characteristics of 239 patients from whom non–multidrug-resistant, methicillin-resistant *Staphylococcus aureus* isolated, stratified by ethnicity.

	Ethnicity			
Variable type, characteristic	Aboriginal (n = 135)	Non-Aboriginal (n = 104)	Unadjusted OR (95% CI)	P
Demographic trait				
Age				
Median, years	30	43		<.01
<18 years	46 (34)	8 (8)	6.2 (2.7–16)	<.01
Remote residence	77 (57)	4 (4)	33 (11–129)	<.01
Female sex	75 (56)	32 (31)	2.8 (1.6–5.0)	<.01
HCA risk present	72 (53)	42 (40)	1.7 (1.0–2.9)	.05
Nosocomial acquisition	26 (19)	13 (13)	1.7 (0.8–3.8)	.16
Comorbidity				
⩾1 Comorbidity	48 (36)	28 (27)	1.5 (0.8–2.7)	.16
Diabetes	36 (27)	11 (11)	3.1 (1.4–7.1)	<.01
Chronic renal disease	14 (10)	1 (1)	11 (1.7–508)	<.01
Scabies	16 (12)	1 (1)	14 (2.1–586)	<.01
Type of infection				
Abscess	41 (30)	49 (47)	0.5 (0.3–0.9)	<.01
Bacteremia	5 (4)	0 (0)	NA	.07
Systemic infection	31 (25)	21 (21)	1.2 (0.6–2.5)	.50
Outcome				
Hospitalization	115 (85)	82 (79)	1.5 (0.7–3.2)	.20
Length of stay, median, days	5	2		.02
Surgery	62 (46)	58 (56)	0.7 (0.4–1.2)	.13
In-hospital mortality	4 (3)	2 (2)	1.6 (0.2–17)	.61
Resistance and virulence features of isolate				
PVL	60 (48)	62 (66)	0.5 (0.3–0.9)	.01
Erythromycin resistance	38 (28)	21 (20)	1.5 (0.8–3.0)	.16
Clindamycin resistance	33 (24)	19 (18)	1.4 (0.7–2.9)	.25

NOTE. Data are no. (%) of patients, unless otherwise indicated. CI, confidence interval; HCA, health-care associated; OR, odds ratio; PVL, Panton-Valentine leukocidin.

lowing hospital discharge are comparable [28]. In Taiwan, there was no difference in the mortality rate for bacteremia due to CA-MRSA strains and that of bacteremia resulting from MSSA [29]. Our case-control study also demonstrated that nmMRSA and MSSA cause similar infections in a comparable group of patients. Similarities existed with respect to disease spectrum and outcomes, as well as most demographic and epidemiological features. This was in marked contrast to the results for mMRSA, which was isolated from a clearly distinct group of patients and resulted in different infections and outcomes.

Multivariate analysis revealed 2 striking differences between patients who had nmMRSA isolated and those who had MSSA isolated. Our finding that those who had nmMRSA isolated were more likely to be female, or rather, less likely to be male, is surprising and intriguing. Previous studies with a comparable design [14, 26, 27, 30, 31] have consistently shown a predominance of males among patients with staphylococcal infections, which, to our knowledge, remains unexplained. After stratifying patients who had nmMRSA isolated according to ethnicity, we found that females comprised more than half of the Aboriginal patients but less than a third of non-Aboriginal patients; this latter figure mirrored that of patients who had MSSA isolated across the study population, with 166 (35%) of 478 being female. Of note, Aboriginal patients were significantly younger, and children (age, <18 years) accounted for 46 (34%) of 135 Aboriginal patients, compared with 8 (8%) of 104 non-Aboriginal patients. Referral bias is unlikely to explain the observed differences in the sex of people requiring treatment in a major tertiary hospital. In our community setting, men with mild infections are less likely than women and children to present to community health clinics, but this is unlikely to be the case for severe infections. It has been postulated that infection with nmMRSA may be more dependent on skin-to-skin or fomite-to-skin transmission, rather than resulting from endogenous colonization [32]. It has also been demonstrated that infants and mothers often share strains of *S. aureus* [33]. In Aboriginal communities, domestic crowding—as many as 7.5 people per bedroom—facilitates the transmission of infectious agents within households

Table 3. Multivariate analysis of variables associated with localized infection versus sepsis

Variable	Unadjusted		Adjusted	
	OR (95% CI)	P	OR (95% CI)	P
Resistance phenotype of isolate				
nmMRSA	0.8 (0.6–1.2)	.45	1.0	
MSSA	0.9 (0.7–1.3)	.62	1.2 (0.8–1.8)	.34
mMRSA	2.0 (1.2–3.5)	.01	1.7 (0.8–3.4)	.14
Age				
Reference	1.0		1.0	.34
Each additional year	1.0 (1.00–1.01)	.41	1.0 (0.98–1.01)	
Sex				
Male	1.0		1.0	.38
Female	1.2 (0.9–1.7)	.24	1.2 (0.8–1.7)	
Ethnicity				
Non-Aboriginal	1.0		1.0	.28
Aboriginal	1.1 (0.8–1.5)	.61	0.8 (0.5–1.2)	
Residence location				
Urban	1.0		1.0	**.05**
Remote	1.5 (1.0–2.1)	**.04**	1.6 (1.0–2.6)	
Comorbidity				
None	1.0		1.0	**.02**
⩾1	1.8 (1.3–2.6)	**<.01**	1.8 (1.1–2.8)	
HCA risk present				
No	1.0		1.0	.50
Yes	1.3 (0.9–1.8)	.12	0.9 (0.6–1.3)	
Type of infection				
Non-SSTI	1.0		1.0	**<.01**
SSTI	0.2 (0.1–0.3)	**<.01**	0.2 (0.1–0.3)	

NOTE. For this analysis, "sepsis" was defined as sepsis, severe sepsis, or septic shock. Bold type indicates significant P values ($P < .05$). CI, confidence interval; HCA, health-care associated; nmMRSA, non–multidrug-resistant, methicillin-resistant *Staphylococcus aureus;* mMRSA, multidrug-resistant MRSA; MSSA, methicillin-susceptible *S. aureus;* OR, odds ratio; SSTI, skin and soft-tissue infection.

[34], and women provide most of the child care. Thus women, through their closer household contact with children, may be more likely to develop nmMRSA infection than men.

Although both MSSA and nmMRSA are more common among Aboriginal people than non-Aboriginal people in the Top End, we have found that remote residence, rather than ethnicity itself, is most strongly associated with infection due to nmMRSA rather than MSSA. This contrasts with the results of studies from the United States, in which ethnicity was associated with nmMRSA infection [30, 31, 35, 36]. The remote areas of the Top End include Aboriginal communities characterized by socioeconomic disadvantage in relation to health, housing, education, and employment [18]. Because women and children were overrepresented among Aboriginal patients who had nmMRSA isolated, our findings support the postulate that factors in remote Aboriginal communities, such as domestic crowding and poor hygiene as well as high rates of scabies, skin sores, and antibiotic use, may contribute to the transmission and emergence of nmMRSA [13]. In addition, children in remote communities repeatedly present to community clinics with respiratory tract and skin infections [37] and are typically treated with penicillin and cephalosporin antibiotics.

The methicillin-resistance phenotype did not predict whether an infection was localized or led to sepsis, and it was not predictive of the clinical spectrum of disease. Unlike the situation in the United States, where virulence and resistance have converged in the predominant USA300 clone [38], there continues to be a diversity of nmMRSA strains circulating in Australia [12]. Our current study and another from the Australian state of Queensland have demonstrated that virulence does not correlate with resistance phenotype in these settings [14]. We have previously established that nmMRSA and MSSA strains from the same clonal complexes circulate in remote Top End communities [10], suggesting that staphylococcal chromosome cassette (SCC) mecIV has independently been acquired by MSSA on multiple occasions in our environment. It has been demon-

strated that the deletion of SCCmecIV in an isogenic mutant of a clinical USA300 isolate had no effect on competitive fitness or virulence [38]. It is therefore unsurprising that we found the clinical spectrum and outcomes of disease due to nmMRSA and MSSA to be similar.

Interestingly, the presence of *pvl* genes in nmMRSA was associated with double the odds of sepsis. There continues to be debate over the importance of Panton-Valentine leukocidin (PVL), and mouse infection models have not consistently shown a major role in pathogenesis [39]. A recent clinical study found no significant difference between PVL-positive and PVL-negative USA400 isolates with respect to their propensity to cause infection or colonization [40]. However, there were only 10 PVL-positive isolates in that study, and there was a trend linking the presence of PVL with increasing severity of disease. Our findings support previous studies that demonstrated that patients with pneumonia or bone and joint infections caused by PVL-positive *S. aureus* were systemically more unwell that those with infections caused by PVL-negative *S. aureus* [41, 42].

nmMRSA has been present in northern Australia and the RDH since at least the early 1990s [4, 43]. Over the past 10–15 years, the percentage of nmMRSA and mMRSA isolates among the overall population of *S. aureus* isolates has increased from 4% [4] to 291 (17%) of 1693 for nmMRSA and from 3% [4] to 137 (8%) of 1693 for mMRSA. However, nmMRSA has not outstripped mMRSA as a nosocomial pathogen at the RDH. In 1991–1995, nmMRSA constituted 40% [43] of nosocomial MRSA isolates, compared with 39 (41%) of 95 in this 2006–2007 case-control study. The finding that nmMRSA and MSSA infection had similar clinical profiles and outcomes calls into question current infection control strategies that target MRSA on the basis of methicillin-resistance phenotype alone. It is possible that the spread of a potentially more virulent, PVL-positive MSSA strain may have more significant consequences than the spread of a PVL-negative nmMRSA strain, and therefore, it should be targeted for more aggressive infection control interventions. Molecular diagnostic methods may facilitate a different approach. One Australian state, Western Australia, has recently attempted to implement a "search and destroy" approach to PVL-positive MRSA strains in the community [44]; this extends existing policies of public health agencies being notified of all MRSA isolations and all MRSA isolates being sent to a reference typing laboratory.

Our study has some limitations. We did not interview patients directly and were unable to assess the impact of factors such as domestic crowding, prior antibiotic use, and the mode of acquisition for SSTIs. Our incidence figures and case-control study pertain only to patients presenting to hospitals and do not reflect the overall community burden of staphylococcal infections. Nevertheless, these hospitals service an area of 510,000 km^2, and our study provides important insights into staphylococcal infections in this large tropical region. Additionally, our calculations are likely to result in an underestimation of the already high incidence of *S. aureus* infection. Among children in some remote communities, the prevalence of pyoderma is 20%, from which *S. aureus* can be cultured in 57% of cases [10]. Hence, in these communities the prevalence of staphylococcal infection among children is $>$11,000 cases per 100,000 population, and the annual incidence of all staphylococcal infections is even higher.

Staphylococcal disease, including that due to nmMRSA, imposes a disproportionate burden on remote Aboriginal communities in northern Australia. Our study demonstrates that female sex and residence in a remote community is associated with nmMRSA infection, and remote residence is likely a marker of social disadvantage that encompasses overcrowding, poor housing conditions, and lack of access to the physical infrastructure needed to maintain skin hygiene. There are well-founded fears that nmMRSA will become a nosocomial pathogen, but primary prevention also requires consideration of public health issues, such as housing and community hygiene. In our situation, nmMRSA is still principally a community pathogen—arising and spreading from within the community setting.

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Appendix V. Fatal community-associated methicillin-resistant *Staphylococcus aureus* pneumonia after influenza

Fatal community-associated methicillin-resistant *Staphylococcus aureus* pneumonia after influenza

Steven YC Tong, Nicholas M Anstey, Gary D Lum, Rachael A Lilliebridge, Dianne P Stephens and Bart J Currie

To the Editor: The report by Risson and colleagues of a fatal case of necrotising pneumonia caused by community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA)[1] appropriately highlights the emerging issue of CA-MRSA infections in Australia,[2] and the possibility that severe *S. aureus* sepsis may follow recurrent furunculosis. We wish to draw attention to the association between severe staphylococcal pneumonia and a preceding influenza-like illness.

In September 2006, a 56-year-old woman of European background with a history of chronic back pain and depression presented to the Royal Darwin Hospital after a 4-day influenza-like illness characterised by cough, fever and sore throat. She then developed dyspnoea and pleuritic chest pain, followed by an abrupt respiratory deterioration.

She was intubated and admitted to the intensive care unit with severe sepsis. A chest x-ray showed widespread bilateral pneumonia. We began broad-spectrum antibiotic therapy with piperacillin–tazobactam and azithromycin as per the hospital's dry-season protocol for severe community-acquired pneumonia. Further history from her husband revealed an episode of boils 1 month previously, which responded to antibiotic therapy. We added vancomycin to the therapy and, when sputum and blood cultures showed MRSA 48 hours after admission, we also added rifampicin and gentamicin. On Day 5 of admission, her clinical condition deteriorated further and we replaced rifampicin and gentamicin with linezolid. Complement fixation testing of serum taken on admission showed an influenza A antibody titre of 128, consistent with recent acute infection. Despite ongoing intensive supportive care, the patient died from refractory respiratory failure 10 days after admission.

Typing of the *S. aureus* isolates from blood and sputum showed that their single nucleotide polymorphism and variable gene profile was consistent with the Queensland clone (ST93-MRSA-IV) of CA-MRSA, and that the Panton–Valentine leukocidin gene was present.

S. aureus has long been a recognised cause of influenza-associated pneumonia. Of concern, two recent reports from the United States identified 25 patients with CA-MRSA associated with severe pneumonia following influenza-like illnesses.[3,4] Most of these patients were young (median age of 21 years[3] and 17.8 years,[4] respectively) and otherwise healthy. Combined mortality in these two studies was 40%.

With an increasing prevalence of CA-MRSA in areas of Australia,[2] CA-MRSA pneumonia should be suspected in patients presenting with worsening respiratory status and sepsis following an influenza-like illness. We stress the importance of annual influenza vaccination for those at increased risk of influenza-related complications.[5]

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Methicillin-resistant *Staphylococcus aureus* in hospitals: time for a culture change

Keith V Woollard

To the Editor: The recent editorial by Collignon and colleagues emphasised the importance of infection control mechanisms in reducing patient harm from antibiotic-resistant organisms.[1] It focused on disinfection of the hands of health care workers in hospitals. However, a vigorous education and surveillance program in a hospital in Victoria failed to achieve compliance among health care workers of even 50%.[2] Top of the list of self-reported factors leading to poor compliance is "skin irritation and dryness associated with the use of hand hygiene agents".[3]

There have been no properly controlled trials, with clinically important endpoints, of currently recommended hand-hygiene practices. With the likely poor compliance rates, such trials would likely fail.

A different approach might be more effective. Reducing skin contact between health care workers, patients and their immediate environment seems logical. Data show that skin contact produces two-step transfer of material in 82% of cases.[4] The Victorian study did include gloving as an alternative to disinfection in measuring hand-hygiene compliance.[2] However, in what might be a backward step, a recent study concluded that physicians should be encouraged to shake hands with patients![5]

Perhaps an educational campaign to avoid skin contact with environmental surfaces and other health care workers, with use of disposable gloves for patient contact, could be the basis of a successful trial to address more effectively the transmission of antibiotic-resistant organisms in hospitals.

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Appendix VI. The emerging problem of community-associated MRSA; Necrotising pneumonia in a 19 month old Aboriginal boy

The emerging problem of community-associated MRSA; Necrotising pneumonia in a 19 month old Aboriginal boy

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Abstract

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infection in children is increasingly common in northern Australia and can be associated with severe infection. An infant with severe CA-MRSA infection causing necrotising pneumonia and rib osteomyelitis is described. Recent literature is reviewed and recommendations for management are provided. In order to restrict the spread of CA-MRSA it is necessary to address the broad issues of socioeconomic disadvantage as well as to implement practical public health measures.

Case report

A 19 month old boy from a remote Aboriginal community in the Northern Territory (NT) presented to Katherine District Hospital (KDH) with a 1 week history of fever and cough and a 24 hour history of shortness of breath.

The past medical history was unremarkable. He was born by normal vaginal delivery at term with a birth weight of 4160 grams. He had had no previous admissions to hospital. Immunisations were up to date and he was developmentally normal.

Examination revealed a miserable, febrile (40° C), tachycardic (heart rate 188 beats per minute) and tachypnoeic (respiratory rate 64 breaths per minute) child. There were decreased breath sounds at the left base of the chest. Investigations revealed a leukocytosis (31.2 X 10^9/L with 77% neutrophils), and a C-reactive protein of 292mg/L. Chest X-ray showed extensive left sided opacification centred in the lower zone with features consistent with pneumonia.

He was commenced on intravenous ceftriaxone. Gram-positive cocci were grown in blood cultures 2 days following admission. Ceftriaxone was changed to flucloxacillin. On day 3 identification and susceptibility testing revealed the isolate to be a non-multi-resistant methicillin-resistant *Staphylococcus aureus* (nmMRSA) and intravenous vancomycin (10mg/kg qid) was started.

The patient improved on vancomycin but spiked high temperatures on days 4 and 5. On day 6, a tender swelling was noticed in the mid-axillary area of the left chest wall. Ultrasound showed a 49mm x 33mm x 39mm hypoechoic collection connecting with the chest wall.

Oral cotrimoxazole (8/40mg/kg BD) was added after infectious diseases consultation and he was transferred to the Royal Darwin Hospital (RDH). Computed tomography of the chest showed consolidation and collapse of the left lower lobe with a loculated pleural collection in the left hemithorax extending through the chest wall, findings consistent with an empyema necessitans complicating lobar pneumonia. There was evidence of osteomyelitis of the sixth rib. Surgical drainage and partial decortication was performed 2 days later, after which the patient made an excellent recovery. Typing of the empyema isolate showed it to be multilocus sequence type 93 (ST93) MRSA, the Queensland clone, and Panton-Valentine leukocidin (PVL) positive.

The patient received a 2 week course of intravenous vancomycin and was transferred back to KDH to complete a total of 6 weeks of oral cotrimoxazole.

CA-MRSA – the emerging problem

CA-MRSA infection in children has been an emerging problem in Australia and overseas with rates of methicillin resistance as high as 76% in some paediatric centres in the USA.[1] The first reports of CA-MRSA in Australia came from the Kimberley region of Western Australia in the 1980s.[2] Subsequently, independent emergence of diverse CA-MRSA strains in geographically distinct regions of Australia has been documented, including strains unique to the NT, NT-MRSA (clonal complex 75).[3,4,5,6]

CA-MRSA typically causes skin and soft tissue infections, however there have been many case reports of severe disseminated infection in children including bacteraemia, necrotising pneumonia, osteomyelitis, and septic venous thrombosis.[7,8]

In a recent longitudinal study of pyoderma in remote Top End Aboriginal communities, *S aureus* was recovered from 58% of skin sores. Methicillin-resistance was detected in 23% of *S aureus* isolates. Of these, 71% were the NT-MRSA clonal complex and 14% were the Queensland clone (ST 93). The next most prevalent isolates were WA-MRSA strains and the Oceania clone (ST 30).[3] CA-MRSA accounts for 15% of *S aureus* isolates recovered from patients presenting to hospitals in the Top End (Steven Tong, personal communication).

This case report illustrates the potential virulence of CA-MRSA and the potential for severe and life threatening infection. An important virulence factor is Panton-Valentine leukocidin (PVL), a bacterial toxin that can mediate leukocyte and tissue destruction. This is expressed by the Queensland and Oceania clones while NT-MRSA and WA-MRSA strains are PVL negative.[3] Most cases of severe CA-MRSA disease in Australia have been due to PVL positive ST93 isolates. Clones positive for PVL have been associated with necrotising pneumonia and death. Of RDH isolates, approximately 50% are PVL positive.[9]

What clinicians need to do

The emergence of methicillin-resistant *S aureus* in NT communities coupled with its potential to cause severe disease poses an emerging public health threat. The following are important points for community clinicians to note:

- There needs to be a low threshold for taking swabs to obtain culture and antimicrobial susceptibility results for skin sores and abscesses that are failing to respond to standard β-lactam therapy.
- Whenever possible, abscesses should be incised and drained.
- CA-MRSA characteristically is susceptible to cotrimoxazole and sometimes to clindamycin. However, we have concerns regarding the empiric use of clindamycin as inducible resistance to clindamycin is present in 22% of RDH isolates (Tong, personal communication), and up to 47% of community isolates. Additionally, clindamycin is not available in Australia in a liquid formulation suitable for children.[7,8]
- The current recommendation is to use oral cotrimoxazole (8/40mg/kg bd) for uncomplicated skin and soft tissue infections caused by CA-MRSA.[10] Cotrimoxazole is widely available, inexpensive, well tolerated in children, and importantly comes as a palatable oral formulation.
- In the setting of suspected severe invasive staphylococcal infection, empirical treatment should include vancomycin until culture and antimicrobial susceptibility results are available. Molecular detection of methicillin-resistance in blood culture isolates may help inform early antimicrobial selection for staphylococcal bacteraemia at the RDH in the near future.

What is needed at the community level

In the United States, the Centres for Disease Control guidelines for clinical management of CA-MRSA emphasise the importance of personal and household hygiene. Wounds should be covered, hands regularly washed with soap and water, and regular bathing maintained. Potentially contaminated items should not be shared, contaminated clothing should be laundered after each use, and environmental surfaces where multiple individuals have bare skin contact cleaned.[11] Unfortunately such recommendations are not currently realistic in Aboriginal communities.

Therefore, in order to restrict the spread and emergence of CA-MRSA in Aboriginal communities, it is necessary to address the broader issues of socioeconomic disadvantage. Overcrowding, poor sanitation, inadequate water supply, poor hygiene, low levels of education and young maternal age have all been shown to be risk factors for skin disease[12,13,14] and are likely to be risk factors for CA-MRSA skin infection. There is no current data to support the use of oral or topical agents to decolonize patients with CA-MRSA. Furthermore, such attempts will almost certainly fail if the personal and household hygiene issues are not addressed first.

Practical public health interventions such as introduction of swimming pools[15] and interventional programs for scabies[16,17,18] have been proven to be effective at decreasing prevalence of pyoderma and should impact on CA-MRSA skin infection if coordinated across communities. A commitment to improve basic living conditions with improved housing, sanitation facilities, and water supply are

fundamental to improving basic hygiene and health.

It is postulated that high rates of antibiotic use in these disadvantaged communities may be promoting the emergence and spread of microbial resistance and the amplification of disease.[19] This threat extends well beyond individual communities as demonstrated by the spread of CA-MRSA clones across Australia and the Pacific. Unless issues of disadvantage are addressed in Aboriginal communities we are likely to see continuing increased rates of microbial resistance.

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Appendix VII: Clinical correlates of Panton-Valentine leukocidin (PVL), PVL isoforms, and clonal complex in the *Staphylococcus aureus* population of northern Autralia

MAJOR ARTICLE

Clinical Correlates of Panton-Valentine Leukocidin (PVL), PVL Isoforms, and Clonal Complex in the *Staphylococcus aureus* Population of Northern Australia

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Background. Regional differences in the prevalence of Panton-Valentine leukocidin (PVL) and PVL isoform–harboring strains as well as in the local population structure of *Staphylococcus aureus* may influence the clinical spectrum of *S. aureus* infections.

Methods. Using a prospective collection of *S. aureus* isolates from northern Australia, we determined differences between infections caused by (1) PVL$^+$ and PVL$^-$ isolates, (2) PVL histidine (H) isoform– and PVL arginine (R) isoform–harboring isolates, and (3) different lineages, including the genetically divergent clonal complex (CC) 75 and the PVL$^+$ CC93.

Results. PVL$^+$ isolates comprised 54% (128/239) of community-associated methicillin-resistant isolates and 40% (95/239) of methicillin-susceptible *S. aureus* (MSSA) isolates. There were 113 H isoform– and 110 R isoform–harboring isolates. PVL was associated with truly community-acquired disease, younger age, and presentation with sepsis. We found no differences in infections due to H isoform–harboring isolates, compared with R isoform–harboring isolates. CC93 was the most prevalent lineage. The genetically divergent CC75 caused clinical disease similar to that of other *S. aureus* clones.

Conclusions. PVL$^+$ and PVL$^-$ infections are clearly distinct. MSSA contributes a large but underrecognized burden of PVL$^+$ disease. Compared with elsewhere in the world, there is a relative abundance of the clade that contains CC93 and CC121 in both northern Australia and Asia.

The emergence of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections beginning in the 1990s has been a global phenomenon. Among the earlier reports were those from the United States [1] and Indigenous Australian communities [2]. Recently, the CA-MRSA epidemic in the United States has been dominated by one clone, USA300, which appears to have undergone a rapid radiation and replaced other CA-MRSA clones [3]. In Australia, where USA300 is rare, there continues to be a diversity of circulating CA-MRSA clones [4]. In northern Australia, 2 lineages are of particular interest. Clonal complex (CC) 75 is phylogenetically diverged but phenotypically similar to other *S. aureus* [5, 6]. The second lineage, CC93, is apparently unique to Australia. Initially described in 2001 [7], CC93 expresses the pore-forming leukotoxin Panton-Valentine leukocidin (PVL) and has spread throughout the nation, becoming the predominant CA-MRSA clone in Australia [4, 8].

An association between the presence of PVL and severe staphylococcal infections (such as necrotizing pneumonia) has been noted worldwide [9, 10]. The vast majority of CA-MRSA isolates in the United States are PVL$^+$ USA300 [3], whereas only 50% of CA-MRSA isolates in Australia are PVL$^+$ [4]. Recent studies have

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demonstrated sequence variations within the PVL gene *lukSF-PV* [11–13]. Molecular modeling suggests that the amino acid substitution of His_{176} to Arg_{176} (H and R isoforms) may affect PVL function [11]; however, clinical studies are lacking. USA300 isolates carry the R isoform, but most other clones isolated outside the United States carry the H isoform [11, 12]. Fifty percent of our PVL^{+} CA-MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates from northern Australia carry the R isoform. The majority of these isolates are CC93.

Thus, the diverse molecular epidemiology of *S. aureus* in tropical northern Australia provides a unique opportunity to investigate whether clinical and epidemiological differences exist between infections caused by (1) PVL^{+} and PVL^{-} isolates, (2) PVL H isoform– and PVL R isoform–harboring isolates, and (3) different lineages, including CC75 and CC93. In addition, we placed the population structure of *S. aureus* in northern Australia within the context of recent findings regarding *S. aureus* in East Asia.

METHODS

The 330-bed Royal Darwin Hospital (RDH) is located in the tropical Top End of the Northern Territory of Australia (Figure 1). It is the tertiary referral center for the capital city, Darwin, along with 2 regional hospitals and >70 remote communities, all together encompassing a population of 190,000 and an area of 510,000 km^2. Both pediatric and adult patients are cared for at the RDH and are included in this study. Indigenous Australians comprise 27% of the total population but account for >50% of the RDH inpatient and emergency department population. Ethical approval for the study was granted by the Human Research Ethics Committee of the Northern Territory Department of Health and Families and the Menzies School of Health Research.

We initially conducted a case-control study that compared infections caused by non–multidrug-resistant MRSA (nmMRSA) with those caused by MSSA at the RDH over a 12-month period beginning April 2006 [14]. For the present study, we matched eligible patients with nmMRSA infection to the next eligible patient with MSSA infection, using sequential laboratory specimen receipt numbers. We collected information on demographics, health care–associated risk factors, comorbidities, clinical details of the infection, treatment details, and outcomes at discharge. Sepsis was defined in adults and children on the basis of the systemic inflammatory response syndrome criteria [15, 16].

The RDH laboratory identified *S. aureus* isolates by standard methods and conducted susceptibility testing using an automated Vitek 2 instrument (version 4.01; bioMérieux) and the Kirby-Bauer disc diffusion method, in accordance with Clinical and Laboratory Standards Institute standards [17]. A disc approximation test (D test) was used to detect inducible clindamycin resistance. Real-time polymerase chain reaction (PCR) verified the identity of nmMRSA isolates by confirming the presence of the *nucA* and *mecA* genes [18]. We phenotypically defined nmMRSA as those resistant to <3 non–β-lactam antibiotic classes and considered these to represent CA-MRSA strains. Antibiotic phenotype has previously been shown to accurately predict CA-MRSA genotypes [19], with validation in recent Australian studies [4, 20].

We genotyped isolates by means of a single-nucleotide polymorphism (SNP) genotyping system based on the multilocus sequence type (MLST) database, as previously elsewhere [18, 21]. In short, a kinetic PCR method was used to interrogate 8 highly discriminatory SNPs, allowing isolates to be assigned into CCs. To confirm isolates as CC93, we also performed a high-resolution melt (HRM) assay interrogating *aroE* position 252 [22]. We determined the presence of the *lukF-PV* gene in all isolates [18, 21] and then used an HRM assay to determine the H and R isoform status of all PVL^{+} isolates. The primers for this HRM assay are PVL527F (5′-CAACAAAACTATATCA-GTGAAGTAGAAC-3′) and PVL527R (5′-TCCCCATTGAACA-CTTTTTGAA-3′). The isoforms were clearly discriminated; the melting temperature of the amplified product for the H isoform was 1°C less than that for the R isoform [23].

Comparisons of infections due to PVL^{+} and PVL^{-} isolates and between CCs were stratified by methicillin resistance (the study included all nmMRSA isolates but only a random subset of MSSA isolates). Statistical significance for crude analysis of dichotomous variables was determined using the χ^2 test or the Fisher exact test; for continuous variables, the Student *t* test or analysis of variance was used. Nonparametric data were compared using the Mann-Whitney *U* test. We conducted multivariate logistic regression analysis with stepwise backward elimination of variables to identify associations with the outcomes of PVL status, PVL isoform status, sepsis, and abscess formation. Variables with a univariate *P* value of <.20 were included in the initial models. The likelihood ratio test was used to assess the statistical significance of candidate risk factors. Two-sided *P* values of <.05 were considered significant. Statistical analysis was performed with Stata software (version 10.1; StataCorp). Phylogenetic analysis was performed on concatenated MLST data, using the neighbor-joining algorithm (Kimura 2-parameter distance estimation) as implemented in MEGA software (version 4.1) [24].

RESULTS

During the study period, 1693 nonduplicate *S. aureus* isolates were recovered from patients at the RDH, with MSSA outnumbering nmMRSA at a ratio of 4.3:1. There were 1265 MSSA (75%), 291 nmMRSA (17%), and 137 multidrug-resistant MRSA (8%) isolates. Patient characteristics, outcomes, and corresponding molecular data from 239 eligible nmMRSA and 239

Figure 1. Map of Australia, with the Top End of the Northern Territory of Australia and the capital city of Darwin highlighted.

matched MSSA isolates were included in the case-control analysis. Twenty-two CCs were represented (Figure 2). Eight of the CCs were represented by both nmMRSA and MSSA isolates. Of the 478 isolates, 223 were PVL^+, of which 113 and 110 harbored the H and R isoforms, respectively. PVL^+ isolates belonged to 8 CCs, with 5 CCs harboring the H isoform and 3 the R isoform. Seven of these 8 CCs had both PVL^- and PVL^+ isolates.

Comparison between PVL^+ and PVL^- isolates. The large numbers of both PVL^+ and PVL^- isolates and their distribution across diverse CCs facilitated meaningful comparisons between infections caused by PVL^+ and PVL^- isolates (Table 1). Univariate analysis for both nmMRSA and MSSA subgroups revealed PVL^+ isolates to affect younger patients with fewer comorbidities and fewer health care–associated risk factors. Overall, 73% (163/223) of PVL^+ isolates were community acquired, compared with only 41% (104/255) of PVL^- isolates ($P < .001$). In contrast, we have previously shown that only 1% of multidrug-resistant MRSA isolates were community acquired in our setting [14]. The proportional incidence of isolates harboring PVL stratified by age group showed a steady decline with age for both nmMRSA and MSSA (Figure 3). Of patients <40 years old, 55% (146/267) had infections caused by PVL^+ isolates, compared with only 36% (77/211) of those >40 years old ($P < .001$). Skin and soft-tissue infections and abscesses were seen more commonly in PVL^+ infections, but there was no difference in bacteremia. Despite a greater proportion of patients requiring surgery, length of stay was shorter for PVL^+ infections. Multivariate logistical modeling revealed that increasing age (adjusted odds ratio [aOR], 0.98 [95% confidence interval {CI}, 0.97–0.99]), the presence of a health care–associated risk factor (aOR, 0.32 [95% CI, 0.20–0.50]), and resistance to fusidic acid (aOR, 0.10 [95% CI, 0.03–0.30]) reduced the odds of a PVL^+ infection, whereas the presence of an abscess (aOR, 9.44 [95% CI, 5.30–16.81]) and methicillin resistance (aOR, 2.50 [95% CI, 1.59–3.94]) increased the odds of a PVL^+ infection.

To determine whether PVL was associated with sepsis, we built an additional multivariate logistic regression model comparing an outcome of sepsis with localized infection. Significant predictors of sepsis were the presence of PVL (aOR, 1.94 [95% CI, 1.21–3.11]), remote residence (aOR, 1.73 [95% CI, 1.09–2.75]), and the presence of at least 1 comorbidity (aOR, 1.66 [95% CI, 1.02–2.70]). A skin and soft-tissue infection reduced the odds of sepsis (aOR, 0.39 [95% CI, 0.21–0.73]). Notably, in contrast to the presence of PVL, methicillin resistance and

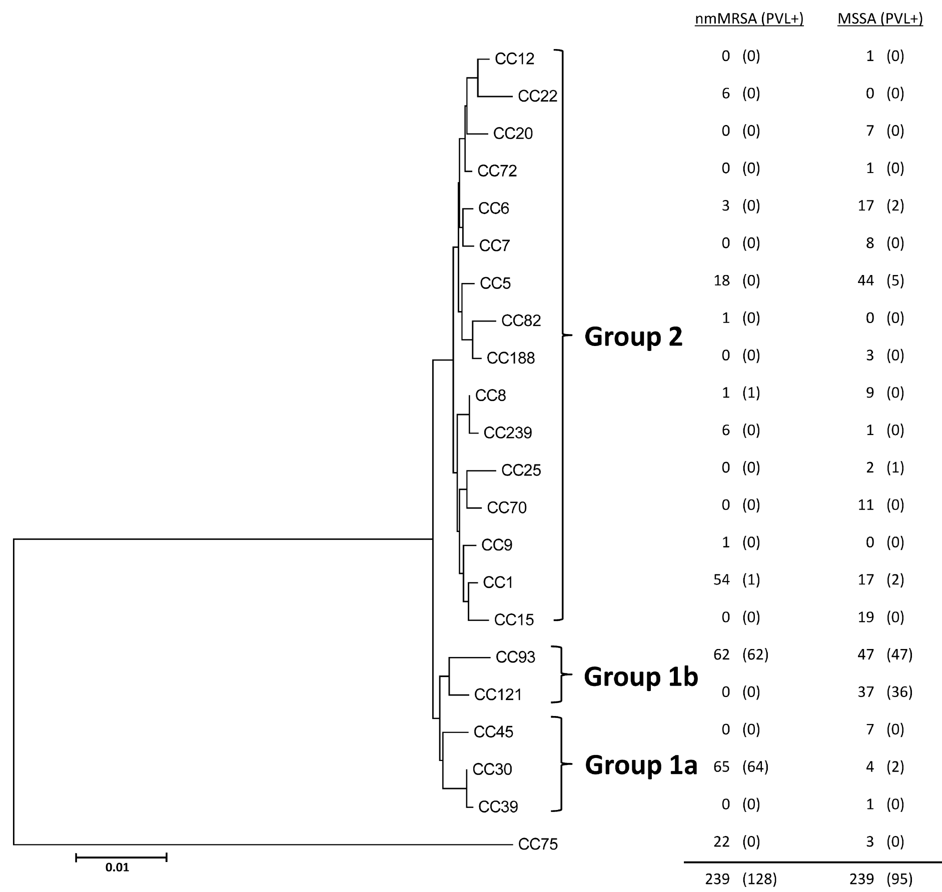


Figure 2. Neighbor-joining tree based on the concatenated sequences of the founder sequence types representing each of the clonal complexes (CCs) in the collection. Three major clades are demonstrated (groups 1a, 1b, and 2), and CC75 is phylogenetically diverged from the rest of *Staphylococcus aureus.* The number of isolates in each CC that are non–multidrug-resistant methicillin-resistant *S. aureus* (nmMRSA), that are methicillin-susceptible *S. aureus* (MSSA), and that harbor Panton-Valentine leukocidin (PVL) are shown.

CC were not associated with sepsis. The presence of PVL was associated with abscess formation on multivariate logistic regression modeling for both nmMRSA (OR, 10.3 [95% CI, 4.5–23.9]) and MSSA (OR, 11.4 [95% CI, 5.4–23.7]).

Comparison between isolates harboring PVL H and R isoforms. There was no difference across all characteristics when comparing infections due to isolates harboring H and R isoforms on univariate analysis (Table 2). A multivariate logistic regression model also found no significant associations.

Population structure and clinical outcomes of different clones. Phylogenetic analysis (Figure 2) demonstrated the divergence of CC75 from all other clones and the clustering of PVL$^+$ isolates within the group 1 clade. Given that we took a random sample of MSSA isolates that is likely to be representative of the overall MSSA population and that MSSA outnumbered nmMRSA at a 4.3:1 ratio, we estimate that MSSA accounts for ~70% of PVL$^+$ isolates. Almost all group 1b clade (CC93 and CC121) isolates were PVL$^+$ and represented 87% (83/95) of all PVL$^+$ MSSA isolates.

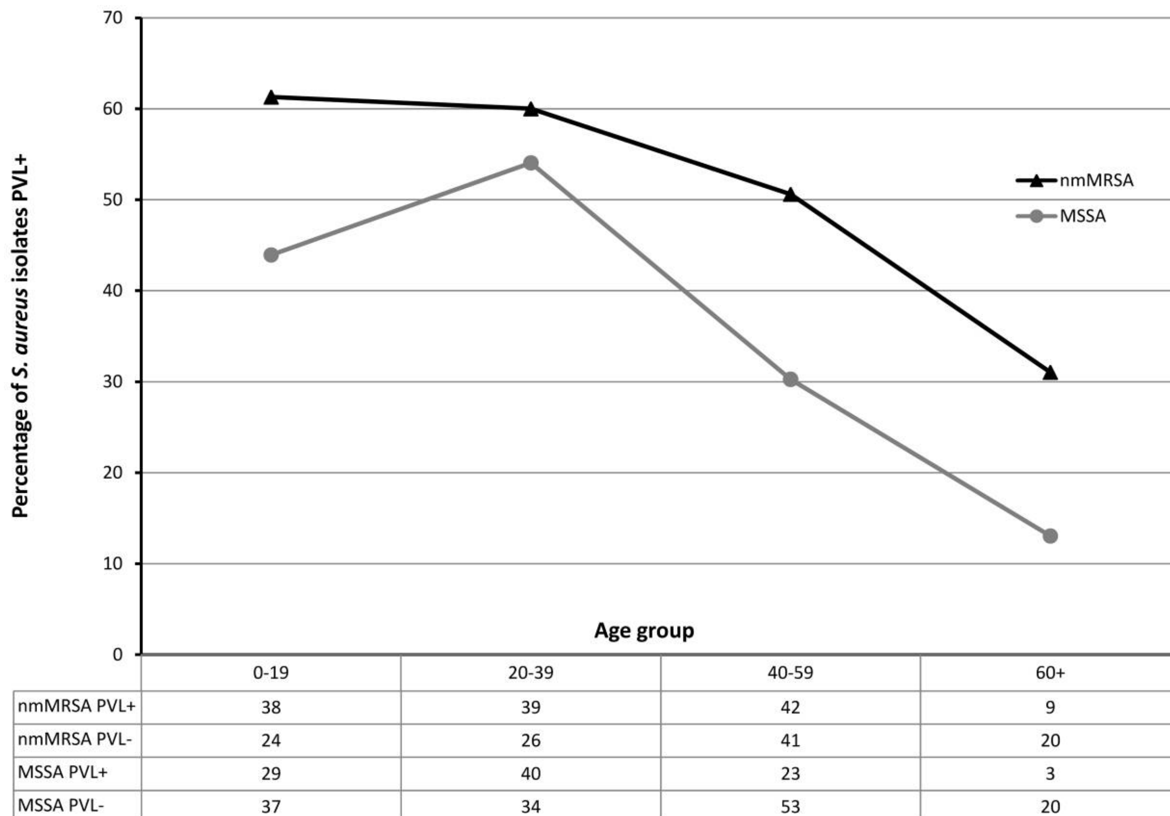
We compared the clinical features and isolate characteristics of CCs that were represented by >20 isolates when stratified by methicillin resistance (Table 3). Together, these lineages comprised 69% (331/478) of all isolates. Across-group comparisons revealed significant differences for patients with regard to age, sex, ethnicity, remote residence, presence of comorbidities, health care–associated risk factors, nosocomial acquisition, presentation with abscess, length of stay, and receipt of surgery. There were also significant differences in antibiotic resistance profile and the presence of PVL. There was no difference in the outcomes of bacteremia and sepsis.

The principally PVL$^+$ clones CC30, CC93, and CC121 affected patients with fewer comorbidities and health care–associated risk factors. Only 7% (14/211) of these infections were acquired in the hospital, indicating a lack of penetration of these community clones into the nosocomial environment. In contrast, CC1, CC5, and CC75 affected patients with more comorbidities and health care–associated risk factors and had higher rates of nosocomial acquisition.

Table 1. Comparison of the Characteristics of Panton-Valentine Leukocidin (PVL)$^+$ with PVL$^-$ Infections, by Methicillin Resistance

	nmMRSA (n = 239)				MSSA (n = 239)			
Variable type, characteristic	PVL$^+$ (n = 128)	PVL$^-$ (n = 111)	Unadjusted OR (95% CI)	*P*	PVL$^+$ (n = 95)	PVL$^-$ (n = 144)	Unadjusted OR (95% CI)	*P*
Demographic traits								
Age, median, years	34	42		.007	30	40		.004
Male sex	76 (59)	56 (50)	0.7 (0.4–1.2)	.167	70 (74)	86 (60)	0.5 (0.3–0.9)	.027
Indigenous	64 (50)	71 (64)	0.5 (0.3–0.9)	.030	46 (48)	74 (51)	0.9 (0.5–1.5)	.653
Remote residence	40 (31)	41 (37)	0.9 (0.5–1.3)	.355	25 (26)	32 (22)	1.3 (0.7–2.8)	.468
Clinical risk factors								
Health care–associated risk	40 (31)	74 (67)	0.2 (0.1–0.4)	<.001	20 (21)	77 (53)	0.2 (0.1–0.4)	<.001
Nosocomial acquisition	10 (8)	29 (26)	0.2 (0.1–0.5)	<.001	3 (3)	25 (17)	0.2 (0.0–0.5)	.003
⩾1 comorbidity	27 (21)	49 (44)	0.3 (0.2–0.6)	<.001	12 (13)	52 (36)	0.3 (0.1–0.5)	<.001
Diabetes	17 (13)	30 (27)	0.4 (0.2–0.8)	.009	6 (6)	29 (20)	0.3 (0.1–0.7)	.005
Chronic renal disease	6 (5)	9 (8)	0.6 (0.2–1.6)	.282	0 (0)	13 (9)	NA	.003
Coronary artery disease	8 (6)	19 (17)	0.3 (0.1–0.8)	.011	1 (1)	10 (7)	0.1 (0.0–1.1)	.065
Injection drug use	1 (1)	1 (1)	0.9 (0.1–14)	.919	0 (0)	1 (1)	NA	.416
Type and severity of infection								
Skin and soft tissue	121 (95)	91 (82)	3.8 (1.5–9.4)	.004	89 (94)	118 (82)	3.3 (1.3–8.3)	.013
Abscess	64 (50)	8 (7)	13.0 (6–29)	<.001	46 (48)	11 (8)	11.0 (5–24)	<.001
Pneumonia	2 (2)	7 (6)	0.2 (0.5–1.2)	.075	1 (1)	7 (5)	0.2 (0.0–1.7)	.141
Bacteremia	5 (4)	5 (5)	0.9 (0.2–3.1)	.818	6 (6)	7 (5)	1.3 (0.4–4.1)	.987
Sepsis	35 (27)	20 (18)	1.7 (0.9–3.2)	.089	24 (25)	30 (21)	1.3 (0.7–2.4)	.423
Outcome								
Length of stay, median, days	2.5	5		.001	2	4		<.001
Hospitalization	105 (82)	92 (83)	0.9 (0.5–1.8)	.863	71 (75)	115 (80)	0.7 (0.4–1.4)	.352
Surgery received	87 (68)	33 (30)	5.0 (2.9–8.7)	<.001	68 (72)	39 (27)	6.8 (3.8–12)	<.001
In-hospital mortality	2 (2)	4 (4)	0.4 (0.1–2.4)	.328	0 (0)	3 (2)	NA	.157
Antibiotic susceptibility								
Erythromycin resistant	9 (7)	50 (45)	0.1 (0.0–0.2)	<.001	28 (29)	12 (8)	4.6 (2.2–9.6)	<.001
Clindamycin resistant	9 (7)	43 (39)	0.1 (0.1–0.3)	<.001	28 (29)	10 (7)	5.6 (2.6–12)	<.001
Fusidic acid resistant	1 (1)	31 (28)	0.0 (0.0–0.1)	<.001	3 (3)	10 (7)	0.4 (0.1–1.6)	.218

NOTE. Data are no. (%) of patients or isolates, unless otherwise indicated. CI, confidence interval; MSSA, methicillin-susceptible *Staphylococcus aureus*; NA, not applicable; nmMRSA, non–multidrug-resistant methicillin-resistant *S. aureus*.



	0-19	20-39	40-59	60+
nmMRSA PVL+	38	39	42	9
nmMRSA PVL-	24	26	41	20
MSSA PVL+	29	40	23	3
MSSA PVL-	37	34	53	20

Figure 3. Percentage of *Staphylococcus aureus* isolates harboring Panton-Valentine leukocidin (PVL) for non–multidrug-resistant methicillin-resistant *S. aureus* (nmMRSA) and methicillin-susceptible *S. aureus* (MSSA), by age group. Raw numbers are shown below the graph.

CC93 was numerically the predominant clone, comprising 26% (62/239) of nmMRSA and 20% (47/239) of MSSA isolates. All harbored the PVL R isoform. There were no significant differences between CC93 nmMRSA and CC93 MSSA. CC75 was principally isolated from Indigenous Australian patients (82% [18/22]; $P = .006$ for the comparison with non-CC75 isolates) and appeared less likely to cause presentations with sepsis. Erythromycin and inducible clindamycin resistance was present in >50% of CC1 and CC121 isolates.

DISCUSSION

There are a number of parallels and contrasts between the community-associated MRSA epidemics described in North America and our experience in northern Australia [25, 26]. In North America, the USA400 clone appeared to be most prominent before 2001 [27] but has since been displaced by USA300 across most of the United States [3]. Both USA300 and USA400 typically express PVL. In northern Australia, despite the presence of community-associated MRSA since at least the early 1990s [28], there continues to be a diversity of strains, with the 3 major clones together representing ~75% of isolates. One of these clones, CC1, is usually PVL$^-$. However, our finding that a significant proportion of both community-associated MRSA and MSSA isolates are PVL$^+$ CC93 reflects the growing emergence of this lineage across Australia since it was first described in 2000 [8]. As with USA300 and USA400, cases of severe staphylococcal disease have been described with CC93 [29, 30].

Early epidemiological studies highlighted the association between PVL and necrotizing pneumonia, furunculosis, and severe bone and joint infections [9, 10, 31]. Evidence supporting the pathogenic role of PVL in mice and rabbit pneumonia models followed [32, 33]. However, other studies have cast doubt on these findings and questioned the role of PVL in the pathogenesis of *S. aureus* infection [34, 35]. Of PVL isoforms, the H and R isoforms appear to be the most significant numerically and by geographic distribution. Molecular models suggest that the R isoform may lead to increased pore formation and, therefore, greater leukotoxicity [11]. In the present study, we have defined epidemiological associations with PVL, as well as with PVL H and R isoforms, in a large prospective sample of patients with infections caused by diverse community-associated *S. aureus* clones. Many previous studies have principally focused on community-associated MRSA; one of the key results of this study is the unanticipated high rate of PVL positivity in MSSA isolates.

Table 2. Comparison of the Characteristics of Panton-Valentine Leukocidin Histidine (H) and Arginine (A) Isoform Infections

Variable type, characteristic	H isoform (n = 113)	R isoform (n = 110)	Unadjusted OR (95% CI)	P
Demographic traits				
Age, median, years	33	30		.337
Male sex	71 (65)	75 (66)	0.9 (0.5–1.6)	.774
Indigenous	56 (51)	54 (48)	0.9 (0.5–1.5)	.641
Remote residence	37 (34)	28 (25)	0.6 (0.4–1.2)	.147
Clinical risk factors				
Health care–associated risk	30 (27)	30 (27)	1.0 (0.5–1.7)	.903
Nosocomial acquisition	5 (5)	8 (7)	1.6 (0.5–5.1)	.423
⩾1 comorbidity	17 (15)	22 (19)	1.3 (0.7–2.7)	.431
Diabetes	11 (10)	12 (11)	1.1 (0.5–2.5)	.879
Chronic renal disease	4 (4)	2 (2)	0.5 (0.1–2.7)	.399
Coronary artery disease	6 (5)	3 (3)	0.5 (0.1–1.9)	.298
Type and severity of infection				
Skin and soft tissue	103 (94)	107 (95)	1.2 (0.4–3.7)	.737
Abscess	60 (55)	50 (44)	0.7 (0.4–1.1)	.125
Sepsis	31 (28)	28 (25)	0.8 (0.5–1.5)	.565
Outcome				
Length of stay, median, days	2	2		.764
Hospitalization	89 (81)	87 (77)	0.8 (0.4–1.5)	.474
Surgery received	76 (69)	79 (70)	1.0 (0.6–1.8)	.894
In-hospital mortality	0 (0)	2 (2)		.161
Isolate characteristics				
mecA positive	64 (58)	64 (57)	1.0 (0.8–1.3)	.816
Erythromycin resistant	21 (19)	16 (14)	0.7 (0.3–1.4)	.324
Clindamycin resistant	21 (19)	16 (14)	0.7 (0.3–1.4)	.325
Fusidic acid resistant	0 (0)	4 (4)		.046

NOTE. Data are no. (%) of patients or isolates, unless otherwise indicated. CI, confidence interval.

Our findings demonstrate a clear difference in the patient population affected and disease between PVL$^+$ and PVL$^-$ isolates, irrespective of methicillin resistance phenotype. Patients with PVL$^+$ isolates were younger by close to 10 years on average and had fewer comorbidities. Despite PVL being significantly associated with sepsis, patients with PVL$^+$ infections had a shorter length of stay. Patients with PVL$^+$ infections were less likely to have health care–associated risk factors, regardless of methicillin resistance phenotype. In other words, even among community-associated strains of *S. aureus*, PVL$^+$ strains were much more likely to be truly community acquired. Only 6% (13/223) of PVL$^+$ isolates were nosocomially acquired, compared with 21% (54/255) of PVL$^-$ isolates and 62% of multidrug-resistant MRSA isolates [14]. PVL$^+$ strains are endemic in our population and cause discharging skin abscesses but have made minimal incursions into the hospital setting. There rightly are concerns that PVL$^+$ community-associated MRSA strains such as USA300 will infiltrate and replace health care–associated genotypes of MRSA [36]. However, it is not clear whether traditional transmission models for MRSA can be applied to CA-MRSA [37]. Our findings suggest that, in our setting, true nosocomial transmission of PVL$^+$ strains (both nmMRSA and MSSA) is uncommon.

Another striking finding was that the age-specific incidence of PVL$^+$ compared with PVL$^-$ infections declined after a peak in the 20–39-year-old age group. Serological studies have demonstrated that PVL$^+$ *S. aureus* infections elicit an anti-PVL immunoglobulin G response [38, 39]. However, no consistent correlation was found between antibody levels and increasing age among individuals without evidence of current *S. aureus* infection [39]. Prospective longitudinal studies would help to determine whether such antibodies provide protective immunity or perhaps contribute to host susceptibility [40] and thus effect the age-specific incidence of PVL$^+$ infection.

We found no difference in the epidemiological and clinical characteristics of infections caused by PVL H isoform– and PVL R isoform–harboring isolates, suggesting to us that the R isoform confers no differential clinical effect. This accords with recent laboratory findings of no difference in leukotoxicity between strains bearing the H isoform and those bearing the R

Table 3. Comparison of Characteristics of Infections Due to Different Clonal Complexes (CCs)

Variable type, characteristic	nmMRSA					MSSA			
	CC1 (n = 54)	CC30 (n = 65)	CC75 (n = 22)	CC93 (n = 62)	Other CC (n = 36)	CC93 (n = 47)	CC5 (n = 44)	CC121 (n = 37)	Other CC (n = 111)
Demographic traits									
Age, median, years[a]	40	33	39	35	48	31	36	30	40
Male sex[b]	27 (50)	40 (62)	14 (64)	36 (58)	15 (42)	36 (77)	35 (80)	25 (68)	60 (54)
Indigenous[c]	39 (72)	33 (51)	18 (82)	31 (50)	14 (39)	23 (49)	20 (45)	22 (59)	55 (50)
Remote residence[d]	26 (48)	22 (34)	11 (50)	18 (29)	4 (11)	10 (21)	9 (20)	15 (41)	23 (21)
Clinical risk factors									
⩾1 comorbidity[d]	21 (39)	13 (20)	6 (27)	15 (24)	21 (58)	6 (13)	16 (36)	5 (14)	37 (33)
Health care–associated risk[d]	36 (67)	21 (32)	12 (55)	19 (31)	26 (72)	10 (21)	23 (52)	10 (27)	54 (49)
Nosocomial acquisition[d]	15 (28)	6 (9)	2 (9)	5 (8)	11 (31)	3 (6)	7 (16)	0 (0)	18 (16)
Type and severity of infection									
Skin and soft tissue[e]	45 (83)	62 (95)	19 (86)	58 (94)	28 (78)	45 (96)	38 (86)	33 (90)	91 (82)
Abscess[d]	5 (9)	36 (55)	1 (5)	27 (44)	3 (8)	21 (45)	7 (16)	18 (49)	11 (10)
Bacteremia	3 (6)	0 (0)	1 (5)	5 (8)	1 (3)	3 (6)	3 (7)	3 (8)	4 (4)
Pneumonia	3 (6)	1 (2)	2 (9)	1 (2)	2 (6)	0 (0)	1 (2)	1 (3)	6 (5)
Sepsis	13 (24)	15 (23)	3 (14)	19 (31)	5 (14)	8 (17)	12 (27)	13 (35)	21 (20)
Outcome									
Length of stay, median, days[f]	6.5	2	2	3.5	4.5	2	3	2	4
Length of stay, mean, days	32	7	9	6	18	9	15	5	20
Hospitalization	50 (93)	54 (83)	14 (64)	51 (82)	28 (78)	33 (70)	33 (75)	29 (78)	91 (82)
Surgery received[d]	19 (35)	43 (66)	5 (23)	42 (68)	11 (31)	34 (72)	14 (32)	26 (70)	33 (30)
In-hospital mortality	2 (4)	0 (0)	1 (5)	2 (3)	1 (3)	0 (0)	0 (0)	0 (0)	3 (3)
Isolate characteristics									
PVL positive[d]	1 (2)	64 (98)	0 (0)	62 (100)	1 (3)	47 (100)	5 (11)	36 (97)	7 (6)
PVL arginine isoform[d]	1 (2)	0 (0)	0 (0)	62 (100)	1 (3)	47 (100)	0 (0)	0 (0)	2 (2)
Erythromycin resistant[d]	32 (59)	1 (2)	6 (26)	8 (13)	12 (33)	8 (17)	2 (5)	20 (54)	10 (9)
Clindamycin resistant[d]	29 (54)	1 (2)	5 (23)	8 (13)	9 (25)	8 (17)	2 (5)	20 (54)	8 (7)
Fusidic acid resistant[d]	29 (54)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	12 (11)

NOTE. Data are no. (%) of patients or isolates, unless otherwise indicated. MSSA, methicillin-susceptible *Staphylococcus aureus*; nmMRSA, non–multidrug-resistant methicillin-resistant *S. aureus*; PVL, Panton-Valentine leukocidin.

[a] $P = .004$, Kruskal-Wallis test.
[b] $P = .004$, χ^2 test.
[c] $P = .007$, χ^2 test.
[d] $P < .001$, χ^2 test.
[e] $P = .041$, χ^2 test.
[f] $P = .001$, Kruskal-Wallis test.

isoform [41]. However, because the majority of isolates harboring the R isoform belonged to CC93, we cannot exclude the lesser possibility that the genomic background or other virulence factors carried by CC93 neutralize a real clinical difference. Notably, the other clone of *S. aureus* that typically harbors the R isoform is USA300. While not as extensive, the prevalence and distribution of CC93 in Australia is also expanding [4, 8]. Whether the R isoform enhances the transmissibility of *S. aureus* compared with the H isoform requires further exploration.

Previous work has demonstrated that all nasal-colonizing *S. aureus* genotypes can cause invasive disease [42, 43]. Our examination of the local population structure of *S. aureus* and associated detailed clinical features revealed several additional points. Both CC1 and CC5, common lineages locally and globally, typically caused disease in older, sicker patients. Carriage studies of *S. aureus* have demonstrated PVL$^-$ strains of CC1 and CC5 to be common nasal colonizers [6, 42, 44, 45]. Our findings suggest that these strains are well-adapted human commensals that mainly cause opportunistic infections in vulnerable hosts.

In contrast, CC93 and the other PVL$^+$ clones affect younger, healthier patients. CC93 was the most common clone identified in our collection, being significantly represented among both nmMRSA and MSSA strains. Previous typing of the staphylococcal protein A gene (*spa*) has revealed similar diversity of *spa* types in both the nmMRSA and MSSA CC93 isolates from northern Australia. Rather than a recent clonal expansion of a

particular CC93 MRSA *spa* type after a single acquisition of staphylococcal cassette chromosome (SCC) mec, the multiple CC93 MRSA *spa* types in a geographically and chronologically localized collection of isolates is better explained by either earlier SCCmec acquisition with subsequent *spa* type diversification or multiple independent acquisitions of SCCmec [22].

An unexpected finding was the importance of CC121, an exclusively MSSA clone in our collection that comprised 15% (37/239) of all MSSA isolates, all but one of which was PVL$^+$. Colonization surveys in remote western Australian Indigenous communities revealed CC121 to comprise 6.7% of MSSA isolates [45], but ours is the first report to attribute clinical disease to this clone in Australia. The effect of CC121 and CC93 in Australia has likely been previously underestimated because of a focus on nmMRSA.

The phylogenetically diverged CC75 lineage is one of emerging interest. Apart from impetigo [18], this is the first report, to our knowledge, that CC75 is capable of causing clinical disease (including bacteremia) similar to that of other *S. aureus* clones. However, 2 observations may indicate a comparatively less virulent phenotype. First, CC75 is underrepresented among clinical isolates, representing only 9% of nmMRSA isolates compared with 71% of nmMRSA isolates recovered from impetigo lesions in remote Indigenous Australian communities [18]. Second, the observed rates of sepsis due to CC75 are probably lower than with other lineages.

Intriguingly, the presence of CC75 has recently been reported in Cambodia and possibly in Indonesia and Malaysia [6]. This may relate to well-described direct population contacts between Macassan traders from Indonesia and northern Australian Indigenous people that predated the European colonization of Australia [46]. Also, a relative abundance of group 1b strains in Asia [6, 44] compared with elsewhere in the world has been noted, and we have found a similar overrepresentation of this clade, with CC121 and CC93 together comprising 26% and 35% of our nmMRSA and MSSA isolates, respectively. In summary, the emerging picture of *S. aureus* in the Top End of the Northern Territory of Australia is of a mixture of globally prevalent lineages (such as CC1, CC5, and CC30) coexisting with lineages that belong to either the highly diverged CC75 or the group 1b clade. CC121 is very widespread in the Asia-Pacific region, whereas CC93 appears to have emerged in Australia.

Limitations of our study include not collecting and typing all MSSA isolates presenting to the laboratory during the study period. However, MSSA isolates were collected in a random manner by sequential matching of laboratory receipt codes throughout the study period and should be representative of all MSSA isolates during this period. Importantly, we have been careful to stratify the analysis by methicillin resistance and have demonstrated that the associations with PVL were consistent for both nmMRSA and MSSA. However, it must be borne in mind that epidemiological associations with PVL should be distinguished from pathophysiological proof of causation. We did not perform SCCmec typing, and the SNP-based typing method does not provide the resolution of full MLST or pulsed-field gel electrophoresis. Thus, we cannot determine differences in the clinical features of some subclones within CCs.

In conclusion, we have determined the clinical disease spectrum caused by prevalent strains of *S. aureus* in northern Australia, including descriptions for CC93 and CC75. While differences in clinical disease existed on the basis of the genetic background as assessed by CC, these differences, including the outcome of sepsis, were principally explained by the presence of PVL. Even among community-associated lineages of *S. aureus*, PVL was a strong predictor of truly community-acquired disease. In our setting, the greater burden of PVL$^+$ disease is carried by MSSA, and focusing on nmMRSA alone will lead to underrecognition and underestimations of the effect of PVL$^+$ clones, such as CC93 and CC121.

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Appendix VIII: Trimethoprim-sulfamethoxazole compared with benzathine penicillin for treatment of impetigo in Aboriginal children: a pilot randomised controlled trial.

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CASE REPORT

Trimethoprim-sulfamethoxazole compared with benzathine penicillin for treatment of impetigo in Aboriginal children: A pilot randomised controlled trial

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Abstract: We conducted a pilot randomized controlled trial comparing trimethoprim-sulfamethoxazole to benzathine penicillin for treatment of impetigo in Aboriginal children. Treatment was successful in 7 of 7 children treated with trimethoprim-sulfamethoxazole and 5 of 6 treated with benzathine penicillin. Trimethoprim-sulfamethoxazole achieved microbiological clearance and healing of sores from which β-hemolytic streptococci and community-associated methicillin-resistant *Staphylococcus aureus* were initially cultured.

Key words: Australia; impetigo; indigenous; randomised controlled trial; trimethoprim-sulfamethoxazole.

Impetigo is highly prevalent in some remote Australian Aboriginal communities and serious complications include sepsis,[1] acute post-streptococcal glomerulonephritis[2] and possibly rheumatic heart disease.[3] Benzathine penicillin G (BPG), to which Group A *streptococcus* (GAS) is susceptible but most strains of *Staphylococcus aureus* are not, is the currently recommended treatment in the remote Aboriginal setting.[4] However, *S. aureus*, including community-associated methicillin-resistant *S. aureus* (CA-MRSA), may be replacing GAS as the dominant cause of impetigo.[5] BPG may no longer be an effective treatment and the intramuscular (IM) injections are painful and poorly tolerated. We conducted a pilot randomised controlled trial comparing IM BPG with oral trimethoprim-sulfamethoxazole (TMP-SMZ) for treatment of impetigo in Aboriginal children. Our objective was to determine if such a study was feasible and acceptable in an Aboriginal community setting, and to perform a preliminary comparison of the efficacy of TMP-SMZ with IM BPG.

Key Points

1. The prevalence of impetigo in children in Aboriginal communities continues to be up to 20%, and *Staphylococcus aureus* appears to be replacing Group A streptococcus as the main causative organism.
2. The currently recommended treatment regimen of intramuscular benzathine penicillin is poorly tolerated by children and may no longer be effective.
3. In this small pilot randomized controlled trial, we have found oral trimethoprim-sulfamethoxazole to be efficacious in treating impetigo in children in a remote Aboriginal community.

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None of the authors have conflicts of interest.

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We visited households in a remote Aboriginal community in the Top End of the Northern Territory, Australia. Children aged between 2 months and 16 years with a diagnosis of purulent or crusted impetigo were eligible for inclusion, but were excluded if they had: (i) received any antibiotic in the previous 7 days, or IM BPG in the previous 30 days; (ii) a known allergy to study medications; or (iii) evidence of sepsis, cellulitis or abscesses. Patients or their care givers provided written consent. The local ethics committee provided ethics approval, and the trial was registered (ACTRN12607000592448).

Participants were randomised to receive either a single dose of IM BPG (45 mg/kg up to 900 mg) or TMP-SMZ (4 + 20 mg/kg up to 160 + 800 mg) orally twice daily for 5 days. The allocation sequence utilised a permuted block design with blocks of four and six. Allocation was concealed by using sealed, sequentially marked, opaque envelopes. We did not stratify for age, gender or severity of impetigo.

The primary outcome was successful treatment of impetigo lesions at day 7 after the commencement of treatment. Treatment was deemed successful if sores were healed or improved in terms of drying of crusts and purulence and decrease in size of lesions, and unsuccessful if unchanged or worse. Assessment was by clinical examination and also by review of photographs by two investigators blinded to treatment allocation. Secondary outcomes were: (i) the bacterial resolution of sores at day 4 and day 7; and (ii) successful treatment at day 4. At baseline and on days 4 and 7, we documented the presence, size, appearance and number of skin sores, took swabs from sores and digital photographs of affected areas. Swabs were placed in Amie's medium and transported back to Darwin for routine culture and antimicrobial susceptibility testing. Analysis was by intention-to-treat.

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Table 1 Treatment given, clinical assessments and microbiological swab results

Age, sex	Treatment, severity	Day 0	Day 4		Day 7	
		Culture	Clinical assessment	Culture	Clinical assessment	Culture
2.1, F	Penicillin Mild	MSSA, GAS	Unchanged	MSSA	Unchanged	MSSA
3.1, M	Penicillin Mild	MSSA, GAS	Cured	No swab taken	Cured	No *Staphylococcus aureus* or βHS
4.9, F	Penicillin Mild	CA-MRSA, GAS	Unchanged	CA-MRSA	Improved	CA-MRSA
3.6, M	Penicillin Severe	MSSA	Improved	No *S. aureus* or βHS	Lost to follow-up	Lost to follow-up
5.4, F	Penicillin Severe	MSSA	Improved	MSSA	Improved	No swab taken
7.1, F	Penicillin Severe	MSSA	Unchanged	MSSA	Improved	MSSA
7.3, F	TMP-SMZ Mild	MSSA, GAS	Improved	MSSA	Improved	No *S. aureus* or βHS
7.1, F	TMP-SMZ Mild	MSSA	Improved	No *S. aureus* or βHS	Cured	No swab taken
4.8, M	TMP-SMZ Severe	MSSA, GCS	Improved	No *S. aureus* or βHS	Improved	No swab taken
4.0, M	TMP-SMZ Severe	CA-MRSA	Improved	MSSA	Improved	No *S. aureus* or βHS
6.5, M	TMP-SMZ Mild	MSSA	Unchanged	No *S. aureus* or βHS	Improved	No swab taken
4.1, M	TMP-SMZ Severe	CA-MRSA	Improved	No *S. aureus* or βHS	Improved	No swab taken
3.0, F	TMP-SMZ Severe	MSSA, GBS	Improved	No *S. aureus* or βHS	Cured	No swab taken

βHS, beta hemolytic *streptococcus*; CA-MRSA, community-associated methicillin-resistant *S. aureus*; F, female; GAS, Group A *streptococcus*; GBS, Group B *streptococcus*; GCS, Group C *streptococcus*; M, male; MSSA, methicillin-sensitive *S. aureus*; TMP-SMZ, trimethoprim-sulfamethoxazole.

Between 23 January and 26 January 2008, we screened 111 children, with 18 potentially eligible for inclusion. Thirteen consented and were randomly assigned to receive TMP-SMZ (7 participants) or IM BPG (6 participants). There was no difference in age, gender or severity of sores between the two groups. *S. aureus* was recovered in skin sore swabs from all 13 participants (three CA-MRSA) and β-haemolytic *streptococci* from 6 (4 GAS). Seven days after randomisation, in all 7 patients assigned to TMP-SMZ, and 5 of 6 patients assigned to IM BPG, treatment was successful (relative risk (RR) of successful treatment in the TMP-SMZ group, 1.2; $P = 0.46$; 95% confidence interval (CI) 0.83, 1.72). See Table 1. Treatment was successful after 4 days in 6 of 7 treated with TMP-SMZ and 3 of 6 with IM BPG (RR 1.7; $P = 0.27$; 95% CI 0.73, 4.03). Microbiological clearance was documented by day 4, in 5 of 7 treated with TMP-SMZ and 2 of 6 with IM BPG (RR 2.1; $P = 0.28$; 95% CI 0.63, 7.30); and by day 7, in all 7 treated with TMP-SMZ compared with 3 of 6 with IM BPG (RR 2.0; $P = 0.07$; 95% CI 0.90, 4.45). Irrespective of treatment received, we did not recover β-haemolytic *streptococci* at day 4 from any patients from whom it was initially recovered. Of the three children with CA-MRSA, clearance was observed by day 7 in both children treated with TMP-SMZ, but not the child given IM BPG. No adverse effects, in particular rash or gastrointestinal upset, from either study medication were observed or reported. There was 78–83% agreement between the clinical assessment and that of blinded assessors.

Both IM BPG and TMP-SMZ were efficacious in healing impetigo in this pilot study. The overall cure or improvement at 7 days in 12 of 13 children, is better than the rate of 61% previously seen with placebo, and equates well with rates of 75–95% with topical or other systemic treatments.[6] Our secondary endpoints suggested the possibility of earlier clinical improvement and microbiological clearance, as well as more successful clearance by day 7 in the TMP-SMZ group. The detected prevalence of impetigo of 16% (18/118 screened) accords well with that found in recent surveys of 10–20% in other Aboriginal communities[5] and is still unacceptably high. Based on the results of this pilot study, we have obtained funding to proceed to a formal randomised controlled trial comparing IM BPG and TMP-SMZ.

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Appendix IX. A randomised controlled trial of alternative treatments to intramuscular penicillin for impetigo in Aboriginal children: Trial protocol

The chief investigators (CI) for this grant were:

CIA	Jonathan Carapetis
CIB	Bart Currie
CIC	Ross Andrews
CID	Malcolm McDonald
CIE	Steven Tong
CIF	Asha Bowen

Background:

- Streptococcal and staphylococcal impetigo is highly prevalent in Aboriginal people living in rural and remote northern and central Australia.
- These skin infections are a major reason for child presentations to remote community clinics, often leading to the prescription of antibiotics; complications include streptococcal and staphylococcal sepsis, post-streptococcal glomerulonephritis, and most likely rheumatic fever.
- We have demonstrated that community based scabies and skin sore control programs can help to reduce the burden of skin infections.
- A key component of case management of skin sores, and of community Healthy Skin programs, is antibiotic treatment of existing sores.
- It is not known if the recommended treatment – intramuscular benzathine penicillin G (BPG) – is efficacious, particularly in light of the emergence of methicillin-resistant *S aureus* as a pathogen. Moreover, intramuscular injections are not ideal for a problem that may affect the majority of children in a community, and we have data that compliance with BPG treatment for impetigo is currently very poor in remote communities. However, the currently recommended oral regimens require multiple daily doses for 10 days, which is impractical (and poorly adhered to) in most Aboriginal communities.
- There is a need to evaluate simple oral regimens for the treatment of impetigo.

Hypothesis: That regimens of 5 days of once-daily oral cotrimoxazole or three days of twice-daily cotrimoxazole will lead to equivalent cure rates of impetigo as a single dose of intramuscular BPG.

Overall study design: This will be an open label randomised controlled trial with three arms.
<u>Arm 1 (Gold Standard)</u>: Single dose of intramuscular benzathine penicillin G
<u>Arm 2 (Alternative treatment 1)</u>: Cotrimoxazole suspension daily for 5 days.
<u>Arm 3 (Alternative treatment 2)</u>: Cotrimoxazole suspension twice daily for three days.

Recruitment of participants: We will recruit in three remote Aboriginal communities participating in the Healthy Skin program. In these communities, all children are regularly screened for skin sores as part of the Healthy Skin Program. Recruitment of study participants for the RCT would be incorporated within the existing screening program.

Primary endpoints: Comparisons of the proportion of children successfully treated on day 7 after the commencement of therapy with 1) benzathine penicillin G vs cotrimoxazole daily for 5 days (arm 2), and 2) benzathine penicillin G vs cotrimoxazole twice daily for 3 days (arm 3).

Secondary endpoints include the effect of each treatment on the clinical and blinded assessment of sores on day 4, the bacterial resolution of sores, prevalence of nasal carriage of *S aureus* and throat carriage of GAS, and evidence of allergy or other reaction to the medication, and acceptability of treatments to participants.

Sample size: A sample size of 198 per group will allow us to demonstrate non-inferiority, with 80% power and a one-sided alpha of .05. We expect to recruit these numbers within three years, with a study start date of early 2009.

Feasibility: We already have an existing East Arnhem Healthy Skin project operating in these communities. We will use existing study staff, including Aboriginal community and health workers, and continue their employment on this study. Essentially, this is an integral part of our existing project, which has already been through extensive community consultation and ethics approvals. A pilot study demonstrated the feasibility and community acceptance of such a study.

Outcomes and significance: This trial has the potential to provide an effective, simple and cheap oral alternative to BPG for impetigo, which could become the universal standard of care whether the patient is in Melbourne or Milingimbi. It would also likely be adopted by the World Health Organization as a standard of care for developing countries. This would lead directly to a reduced burden of impetigo and its complications. It would also open the way for studies to explore even simpler regimens.

Aims

Primary aim:

To determine if simple, cheap regimens of oral antibiotics (namely 5 days of once-daily oral cotrimoxazole or three days of twice-daily cotrimoxazole) will lead to equivalent cure rates of impetigo as the current gold standard treatment (a single dose of intramuscular benzathine penicillin G) in Aboriginal children.

Secondary aim:

To determine the relative contributions of *Staphylococcus aureus* (methicillin susceptible and methicillin resistant) and group A streptococcus to the aetiology of impetigo in Aboriginal children.

Background

Key points of research background:

- **Streptococcal and staphylococcal impetigo is highly prevalent in Aboriginal people living in rural and remote northern and central Australia.**
- **These skin infections are an important health problem:**
  - **They are the main reason for child presentations to clinics in remote communities, and usually lead to prescription of antibiotics +/- scabicides.**
  - **They are the major risk factor for streptococcal and staphylococcal sepsis, both of which occur at dramatically elevated rates in Aboriginal people.**
  - **They are the cause of almost all cases of post-streptococcal glomerulonephritis, which occurs at the highest rates in the world in Aboriginal children, and is a contributing factor to end-stage renal disease.**
  - **They have a causative role, either directly or indirectly, in rheumatic fever, which occurs at the highest rates in the world in Aboriginal people.**
- **We have demonstrated that community based scabies and skin sore control programs can help to reduce the burden of skin infections.**
- **A key component of case management of skin sores, and of community Healthy Skin programs, is antibiotic treatment of existing sores.**
- **It is not known if the recommended treatment – intramuscular benzathine penicillin G – is efficacious, particularly in light of the emergence of methicillin-resistant *S aureus* as a pathogen. Moreover, intramuscular injections are not ideal for a problem that may affect the majority of children in a community, and we have data that compliance with BPG treatment for impetigo is currently very poor in remote communities. However, the currently recommended oral regimens require multiple daily doses for 10 days, which is impractical (and poorly adhered to) in most Aboriginal communities.**

Over the past decade, we and others have demonstrated that skin infections, most commonly impetigo caused by group A streptococcus (GAS) and *Staphylococcus aureus*, are extremely common in Aboriginal people living in rural and remote northern and central Australia. Seventy percent or more of children, and up to one-third of adults, in some communities may be affected at any one time.[1, 2] By the age of one year in some communities, 63% and 69% of children have presented with scabies and skin sores respectively.[3] Antibiotic treatment for these skin infections undoubtedly contributes to the spreading epidemic of antibiotic resistance, particularly among pneumococci and *S. aureus*, in Aboriginal communities.[4]

Impetigo may lead to serious complications. Impetigo is the major causative factor in bacteraemia due to GAS and *S. aureus* in Aboriginal people. These infections are severe and frequently fatal.[5-7] Two studies from the NT and North Queensland have found that GAS bacteraemia in Aboriginal people occurs at five to eight times that of non-Aboriginal people living in the same region.[6, 7] Northern Territory studies have found that skin infection is the single most important risk factor for both GAS and *S. aureus* sepsis in Aboriginal people.[5, 7]

Acute post streptococcal glomerulonephritis (APSGN), an inflammatory disease of the kidneys, periodically causes outbreaks involving hundreds of children in multiple Top End communities.[8, 9] The risk of developing APSGN during an epidemic is increased by five for children with skin sores.[10] A recent study followed up a cohort of Aboriginal children in the NT an average of 15 years after two APSGN epidemics had struck their communities and found APSGN in childhood increased the risk of adult renal disease by six times.[11]

Streptococcal skin infection is also linked with acute rheumatic fever (ARF) and rheumatic heart disease (RHD). ARF and RHD occur at the highest rates in the world in the Aboriginal population, and are important causes of premature mortality.[12-14] Whereas the cause of ARF has traditionally been attributed to GAS infection of the upper respiratory tract, recent work by us and others has raised the possibility that skin infection with GAS may be involved in the pathogenesis of ARF.[15-17] The possibility that GAS skin infection may have a role in ARF pathogenesis is supported by a recent large randomised controlled trial in New Zealand where a sore throat-based primary prevention of ARF did not significantly reduce ARF incidence. In the seven cases of ARF with a preceding sore throat, from which a GAS was isolated, all of the strains belonged to serotypes normally associated with skin, rather than throat, infection - emm 53, 58, 74, 75, 76, 92 and 99 ([18] and Prof D Lennon, unpublished data presented at Lancefield Streptococcal Meeting, Cairns 2005).

We have adapted a successful model from Panama and used this effectively in several remote NT communities to control high rates of scabies.[1, 19] These community-based "Healthy Skin" programs are the basis of the current recommendations of the NT Dept of Health and Community Services for scabies and skin sore control, and also the subject of our current East Arnhem Healthy Skin Program (EAHSP). In the EAHSP, underway since 2005 in six remote communities, we are attempting to demonstrate the feasibility and practicality of regional Healthy Skin programs. As of August 2007, there had been 6047 assessments conducted on 2329 children. Although there had been an absolute reduction of 18.5% (95% CI 20.9%,16.0%) in the monthly period prevalence of impetigo over the study period, the prevalence remained unacceptably high at 27.6% (95% CI 26.1%,29.1%). (MSHR, Healthy Skin Report, 17 December 2007).

A key component of management of impetigo at the individual or community level is the need to treat affected individuals with antibiotics. The currently-recommended antibiotic for impetigo in Aboriginal communities is intramuscular benzathine penicillin G (IM BPG).[20, 21] The advantage of BPG treatment is that it requires only a single injection, which is particularly useful in Aboriginal communities where adherence to oral treatment regimens is often problematic.[22] One major disadvantage of BPG is that the injection is painful. This reduces the acceptability of BPG particularly when impetigo lesions are found in the majority of children in a community, as in a Healthy Skin screening program. It is not surprising that recommendations to administer an injection to large numbers of affected children are not well received by health staff or parents.

Unpublished data from the EAHSP, show that there is reluctance on the part of community staff to recommend BPG treatment for children with impetigo, and that the majority of children for whom it is recommended do not actually receive the treatment. For example, only 13% (110/838) of children with crusted or purulent sores, and 21% (63/308) of children with severe impetigo ($\geq$5 sores) detected at screening had BPG recommended. Only 47% (53/132) of children for whom BPG was recommended during screening actually received the treatment. The result was that, of the 308 children with severe pyoderma who without doubt should have been treated,[21] only 10% (31/308) actually received BPG (T Kearns, EAHSP, unpublished data).

Current recommendations for oral treatments are not acceptable alternatives to IM BPG. Courses of oral flucloxacillin or roxithromycin two to four times per day for 10 days are rarely completed, especially in remote communities. A shorter regimen, reducing both the frequency of dosing and the duration of the course, is more likely to improve treatment adherence.[23] A simplified oral regimen that is effective and requires just a few days of either daily or twice-daily doses would be a major advance in treatment and control of skin infections and their complications.

Studies from tropical settings during the 1970s demonstrated the excellent clinical response of impetigo lesions to treatment with BPG, to which GAS but not *S. aureus* are susceptible.[24-27] Any detection of *S. aureus* was explained by its role as a secondary wound coloniser rather than as a predominant infectious agent.[2, 24] However, more recent data from Hawaii, Israel and Sydney provide evidence that *S. aureus* has replaced GAS as the dominant cause of impetigo.[28-30] In a recent study we undertook in three remote communities in the Top End of the NT, *S. aureus* was recovered from skin sores of 221 of 375 children with pyoderma (59%) and was found in company with group A beta-haemolytic streptococcus on 110 occasions (29%).[31] Methicillin-resistance was detected in 23% of *S. aureus* isolates. The antibiogram showed no cotrimoxazole, tetracycline, gentamicin or rifampicin resistant isolates, a pattern typical of community-associated methicillin-resistant *S. aureus* (CA-MRSA).

We do not know if BPG is effective in treating impetigo in Aboriginal communities. There have been no studies of its efficacy in this country, and the most recent studies from other countries were performed three decades ago. After its administration, it is rare for Aboriginal community clinics to follow up children with impetigo, so we do not even have consistent anecdotal impressions of its effectiveness. Moreover, with *S. aureus* possibly emerging as a major pathogen in impetigo in Aboriginal communities, BPG may no longer be an effective treatment.

Because of the extremely high burden of impetigo in remote communities, with its associated morbidity and mortality, and the expanding focus on large scale community-based treatment and control programs, there is an urgent need to develop a simple oral regimen that will have a high chance of leading to good adherence among Aboriginal people, and will also be effective against the emerging threat of CA-MRSA.

We propose to conduct a randomised controlled trial comparing two simple, oral antibiotic regimens with the existing standard treatment (BPG) in communities that already participate in our regional East Arnhem Healthy Skin program.

<u>Choice of oral regimens</u>: Because of the potential role of *S. aureus* in aetiology of skin sores, it is important to choose an antibiotic with activity against both staphylococcal and streptococcal infections. Topical antibiotics such as mupirocin have been used successfully to treat skin sores,[32, 33] but are inappropriate for widespread use in remote Aboriginal communities because of documented emergence of antimicrobial resistance.[34]

The Australian Therapeutic Guidelines on antibiotics suggest the use of oral dicloxacillin, flucloxacillin, cephalexin or roxithromycin for staphylococcal skin infections.[20] The recommended regimen for flucloxacillin is four times daily for 10 days; a regimen unlikely to be taken to completion by patients in any setting, let alone in remote Aboriginal communities.[22] Paediatric liquid preparations of flucloxacillin are particularly unpalatable. Moreover, almost 25% of *S. aureus* isolates from impetigo in these communities is CA-MRSA,[31] which would not respond to flucloxacillin.

A previous study found GAS resistance to cotrimoxazole[35] but used flawed methodology; utilising Mueller-Hinton agar containing 5% sheep blood rather than Mueller-Hinton with lysed blood agar (thymidine inhibition is likely to be the reason for discordance using the different methods). We tested 100 NT GAS isolates and 20 NT CA-MRSA isolates with E-tests. All CA-MRSA isolates and 96% of GAS isolates were susceptible to cotrimoxazole (Table 1). Aside from an ideal spectrum of activity, cotrimoxazole has the added advantage of a long duration of action. Its half life is 10-11 hours (compared to 2 hours for flucloxacillin and <1 hour for cephalexin)[36] and its main active ingredient, trimethoprim, is recommended for daily dosing in cystitis,

Table 1. Cotrimoxazole MICs for Northern Territory GAS and CA-MRSA isolates

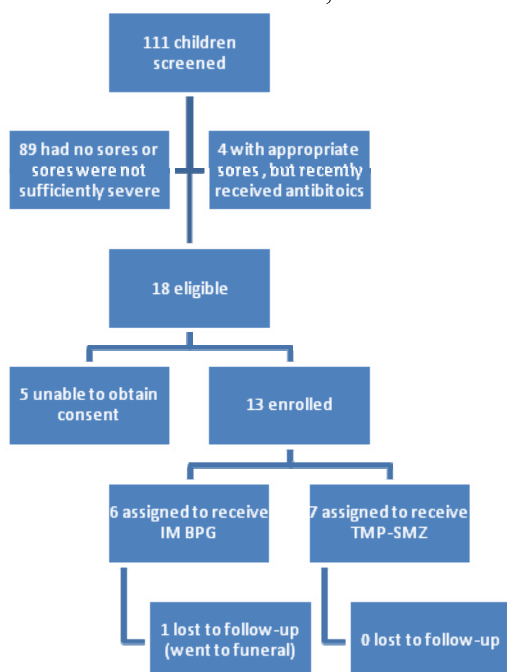
Organism	Cotrimoxazole MIC (mg/L)*	Number (%)
NT Group A streptococcus (n=100)	≤2	96 (96%)
	4	2 (2%)
	>4	2 (2%)
NT CA-MRSA (n=20)	≤ 0.2	20 (100%)

* There are no cotrimoxazole break points for GAS. It is likely that ≤2 mg/L will be susceptible.

pyelonephritis and epididymo-orchitis.[20] Cotrimoxazole has excellent skin penetration,[37] is being increasingly favoured for treatment of CA-MRSA infections, including skin sepsis,[36] and such use is now a recommendation for treatment of CA-MRSA skin infections in the Australian and United States guidelines.[20, 38] Daily dosing would improve the likelihood of adherence and would also be ideal for supervised treatment if this was necessary. Other advantages are its low cost and appealing taste.

Cotrimoxazole has an excellent safety profile. It is the most widely-used antibiotic in the world, and remains the recommended treatment for many non-severe infections including otitis media and mild pneumonia in developing countries.[39, 40] Serious side effects are rare. The reported risk of Stevens Johnson Syndrome is extremely low: occurring in 0.4 per 100,000 exposures to cotrimoxazole in children <15 years old in Malawi[41] and 2.8 per 100,000 patients prescribed cotrimoxazole in a general practice based study in the United Kingdom.[42]

Pilot study: As a proof of principle that cotrimoxazole could be an effective skin sore treatment in remote NT communities, and to determine if recruitment and retention would be a problem, we conducted a pilot to test the trial protocol in January 2008 at Galiwinku, Elcho Island. The pilot included allocation concealment and blinding of the outcome assessment. It was registered with the Australian Clinical Trials Registry (ACTRN12607000592448), and obtained community consent and ethics approval. We screened 111 children by household visit over 3 days, enrolling 13 of 18 eligible children (i.e. with purulent or crusted sores and not excluded on other grounds). The five children eligible but not enrolled had either not returned for randomisation after the initial eligibility assessment (3) or their parents were unavailable (2). No parents objected to randomisation and no parents withdrew consent after being randomised. The control group received the currently recommended regimen of IM BPG 45mg/kg. The intervention group were assigned cotrimoxazole 4 + 20 mg/kg twice daily for 5 days.



All 13 participants cultured *S. aureus* (CA-MRSA in 3) at the initial visit, and 6 also grew β-haemolytic streptococci. No staphylococcal isolates demonstrated resistance to cotrimoxazole. Seven days after randomisation, all 7 patients assigned to cotrimoxazole, and 5 of the 6 patients assigned to IM BPG were successfully treated (RR 1.2; 95% CI 0.83 to 1.72). Treatment was deemed successful after only 4 days in 6/7 treated with cotrimoxazole and 3/6 treated with IM BPG (RR 1.7; 95% CI 0.73 to 4.03). Microbiological clearance was documented at day 4, in 5/7 treated with cotrimoxazole and 2/6 treated with IM BPG and at day 7, in all 7 receiving cotrimoxazole compared to 3/6 receiving IM BPG.

Cotrimoxazole was directly administered for 59 of 70 doses (84%). Five of 7 children were directly observed for ≥8 of 10 doses, and we were confident that only 1 child did not receive all 10 doses. No adverse effects from either medication were observed or reported. Health-care staff expressed a preference for administrating oral cotrimoxazole rather than often painful intramuscular injections. There were a total of 36 assessments of sores for the 13 subjects and there was 78-83% agreement between the field assessment and that of the blinded assessors.

The pilot study demonstrated that both IM BPG and cotrimoxazole were able to heal sores in a remote Aboriginal community. The overall success rate was better than 61% previously seen with placebo ($p = 0.02$),[32] and equates well with rates of 75-95% with topical or other systemic treatments.[32] Our recruitment rate of 72% (13/18) would likely be improved with more extensive community wide consultation and education. One participant was lost to follow-up, and in a larger

study, it is likely that the rate of loss to follow-up will realistically be between 5-10%. Our secondary endpoints suggested earlier clinical improvement and microbiological clearance, as well as more successful microbiological clearance at day 7, in the cotrimoxazole group.

For the definitive trial, we have chosen to compare three groups. Arm 1 will be the current gold-standard treatment – BPG. The others are two different regimens of cotrimoxazole because we believe the 5-day, twice daily regimen in our pilot study is still too long and complex to result in good adherence in practice. Each of our chosen regimens has advantages over the currently-recommended oral antibiotic regimens in terms of fewer total doses, fewer doses per day, and fewer total days of treatment:

Arm 2 (Daily cotrimoxazole for 5 days): The dose selected is that recommended in the Australian Antibiotic Guidelines for daily dosing of trimethoprim for pyelonephritis and epididymo-orchitis. It is our experience that this dose is well tolerated in children. The 5 day course, particularly administered once-daily, is clearly an improvement over a 7-10 day antibiotic course 2 to 4 times per day. Daily dosing allows the option of supervised dosing.

Arm 3 (Twice-daily cotrimoxazole for 3 days): This is a more ambitious arm, but if successful would be a major advance. Most clinical staff would be reasonably optimistic about adherence to a 6-dose regimen over 3 days. Participants in Arms 2 and 3 receive the same total daily dose of cotrimoxazole. Note that 6 of 7 patients in our pilot study were successfully treated when reviewed at day 4, providing further evidence that this brief course may be sufficient.

Research Plan – Methods and Techniques to be Used

Hypothesis That regimens of 5 days of once-daily oral cotrimoxazole or 3 days of twice-daily cotrimoxazole will lead to equivalent cure rates of impetigo as a single dose of intramuscular BPG.

Overall study design: This will be an open label randomised controlled trial (RCT) with three arms blinded for outcome assessment.

Inclusion criteria:

1. Age 2 months to 14 years.
2. Diagnosis of purulent or crusted impetigo by criteria outlined in the Booklet “Recognising and Treating Skin Conditions” (EAHSP, Menzies School of Health Research 2006).

Exclusion criteria:

1. Receipt of any antibiotic in the previous seven days, or receipt of BPG in the previous 30 days.
2. The presence of impetigo lesions that are only “flat-dry” (i.e. lesions that are not purulent or crusted) by criteria outlined in the Booklet “Recognising and Treating Skin Conditions” (EAHSP, Menzies School of Health Research 2006).
3. Known allergy to penicillin, sulphonamides or trimethoprim, or other constituents of study medications.
4. Immunocompromised.
5. Acute or chronic dermatosis.
6. Previous participation in the trial.
7. Evidence of sepsis, cellulitis, bullous impetigo, boils or carbuncles.
8. Intention by the patient or parent to use topical antibiotics.

Recruitment of participants: We will recruit in three communities participating in the EAHSP – Milingimbi, Galiwinku and Yirrkala. In these communities, all children are regularly screened for skin sores as part of the EAHSP by Aboriginal Community Workers who have undertaken a VET Certificate II level training program in Healthy Skin. Recruitment of study participants for the RCT would be incorporated within the existing screening program, subject to informed consent. Children with impetigo, and their parents/guardians, will be approached for written, informed consent. Consenting children will be assessed for eligibility.

Randomisation: Eligible children will be randomised by the use of sealed, sequentially marked, opaque envelopes.[43] Randomisation will be stratified by community and sore severity, to ensure an

even distribution of community representation and severe or mild sores in each group. Sore severity will be classified as "mild" (<5 sores, of which no more than one is purulent) or "severe" (≥5 sores or ≥2 purulent sores). In each community two sets of randomisation envelopes will be provided – one for mild and one for severe sores. Each set will block randomise children in random batch sizes of 6 or 12 using sealed envelopes. Unfortunately, centralized telephone based randomization is not practical due to unreliable telephone reception in remote communities.

Treatment:

Arm 1 (Gold Standard)	Arm 2 (Alternative treatment 1)	Arm 3 (Alternative treatment 2)
Single dose intramuscular benzathine penicillin G 45 mg/kg up to maximum of 900mg (1.2 million units)	Cotrimoxazole suspension 8 + 40 mg/kg (max 320 + 1600 mg) daily for 5 days.	Cotrimoxazole suspension 4 + 20 mg/kg (max 160 + 800 mg) twice daily for three days

In arms 2 and 3, we will attempt to observe all doses. If a dose is unable to be observed, we will document from the caregivers the next day as to whether the dose was given.

Additional treatments:

- Topical antibiotics will not be permitted.
- If evidence of associated scabies, topical permethrin cream will be prescribed as per local treatment guidelines.
- Other treatments, including traditional medications or other treatments, will not be prescribed, and their use will be discouraged. At follow-up reviews on Day 4 and 7, we will record the use of these alternative treatments. Use of alternative treatments will be treated as a possible confounder or effect modifier in the analysis.

Blinding: Due to the differing methods of administration of medication, both the patient and the field worker would know to which treatment group the child belongs. To minimise bias in assessments at follow-up, outcomes of treatment will be assessed by two independent investigators (blinded to the group assignment and the field worker's assessment) using digital photographs of the same sores taken at baseline, day 4 and day 7 according to a standard protocol (see below). Where disagreements between the blinded assessors occur, they will meet and be asked to come to a consensus. This final blinded assessment will be used for analysis of outcomes.

Endpoints: Among 16 trials reviewed for a meta-analysis on the treatment of skin sores, the most common outcome measurement stratified sores into 4 categories either: 'healed', 'improved', 'unchanged' or 'worse'.[32] This approach provides detail for the comparison of treatments and their effects while also allowing for a simple binomial analysis of each intervention by combining the categories; 'healed' with 'improved' (success) and 'unchanged' with 'worse' (failure). *The primary outcomes will be a comparison of the proportion of children successfully treated on day 7 after the commencement of treatment with 1) benzathine penicillin G vs cotrimoxazole daily for 5 days (arm 2), and 2) benzathine penicillin G vs cotrimoxazole twice daily for 3 days (arm 3).* Day seven was recommended by the authors of the meta-analysis of treatment of impetigo because rapid clinical recovery is desirable.[32] Comparison of arm 2 and arm 3 will not be an *a priori* comparison. If both arm 2 and arm 3 outcomes are no worse than BPG, then both could be recommended for future use. Assessments will be clearly guided by our established tools for classification of skin sores, including a flipchart with photographic examples of how to classify sores. Where there are multiple sores per person, photographs will be taken of several areas and encompass as many sores as is practically possible. We anticipate few problems with overall assessment of improvement or worsening of skin sores as it is rare for there to be discordant resolution in one person. We used this methodology in our pilot study without any problems.

<u>Secondary endpoints:</u>

a) clinical and blinded assessment of sores at day 4 to determine early differences in outcome.

b) effect of each treatment on the bacterial resolution of sores. We will take swabs from up to 2 purulent sores for each patient at baseline with repeat swabs from the same sores at days 4 and 7. The day 4 endpoint was chosen because it is our experience that bacteriological eradication may be rapid and precedes clinical cure.

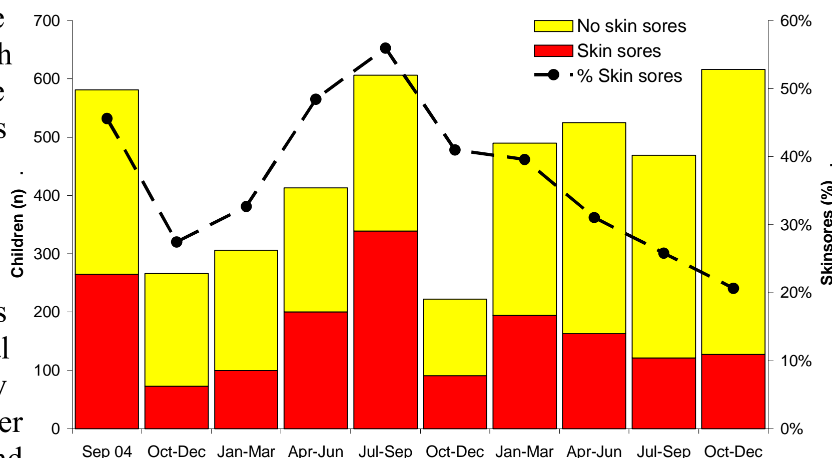
c) prevalence of nasal carriage of *S. aureus* (including a comparison of the prevalence of methicillin-resistant MRSA at baseline and day 7) and throat carriage of GAS at baseline and day 7, to determine the effectiveness of each treatment on eradication of these organisms.

d) evidence of allergy or other reaction to the medication, and acceptability of treatments to participants (or parents) using a simple questionnaire.

In the three months following enrolment, the research team will monitor for other potential secondary endpoints for each enrolled individual, such as streptococcal/staphylococcal sepsis, post-streptococcal glomerulonephritis and rheumatic fever. Data sources will be community clinic files, available hospital separation data and the notifiable disease database (where relevant). Numbers are expected to be extremely small.

Sample size: This will be a non-inferiority study, designed to demonstrate that the alternative treatment arms give equivalent cure rates to the gold standard (BPG) arm. We have defined non-inferiority as a cure rate no more than 10% less than that of the gold standard arm, and have assumed a cure rate of 80% in the gold standard arm. Based on our expectation that the true difference between the alternative and gold standard arms is zero, a sample size of 198 per group will allow us to demonstrate non-inferiority, with 80% power and a one-sided alpha of 0.05.

Assuming a 10% drop out rate (we expect good follow-up over a 7 day period, as demonstrated in our pilot study) we will aim to recruit 660 participants. Over the duration of the EAHSP, we conducted an average of 158 skin assessments each month (Figure). For the purposes of the RCT, we estimate 150 assessments will be conducted each month, 25% will have impetigo (38) and, of these, 62% (23) will be considered severe. Assuming 80% of potentially eligible subjects consent to participate in the trial (consistent with our pilot study findings and experience in other trials in Aboriginal communities) and allowing 10% loss to follow-up, we expect to be able to recruit the desired sample of 198 subjects per group within 3 years (18 subjects per month), and possibly quicker.



Data collection: Information collected at <u>Day 0</u> will include: name, address, sex, and date of birth. Some of these data will also serve for baseline analysis along with other potential confounders such as household size, number of siblings and the presence of co-infections of the skin such as tinea and scabies. For the purpose of confidentiality as well as identification, each child will be issued with a unique identifying code. Quantification and description of the sores at baseline would occur using our standard screening form. Digital photographs will be taken of the affected area of the body according to a specified protocol that includes using a study camera with automatic focus and always using a flash, training of the user, taking photos from a standard distance and use of a measurement reference. Swabs from up to 2 purulent sores will be taken, as will throat and nose swabs, from each child. These will be placed in paper bags and transported with desiccant in a cold box via air to our laboratory in Darwin where they will be cultured and identified using standard techniques. We have validated this procedure in Aboriginal communities and confirmed that it is

almost as sensitive as direct plating.[44] All isolates will undergo antibiotic susceptibility testing using disk diffusion and resistant isolates will have MICs tested using the E test method.

On Day 4, sores will be clinically assessed as described on page 6, measured, photographed and skin sore swabs taken as per day 0. We consider Day 4 as an appropriate mid-point to assess the progress of sore resolution. On Day 7, assessments will be as per day 4, with skin sore, nasal, and throat swabs repeated as per day 0. The research team will endeavour to retrieve medicine bottles and record the amount used by each participant.

Data analysis: The primary outcome is binomial; therefore the test for two proportions will be used. Confidence intervals and p-values will be calculated using the normal distribution. Primary analysis will be by intention-to-treat. A secondary 'per protocol' analysis will also be conducted. For analyses of secondary outcomes, the test for two proportions would also be used. The proportion of bacterial cure rates will be compared across the treatment groups as will the proportion of bacterial carriage rates. Outcomes will be based on the blinded assessment in these analyses, but we will also calculate the agreement percentages and kappa statistic to analyze inter-observer agreement of treatment success between the blinded and unblinded assessors.

Safety considerations: All participants will be monitored for adverse events, particularly allergies to the antibiotics. Children will be observed in the clinic for 30 minutes after the first dose of antibiotics. Parents of participating children will be encouraged to return to the clinic if they are concerned about any adverse reactions or side-effects. Children in the study who develop suspected cotrimoxazole-related drug allergy and still have active pyoderma will be offered standard treatment with BPG. Children experiencing any serious side-effects, including Stevens-Johnson syndrome, will be withdrawn from treatment, although most will have completed the course by the onset of clinical features. In these communities, any children considered to be seriously ill, from any cause, are routinely and immediately airlifted to Darwin for paediatric assessment. These cases will also be notified to the independent safety monitoring committee (ISMC), below.

Although our pilot study has demonstrated proof of principle, and suggests that cotrimoxazole will be at least equally effective to BPG, we acknowledge the need for careful monitoring during the trial. Therefore, we will appoint an independent safety monitoring committee (ISMC) consisting of three members: an expert in clinical paediatric infectious diseases, an expert in clinical trials, and a biostatististician. Any adverse events occurring in a temporal relationship to the administration of the study medications will be reported to the ISMC. In addition the ISMC will be asked to review results of the first 180 participants, expected approximately 12 months after commencement of recruitment. At that time, if there is clear evidence of failure of one or both cotrimoxazole treatment arms, the committee will have the power to suspend the trial based on criteria determined *a priori*. The p values for this interim analysis will be stringently set at three standard deviations (one sided $p<0.002$) to minimize the risk of wrongly assigning inferiority to cotrimoxazole at this early stage of the study. With 60 participants in each arm, the interim analysis will have 83% power to detect a reduction in success rate from 80% in the BPG arm to 45% in one of the cotrimoxazole arms. If there is an apparent trend to reduced efficacy of cotrimoxazole, but not sufficient evidence to suspend the trial, the ISMC will have the power to order a further interim analysis during the study. Cotrimoxazole resistance in *S. aureus* isolates will be monitored as a cumulative proportion as well as a three-monthly rolling average. The ISMC will review this beginning at six months into the study and then every three months, with power to suspend the trial if there is a significant and consistent increase in cotrimoxazole resistance.

Timeline: We have allocated three months for community consultation in 2009, much less than we would normally do because there has already been substantial discussions with communities about the need for the study, and indeed have already performed a pilot version in one, Galiwinku. During this time we will seek to finalise ethics committee approvals and other outstanding matters.

	2009		2010		2011		2012	
Community Consultation/Ethics	■							
Conduct RCT		■	■	■	■	■	■	
Analysis, feedback, dissemination								■

Ethics approval: The research protocol will be presented to community councils and leaders to discuss the potential risks and benefits associated with involvement in the trial. Upon community endorsement, ethics approval for the trial will be sought from the Human Research Ethics Committee of the NT Dept of Health and Community Services and the Menzies School of Health Research. Participant information sheets and appropriate explanatory material will be devised in picture form and local languages as per our current consent process for the Healthy Skin program. Individual written consent to participate in the trial will be sought from the parent or legal guardian of each child. However, children could refuse treatment as per NH&MRC guidelines.

Outcomes and Significance: Notwithstanding the inherent difficulties and expense of conducting large interventional studies in a remote Aboriginal community setting, our pilot study suggests that it is feasible and culturally acceptable. These communities grasp that despite improvements in skin health of Aboriginal children over that past 10 years, impetigo and its complications continue to contribute a significant burden of disease and that the standard treatment of IM BPG has the significant drawback of being painful and unpleasant to administer. Therefore many, and perhaps most, affected children are left untreated in some communities. In communities where children are properly treated with BPG, the prospect of many children receiving injections at the same time is a major barrier to community acceptance of Healthy Skin programs. We urgently need an effective, simple and cheap oral alternative to BPG for impetigo.

This trial has the potential to provide that alternative. If either or both of the comparison arms proves equivalent to BPG, this will quickly become the standard of care for impetigo in rural and remote communities. Moreover, this could become the universal standard of care (i.e. for all impetigo regardless of whether it occurs in Melbourne or Milingimbi) given that cotrimoxazole is effective against methicillin-susceptible *S. aureus*, most CA-MRSA and GAS. Our team includes members of the writing group for the Australian Antibiotic Guidelines and the Central Australian Rural Practitioners Association Manual, so we will be able to ensure that the results are quickly translated into clinical practice guidelines. Through our links with the World Health Organization (WHO), we can also ensure that the findings translate into guidelines for international practice. The WHO strategy for primary care of young children in developing countries is Integrated Management of Childhood Illness (IMCI). IMCI is an algorithm-based approach that allows lowly-trained health workers to identify, treat and/or refer children with a range of important clinical features. Skin infections have not been part of IMCI until recently, when many countries have expressed an interest in their inclusion. We are part of a consortium that is currently developing and trialing a Skin-IMCI module. The ability to include a simple regimen of the most widely used childhood antibiotic in developing countries (cotrimoxazole) for impetigo in a Skin-IMCI module would greatly increase its chance of acceptability by most countries, and effectiveness in practice.

If successful, this trial could ensure that children (and adults) with impetigo are properly treated, whereas presently they often receive no treatment. The complications of impetigo are dramatic. They include kidney disease (glomerulonephritis and its role in chronic renal disease), rheumatic fever (leading to rheumatic heart disease), and sepsis (streptococcal and staphylococcal), The potential ramifications of this study include a reduced burden of all of these complications.

Should either or both of the alternative treatment arms be successful, it will also raise the possibility of exploring even simpler regimens. For example a two or three-day course of daily cotrimoxazole could be even better. However, we feel it is important to first study regimens that are an improvement on current recommendations while still standing a good chance of success.

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Appendix X. High-resolution melting analysis of the *spa* locus reveals significant diversity within sequence type 93 methicillin-resistant *Staphylococcus aureus* from northern Australia

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High-resolution melting analysis of the *spa* locus reveals significant diversity within sequence type 93 methicillin-resistant *Staphylococcus aureus* from northern Australia

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Abstract

High-resolution melting analysis is an inherently robust, easy and inexpensive approach to the examination of genomic regions containing single-nucleotide polymorphisms and hypervariable loci. *Staphylococcus aureus* sequence type (ST) 93 is a singleton, Panton–Valentine leukocidin-positive clone unique to Australia. A high-resolution melting-based method for the identification of ST93 was developed, and a similar approach was used to reveal diversity within the *spa* locus of this lineage. Statistical and graphical methods that account for instrumental and operator-dependent variation in high-resolution melting curves were developed, to allow greater confidence and reproducibility in deciding whether another curve is truly different from the baseline curve of an amplicon with known sequence. The data support a very early acquisition, or multiple independent acquisitions, of SCC*mec* by ST93 methicillin-susceptible *S. aureus* (MSSA), and the coexistence of MSSA and methicillin-resistant *S. aureus* versions of the same lineage within northern Australia.

Keywords: High resolution melting, *Staphylococcus aureus*, *SPA*, ST93

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Introduction

In the evolving Australian epidemics of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA), the most common multilocus sequence types (MLSTs) are 1, 93 and 30 [1]. Sequence type (ST) 93 methicillin-resistant *S. aureus* (MRSA), first described in 2000 [2], is an MLST singleton clone, is typically Panton–Valentine leukocidin (PVL)-positive, and is a major cause of severe CA-MRSA disease in Australia [2–4]. The other common PVL-positive clone, ST30-MRSA, probably arrived in Australia via large population migrations in the 1980s [5]. ST93-MRSA is the most rapidly expanding CA-MRSA clone throughout Australia [1], and now constitutes 33% of Australian CA-MRSA isolates (13th International Symposium on Staphylococci and Staphylococcal Infections (ISSI, 2008), Abstract 110), and has also been exported to the UK by travellers (13th ISSI, 2008, Abstract 604). Previous investigators found little genetic diversity within ST93-MRSA, suggesting that it arose from one PVL-positive subtype of ST93 methicillin-susceptible *S. aureus* (MSSA) [6].

The polymorphic X region of the staphylococcal protein A (*spa*) gene rapidly evolves, and *spa* sequence typing can elucidate patterns of diversity within sequence types. High-resolution melting (HRM) analysis, a new approach to analysing genetic variation, is cost-effective, simple, and robust. It has been shown to be highly effective for resolving *spa* types [7]. Although empirical methods to define HRM curves as the same or different have been used, such methods have not been based upon statistical analysis [7].

Our aims were to: (i) develop an HRM-based method for the rapid identification of ST93; and (ii) determine the *spa* locus diversity of ST93-MRSA, ST93-MSSA and ST30-MRSA isolates from northern Australia. Of particular interest was whether ST93-MRSA represents a highly conserved clone with a very recent origin as compared with ST93-MSSA.

Materials and Methods

Isolates were collected in community-based studies of impetigo conducted in remote Aboriginal communities from 2003 to 2005 [8], and in a prospective study of CA-MRSA infec-

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TABLE 1. High-resolution melting (HRM) curves and corresponding *spa* types and repeats

	HRM curve (no. of isolates)	*spa* genotype (no. sequenced)	*spa* repeats
ST93-MRSA[a] (Fig. 1c)	HRM curve 1 (23)	t202 (8)	11-17-23-17-17-16-16-25
	HRM curve 3 (2)	t1819 (2)	11-17-23-17-16-16-25
	HRM curve 4 (1)	t4675 (1)	11-17-17-17-16-16-25
	HRM curve 5 (4)	t1811 (4)	11-17-17-16-16-25
ST93-MSSA (Fig. 1d)	HRM curve 1 (26)[a,b]	t202 (5)	11-17-23-17-17-16-16-25
	HRM curve 2 (1)[c]	t4178 (1)	11-17-23-17-17-16-16-16-25
	HRM curve 6 (2)[c]	t4699 (2)	11-17-16-16-25
	HRM curve 10 (1)[b]	t4698 (1)	04-16-16-25
ST30-MRSA[a] (Fig. 1e)	HRM curve 7 (27)	t019 (7)	08-16-02-16-02-25-17-24
	HRM curve 8 (2)	t138 (2)	08-16-02-25-17-24
	HRM curve 9 (1)	t4700 (1)	08-275-02-16-02-25-17-24

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*; ST, sequence type.
[a]All ST93-MRSA, ST30-MRSA and 21 ST93-MSSA isolates were from the 2006–2007 Royal Darwin Hospital collection.
[b]Six ST93-MSSA isolates were from the 2003–2005 community-based studies.
[c]Three ST93-MSSA isolates were from the 1991 Royal Darwin Hospital collection.

tions at the Royal Darwin Hospital (RDH) from 2006 to 2007 (47th ICAAC, 2007, Abstract L1141); three ST93-MSSA isolates were collected in 1991 at the RDH (Table 1). The community studies had revealed the highly divergent clonal complex (CC) 75 to be the predominant *S. aureus* clone in remote communities in northern Australia [8].

A robust and efficient method for identifying ST93 was developed. An ST93-specific single-nucleotide polymorphism (SNP), *glpF* 165, was identified in the *S. aureus* MLST database using the software Minimum SNPs [9]. This is a three-state SNP, with the 'C' allele completely specific for ST93, the 'A' allele completely specific for CC75, and the 'T' allele possessed by all other known STs. To add redundancy, we also used the MLST database-derived SNP *aroE* 252, which has been determined to be diagnostic for ST93. This SNP also has three allelic states [10], with the 'G' allele being unique to ST93. Primers were designed to generate amplicons containing these SNPs.

The diversity of ST93 was assessed by performing HRM analysis of the *spa* locus of 30 isolates each, identified as ST93-MRSA and ST93-MSSA. Additionally, 30 isolates of PVL-positive ST30-MRSA from northern Australia, previously characterized using a kinetic PCR method to examine eight SNPs [10], were similarly analysed.

We used a Rotor-Gene 6000 instrument (Corbett Life Science) for the HRM analysis. DNA was extracted using the QIAamp DNA minikit protocol for Gram-positive bacteria with lysostaphin. Each reaction contained 5 μL of Platinum SYBR Green qPCRSuperMix-UDG (2x; Invitrogen Life Technologies), 0.5 μM each primer and 1 μL of a 1 : 5 dilution of the DNA template in a final volume of 10 μL. The real-time PCR thermocycling parameters were: 50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 5 s and 60°C for 30 s; 72°C for 2 min; and 50°C for 20 s; this was followed by HRM ramping with fluorescence data acquisition at 0.05°C increments. The primers and HRM ramping temperatures are shown in Table 2. Reactions were routinely carried out in duplicate. The exported HRM curves are available as Supporting Information. The *spa* locus was sequenced in representative isolates that yielded the different curves, and *spa* types were assigned according to the *spa* database [11].

TABLE 2. Primers and ramping temperatures for high-resolution melting reactions

Primer name	Primer sequence (5′–3′)	Ramping temperatures (°C)
glpF 165F	ACCCAGCGGTGTCTTTAGCTCTTGCAT	72–84
glpF 165R	ATACAATCGTTGCTCCGACA	
aroE 252F	GTGGATAGGGTATAATACAG	68–81
*aroE*252R	ACCTGCGCCCAAAATTAAAA	
spa 1095F	AGACGATCCTTCGGTGAGC	75–87
*spa*1517R	GCTTTTGCAATGTCATTTACTG	

We defined 95% CIs for the HRM curves corresponding to isolates of the same sequenced *spa* type by exporting the normalized fluorescence data of the HRM curves to calculate the mean and standard deviation (SD) at each temperature of the melting protocol. This was only performed when there were at least six isolates of the same *spa* type. At each temperature, the 95% CIs were calculated as the mean ± (1.96 × SD). We generated difference graphs by subtracting this mean normalized fluorescence from the normalized fluorescence of each curve at each temperature, and defined the 95% CIs for the difference graph as 0 ± (1.96 × SD).

Results

We developed HRM-based methods for the examination of both SNP regions, SNP *glpF* 165 and SNP *aroE* 252, using

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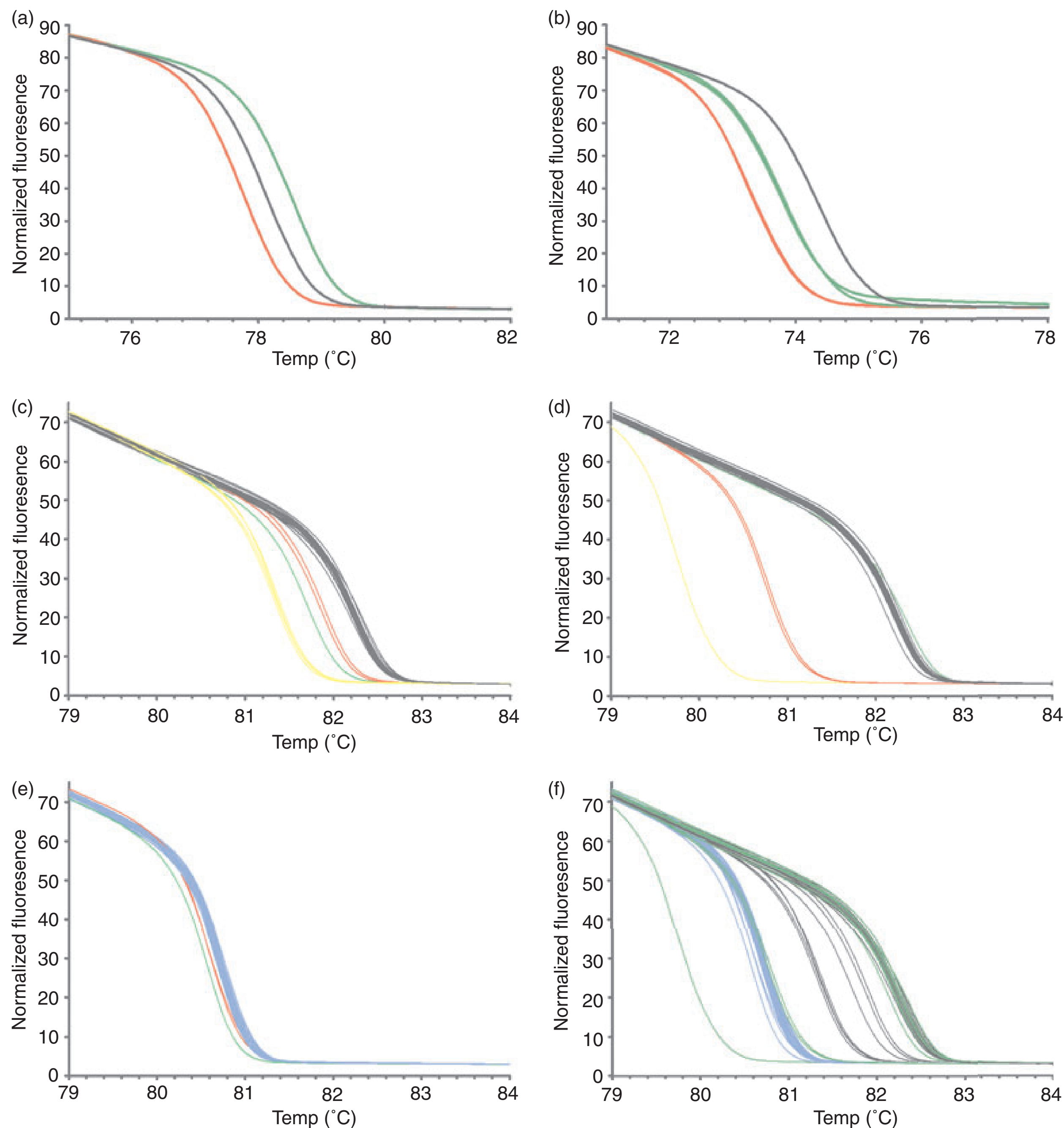

FIG. 1. High-resolution melting curves of the *glpF* 165, *aroE* 252 and *spa* loci (c–f). (a) Representative curves corresponding to each allele at *glpF* 165: C (black) is specific for ST93, and A (green) is specific for CC75. (b) Representative curves for the *aroE* 252 region: the black curve is specific for sequence type (ST 93. (c) ST93 methicillin-resistant *Staphylococcus aureus* (ST93-MRSA) showing four curves: curve 1 (black), curve 3 (red), curve 4 (green), and curve 5 (yellow). (d) ST93 methicillin-susceptible *S. aureus* (ST93-MSSA) showing four curves: curve 1 (black), curve 2 (green), curve 6 (red), and curve 10 (yellow). (e) ST30-MRSA showing three curves: curve 7 (blue), curve 8 (red), and curve 9 (green). (f) ST93-MRSA (black), ST93-MSSA (green) and ST30-MRSA (blue) combined.

isolates of known ST. For each SNP region, three easily discriminated curves were obtained (Fig. 1a,b). During the development of the *glpF* 165 assay, some unexpected results were obtained. The initial primer set allowed amplification of a product that generated identical HRM curves for ST93 and CC75, even though CC75 is highly divergent from all other *S. aureus*, and the two PCR products differed at seven positions. Examination of the seven SNPs indicated a neutral

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balance of hydrogen bonds, with an identical GC content. Redesign of the primers (*glpF* 165F and *glpF* 165R) allowed clear discrimination of ST93 and CC75, with the amplified regions now having differing GC contents. Of the possible amplified *aroE* 252 regions, ST93, among all the STs, has a unique GC content as well as the highest melting temperature, and therefore generates a unique HRM curve. We were unable to consistently generate an *aroE* amplicon for CC75, and further work to better characterize this locus for CC75 is proceeding. It was concluded that examining the *glpF* 165 and *aroE* 252 SNP regions by HRM analysis is a rapid and robust method for identifying ST93. It has the added benefits of incorporating redundancy and allowing the identification of CC75, which is another major northern Australian clonal complex.

HRM analysis of the *spa* locus revealed four and three clearly distinct curves for ST93-MRSA and ST93-MSSA isolates, respectively (Fig. 1c,d and Table 1). Of 14 sequenced ST93 isolates associated with HRM curve 1, 13 had the same *spa* type of t202, and one contained an additional 24-bp repeat, t4178. Closer examination of the t4178 HRM difference curve, with the mean t202 curve as the baseline, revealed part of the curve sitting outside the 95% CI curve, suggesting that the t4178 and t202 HRM curves are indeed different (Fig. 2a). The five other curves correlated with different *spa* types. Comparison of the raw data of HRM curves of two ST93-t202 isolates produced in another laboratory [7] showed that they were consistent with our t202 curves, although a part of the curve at 72°C, well away from the melting temperature, was outside the 95% CI (Fig. 2a). Within ST30-MRSA, three slightly different curves could be distinguished on both the normalized fluorescence and the difference curves (Figs 1e and 2b). The *spa* loci of seven isolates with HRM curve 8 were sequenced; all had the same *spa* type of t019. The two other curves correlated with different *spa* types (Table 1). Fig. 1f shows curves from all three groups of isolates.

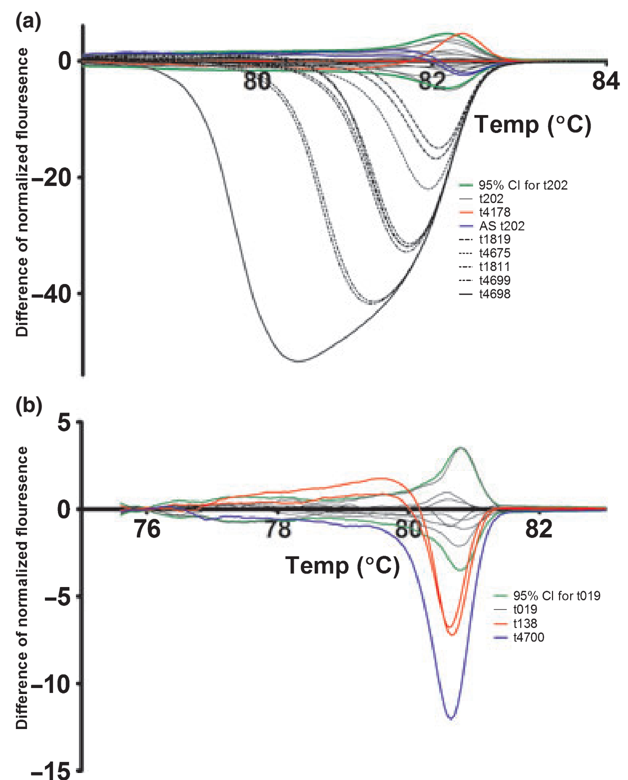

FIG. 2. Difference graph of high-resolution melting curves of *spa* loci. (a) Curves for sequence type (ST) 93 isolates. The mean of the known t202 curves was used as the baseline. The green lines indicate the 95% CIs for the 13 t202 curves, and the thin black lines indicate each t202 curve. The blue lines (AS t202) refer to t202 curves derived from the raw data of the study by Stephens *et al.* [7], and sit within the 95% CI, whereas part of the t4178 (red) curve sits outside the 95% CI. (b) Curves for ST30 isolates. The mean of the known t019 curves was used as the baseline. The green lines indicate the 95% CIs for the seven t019 curves, and the thin black lines indicate each t019 curve.

Discussion

All of the ST93-MRSA isolates were from the 2006–2007 hospital-based study, and the finding of four *spa* types within these 30 isolates revealed surprising *spa* type diversity for a group of chronologically and geographically localized isolates from a hospital serving a population of only 176 000. Similarly localized [12–14], and some even larger [15,16], studies of CA-MRSA revealed no *spa* type diversity among ST30, ST80 and USA300 isolates. The diversity in ST93-MRSA more closely mirrors that seen in studies across broader geographical regions and time periods, where single STs contained up to five *spa* types [17–20]. Larsen *et al.* [19,20] linked this diversity within ST80 and USA300 to multiple international importations of these strains into Denmark. Such importation could explain the diversity that we demonstrated in the widely distributed ST30-MRSA, but not that in ST93-MRSA.

The diversity in both ST93-MRSA and ST93-MSSA does not support the hypothesis that ST93-MRSA is undergoing an explosive clonal expansion derived from a recent single instance of SCC*mec* acquisition. Rather, the data support an early acquisition of SCC*mec*, with subsequent rearrangements of the *spa* sequence or multiple independent acquisitions of SCC*mec*, and coexistence of MSSA and MRSA versions of the same lineage. A recent study of ST5 revealed

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that SCC*mec* has probably been acquired repeatedly in geographical regions within ST5, and predicted that MRSA haplotypes within a geographical region should also be present in MSSA from that region [21]. Our findings support this prediction; for instance, the commonest *spa* type, t202, is present in ST93-MRSA from RDH and in ST93-MSSA from both RDH and community study collections. Interestingly, all 21 ST93-MSSA isolates from the 2006–2007 hospital collection were *spa* type t202, raising the possibility of a current epidemic of ST93-MSSA-t202, as compared with endemic ST93-MRSA. However, these 21 isolates were not linked epidemiologically in terms of time or patient residence, and only one was nosocomially acquired.

HRM analysis is a single-step closed-tube reaction. It is inherently robust, easy, and inexpensive to optimize and perform. It can also be used to examine hypervariable loci [7]. The derivation of SNP sets from sequence alignments using the software Minimum SNPs, and the development of HRM-based assays for the examination of these SNPs, is a straightforward and efficient approach for assembling genotyping procedures for specific purposes. We have been able to accurately discriminate two major clones in northern Australia from all other clones and also from each other.

HRM-mediated *spa* examination can differentiate closely related *spa* types, and provides further resolving power for genotyping. It is less expensive than full *spa* sequencing; each sequencing reaction in our institution costs US $20, as compared with US $0.50 for reagents for each HRM run. The Rotor-Gene 6000 software and previous publications have used a single HRM curve of an amplicon of known sequence as the baseline to generate difference curves. However, despite the robust methods, there is inevitably instrumental and operator error, resulting in slight variations in HRM curves generated from identical amplicons. We have developed a simple technique that incorporates this variation and allows greater confidence and reproducibility in deciding whether another curve is truly different from the baseline curve of known sequence. With appropriate calibration of instruments, it should also allow portability of the HRM curves and interlaboratory comparison, as demonstrated in this article.

Acknowledgements

These findings were presented in part at the 13th International Symposium on Staphylococci and Staphylococcal Infections, 7–10 September 2008, Cairns, Australia, Abstract 709. We thank the RDH microbiology laboratory for its assistance, and F. O'Brien for providing the 1991 RDH isolates. This publication made use of the *spa* typing website (http://www.spaserver.ridom.de/), which is developed by Ridom GmbH and curated by SeqNet.org (http://www.SeqNet.org/).

Transparency Declaration

S. Y. C. Tong is an Australian National Health and Medical Research Council PhD scholar (NHMRC grant 436033), and financial support was also provided by the Co-operative Research Centre for Aboriginal Health. P. M. Giffard is an inventor on a patent application that describes the software "Minimum SNPs" and is eligible for royalty payments if this is commercialized. The other authors have no competing interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Exported numerical data defining the HRM curves generated in this study.

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Appendix XI: Rapid detection of H and R Panton-Valentine leukocidin isoforms in *Staphylococcus aureus* by high-resolution melt analysis

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Rapid detection of H and R Panton–Valentine leukocidin isoforms in *Staphylococcus aureus* by high-resolution melting analysis☆

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Abstract

We designed a single-step, closed-tube, real-time polymerase chain reaction and high-resolution melting assay to simultaneously detect the presence of the Panton–Valentine leukocidin gene and discriminate histidine and arginine isoforms. Of 223 *Staphylococcus aureus* isolates from northern Australia, isoforms clustered by clonal complex (CC). All CC93 isolates harbored the arginine isoform.
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Keywords: Community-associated methicillin-resistant *Staphylococcus aureus*; Australia; Panton–Valentine leukocidin; *Staphylococcus aureus*; High-resolution melting

1. Introduction

The majority of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains produce Panton–Valentine leukocidin (PVL), which is encoded by the cotranscribed genes *lukS-PV* and *lukF-PV* (Kaneko et al., 1998). Recent studies have demonstrated sequence variations within *lukSF-PV* (Dumitrescu et al., 2008; O'Hara et al., 2008; Wolter et al., 2007). In particular, a single nucleotide polymorphism (SNP) at nucleotide site 527 leads to an A → G His_{176} to Arg_{176} substitution, the H and R isoforms. USA300 (clonal complex [CC] 8) and USA400 (CC1) isolates have been found to carry the R isoform, but most clones isolated outside the United States carry the H isoform (Dumitrescu et al., 2008; O'Hara et al., 2008). To date, the only isolate with the R isoform not from CC1 or CC8 is an Australian isolate of CC93 (Dumitrescu et al., 2008). CC93 is the most rapidly expanding CA-MRSA clone in Australia and a major cause of severe CA-MRSA disease (Nimmo et al., 2006). Molecular modeling suggests these isoforms may affect PVL function (O'Hara et al., 2008), and a rapid means of discriminating these isoforms, without the need for sequencing, would facilitate clinical studies (Tong et al., 2010).

High-resolution melting (HRM) analysis is a newly emerging technology that employs the ability to monitor with fluorescence the separation of 2 strands of DNA with increasing temperatures (Erali et al., 2008). The combination of real-time polymerase chain reaction (PCR) and HRM can be used to determine the presence or absence and also sequence variants of a gene. We designed a high-throughput HRM-based assay to simultaneously detect the presence of *lukSF-PV* and discriminate the H and R isoforms. We determined the distribution of H and R isoforms in a collection of hospital-based isolates from the tropical top end of northern Australia.

Isolates were collected as part of a case-control study, comparing CA-MRSA to methicillin-susceptible *S. aureus*

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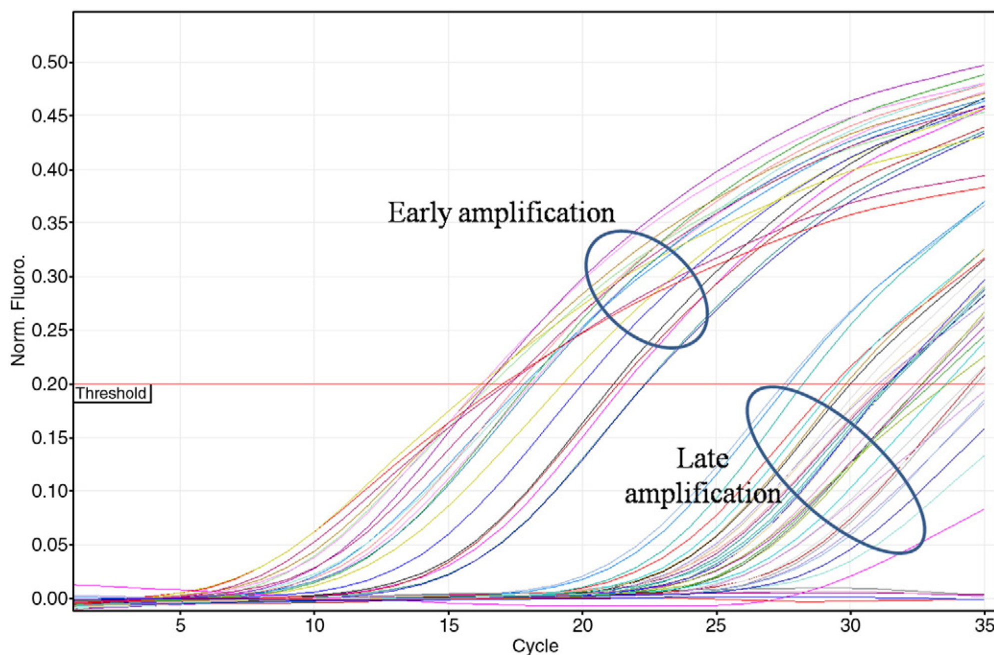

Fig. 1. Real-time PCR amplification curves of PVL-positive and negative isolates. The PVL+ isolates all have a cycle threshold of <23, and PVL isolates have a cycle threshold of >27.

(MSSA) infections at the Royal Darwin Hospital, Darwin, Australia (Tong et al., 2009a). For this study, isolates from eligible CA-MRSA patients were matched 1:1 to the next eligible MSSA patient using sequential laboratory numbers. DNA was extracted using the QIAamp® DNA minikit (QIAGEN, Melbourne, Australia) protocol for Gram-positive bacteria with lysostaphin. We typed isolates into CCs using an SNP genotyping system based on the multilocus sequence type database (Huygens et al., 2006). To confirm isolates as CC93, we also performed an HRM assay interrogating *aroE* position 252 (Tong et al., 2009b). Pulsed-field gel electrophoresis was performed on selected isolates (O'Brien et al., 2006).

Previously, our laboratory detected *lukSF-PV* by amplifying a region of the *lukF-PV* gene (Huygens et al., 2006). For this HRM assay, we designed primers to amplify a 57-bp fragment of *lukS-PV* spanning nucleotide position 527 with primers: PVL527F (5′-CAACAAAACTATATCAGT-GAAGTAGAAC-3′), PVL527R (5′-TCCCCATTGAA-CACTTTTTGAA-3′). PCR fragments containing the 57-bp HRM assay amplicon were obtained using flanking primers PVLlukS1F (5′-TGGTCCATCAACAGGAGGTA) and PVLlukS1R (5′-TGGGGGTAATTCATTGTCTG-3′), and these were commercially sequenced (Macrogen, Seoul, Korea) on an ABI3730XL (Applied Biosystems, Seoul, Korea) machine.

HRM reactions were performed on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) system. Each HRM reaction contained 5 μL of Platinum® SYBR® Green qPCRSuperMix-UDG (2×, Invitrogen Life Technologies, Melbourne, Australia), 0.5 μM of each primer, and 1 μL of a 1:5 dilution of the DNA template in a final volume of 10 μL. The thermocycling parameters were as follows: 50 °C for 2 min, 95 °C for 2 min, 1 cycle each of 95 °C for 2 s, and decreasing annealing temperatures from 65 to 61 °C in 1 °C decrements for 10 s, 35 cycles of 95 °C for 2 s and 60 °C for 10 s, 72 °C for 2 min, and 50 °C for 20 s, followed by HRM ramping from 64 to 74 °C with fluorescence data acquisition at 0.3 °C increments. Raw HRM curves were normalized by the Rotor-Gene 6000 software, using normalization regions of 64.5 to 65.0 °C and 73.2 to 73.7 °C.

There were 239 CA-MRSA and 239 MSSA isolates collected for analysis. Initial testing for *lukF-PV* on real-time PCR revealed 223 positive and 255 negative isolates. Of the 223 *lukF-PV*–positive isolates, 128 were CA-MRSA and 95 MSSA. These were analyzed with the HRM assay, together with 40 negative isolates that were chosen for representatives across different CCs. All 223 *lukF-PV*–positive isolates amplified with the HRM primers, and the 40 *lukF-PV*–negative isolates either amplified late or not at all (Fig. 1). At a normalized fluorescence threshold of 0.2, all positive isolates amplified before cycle 23 and all negative isolates amplified after cycle 27. Late amplifying isolates are likely to harbor *lukD*, which has some sequence homology with *lukS* at the primer sites. A cycle threshold cutoff of 25 provided 100% sensitivity and 100% specificity for detecting *lukSF-PV*.

The HRM assay discriminated between the H and R isoforms with melting temperatures differing by 1 °C. These differences are clear on both the HRM normalized curves and the standard d*F*/d*T*-based melting profile (Fig. 2). These results for 10 H and 10 R isoform isolates were confirmed by DNA sequencing. An additional SNP at nucleotide position 663, which is found in the H1 and H2

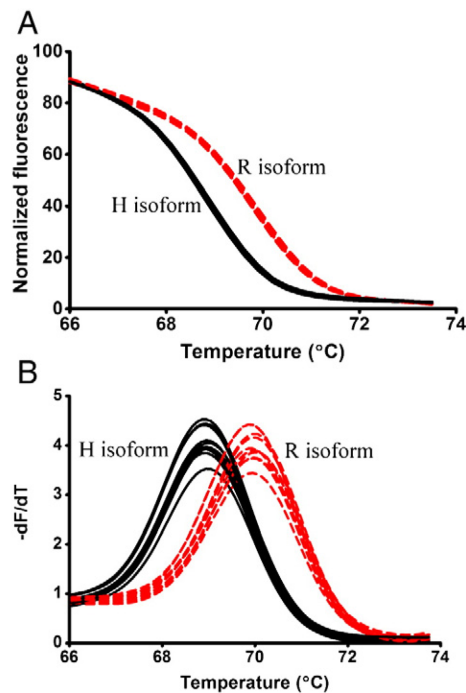

Fig. 2. HRM (A) and standard melting temperature (B) curves for isolates of known sequence. Histidine (H) isoforms (black, unbroken lines, n = 10) and arginine (R) isoforms (red, broken lines, n = 10).

subtypes (O'Hara et al., 2008), was present in all the H isoforms sequenced. This SNP is outside the region interrogated by the HRM assay and thus does not impact upon the HRM curves.

Overall, there were 110 H isoforms and 113 R isoforms. All 109 CC93 isolates, 62 CA-MRSA and 47 MSSA, were R isoforms. There were 4 other R isoform isolates: 3 CC1s and 1 CC8. Using pulsed-field gel electrophoresis, we characterized the CC8 and CC1 isolates as USA300 and USA400, respectively. The majority of H isoforms were CC30 (66 isolates) and CC121 (36 isolates); the remainder were CC5 (5 isolates), CC6 (2 isolates), and CC25 (1 isolate).

In addition to the first description of the presence of USA300 in the Northern Territory, we have found a major epidemic Australian clone, CC93, to universally harbor the R isoform. Unlike USA300, CC93 does not contain the arginine catabolic mobile element, which may be central to the transmissibility of USA300 (Diep et al., 2008). Whether the R isoform of PVL confers epidemic properties to these respective clones is yet to be determined. In contrast to O'Hara et al. (2008), but in agreement with the findings of Dumitrescu et al. (2008), we found no correlation between PVL isoform and the presence of *mecA* (P = 0.82). This is well illustrated by CC93, where both CC93-MRSA and CC93-MSSA harbor the R isoform. The most likely explanation is that the presence of the R isoform predates the acquisition of *mecA*.

In summary, we have developed and utilized a high-throughput, single-step, closed-tube HRM-based assay, which simultaneously detects the presence of PVL and discriminates its 2 major isoforms.

Acknowledgments

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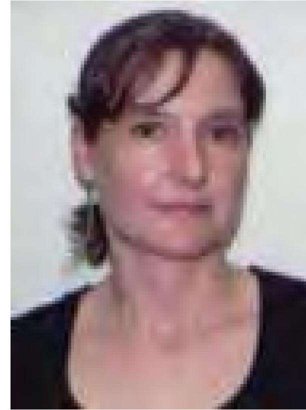
Appendix XII. CA-MRSA: emerging remotely

CA-MRSA: emerging remotely



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Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) was first described in remote Indigenous populations in Australia over 20 years ago. The burden of staphylococcal disease, including *S. aureus* bacteraemia, disproportionately affects Indigenous populations and is likely related to socio-economic disadvantage. Factors such as domestic crowding, poor hygiene and high rates of scabies, skin sores and antibiotic use contribute to the transmission and emergence of CA-MRSA. Studies focusing on two clones, sequence type (ST) 93 and clonal complex (CC) 75, provide supportive evidence for the emergence of methicillin-resistance in Indigenous communities.

Despite the initial report of CA-MRSA originating from remote Indigenous communities in the Kimberley region of Western Australia [1], the burden of staphylococcal disease and the emergence of CA-MRSA in Indigenous communities has been underappreciated. CA-MRSA has been present in the Northern Territory (NT) and Western Australia (WA) from at least the late 1980s [2, 3]. Between 1991 and 1995, isolations of CA-MRSA in the NT already outnumbered isolations of hospital-associated MRSA [4]. The highest incidence of MRSA notifications during the 1990s in WA were consistently from the remote Kimberley and Goldfield regions [5].

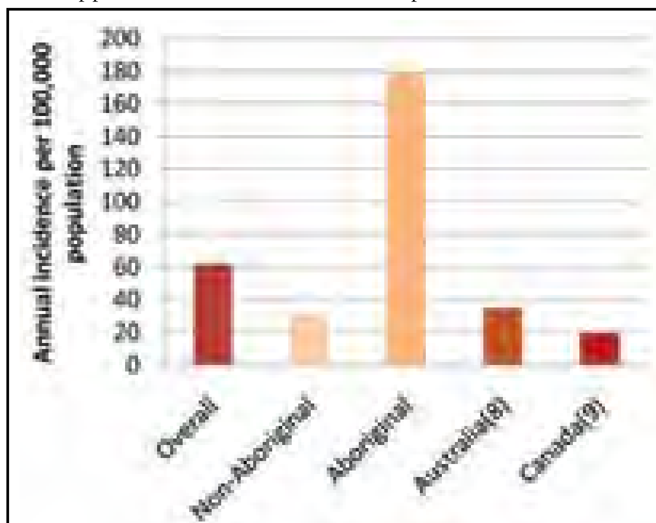

Figure 1: Annual incidence of *Staphylococcus aureus* bacteraemia in the Top End and comparative rates for Australia and Canada (references in brackets).

More recent studies have revealed the burden of disease due to *S. aureus* and CA-MRSA continues to be high in Indigenous communities. Pyoderma was found in 38% of children in three remote Top End communities with *S. aureus* recovered from 59% of pyoderma lesions and methicillin-resistance detected in 23% of these isolates [6]. The annual incidence of *S. aureus* bacteraemia in the Top End Indigenous population is 172 cases per 100,000, while the incidence in the non-Indigenous population of 30 cases per 100,000 (rate ratio 5.8 [95% confidence interval, 3.8-8.9]) is similar to that of the rest of Australia [7] (Figure 1 [8, 9]). This difference in the incidence rates of *S. aureus* bacteraemia demonstrates that the health disparity between Indigenous and non-Indigenous Australians includes the burden of staphylococcal disease.

However, Indigenous ethnicity itself does not appear to explain this difference. Notably, there was a strong correlation between incident isolation of *S. aureus* and measures of socio-economic disadvantage and remoteness in the Top End [7]. A case-control study comparing CA-MRSA with MSSA found that rather than Indigenous ethnicity, female sex (adjusted OR 1.5) and remote residence (aOR 1.8) were associated with CA-MRSA on multivariate logistic analysis [7]. It is likely that factors in remote Indigenous communities, such as domestic crowding, poor hygiene and high rates of scabies, skin sores and antibiotic use, contribute to the transmission and emergence of CA-MRSA [10]. A possible explanation for the association of CA-MRSA with female sex is that children have a particularly high prevalence of skin disease and it is women who provide most of the childcare.

Two *S. aureus* lineages, ST93 and CC75, are of particular interest and provide insight into the emergence of CA-MRSA from Indigenous populations. ST93, known as the Queensland clone, was first described in a Caucasian group of patients in

Queensland [11]. Subsequent studies, though, have led to the conclusion that it has probably emerged from Indigenous communities [12]. ST93 harbours Panton-Valentine leukocidin, a pore-forming toxin linked to severe disease manifestations [13]. Of concern, it is also the most rapidly expanding clone of CA-MRSA in Australia [14, 15]. The spread of ST93-MRSA had been thought to be due to a rapid clonal expansion following a recent single instance of acquisition of SCC*mec,* the mobile genetic element which mediates methicillin-resistance [16]. However, analysis of the *spa* gene, a hypervariable repeat region of the genome, has demonstrated diversity of *spa* types in both ST93-MSSA and ST93-MRSA isolates from the Top End [17]. This diversity within ST93-MRSA is not consistent with an explosive clonal expansion. Rather, the data support an early acquisition of SCC*mec* with subsequent rearrangements of the *spa* sequence or multiple, independent acquisitions of SCC*mec.* Therefore, there is a reasonable likelihood that there is ongoing emergence of ST93-MRSA from circulating ST93-MSSA strains in Indigenous communities.

In contrast to the geographic expansion of ST93 within Australia, CC75 has only been isolated from the Top End to date. CC75 is the dominant strain of CA-MRSA in Top End communities, where 71% of CA-MRSA strains recovered from pyoderma lesions were CC75 [6]. CC75 is of great interest in that phenotypically it resembles *S. aureus,* but phylogenetic analysis indicates significant genotypic divergence from the rest of *S. aureus* [18, 19]. Analysis of the MLST loci revealed remarkable diversity within CC75 isolates, even when isolated from within the same small human population in the NT [18]. It appears that CC75 is not a clone of *S. aureus* but a distinct taxon in its own right. Whether CC75 should be formally reclassified is problematic, given the lack of a diagnostic phenotype, but the phylogenetic justification for reclassification is strong. Both CC75-MRSA and CC75-MSSA were found to co-circulate with evidence of multiple acquisition events of SCC*mec* by CC75-MSSA [6]. Thus, this is an example of ancient MSSA strains and their direct SCC*mec*-harbouring descendents coexisting in an isolated environment where there are many factors present to drive the emergence of resistance.

Most *S. aureus* collections originate from affluent regions of the world and our knowledge of the population structure of *S. aureus* is therefore skewed. Remarkably, CC75 has recently also been found in Cambodia and possibly Malaysia and Indonesia [19]. It is fascinating to speculate that this highly divergent lineage of *S. aureus,* which has been found in neglected human populations in terms of staphylococcal research, may be associated with population movements in the Asia-Pacific region. Strikingly, within Australia, CC75 appears only to be found in the Top End, where the Indigenous population is unique in that it had contact with Maccassan traders in historical times.

In summary, in northern Australia, both the conserved and radiating ST93 and the diverse, divergent and endemic CC75, have likely been subject to multiple introductions of SCC*mec.* Further research into the population structures of CC75 and ST93 are likely to provide ongoing insights into the emergence of methicillin-resistance in community strains of *S. aureus.* This understanding should fuel the need to address issues of socio-economic disadvantage in Indigenous communities, to reduce the impact of staphylococcal disease and the emergence of resistance.

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