Phenotypic and genotypic characterisation of $qacA$-containing *Staphylococcus aureus* in New Zealand

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Abstract

*Staphylococcus aureus* is a significant human pathogen and is the primary cause of skin and soft tissue infections. Antimicrobial resistance is an ongoing concern, with increasing reports of multi-resistant *S. aureus* infections which have limited options available for treatment. New Zealand has high incidence rates of infections caused by *S. aureus*, with the predominant circulating clone often being resistant to the topical antibiotics fusidic acid and mupirocin. Healthcare facilities have relied on chlorhexidine as the main antimicrobial agent used to prevent and control infections caused by common pathogens including *S. aureus*. Chlorhexidine resistance in *S. aureus* has been associated with *qacA*, a gene which encodes for a multi-drug efflux pump.

The objectives of this study were to (i) determine prevalence of *qacA* in New Zealand *S. aureus*; (ii) correlate prevalence of *qacA* gene in New Zealand *S. aureus* strains with clonal lineages, susceptibility profiles, and patient demographics; and (iii) examine the genetic context of the *qacA* gene in circulating *S. aureus* clones.

The *qacA* prevalence in New Zealand *S. aureus* isolates is 7%. Concerningly, presence of the *qacA* gene is associated with the predominant clonal lineage *spa* type t127 strain found in New Zealand. The predominant New Zealand t127 strain is synonymous with fusidic acid resistance and, in 45% of cases, mupirocin resistant. The combination of *qacA* with fusidic acid and mupirocin resistance in *S. aureus* has the theoretical potential of being resistant to the three most common topical treatment options used for skin and soft tissue infections.

We were unable to find a statistically significant difference in phenotypic susceptibility to chlorhexidine when testing isolates with and without *qacA*. Despite being unable to find reduced susceptibility in *qacA* carrying strains, these strains continue to be associated with failed decolonisation strategies in case control studies. Further work could look at the development of an assay in different milieu to detect phenotypic expression of *qacA* which leads to reduced susceptibility to chlorhexidine.

The *qacA* gene was found on a ~28 kb plasmid, co-located with genes that confer resistance to penicillins and mupirocin (*mupA*). The representative plasmid (pNZAK1)
closely resembles the ubiquitous pMW2 plasmid. Interestingly, \textit{mupA} and the \textit{qac} operon are found on a \textasciitilde7 kb insertion sequence. A nucleotide BLAST search of this sequence indicates that this genetic arrangement is specific to the ST1, t127 clonal lineage in New Zealand. Although not closely located in the genome, \textit{qacA} and the fusidic acid resistance gene \textit{fusC} are both associated with the predominant t127 clone. These findings suggest that the heavy use of topical antibiotics and amoxicillin consumption in New Zealand may have contributed to the marked prevalence of \textit{qacA} in New Zealand \textit{S. aureus}.

Taken together, antimicrobial use, including chlorhexidine, has the theoretical potential to select out multi-resistant \textit{S. aureus} leading to the predominance and success of the t127 clone and consequently, to the high rates of infection found in New Zealand. Thus, consideration should be given to the widespread use of chlorhexidine and the unintended consequence this has on health-care related infections.
Acknowledgments

This work could not have begun, or completed, without the help and support of a small army. I would especially like to thank those who have made a notable contribution to this work.

My sincerest thanks to my supervisor, Dr Deborah Williamson, who recognised that I needed to challenge myself, and was instrumental to my personal and professional development in this field. None of this would have been possible without that initial push. Your drive, determination and, most of all, wisdom in all things microbiology and genomics has been aspirational. Thank you for your words of encouragement and most importantly, your time.

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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARL</td>
<td>Antibiotic Reference Laboratory, ESR</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BHQ</td>
<td>Black Hole Quencher™</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CC</td>
<td>Clonal complex</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CHX</td>
<td>Chlorhexidine</td>
</tr>
<tr>
<td>Cip</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
</tr>
<tr>
<td>Cq</td>
<td>Quantification cycle</td>
</tr>
<tr>
<td>DHA</td>
<td>Drug H⁺ antiporter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECOFF</td>
<td>Epidemiological cut off value</td>
</tr>
<tr>
<td>Ery</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>ESR</td>
<td>Institute of Environmental Science and Research</td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
</tr>
<tr>
<td>FAM</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>Fus</td>
<td>Fusidic acid</td>
</tr>
<tr>
<td>g, mg</td>
<td>gram, milligram</td>
</tr>
<tr>
<td>HCAI</td>
<td>Healthcare associated infection</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>JCM</td>
<td>Japan Collection of Microorganisms</td>
</tr>
<tr>
<td>kb Mb</td>
<td>kilobase pair, megabase pair</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>L, mL, µL</td>
<td>litre, millilitre, microlitre</td>
</tr>
<tr>
<td>M, mM, µM</td>
<td>molar, millimolar, micromolar</td>
</tr>
<tr>
<td>MBC</td>
<td>Minimum bactericidal concentration</td>
</tr>
<tr>
<td>MBC₅₀, MBC₉₀</td>
<td>MBC result at 50th percentile, MBC result at 90th percentile</td>
</tr>
<tr>
<td>MFS</td>
<td>Major facilitator superfamily</td>
</tr>
<tr>
<td>MGE</td>
<td>Mobile genetic element</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;, MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>MIC result at 50&lt;sup&gt;th&lt;/sup&gt; percentile, MIC result at 90&lt;sup&gt;th&lt;/sup&gt; percentile</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence typing</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant &lt;i&gt;S. aureus&lt;/i&gt;</td>
</tr>
<tr>
<td>MSSA</td>
<td>Methicillin-susceptible &lt;i&gt;S. aureus&lt;/i&gt;</td>
</tr>
<tr>
<td>Mup</td>
<td>Mupirocin</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NIL</td>
<td>Nosocomial Infections Laboratory, ESR</td>
</tr>
<tr>
<td>NZDep</td>
<td>New Zealand Deprivation Index</td>
</tr>
<tr>
<td>NZRM</td>
<td>New Zealand Reference Culture Collection Medical Section, ESR</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulse-field gel electrophoresis</td>
</tr>
<tr>
<td>PSI</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>PVL</td>
<td>Panton-Valentine leukocidin</td>
</tr>
<tr>
<td>RM</td>
<td>Restriction modification</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SAB</td>
<td>&lt;i&gt;S. aureus&lt;/i&gt; bacteraemia</td>
</tr>
<tr>
<td>SCC</td>
<td>Staphylococcal cassette chromosome</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSTI</td>
<td>Skin and soft tissue infection</td>
</tr>
<tr>
<td>TMS</td>
<td>Trans-membrane segment</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume ratio</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

1.1 *Staphylococcus aureus*

*Staphylococcus aureus* is a commonly encountered human and animal pathogen which is associated with skin and soft tissue infection (SSTI) and invasive disease such as pneumonia and blood stream infections. *S. aureus* is part of the Staphylococcaceae family, a family of Gram-positive bacteria, known for their ‘grape-like’ clusters in Gram stains (1). The Latin term *aureus*, used for this species, is attributed to the golden colour of the organism. *S. aureus* are facultatively anaerobic, catalase and coagulase positive, and β-haemolytic on Columbia sheep blood agar (1).

1.1.1 Spectrum of *S. aureus* infections

*S. aureus* infections can manifest in several forms and are not limited to an organ system. *S. aureus* are known to cause SSTIs such as furuncles or carbuncles, impetigo bullosa, pyomositis, and scalded skin syndrome (2). Healthcare-associated infections (HCAI) such as surgical wound infections, prosthetic device infections, and pleuropulmonary infections like pneumonia are also strongly associated with *S. aureus* (3) and highlights the importance of implementing an effective infection control procedure. *S. aureus* bacteraemia (SAB) is often a consequence of different primary clinical foci (3). Case fatality rates for SAB have dropped since the introduction of β-lactam antibiotics for treatment from 80% to 15-50%. While this rate still seems elevated, several factors prevent this number from being lower. These factors include a changing, aging population and patients with existing co-morbidities, as well as difficulties associated with the management and treatment of complex *S. aureus* infections (3).

1.1.2 Pathogenesis and virulence

*S. aureus* colonisation is common in healthy individuals and nasal carriage is found in approximately 30% of all adults (4). However colonisation is not exclusively nasal. *S. aureus* has been found on the skin, perineum, and the pharynx (4). In addition, studies have also found carriage of *S. aureus* within the gastrointestinal tract, vagina, and axillae. Carriage rates can be variable as some adults are shown to be transient carriers, with different *S. aureus* strains isolated on different occasions, whereas persistent carriers tend to carry one strain (4). As an opportunistic pathogen, colonisation with *S. aureus* requires
only a breach to the host’s defence mechanism, making this event the primary catalyst for infection (2,5,6). Therefore, those who are persistent carriers of *S. aureus* are naturally more at risk of developing *S. aureus* infections with the same colonised strain (4).

Multiple virulence factors (summarised in Table 1) contribute to the success of *S. aureus* in establishing infection. These factors are involved in the adhesion, invading, and binding of *S. aureus* to host tissue (2,7). Furthermore, *S. aureus* has factors which evade host defences (8); and factors that are involved with dissemination of *S. aureus* to other sites resulting in septicaemia or toxin mediated disease (5).
## Table 1. Summary of *S. aureus* virulence factors

Table taken from Gordon and Lowy 2008 (7)

<table>
<thead>
<tr>
<th>Type of virulence factors</th>
<th>Selected factors</th>
<th>Associated genes</th>
<th>Associated clinical syndromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Involved in attachment</td>
<td>MSCRAMMs</td>
<td><em>clfA, clfB, fnbB, cna, sdr, bbp</em></td>
<td>Endocarditis, osteomyelitis, septic arthritis, and prosthetic-device and catheter infections</td>
</tr>
<tr>
<td>Involved in persistence</td>
<td>Biofilm accumulation, small-colony variants, and intracellular persistence.</td>
<td><em>Ica locus, hemB mutation</em></td>
<td>Relapsing infections, cystic fibrosis, and syndromes as described above for attachment</td>
</tr>
<tr>
<td>Involved in evading/destroying host defences</td>
<td>Leukocidins, capsular polysaccharides, protein A, CHIPS, Eap, and phenol-soluble modulins</td>
<td><em>lukS-PV, lukF-PV, hlg, cap5 and 8 gene clusters, spa, chp, eap, psm-α gene cluster</em></td>
<td>Invasive skin infections and necrotising pneumonia (CA-MRSA strains that cause these are often associated with capsular polysaccharides)</td>
</tr>
<tr>
<td>Involved in tissue invasion/penetration</td>
<td>Proteases, lipases, nucleases, hyaluronate lyase, phospholipase C, and metalloproteases</td>
<td><em>V8, hysA, hla, plc, sepA</em></td>
<td>Tissue destruction and metastatic infections</td>
</tr>
<tr>
<td>Involved in toxin-mediated disease and/or sepsis</td>
<td>Enterotoxins, toxic shock syndrome toxin-1, exfoliative toxins A and B, α-toxin, peptidoglycan, and lipoteichoic acid.</td>
<td><em>sea-g (no sef), tstH, eta, etb, hla</em></td>
<td>Food poisoning, toxic shock syndrome, scalded skin syndrome, bullous impetigo, and sepsis syndrome</td>
</tr>
</tbody>
</table>
1.1.3 Methicillin-resistant *S. aureus* and associated multi-resistance

Since the introduction of antibiotics, resistance in microbes can be found to all new antibiotics shortly thereafter (9). Penicillin, a β-lactam antibiotic, was introduced in the 1940s as an antibiotic which could effectively treat staphylococcal infections. However resistance emerged within a couple of decades in at least 80% of all community associated and hospital acquired staphylococcal isolates. Similarly in the 1960s, the introduction of another β-lactam antibiotic, methicillin, as an anti-staphylococcal alternative, soon gave rise to methicillin-resistant *S. aureus* (MRSA) rendering methicillin inadequate as a treatment option for all *S. aureus* infections (9). In addition, mechanisms of resistance to other classes of antibiotics including fluoroquinolones, aminoglycosides, and macrolides have been extensively described, and glycopeptide resistance remains an ominous threat (10). The most recently released β-lactam antibiotic, ceftaroline, was marketed as an anti-MRSA antibiotic in the post-vancomycin era, however emerging resistance to ceftaroline has already been reported with high minimum inhibitory concentrations (MICs) observed at levels which are classed as non-susceptible (11).

Bacteria that develop or acquire resistance mechanisms have a selective advantage in the presence of antibiotics. These resistance mechanisms include efflux pumps, target site modification, and destruction of the antibiotic itself (12). Antibiotic destruction is best exemplified in penicillin resistance whereby the β-lactamase hydrolyses the β-lactam ring found in penicillin, preventing penicillin from binding to penicillin-binding proteins (PBPs). PBPs are enzymes found on the cell membrane of *S. aureus* and aid the transpeptidation reaction which links the peptidoglycan chains together in the cell wall. β-lactamase production is encoded by the *blaZ* gene which is regulated by repressor proteins BlaI and BlaR1 (Figure 1). Upregulation of β-lactamase production is through interaction of the membrane protein BlaR1 with penicillin. This binding results in cleavage of protein BlaR1 resulting in BlaR2 interacting with BlaI. This interaction prevents BlaI from binding to the operator region which allows access for RNA polymerase to bind to the operator region and begin synthesis of the β-lactamase from *blaZ*. This increased expression of the β-lactamase results in penicillin resistant strains of *S. aureus* (9).
In the case of methicillin resistance, the *mecA* gene encodes for penicillin-binding protein 2a (PBP2a), in which the target PBP has been modified, preventing β-lactam antibiotics from binding to the PBP2a. The change from PBP to PBP2a in MRSA is enough to allow survival to high concentrations of β-lactam antibiotics. Classically, methicillin resistance in *S. aureus* were associated with hospital-acquired strains, however, in recent years the incidence of community-associated MRSA infection has been increasing (13). The rise in incidence of community-associated MRSA infections clearly indicates that multi-resistance in *S. aureus* and MRSA is no longer an exclusively nosocomial issue, and that prevention of infection also needs to target the community.

![Diagram](image-url)  
*Figure 1. β-lactamase expression and regulation in S. aureus by Lowy et al.* β-lactamase expression is upregulated when penicillin is bound to BlaR1. This leads to a series of reactions resulting in the binding of RNA polymerase to the *blaZ* gene. β-lactamase protein hydrolyses and inactivates the β-lactamase ring in Penicillin (14).

Efflux mediated resistance mechanisms are recognised mainly for resistance to fluoroquinolones, antiseptics, and disinfectants. Efflux mediated resistance can be either chromosomally- or plasmid- encoded. Efflux systems can confer resistance specific to a single compound, or to several classes of antimicrobial compounds (15). The specificity of a single efflux pump to a wide range of substrates often leads to resistance to multiple compounds making efflux pumps an advantageous and efficient acquisition for bacteria. In most cases, efflux pumps work by migrating the noxious substrates from within the cell extracellularly using proton motive force or the sodium membrane gradient to
energise the pump (15). Antibiotics like fluoroquinolones, phenicols, and topical antibiotics like fusidic acid and mupirocin have all been recognised as substrates of chromosomally-encoded efflux systems (15). Disinfectants including chlorhexidine, cetrimide, ethidium bromide, and benzyl ammonium chloride are recognised substrates of plasmid-encoded efflux systems (9,12,15).

1.1.4 New Zealand epidemiology of *S. aureus*

The incidence of *S. aureus* infections in New Zealand was well documented by Williamson et al. who looked at longitudinal trends in data collected over a 12 year period. Incidences were calculated to observe trends in different demographics including gender, ethnicity, socioeconomic status using NZDep (NZ deprivation index) score, and geographic regions. The paper found New Zealand incidence rates over the 12 years to be 127 per 100,000 population per year (16). A notable increase was found in the incidence of SSTI which climbed from 81 cases per 100,000 population to 140 cases per 100,000 population. SSTI incidence was highest in children less than 5 years of age (242 cases/100,000 population/year) and incidence of sepsis and pneumonia is higher in those aged 70 or over (62 and 24 cases/100,000 population/year respectively) (16). Incidence rates were higher for Māori and Pacific Peoples as well as those who were from areas with a higher NZDep score. This disparity in incidence rates seen between Māori and Pacific Peoples compared to other ethnicities was still present when adjusted for socioeconomic deprivation (16).

Surveillance of antibiotic resistance of circulating strains of *S. aureus* in New Zealand is regularly performed by the Antibiotic Reference Laboratory (ARL) at the Institute of Environmental Science and Research (ESR). Antibiotic susceptibility information is collected annually from multiple centres across the country and published publicly. In 2013, MRSA made up 10.2% of all clinical *S. aureus* isolates reported (17). This rate sits well below reports of over 50% prevalence in the United States of America (USA) in 2007 and 21% in a combined Europe prevalence survey in 2008 (18,19). In Australia the proportion of *S. aureus* that are MRSA is 17.9% (20). Despite New Zealand rates being lower than USA, Europe and Australia, New Zealand MRSA rates have steadily risen from 7.5% in 2003. The 2014 annual MRSA survey published by ESR, reports that the national period-prevalence has risen from 12.9 per 100,000 in 2005 to 23.8 per 100,000 resulting in an 85% increase. In the last four years this number has stabilised, however
the proportion of strains included in the annual studies has seen a dominant presence of the fusidic acid-resistant strain AK3, a community-associated strain of MRSA (Figure 2) (21).

The emergence of fusidic acid resistant isolates as the predominant circulating strain has been described in previous surveillance of both MRSA and MSSA. The trend closely resembles the increased prevalence of mupirocin-resistant strains when mupirocin was available as an over the counter topical antibiotic (22). Fusidic acid has been available as an intravenous, oral, and topical antibiotic since the 1960s in Europe and Australia, and in the late 1980s in Canada, yet rates of resistance have remained low in Europe and Canada despite usage in the clinical setting (23–25). In New Zealand, the rate of fusidic acid resistance has been steadily increasing, and is associated with prescribing habits of the topical fusidic acid ointment, which is fully subsidised by the NZ Ministry of Health. This goes hand in hand with the decreasing mupirocin resistance trend and corresponds to the mupirocin ointment being only partially subsidised and being the more expensive option to treat skin and soft tissue infections in the community. The emergence of resistance to fusidic acid in NZ strains is predominantly driven by two clones, the MSSA spa type t127 CC1 (clonal complex 1) and for MRSA, spa type t002 CC5, both of which are associated with fusC gene carriage (26).
Figure 2. New Zealand MRSA distribution by year and clonal lineage taken out of Annual survey of MRSA, 2014, ESR.
Emergence of AK3 MRSA (dark blue) was evident from 2006 onwards. In 2011 AK3 MRSA were the predominant strain of MRSA in New Zealand. AK3 MRSA are known to be fusidic acid-resistant (27).
1.2 **S. aureus on a molecular level**

*S. aureus* and their respective genes and genomes have been widely studied due to the clinical impact of the organism on health as a whole. Before molecular methods, typing of strains relied on phenotypic characteristics like antibiotic resistance profiles or phage typing. Development of new technological advances have provided epidemiologists and scientists with a novel approach to grouping *S. aureus* strains by incorporating a variety of typing methods on the molecular level by looking at genes and gene expression. Whole genome sequencing has now become an important development in microbiology research and surveillance as information generated per isolate can provide more data than what can be found through a typical typing system. To date, 80 complete and annotated genomes of *S. aureus* are available in the GenBank database to provide a context or a reference for any projects involving next generation sequencing. This information is invaluable as it allows for continuous progression in the understanding of emerging trends in *S. aureus* infections.

### 1.2.1 Molecular typing methods

Typing methods are valuable from an epidemiological perspective as they provide information beyond what can be detected phenotypically in a diagnostic laboratory. Typing is performed for public health surveillance to investigate any emerging trends in population health relating to *S. aureus*. Typing is also critical for outbreak investigations by being able to discriminate between related and unrelated strains to inform a public health response if required.

Pulse-field gel electrophoresis (PFGE), or macrorestriction pattern analysis, was the initial method used across reference laboratories to type strains on a molecular level. This method replaced bacteriophage typing at the Centre for Disease Control and Prevention in the late 90s and was found to have better discriminatory power as most *S. aureus* were typable through this method (28). Restriction enzymes (commonly SmaI – a type 2 enzyme derived from *Serratia marcescens*) digest genomic *S. aureus* DNA in an agarose plug by cutting at restriction sites creating large fragments of DNA (29). The large fragments are then separated through PFGE and visualised as banded patterns on agarose gels. Differences in banding patterns may be the result of inclusion or exclusion of repeat sequences, or additional mobile genetic elements.
Multi-locus sequence typing (MLST) uses sequence analysis to determine sequence types by looking at a group of core genes or loci within the genome. Using a central MLST database for *S. aureus* (https://pubmlst.org/SAureus/) the sequences of the PCR products of seven housekeeping genes are able to determine allelic profiles for each loci. These seven loci genes are *arcC, aroE, glpF, gmk, pta, tpi,* and *yqiL.* The *S. aureus* MLST database converts the combination of these allelic profiles into sequence types (29). As the genes targeted in this method are core variants, any mutations or changes in nucleotide sequence are minimal and sequence typing results are mostly unaffected by mobile genetic elements. This makes MLST a useful tool for tracing the evolution of specific lineages (8). BURST (Based Upon Related Sequence Types) analysis can group sequence types into clonal complexes showing further relatedness between lineages (8).

Typing of the *spa* gene is an alternative sequence-based method used for typing *S. aureus* strains. This method amplifies the polymorphic X region of the staphylococcal protein A gene. The polymorphic X region is a series of 24 bp repeats, with well conserved sequences on either side of the region (8,30). The *spa* gene is considered one of the core variant genes, and the highly variable polymorphic X region allows for *S. aureus* to be typed into different types depending on the repeat pattern of the 24bp repeats. Each variation of the 24bp repeat is given a repeat number and the combination and order of these repeats denotes the *S. aureus* *spa* type (29). The Ridom Spa Server (http://www.spaserver.ridom.de) lists different *spa* types and repeat patterns and associated MLST sequence types and clonal complexes.

Each of the previously mentioned typing methods have advantages and disadvantages. Application of the different typing systems largely depends on the nature of surveillance required. PFGE is ideal for outbreak settings as analysis takes into account the whole genome and comparisons between strains are highly discriminatory. For example, large differences in banding patterns in a small outbreak setting can immediately rule out unrelated strains (29). However, PFGE is less useful in large surveillance projects as the method lacks clonal lineage information. Thus, MLST and *spa* typing have been widely adopted as typing methods for surveillance of *S. aureus.* Typing of the *spa* gene has shown good correlation with sequence types and clonal complexes making it an ideal alternative to MLST (31). This method is considered more efficient than MLST as *spa* typing requires one PCR and sequencing reaction compared with the seven PCR and
sequencing reactions required for MLST. Misclassification is a weakness of *spa* typing as specificity may be compromised because of targeting one loci. Microarray-based analysis is another method which essentially works as a multiplex PCR which screens for different markers and genes of interest. Microarray-based analysis has also shown to give good correlation to clonal complexes (32).

The most significant change in molecular analysis of microbes is the introduction of whole genome sequencing (WGS). WGS of *S. aureus* isolates has the ability to perform all the functions of the above methods with the exception of PFGE. WGS is able to provide information on genes carried by the strains, and sequencing results for both MLST and *spa* typing. The same reaction can also define SCCmec complexes and has the ability to discriminate between clonal lineages as well as PFGE can, by comparing single nucleotide polymorphisms (SNPs) in the core genome of *S. aureus* strains (29). This higher resolution of molecular typing has been utilised by public health laboratories for surveillance and outbreak investigations. Data interpretation, technical expertise, and turnaround time in WGS is still a limiting factor in the application of this technology in diagnostic routine medical microbiology. As prices for this service continue to drop, vital data generated can provide thorough understanding of the phenotypic and genotypic behaviour of the isolate or isolates of interest. WGS data can also assess for resistance genes retrospectively if newfound antibiotics are introduced to the market, by looking for previously undiscovered genes (33). Another advantage of WGS, which the other typing methods do not provide is analysis of the accessory genome and flexibility of analysis of the whole genome.

1.2.2 *S. aureus* genome

The *S. aureus* genome is approximately 2.8 Mb in size (6,29,34). The core genome of *S. aureus* makes up approximately 75% of the whole genome and is highly conserved throughout the species. The core genome is mostly made of genes which are responsible for housekeeping and metabolic functions of the organism. The core genome also contains virulence factors specific to *S. aureus* which make this organism more pathogenic than other staphylococcal species (34).

Within the core genome are a group of genes called core variant (CV) genes. CV genes are genes with different variants in their sequence which have evolved over time through point mutations or recombinations (34,35). These variants are often used to type clonal
lineages of *S. aureus* as they form part of the core genome but are different enough to separate into different groups. *S. aureus* typing methods, like MLST for example, target these core genes to provide an allelic profile which confers a sequence type (34).

All remaining parts of the genome not belonging to the core genome, are termed the accessory genome (34). The accessory genome makes up approximately 25% of the entire genome. The accessory genome component of the genome differs from strain to strain and is often incorporated into the genome through mobile genetic elements. While the core genome is known for the basic metabolic functions of *S. aureus*, the accessory genome is largely associated with antibiotic resistance and virulence factors (34). Mobile genetic elements are a large part of what makes up an accessory genome, and because of the association of antibiotic resistance with highly pathogenic strains, a lot of research has been conducted around the genes that are carried on mobile genetic elements.

### 1.2.3 Mobile genetic elements

Mobile genetic elements (MGEs) are portions of DNA which make up part of the accessory genome. MGEs found in *S. aureus* include bacteriophages, pathogenicity islands, staphylococcal cassette chromosomes, transposons, and plasmids. It is also important to note that the transfer of MGEs to lineages are highly specific.

Horizontal gene transfers and exchange rates of genes are often higher in the same lineages than between different lineages (35,36). The restriction modification (RM) system Sau1 type 1 is responsible for protecting genetic information from incompatible transfers of genes between clonal lineages. Foreign DNA will be recognized and then digested to prevent the genes from incorporating into the genome. This system of restriction enzyme complexes is encoded by genes *hsdR*, *hsdM*, and *hsdS*. HsdS acts as the specificity subunit, and is lineage specific. HsdS modifies and digests DNA at different sites in different lineages playing a central role in recognising foreign DNA ensuring that horizontal gene transfers are lineage specific (36). Consequently, the RM system is important for the promotion and thriving of certain lineages of *S. aureus* by regulating the horizontal gene transfers of MGEs.

The following describes the different MGEs found in *S. aureus* and is summarised in Table 2.
Bacteriophages and pathogenicity islands

*S. aureus* can carry from one to four bacteriophages within their cells (35). Virulence factors like Panton-Valentine leukocidin genes and enterotoxin A can be found on bacteriophages (35). Bacteriophages within the *S. aureus* cell can be grouped into five families by analysing the homology of the integrase genes (36). Integrase genes determine integration of sequences into insertion sites. No more than one of each family is found within a single *S. aureus* cell, possibly due to competition for insertion sites or phage immunity (34). *S. aureus* pathogenicity islands (SaPIs) are essentially bacteriophages which lack genes to produce the capsid head or tails essential for gene transfer. Therefore SaPIs utilise helper phages to transfer genetic material. SaPIs are usually small sequences which can be packed up neatly into miniature phage heads (8,34,35). Small sequences also mean that SaPIs transfer at a relatively high rate compared to other MGEs.

Staphylococcal cassette chromosome

The staphylococcal cassette chromosome (SCC) element is most often associated with SCC*mec* which is known to carry the gene for methicillin resistance, *mecA*. Although considered mobile, they are relatively large for MGEs and do not transfer as readily as the others. The SCC inserts into the *orfX* gene in *S. aureus* and gets incorporated into the *S. aureus* chromosome (34,35). Several classes of SCC*mec* have been identified and with particular interest in SCC*mec* IV which is found in most community-associated strains of MRSA. SCC*mec* IV has been shown to spread more rapidly than than classes I – III. Later classes of SCC*mec* seem more mobile, potentially because of a reduction in size (36). For example, SCC*mec* IV and VI are 24.0 kb in size and class V is 27.6 kb. Other classes of SCC*mec* range from 32 kb in class VIII to 53 kb in class II. This difference in size observed between the SCC*mec* classes may support the mobility of some classes over others. Other mechanisms of resistance found on SCC are mercury resistance and fusidic acid resistance, often referred to SCC*mercury* and SCC*fus* respectively (Table 2) (37,38).
Transposons and plasmids

Transposons are short sequences of DNA which often get incorporated into chromosomes, SCC, or plasmids. Transposons often contain information to confer a single resistance mechanism (37). Penicillinase genes and macrolide resistance genes are carried on Tn552 and Tn554 respectively. However, larger transposons have been shown to code for resistance to aminoglycosides, trimethoprim, and tetracycline (36). Plasmids are known for their ability to carry multiple genes for antibiotic resistance, in addition to genes which encode resistance to detergents and antiseptics. Most of the resistance genes found on plasmids are incorporated from acquired transposons (34). Smaller plasmids replicate by rolling circle mechanism, however larger plasmids replicate via the theta mechanism (34). Mechanisms of transfer for plasmids are mostly through transduction although some plasmids carry tra genes for conjugative transfer (34,36). The rep genes which encode for replication proteins can be used to classify plasmids into plasmid groups. McCarthy et al. found that plasmid families are lineage associated and this supports the argument that selective pressures which select for genes in the plasmids are responsible for the success of certain clones (39).
### Table 2. Properties of *S. aureus* MGEs

<table>
<thead>
<tr>
<th>MGEs</th>
<th>Description</th>
<th>Size</th>
<th>Associated genes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteriophage</strong></td>
<td>• Incorporation into genome via transduction&lt;br&gt; • can be lytic, temperate or chronic</td>
<td>&lt;20 - 125 kb</td>
<td><em>luk-PV</em> (PVL toxin), <em>SEA</em> toxin</td>
<td>(40)</td>
</tr>
<tr>
<td><strong>Pathogenicity islands (SaPI)</strong></td>
<td>• Similar to phages, but do not have genes for phage construction</td>
<td>~15 - 20 kb</td>
<td><em>tst1</em> (toxic shock syndrome), super antigens</td>
<td>(35)</td>
</tr>
<tr>
<td><strong>SCC</strong></td>
<td>• Integration into open reading frame sequence (<em>orfX</em>) region&lt;br&gt; • Classification of SCCmec is a combination of <em>ccr</em> and <em>mec</em> complexes</td>
<td>21 – 67 kb</td>
<td>SCCmec (methicillin resistance), SCCme3, SCC476 (fusidic acid resistance), SCCmencury</td>
<td>(37,38,41)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td>• Plasmids transfer by conjugation or transduction.&lt;br&gt; • <em>rep</em> genes aide in replication and <em>tra</em> genes aide in conjugative transfer transfer</td>
<td>Type I - 1-5 kb, Type II - 15-40 kb</td>
<td><em>bla</em>Z (penicillinase), gentamicin, trimethoprim, disinfectants, heavy metal ion resistance.</td>
<td>(39)</td>
</tr>
</tbody>
</table>
1.3 Chlorhexidine in healthcare

Chlorhexidine was first described in 1954 and subsequently adopted as an antiseptic used in healthcare as a preventative measure to the rising incidence of nosocomial infections. The water-soluble form, chlorhexidine digluconate, is extensively utilised because of its residual activity, which produces a long lasting, broad spectrum, bactericidal effect. In addition, chlorhexidine digluconate use has had low reports of toxicity or irritation by health-workers and patients when applied to the skin (42,43). These properties make this antimicrobial useful for universal decolonisation procedures. Huang et al. have found that the universal decolonisation approach with chlorhexidine is more effective than targeted decolonisation and screening-isolation strategies for the reduction of bloodstream infections with any pathogen, including MRSA (44).

1.3.1 Spectrum of activity and mechanism of action

Chlorhexidine digluconate is a broad-spectrum agent with the most activity observed against Gram-positive bacteria (42,43). Chlorhexidine digluconate also has activity against Gram-negative bacteria as well as anaerobes, yeasts, and viruses. Chlorhexidine is mycobacteriostatic, however the mechanism for this is unknown (43). Despite being a broad-spectrum agent, chlorhexidine is unable to kill spores, as it cannot prevent germination of spores, but does however, inhibit the outgrowth of the spore (42). Chlorhexidine in ethanolic solutions can be tuberculocidal, and sporocidal when used at 98-100°C. Chlorhexidine is also known to be less effective in the presence of organic matter like serum, blood, or pus. The effectiveness of chlorhexidine can also be affected by pH with the optimum range being 5.5 – 7.0 (42,45).

The mechanism of action of chlorhexidine on bacteria depends largely on the concentration of chlorhexidine digluconate. In low concentrations (<10 µg/mL) chlorhexidine cations in a chlorhexidine digluconate solution are adsorbed onto the surface of the bacterial cell by interacting with negatively charged components of the cell surface (42,45). This adsorption weakens the cell membrane structure, leading to leakage of cytoplasmic contents (42,45). This change is considered irreversible damage and occurs rapidly. However, in high concentrations (>90 µg/mL) coagulation of the cytoplasmic contents results in rapid cell death, though without cellular leakage (42,45). Therefore, low concentrations of chlorhexidine digluconate are considered to be “bacteriostatic” and high concentrations are deemed “bactericidal”. Due to the
differences in the mechanism of action, high concentrations of chlorhexidine are widely used for antisepsis and disinfection (45).

### 1.3.2 Uses and application in healthcare

Chlorhexidine is used in many clinical settings for the prevention of infection. Repeated use of chlorhexidine has shown to be more effective at reducing skin flora than povidone-iodine due to the long-lasting effect of the agent (46). Primarily, chlorhexidine is used as an antiseptic and disinfectant, often found in surgical scrub solutions or in handwashes. Chlorhexidine in antiseptics are often used at a concentration of 4% and in combination with alcohol for hand disinfection (46). Skin disinfection with chlorhexidine of patients and health-workers have also been incorporated into surgical preparation procedures.

The use of chlorhexidine is more beneficial for the prevention of HCAI when used for maintenance and insertion of indwelling devices than when used for pre-operative bathing and surgical site preparation. Preparation of the catheter site pre-insertion has been shown to reduce intravenous catheter colonisation in neonates when compared to povidone-iodine preparation by 50% (47). Similarly, preparation of the pre-insertion site of intravenous catheters was also found to reduce the catheter-related blood stream infections (CRBSI) by a rate of 49% (95% CI 0.28 to 0.88) as determined by a meta-analysis study of 4,143 catheters (48). In response to these observed reductions in CRBSI, the Centre for Disease Control and Prevention Guidelines for the Prevention of Intravascular Catheter-Related Infections, 2011 have recommended the use of chlorhexidine at a concentration greater than 0.5% for skin preparation prior to insertion of central venous and peripheral arterial catheters (49). The guidelines also recommend an alcoholic chlorhexidine gluconate solution to be applied to the skin prior to peripheral venous catheter insertion (49).

Chlorhexidine’s ability to work rapidly and drastically reduce skin flora for prolonged periods have led to the use of chlorhexidine to prevent cross-contamination of pathogens between patients in hospital environments. Hand-washing with chlorhexidine can reduce skin flora by 86% - 92% (46). This reduction was also observed to be sustained for up to 6 hours (46). Therefore, chlorhexidine has been adopted for decolonisation strategies, particularly for MRSA in inpatients of wards with high colonisation pressure, like those in intensive care units (ICUs). Decolonisation procedures using skin antisepsis alone cannot eradicate MRSA from the patient. Thus, whole-body washing with chlorhexidine
is often combined with intranasal mupirocin. Studies which looked at the efficacy of
decolonisation for the eradication of _S. aureus_ carriage had a success rate of 69% in
haemodialysis patients at 12 weeks, and 74% at 3 months in a multi-centre study in
Toronto, Canada (50). The latter study also used doxycycline and rifampin in their
decolonisation procedure. Another study from the same paper also found that the use of
chlorhexidine and mupirocin on ICU inpatients was able to reduce the incidence of _S.
aureus_ infection by 66% over a four year period (50).

A secondary advantage of chlorhexidine use in healthcare is the reduction of
vancomycin-resistant Enterococci (VRE) acquisition in ICU patients and environmental
VRE contamination (46,51). The broad spectrum of chlorhexidine makes it an
advantageous addition in the prevention of nosocomial infections, particularly with the
increase in incidence of multi-resistant organisms in hospitals (46). Chlorhexidine is also
used in oral health and found in mouthwashes, dental gels, and toothpastes. Use of
chlorhexidine in oral health has shown to inhibit dental plaque formation due to the
bacteriostatic and residual activity properties of chlorhexidine (43).

### 1.3.3 Bacterial susceptibility to chlorhexidine

Resistance to chlorhexidine can be either innate or acquired. Innate resistance to
chlorhexidine in bacteria is primarily due to the structure of the outer layers of the cell
wall. Despite being known as a broad spectrum agent, chlorhexidine is not effective
against all organisms. As previously mentioned, chlorhexidine is known to be not as
effective against bacterial spores or mycobacteria when compared to other Gram-positive
bacteria (42). Spores are surrounded by a cortex which is a barrier that prevents the entry
of chlorhexidine into the cell. Mycobacteria possess a waxy cell wall which is highly
hydrophobic that provides a barrier to protect the cell from chlorhexidine (42).

Resistance to chlorhexidine can also come in the form of physiological or phenotypic
changes in the organism, or in groups of organisms (42). Biofilms are prime examples of
this, with organisms collectively showing increased resistance to chlorhexidine.
Populations of species within a biofilm grow at different rates due to the change in the
uptake of nutrients. Slower growing bacteria, in particular, exhibit increased resistance
to antimicrobial agents and disinfectants like chlorhexidine (52). However in most
instances, when an organism is removed from the biofilm and grown in vitro, there is no
notable decrease in susceptibility when compared to the normal levels of susceptibility.
observed in the wild-type population (52). Mechanisms which lead to increased resistance to disinfectants like chlorhexidine have been hypothesised to be due to the following reasons: (i) reduced access of the disinfectant to the organism within the biofilm possibly due to the physical access or chemical interaction of the disinfectant with the biofilm itself, (ii) the modulation of the environment surrounding the organism, (iii) the production of enzymes which degrade or neutralise the disinfectant by neighbouring bacteria, or (iv) exchange of genetic information between the microorganisms leading to enhanced tolerance to the antiseptic or disinfectant (42). Biofilms provide a protective environment in which survival is achieved despite exposure to high levels of disinfectant used for preservation or antisepsis. *Serratia marcescens*, a Gram-negative bacillus known to cause ventilator associated pneumonia, has been known to survive in 2% chlorhexidine in a thick biofilm within the walls of storage containers (53).

Acquired bacterial resistance to chlorhexidine is of greater concern than innate resistance due to the uptake of resistance mechanisms which promote survival of resistant bacteria (42). Acquired resistance mechanisms are often related to the incorporation of resistance genes found on plasmids. Mechanisms of acquired resistance to chlorhexidine can be due to inactivation, or through efflux. It is important to note that acquired resistance can often mean an increased MIC from the norm and can also be interpreted as increased tolerance to chlorhexidine. Despite an increase in MIC, an organism can still be considered susceptible to levels of chlorhexidine commonly used in clinical applications.

Plasmid mediated resistance in staphylococci is considered to be of greatest concern due to the use of chlorhexidine for decolonisation or eradication of MRSA or *S. aureus* from patients in high-risk wards. The genes which have been recognised to confer reduced susceptibility to chlorhexidine are the *qacAB* family of genes (54). Multi-drug resistance determinant *qacA* is commonly located in the pSK1 family of multi-resistant plasmids which also encodes resistance to β-lactams and heavy metals (54). Thus, selection for the *qacAB* resistance genes with chlorhexidine has implications on other resistance genes or gene networks that are found within the same plasmid or chromosome respectively. This will be discussed in detail further in this chapter. The *qacA* gene can be found in coagulase-negative staphylococci as well as *S. aureus* and confers resistance to diamidines, acridines, ethidium bromide, and quaternary ammonium compounds in addition to chlorhexidine salts (42).
1.4 The \textit{qacA} gene and QacA protein

The \textit{qacA} gene encodes QacA, an efflux mediated pump. The name derives from quarternary ammonium compounds, due to the resistance observed to this group of compounds. The significance of this protein is that it has been identified in \textit{S. aureus} and has the ability to confer resistance to several different compounds typically used in healthcare settings as antiseptics or disinfectants.

1.4.1 The \textit{qacA} gene and expression

QacA was first described as a multi-drug efflux pump by Tennent et al. in 1989 (55). The \textit{qacA} gene was found within pSK1; a plasmid which also carries other resistance mechanisms to other classes of antibiotics, namely the aminoglycosides; gentamicin, tobramycin, and kanamycin as well as resistance to trimethoprim (56). The same plasmid also conferred resistance to the disinfectants: acriflavine, ethidium bromide, and quarternary ammonium compounds such as cetrimide and diamidines (56). In 1985 Tennent et al. were able to identify this disinfectant resistant phenotype down to carriage of a specific locus within the pSK1 plasmid family (56).

The Tennent et al. study was related to the emergence of an outbreak strain of nosocomial, multi-resistant \textit{S. aureus} in Australia in the late 1970s. This outbreak strain was resistant to methicillin, streptomycin, erythromycin, clindamycin, tetracycline, gentamicin, and kanamycin. This strain also had variable resistance to rifampicin and fusidic acid. Treatment for these strains relied on vancomycin, a “last resort” option for \textit{S. aureus} infections (57). Up to 50\% of the outbreak strain isolates had plasmids from the pSK1 plasmid family and was of great concern as this strain was associated with increased resistance to the widely used antiseptic/disinfectant formulas at the time (57).

The study and characterisation of the \textit{qacA} gene and associated repressor gene \textit{qacR} is well described by Rouch et al. (58). Their paper studied a distinct 2542bp nucleotide sequence within the pSK1 plasmid which contained the gene which encoded resistance to the disinfectant compounds like ethidium or acriflavine and quarternary ammonium type antiseptics (58). Rouch et al. found two open reading frames (ORFs) within this sequence belonging to the \textit{qacA} and \textit{qacR} genes which encode for the proteins for QacA and QacR respectively (58).
The Rouch et al. study identified that the first of the two ORFs found within the nucleotide sequence belonged to \textit{qacA}. This sequence was deduced to be \textit{qacA} as the sequence length matched the molecular weight of the previously published papers describing the QacA protein (55,56). The start codon for \textit{qacA} was identified as CUG which was found in close proximity to the ORF. The CUG start codon is considered atypical and is usually an indicator that an external control step is required to regulate the expression of \textit{qacA} (58).

The second ORF in the Rouch et al. study was later identified to belong to \textit{qacR}, the gene that encodes for the repressor protein QacR (58,59). QacR represses expression of \textit{qacA} by binding to a palindromic sequence found between \textit{qacR} and \textit{qacA} termed IR1 (inverted repeat 1). QacR’s protein structure has a helix-turn-helix motif found on the N-terminal of the protein which binds to IR1 resulting in the direct interaction of QacR to the operator DNA sequence of the \textit{qacA} gene. This binding inhibits the transcription of \textit{qacA} (Figure 3) (58–60). IR1’s palindromic sequence consists of two 15bp arms which are separated by six bases. These six bases between the sequences play little or no part in the binding of the QacR protein (60).

The \textit{qacA} gene expression is promoted when the diffusible repressor protein QacR is bound to substrates that QacA and QacR have in common (61). It is thought that the ligand binding portion of the QacR contains several amino acids that these substrates could bind to, conforming in a change of shape to the QacR protein structure. This change in structure prevents QacR from binding to IR1 thus inducing the expression of \textit{qacA} (60,61). A second inverted repeat sequence IR2 was also hypothesised to be a secondary regulatory protein which indirectly regulates the expression of \textit{qacA} by regulating the expression of \textit{qacR} (61).
Figure 3. Regulation of qacA expression by Grkovic et al.
QacR forms a drug complex with the substrate freeing the start codon for the production of QacA (62).

1.4.2 QacA protein function and structure

The QacA protein is part of the major facilitator superfamily (MFS) of transport proteins (58,60,61,63–65). MFS transport proteins can exhibit uniport, symport, or antiport systems for transporting substrates across the membrane. The coverage of substrates by MFS is wide and varied; and is integral to the functioning of all living things. Drug resistance, in particular, is an important function of MFS in bacteria (66). DHA proteins are drug H⁺ antiporter proteins meaning that the drug substrate is transported out as H⁺ ions are transported into the cell (60,66). This mode of transport can also be termed proton motive force and relies on a pH gradient to assist in the efflux of the substrate (61,63,66). QacA is categorised into class DHA2 which are DHA transporters which have 14 trans-membrane segments (TMS) (61).

The QacA protein is 55kDa in size and is constructed from a 514 amino acid sequence. MFS proteins like QacA have highly conserved amino acid motifs that are important for the structure and function of the protein (60). These highly conserved regions throughout the structure aid the functionality of the transport mechanism which controls the flow of the substrate either in or out of the cell (65). Regions which are not highly conserved...
among MFS account for the substrate recognition component of the protein. Due to the wide array of substrates that QacA can transport, it is hypothesised that the binding site of QacA lacks elements that are specific to each of the individual substrates (60). The alternate theory of QacA specificity is that the transporter has a flexible binding site which can accommodate for the differences in substrate structure. Another hypothesis is that there may be specific areas of the binding site which bind to parts of the substrate which all share a similar feature – this being the cationic nature of the compounds (60).

1.4.3 QacA substrates

QacA’s multi-drug specificity is an important characteristic, with the potential to confer resistance to several antiseptics and disinfectants. Acquisition of this resistance mechanism may contribute to the sustained high incidence rates of *S. aureus* infections despite heavy disinfectant and antiseptic use.

More than 30 substrates have been found to be recognised by QacA, all of which share common structural features (60). The common denominator of these substrates is that all are cationic and lipophilic. Mitchell et al. investigated the different properties of monovalent and divalent antimicrobial cations that were considered potential substrates of QacA and QacB. Both QacA and QacB had been observed to confer an increase in MIC to chlorhexidine. However, QacA had shown a greater increase than QacB exhibiting MICs of 12 μg/mL and 6 μg/mL respectively from a control value of 1 μg/mL (67). No change in MIC was noted for alexidine or chlorguanide, an aliphatic derivative and monovalent derivative of chlorhexidine respectively. It was also observed that QacA had shown increased MICs for all the diamidine compounds tested, where QacB did not. This may be due to the second positively charged cation in the diamidine structure which is recognised by the negatively charged amino acid substitution seen in QacA that is not found in QacB. This amino acid substitution occurs in amino acid 323 found in the TMS 10 (Figure 4) (60,63–66).

The structural and sequence similarity between *qacA* and *qacB* and their encoded proteins stems from the theory that *qacA* evolved from *qacB*, as the latter was found in plasmids from circulating strains of *S. aureus* from the 1950s. The *qacA* gene differs from *qacB* by 7 nucleotides in the sequence resulting in 7 amino acid substitutions. However the resulting protein encoded by *qacA* differs phenotypically from *qacB* solely because of the amino acid substitution found on amino acid 323 (63). This small but significant
change from the neutral amino acid alanine in QacB to negatively charged aspartate in QacA provides a secondary and strong binding site at amino acid 323 for divalent aromatic cations (60,63). This substitution expands the range of substrates which can be transported by the efflux protein. Other negatively charged binding sites have been shown to be in TMS 1, amino acid 34 and the negatively charged arginine in amino acid 114 in TMS 4, although the latter occurs in a highly conserved region seen in the DHA proteins (Figure 4). Although there are three negatively charged sites in the protein, studies have shown that trivalent cations are not recognised substrates of QacA (63).

Figure 4. Trans-membrane diagram of QacA from Paulsen et al. showing α helical structure of TMS 10
Proteins in black are residue differences between QacA and QacB. The proline residues (black P) in TMS 10 may be involved in the flexibility or conformational changes in the structure to accommodate for a variety of substrates for QacA (68).
1.5 Prevalence of *qacA* gene and chlorhexidine resistance studies

Given that chlorhexidine, a widely used antiseptic in healthcare, is a substrate of *qacA*, several studies have focused on the prevalence of *qacA* carriage and phenotypic characteristics of *qacA* carrying strains. In the era of HCAI with increasingly resistant strains of *S. aureus*, chlorhexidine has become an important compound for the prevention of infection in the clinical setting.

1.5.1 Prevalence of *qacA*

Prevalence of the *qacA* gene in *S. aureus* studies have been conducted worldwide with variable carriage rate results. Difficulties in comparing the rates of *qacA* carriage is due to study design and inclusion criteria of the study population of *S. aureus*. Screening for *qacA* gene inevitably led to the screening for the *qacB* gene as well because of the similarities in the gene sequence and primer selection for polymerase chain reaction methods (PCR). Therefore a majority of studies stylised the two genes as *qacA/B* to describe their gene of interest. Some methods were able to separate the two genes by using probes which bind to a short sequence containing one of the seven nucleotide differences between *qacA* and *qacB* in a real-time PCR reaction (69). Alternatively, sequencing of *qacA/B* PCR products also enabled studies to detect whether the gene amplified was *qacA* or *qacB* (64,69–73). The findings of the study are summarised in Table 3 below.

There are more prevalence of *qacA* in MRSA studies than MSSA due to the increasing concern about HCAIs, infection control, and decolonisation of at-risk patients. A number of studies looked for mupirocin resistance genes alongside *qacA/B* as mupirocin, a topical antibiotic, and CHX are used together for decolonisation (74–77). Within the studies, MRSA *qacA/B* carriage has been reported from as low as none detected to as high as 94.7% in an Australian study (78). Prevalence was lower in MSSA isolates with the highest prevalence rate reported in Japan at 7.5% (71). Higher rates reported for MRSA are likely to be due to more studies involving MRSA, or study designs which included highly resistant MRSA strains. Selection pressures from antibiotic use or antiseptic and disinfectant use, could still be the driving force behind a higher prevalence of *qacA/B* in MRSA, given that MRSA are historically a healthcare associated issue.
Table 3. Summary of qacA prevalence studies

<table>
<thead>
<tr>
<th>Location of study</th>
<th>Single/multi centre</th>
<th>Year of isolate collection</th>
<th>Total SA</th>
<th>qacA/B prevalence (%)</th>
<th>Clinical / non-clinical</th>
<th>Hospital / community¹</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MRSA</td>
<td>MSSA</td>
<td>Combined</td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>Single</td>
<td>1993-2001</td>
<td>522</td>
<td>32.6</td>
<td>7.5</td>
<td>23.6</td>
<td>Clinical</td>
</tr>
<tr>
<td>Glasgow, Scotland²</td>
<td>Multi</td>
<td>Not specified</td>
<td>88</td>
<td>15.8</td>
<td>0</td>
<td>11.4</td>
<td>Clinical</td>
</tr>
<tr>
<td>Turkey</td>
<td>Single</td>
<td>Not specified</td>
<td>100</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>Clinical</td>
</tr>
<tr>
<td>Taiwan</td>
<td>Single</td>
<td>2008-2009</td>
<td>156</td>
<td>43.8</td>
<td>3.3</td>
<td>28</td>
<td>Clinical</td>
</tr>
<tr>
<td>Shanghai, China</td>
<td>Single</td>
<td>2005-2010</td>
<td>610</td>
<td>4.2</td>
<td>4.8</td>
<td>4.4</td>
<td>Clinical</td>
</tr>
<tr>
<td>Shanghai, China</td>
<td>Single</td>
<td>2011</td>
<td>608</td>
<td>15.7</td>
<td>3.6</td>
<td>11.8</td>
<td>Clinical</td>
</tr>
<tr>
<td>Malaysia</td>
<td>Single</td>
<td>2011</td>
<td>259</td>
<td>70.5</td>
<td>1.8</td>
<td>27</td>
<td>Clinical and carriage</td>
</tr>
<tr>
<td>Spain</td>
<td>Single</td>
<td>1997-2006</td>
<td>111</td>
<td>.³</td>
<td>2.7</td>
<td>2.7</td>
<td>Carriage</td>
</tr>
<tr>
<td>China</td>
<td>Multi</td>
<td>2008-2012</td>
<td>499</td>
<td>7.8</td>
<td>0.6</td>
<td>5.2</td>
<td>Clinical and environmental</td>
</tr>
</tbody>
</table>

¹ Abbreviations H, hospital isolates were used in the study; C, community isolates were used in the study; HC, hospital and community isolates were used in the study
² Isolates included Vancomycin-resistant strains from Japan
³ Denotes that no strains tested or unspecified for this category

Abbreviations H, hospital isolates were used in the study; C, community isolates were used in the study; HC, hospital and community isolates were used in the study

Isolates included Vancomycin-resistant strains from Japan

Denotes that no strains tested or unspecified for this category
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<th>Total SA</th>
<th>qacA/B prevalence (%)</th>
<th>Clinical / non-clinical</th>
<th>Hospital / community¹</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Zealand</td>
<td>Multi</td>
<td>2014</td>
<td>751</td>
<td>4.4 7.3 7.1</td>
<td>Clinical</td>
<td>HC</td>
<td>This study</td>
</tr>
<tr>
<td>USA</td>
<td>Single</td>
<td>2004-2009</td>
<td>281</td>
<td>18.5 - 18.5</td>
<td>Clinical paediatric</td>
<td>H</td>
<td>(85)</td>
</tr>
<tr>
<td>Predominantly USA⁴</td>
<td>Multi</td>
<td>2011</td>
<td>1962</td>
<td>0.9 - 0.9</td>
<td>Clinical</td>
<td>H</td>
<td>(86)</td>
</tr>
<tr>
<td>Korea</td>
<td>Multi</td>
<td>2006-2009</td>
<td>446</td>
<td>- - 9</td>
<td>Clinical</td>
<td>H</td>
<td>(76)</td>
</tr>
<tr>
<td>California, USA</td>
<td>Multi</td>
<td>2008-2011</td>
<td>829</td>
<td>0.6 - 0.6</td>
<td>Carriage</td>
<td>LTCF⁵</td>
<td>(74)</td>
</tr>
<tr>
<td>Malaysia</td>
<td>Single</td>
<td>2009</td>
<td>60</td>
<td>83.3 - 83.3</td>
<td>Clinical and carriage</td>
<td>H</td>
<td>(87)</td>
</tr>
<tr>
<td>USA and Germany</td>
<td>Multi</td>
<td>2009</td>
<td>689</td>
<td>- - 0.7</td>
<td>Clinical and environmental</td>
<td>H</td>
<td>(88)</td>
</tr>
<tr>
<td>Australia⁶</td>
<td>Single</td>
<td>2000-2009</td>
<td>151</td>
<td>65-94.7 - 65-94.7</td>
<td>Clinical</td>
<td>H</td>
<td>(78)</td>
</tr>
<tr>
<td>Toronto, Canada</td>
<td>Multi</td>
<td>2005-2009</td>
<td>334</td>
<td>2 - 2</td>
<td>Clinical and carriage</td>
<td>H</td>
<td>(72)</td>
</tr>
</tbody>
</table>

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⁴ Isolates included strains from Iraq, Afghanistan, Europe, and the Caribbean
⁵ Long term care facility
⁶ Prevalence was recorded as individual results for each year of the study
<table>
<thead>
<tr>
<th>Location of study</th>
<th>Single/multi centre</th>
<th>Year of isolate collection</th>
<th>Total SA</th>
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<th>Clinical / non-clinical</th>
<th>Hospital / community¹</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MRSA</td>
<td>MSSA</td>
<td>Combined</td>
<td></td>
</tr>
<tr>
<td><strong>Belgrade, Serbia</strong></td>
<td>Single</td>
<td>2007-2009</td>
<td>100</td>
<td>32</td>
<td>2</td>
<td>17</td>
<td>Clinical</td>
</tr>
<tr>
<td><strong>Edinburgh, Scotland</strong></td>
<td>Multi</td>
<td>2006</td>
<td>120</td>
<td>8</td>
<td>-</td>
<td>8</td>
<td>Clinical</td>
</tr>
<tr>
<td><strong>Brazil</strong></td>
<td>Multi</td>
<td>2002-2003</td>
<td>74</td>
<td>80</td>
<td>-</td>
<td>80</td>
<td>Unspecified</td>
</tr>
<tr>
<td><strong>Tokyo, Japan</strong></td>
<td>Single</td>
<td>2003</td>
<td>65</td>
<td>52</td>
<td>-</td>
<td>52</td>
<td>Unspecified</td>
</tr>
</tbody>
</table>

¹ Includes hospice facility
Interestingly, the prevalence studies in MRSA strains conducted in USA and Canada showed low carriage rates of \textit{qacA/B}, reporting prevalence of less than 2% (72–74). The only exception to this is a study conducted by Johnson et al. where prevalence is reported at 19% (85). However, this was the only study which used clinical isolates from paediatric patients (85). The low prevalence rates compared to the rest of the world may indicate that clonal lineage or circulating strains may play a part in the carriage and spread of \textit{qacA/B} genes and is not necessarily due to selection pressure.

Most studies were not able to provide a current \textit{status quo} of the prevalence of \textit{qacA/B} carrying \textit{S. aureus}, often using isolates collected over several years. Only one study showed the carriage rate in an unbiased sampling of all \textit{S. aureus} clinical isolates from a hospital in Shanghai, which found carriage rates to be 11.8% (81). Despite the many studies on prevalence, little is known about the prevalence of \textit{qacA/B} in the community.

1.5.2 \textbf{Associated clonal lineages with \textit{qacA/B}}

Carriage studies of \textit{qacA/B} have looked for the presence of other co-resistance genes with particular interest in mupirocin-resistant isolates of MRSA. This is due to decontamination procedures which utilises the two topical agents for the eradication and decontamination of \textit{S. aureus} colonised individuals pre-operatively (75,77). McDanel et al. were unable to find co-resistance between high level mupirocin resistance and \textit{qacA/B} carriage amongst MRSA isolates from colonised nursing home residents in California, USA (74). McGann et al. described a multi-plex PCR which detects \textit{mupA} and \textit{qacA/B} genes in MRSA, however the study did not indicate whether any co-detection of \textit{mupA} and \textit{qacA/B} was found in any of the isolates tested (86). The development of screening tests and increased monitoring of co-resistance are important to track emerging trends to these topical agents. These two studies did not include molecular typing and therefore no correlation was made between gene carriage and clonal lineages. Furthermore, these studies also had low numbers of positive isolates, thus molecular typing on these isolates would not have indicated a statistically significant correlation of \textit{qacA/B} carriage with any clonal lineages.

Other studies which included molecular typing showed that \textit{qacA/B} carriage was seen more frequently in ST239 \textit{S. aureus} which is part of clonal complex 8 (CC8) (76,80,81). In Sydney, Australia, Ho et al found that 94.5% of \textit{qacA/B} containing strains of MRSA were ST239 (78). Other published reports from China, Korea, and Taiwan also found
that a high proportion of *qacA/B* containing strains belonged to ST239 and ST5 (CC5), namely *spa* types t037 and t002 respectively (76,80,81).

ST239 MRSA in New Zealand is commonly known to medical microbiology laboratories as Akh4, and is a multi-resistant, hospital-acquired MRSA (27). These strains are typically resistant to ciprofloxacin, clindamycin, co-trimoxazole, erythromycin, gentamicin, and tetracycline. The predominance of fusidic acid-resistant strains of AK3 MRSA (ST5) in recent years have driven down the numbers of other MRSA, thus ST239 Akh4 make up a very small percentage of MRSA in New Zealand.

### 1.5.3 Chlorhexidine MIC and MBC

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) to CHX were carried out alongside most of the *qacA/B* prevalence studies to detect for phenotypic expression. Phenotypic differences are critical for determining whether CHX use can drive the selection for resistant genotypes. For the purposes of this section, I will refer to all antibiotics, agents of interest, antiseptics, or disinfectants as antimicrobial(s).

MIC refers to the concentration of the antimicrobial at which growth is no longer visible. MBC refers to the concentration at which 99.9% of the colony forming units are killed off after sustained exposure, normally by removing the organism from the antimicrobial and growing any remaining viable colonies in media absent of the antimicrobial (93).

Currently, there is a lack of standardisation around methods for testing MIC and MBC to biocides in common pathogens. A report entitled “Antibiotic Resistance Effects of Biocides” prepared by the Scientific Committee on Emerging and Newly Identified Health Risks brought attention to the need for a standardised method to assess biocide resistance trends. Although prepared for the European Commission, the problem areas identified in the report are of global significance (94). In response to this report Morrisey et al. set out to identify epidemiological cut off values (ECOFFs) for several biocides including CHX (95). ECOFFs provide an indication of the MIC or MBC to the antimicrobial of which 99.9% of wild type strains (strains lacking the gene conferring resistance to the agent) sit below. Therefore, if a given organism was found to have an MIC or MBC above the ECOFF, it should be considered to have an abnormally high resistance to the antimicrobial, and to contain gene(s) conferring resistance to the
antimicrobial, and outside of what would be considered to be the “natural” level of resistance.

Assigning ECOFFs for organisms and antimicrobials, or deciding on a value at which an organism is considered resistant or non-susceptible, requires methodologies to be standardised in order for values to be comparable. Several methodologies have been utilised for S. aureus and CHX. The popular choice for MIC methodology is described in the Clinical Laboratory Standards Institute document CLSI M26-A (96). MBC methodologies were extremely variable although the most popular choice was as outlined by CLSI M26-A. Alternative methods varied from including a time-kill study with S. aureus strains exposed to CHX at set time intervals (97), or variations from the CLSI methodology (70,72,86). Unfortunately CLSI methodology is designed for antimicrobial use in clinical applications therefore the method may be a questionable way of determining MICs and MBCs for antiseptics or disinfectants (95).

Due to the variation in methods, reporting of MIC and MBC results were difficult to summarise. Different methods yielded very different results, and as there are no interpretive standards denoting a resistant or non-susceptible concentration, reporting of the results proved problematic to collate. The findings are briefly summarised in Table 4.

MIC/MBC findings have been mixed with no clear association established between qacA/B carrying S. aureus strains and those which do not carry the gene. This could be put down to methodology or the expression of qacA/B in different milieu. Some are of the opinion that the MBC reported are several thousand fold lower than what would be used in the hospital and any increased MBCs in qacA/B is negligible (98,99).
Table 4. Summary of MIC and MBC methods and results for *S. aureus* and chlorhexidine studies

<table>
<thead>
<tr>
<th>Method</th>
<th>Summary of MIC and MBC result</th>
<th>References</th>
</tr>
</thead>
</table>
| CLSI method for MIC and MBC              | • MIC results were grouped between 2 – 4 mg/L, and rarely reported above 4 mg/L for *qacA/B* carrying isolates. MICs for control strains ranged from 1 – 2 mg/L.  
• MBC results ranged from 8 – 32 mg/L for all isolates.                                                                                       | (69,73,74,86,95,100) |
| Variation of CLSI method for MBC         | • MBCs ranged from 8 – 16 mg/L  
• One study reported concentrations as percentages with MBCs of 0.007% for *qacA/B* carrying strains and 0.002% for control strains.                                                                                   | (70,73)             |
| MBC with time-kill and neutralisation step | • “Kill-rate” shown to be between 8 – 64 mg/L at different exposure times.  
• MRSA strains had MBCs from 2500 mg/L to >5000 mg/L at different exposure times,  
MSSA strains ranged from <4.9 mg/L at longest time exposure, to >2500 mg/L at the shortest time exposure.                                                                                             | (97,101)            |
1.6 Rationale for this study

*S. aureus* disease continues to be of public health importance in New Zealand. *S. aureus* infections and related medical complications place a significant clinical and economic burden on the healthcare system. *S. aureus* are also becoming increasingly difficult to treat as antimicrobials are not being developed at the rate at which resistance emerges. Collectively, this compounds the current burden posed by *S. aureus* disease to the health sector. New Zealand’s high incidence of *S. aureus* disease and incidence disparity between ethnic groups indicates a need for greater understanding of underlying factors that contribute to high rates of disease.

The prevention of infection has relied on the use of chlorhexidine for antisepsis in healthcare. Widespread use of chlorhexidine in the clinical setting has the theoretical potential to select out multiply-resistant strains of *S. aureus* due to the association of *qacA* with other resistance genes co-located on the plasmid. The location of *qacA* on the mobile plasmid can result in widespread dissemination of these co-located genes, particularly if carriage is found in the predominant clones of *S. aureus* in New Zealand.

Prevalence studies of *qacA* and concurrent phenotypic susceptibility to chlorhexidine in New Zealand *S. aureus* have yet to be conducted. In conjunction with the 2014 *S. aureus* survey conducted by ARL, ESR, this study sets out to be the first to determine the *status quo* of chlorhexidine susceptibility in New Zealand *S. aureus*.

Accordingly, the aims of this study are to investigate the following hypotheses:

I. That the prevalence of *qacA*-containing strains of *S. aureus* would be higher in MRSA and multi-resistant healthcare-associated clonal lineages of *S. aureus*.

II. That *qacA*-containing strains of *S. aureus* have reduced susceptibility to the antiseptic chlorhexidine compared with non-*qacA*-containing strains of *S. aureus*, and that exposure to chlorhexidine can select for more resistant phenotypes of *S. aureus*.

III. That *qacA* gene in New Zealand strains of *S. aureus* are co-located on a plasmid with multiple antimicrobial resistance genes.
Chapter 2. New Zealand *S. aureus* isolates used in study

2.1 ESR *S. aureus* surveillance isolates and data

The survey descriptions, methods, and data described in this chapter were carried out and published by ARL and the Nosocomial Infections Laboratory (NIL), ESR. The isolates and data collected from these surveys were used for this study. The work done for this study was designed to enhance and support the published surveillance data by using phenotypic and molecular epidemiological analysis including the use of whole genome sequencing to investigate chlorhexidine resistance in New Zealand *S. aureus* isolates. Chapter three describes the work that was carried out for this study.

2.2 New Zealand *S. aureus* 2014 survey

The 2014 New Zealand *S. aureus* surveillance report titled “Demographics, antimicrobial susceptibility and molecular epidemiology of *Staphylococcus aureus* in New Zealand, 2014” was prepared by ARL and NIL, ESR (102). The survey was conducted as a point-prevalence survey in 2014. The report was prepared for the New Zealand Ministry of Health and is publicly accessible online. This report follows the preceding New Zealand *S. aureus* survey which was conducted in 1999 (22). In addition to the data generated from the 1999 survey, the 2014 report also included molecular characterisation of clonal lineages, as well as molecular screening for Panton-Valentine leukocidin producing strains and epidemiological information to determine demographics of New Zealand *S. aureus* isolates.

2.2.1 Sampling of *S. aureus* survey isolates

Submitting medical diagnostic laboratories were requested to refer all clinical isolates of *S. aureus* to ESR over a 3 day period, from 18-20 March 2014 inclusive. *S. aureus* which were obtained for patient or health-worker screening purposes were not included in this survey. For each isolate which was referred, submitting laboratories were also asked to provide epidemiological information about the patient. These included national health index numbers (NHI), date of birth, hospitalisation status and whether the patient resided in long term care facility. Information about the *S. aureus* isolate was also requested, namely the source of infection and penicillin susceptibility of the isolate.
Labtests, which services the community of the wider Auckland region were asked to give a representative sub-sample (approximately 40) of all their clinical *S. aureus* as the total numbers would lead to over-representation of that region. Duplicate isolates from the same patient were not accepted and for laboratories which submitted 30 or more isolates, stratified sub-sampling was performed by age group and by volume of isolates by referring laboratories. Nelson Hospital Laboratory was exempt from the above exclusion criteria as isolates were receipted post sub-sampling. A total of 751 isolates were sub-sampled from 1185 of *S. aureus* isolates referred for the survey from 29 submitting medical diagnostic laboratories.

Epidemiological information was gathered using the NHI number and was used to determine the District Health Board, ethnicity, recent hospitalisation history, and deprivation index score (NZDep2006) of the patients. Access to this information was approved by the Northern Regional Ethics Committee (Ethics ref: 14/NTA/109). This data was used to determine demographic information about *S. aureus* disease in New Zealand. This epidemiological information was used in our study to determine association of qacA carriage with hospitalisation.

### 2.2.2 Phenotypic susceptibility testing of *S. aureus*

Phenotypic susceptibility testing of isolates was performed by the ARL, ESR. Antimicrobials selected for testing included cefoxitin, ceftaroline, cephalothin, ciprofloxacin, clindamycin, co-trimoxazole, doxycycline, erythromycin, fusidic acid, gentamicin, mupirocin, rifampicin, and vancomycin. Agar dilution methods, using doubling dilutions, were performed according to Clinical and Laboratory Standards Institute (CLSI) standards to determine the minimum inhibitory concentrations (MICs) (103). MICs were used for interpretation according the CLSI guidelines (104). Fusidic acid and mupirocin were not included in CLSI guidelines and interpretation of these two antibiotics used European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (105).

Inducible clindamycin resistance was tested using CLSI disc diffusion methods and only in the instance when erythromycin tested resistant and clindamycin tested susceptible through agar dilution methods (104). Inducible clindamycin resistance is observed by the flattening of the clindamycin zone between the clindamycin disc and erythromycin disc.
Phenotypic susceptibility data obtained for this survey was used to determine any correlation between qacA carriage and antimicrobial resistance profiles.

### 2.2.3 Molecular analyses used in survey relevant to this project

Clonal lineages were determined using spa typing as outlined in the *S. aureus* survey. The polymorphic X region of the spa gene was amplified using PCR and sequenced by the Sequencing Laboratory at ESR with the ABI 3130XL Sequencer. Sequences were assigned repeat patterns which dictate the spa type using Ridom StaphType software version 2.2.1 (Ridom GmbH, Würzburg, Germany). Methods for this protocol were performed as previously described by Strommenger et al. (106). The spa typing information for the isolates was used in this study for clonal association with qacA.

Pulsed-field gel electrophoresis (PFGE) was performed on all isolates which were uncharacterised via spa typing. PFGE was performed using a previously described method using SmaI-digested genomic DNA (28). Analysis of PFGE banding patterns was performed using the BioNumerics software version 6.6. Further analysis of predominant spa types used multilocus sequence typing (MLST) to determine sequence types by targeting loci *arc, aroE, glpF, gmk, pta, tpi*, and *yqiL* and according to protocols outlined in the *S. aureus* MLST website. BioNumerics software (http://www.applied-maths.com) was used to analyse sequences and sequence types were determined by using the *S. aureus* MLST website interface.

Screening for the PVL gene was performed using a duplex real-time PCR assay, targeting the *lukS-PV* gene, and the *nuc* gene (thermostable nuclease gene specific to *S. aureus*). The description of this assay and corresponding quality control measures are published in the aforementioned report (102).

### New Zealand *S. aureus* 2014 survey data

As previously mentioned in section 2.2.1 a total of 1185 isolates were submitted as part of the New Zealand *S. aureus* 2014 survey. 751 isolates were sub-sampled from 1185 and this sub-sample made up the sample for which data was generated for this study.

*S. aureus* that were defined as hospital-acquired were isolates from patients that had been in a hospital in the three months prior to the time of specimen collection. Those which had no history of hospitalisation in the previous 3 months were defined as
community-associated *S. aureus*. Hospitalisation status was recorded for 679 out of the 751 isolates with 72 isolates submitted without hospitalisation information. One hundred and seventy-eight out of 679 (26.2%) isolates were hospital-acquired *S. aureus*, and the remaining 501 (73.8%) isolates were classified as community-associated.

Sixty-eight out of 751 (9.1%) isolates were methicillin-resistant. Forty-nine out of the 68 MRSA in the survey had co-resistance to other antibiotics with the predominant antibiotic susceptibility pattern amongst MRSA were fusidic acid-resistant (39.7% n=27).

Amongst methicillin-susceptible strains, 464 out of 683 (67.9%) were susceptible to all antibiotics tested in the survey. Fusidic acid resistance also featured heavily among MSSA with 143 out of 683 (20.9%) isolates which were fusidic acid-resistant. The most common resistance pattern amongst MSSA was fusidic acid mono-resistance (12.1%), followed by resistance to fusidic acid and mupirocin (6.9%).

Eighty-nine out of 751 (11.9%) *S. aureus* isolates tested as non-susceptible (interpreted as intermediate or resistant) to erythromycin, and 34 (4.5%) isolates were non-susceptible to ciprofloxacin. Non-susceptible antibiotic resistance was also observed to doxycycline, clindamycin, rifampicin, and gentamicin. All 751 isolates were susceptible to cephalothin, co-trimoxazole, and vancomycin.

The predominant clonal lineage among MRSA was *spa* type t002, which is also known as the AK3 MRSA strain. This strain made up 57% of all MRSA in the survey. Other MRSA strains from the 2014 survey included nine (11.7%) EMRSA-15 (*spa* types t032, t852 and t7964), eight (10.2%) WSPP (*spa* types t019, t975 and t1133), four (5.8%) WR/AK1 (*spa* type t127), and four (5.8%) USA300 (*spa* type t008 and t024). The remaining strains were either the Queensland strain (*spa* type t3949) or had no designated MRSA strain (*spa* type t7776, t375 and t311).

As MSSA do not have designated strain names clonal lineage relied only on *spa* types and associated *spa* clonal complexes. The two most common strains were *spa* type t127 (14.7%) and t189 (10.5%). Each of the remaining *spa* types represented less than 5% of the *S. aureus* 2014 data set.
2.3 New Zealand 2015 Annual MRSA Survey

The Annual MRSA survey report has been prepared by the ARL and NIL, ESR since 2005 (107). Prior to this, reports on multi-resistant MRSA were prepared annually for the Ministry of Health. The move to the annual MRSA survey was to capture underlying emerging trends not captured by the reports on multi-resistant MRSA alone. The design of the survey was to gain a cross-sectional view of all MRSA isolated in New Zealand in a given time period.

2.3.1 Sampling of MRSA

Routinely microbiology diagnostic laboratories in New Zealand were asked to refer all MRSA isolated to ARL and NIL in the month of August 2015. Laboratories which were unable to submit within the specified dates were asked to submit isolates over 31 consecutive days in August 2015. MRSA submitted included both clinical samples and screening samples.

Laboratories were also asked to supply epidemiological information which included location of the patient; date of birth; and history of, and current hospitalisation status of the patient. In addition, information was provided on the site of where the MRSA was isolated, infection or colonisation status of the isolate, and whether the isolate was a screening specimen or a diagnostic specimen. Isolates and corresponding information from this survey were used for this study for qacA prevalence studies in New Zealand MRSA.

2.3.2 Phenotypic susceptibility testing

Phenotypic susceptibility testing was performed on isolates when required to confirm strain type using antibiotic resistance profiles and to supplement susceptibility information from referring laboratories. Susceptibility methods followed the protocol for disc diffusion outlined in CLSI and interpreted using the CLSI criteria (103,104). Fusidic acid- and mupirocin-resistance was interpreted using EUCAST criteria (108).

2.3.3 Molecular analyses used in survey relevant to this project

Isolates submitted for the 2015 MRSA survey were typed using methods outlined in section 2.2.3. All isolates underwent spa typing. For all necessary isolates, MLST for S. aureus and PFGE were done for strain typing if untypable using spa typing. The spa
typing information obtained from this survey was used for clonal association studies with \( qacA \).

A multi-plex PCR was used for the detection of \( mecA \), \( mecC \), \( S. \, aureus \) control \( nuc \), and PVL gene targeting \( lukS-PV \). Confirmation of MRSA was the presence of either \( mecA \) or \( mecC \), and the \( nuc \) gene. Isolates submitted as MRSA which did not have \( mecA \), \( mecC \), or \( nuc \) were not included in the survey.

### 2.3.4 2015 Annual MRSA survey data

There were 1222 isolates submitted to ARL for the 2015 Annual New Zealand MRSA Survey. These isolates represent all \( S. \, aureus \) isolated from clinical and screening specimens collected over a one month period. Only 1218 of these isolates were available to determine prevalence of \( qacA \) in the MRSA population.

Strain assignment was performed on all isolates to group into common MRSA strains found in New Zealand. One hundred and eight (8.9%) strains had \( spa \) types or susceptibility patterns which did not match a recognised MRSA strain. The most common strain was AK3 (\( n=631 \), 52%), followed by WR/AK1 (\( n=139 \), 11%), and the Queensland clone (\( n=123 \), 10%). Sixty-eight strains were associated with USA300 (6%), 66 strains with WSPP (5%) and 62 strains with E-MRSA-15 (5%). The remaining strains made up less than 2% of the strains available for screening.

The most common \( spa \) type for AK3 was t002 (\( n=535 \)), t127 (\( n=100 \)) for WR/AK1, and t3949 (\( n=86 \)) for the Queensland clone strain.
Chapter 3. Methods and materials

3.1 Bacterial control strains and primers

3.1.1 Bacterial strains
The primary data set used was the sub-sampled set of 751 S. aureus isolates from 1185 submitted for the 2014 National S. aureus survey (102). Accession numbers assigned by ARL, ESR were used throughout the study as a primary identifier.

Additionally, 1218 isolates referred to ARL for the 2015 survey were also screened for the presence of qacA (107). Similarly, accession numbers assigned by ARL, ESR for these isolates were used throughout the study as a primary identifier.

Additional control strains used for the study are described below in Table 5. Strains were acquired from the Antibiotic Reference Laboratory, ESR (ARL;ESR); New Zealand Reference Culture Collection Medical Section, ESR (NZRM;ESR); and Japan Collection of Microorganisms, RIKEN BioResource Center (JCM).

3.1.2 Primers and probes
Primers and probes for the real-time PCR component of this study were modified from the McGann et al. paper (79). Primers and probes targeted qacA and mupA as genes of interest, and femA was used as an internal control for each reaction. Primers and probes were purchased from BioSearch Technologies (Novato, California, United States). Probe modifications were chosen to ensure wavelength was as close to the 5’ modification used in the paper. The TYE665 modification for qacA used in the McGann et al. paper was changed to Quasar 705 from Quasar 670 as the emission spectra would be more distinct and had less overlap with the CAL Fluor Red modification of mupA. The primers shared melting points between 46.5°C and 57.5°C. Probes shared melting points between 58.5°C and 60.5°C.

Primers were reconstituted with UltraPure™ H₂O (Invitrogen) to make 100 µM stock primer solution, of which 10 µM concentrations of forward and reverse primer mix and 10 µM probe for each target gene were used as working concentrations.
Table 5. Control strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZRM 2205</td>
<td><em>Staphylococcus epidermidis</em> ATCC 14990 – Type strain</td>
<td>NZRM; ESR</td>
</tr>
<tr>
<td>ARS 99/577</td>
<td>S. aureus Submitted as part of 1999 national S. aureus survey with qacA, fusC, and mupA.</td>
<td>ARL; ESR</td>
</tr>
<tr>
<td>ARS 99/580</td>
<td>S. aureus Submitted as part of 1999 national S. aureus survey with qacA, fusC, and mupA.</td>
<td>ARL; ESR</td>
</tr>
<tr>
<td>ARS 99/581</td>
<td>S. aureus Submitted as part of 1999 national S. aureus survey with qacA, fusC, and mupA.</td>
<td>ARL; ESR</td>
</tr>
<tr>
<td>JCM 16554</td>
<td>S. aureus Small multi-drug resistance (<em>smr</em>) on a self-transmissible plasmid pTZ20</td>
<td>JCM</td>
</tr>
<tr>
<td>JCM 16555</td>
<td>Methicillin-resistant S. aureus pTZ2077 encoding multidrug efflux gene qacA</td>
<td>JCM</td>
</tr>
<tr>
<td>JCM 16556</td>
<td>Methicillin-resistant S. aureus pTZ2162 encoding multidrug efflux gene qacBIII</td>
<td>JCM</td>
</tr>
<tr>
<td>Target</td>
<td>Primers/Probe</td>
<td>Sequence (5'-3')</td>
</tr>
<tr>
<td>--------</td>
<td>---------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>femA</td>
<td>Primers</td>
<td>GGCATTGACCGTTATAATTTC/CCAACATATTCAATAATTTCAGCA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>AACTACACCAGCATCTTCAGCAGCATC</td>
</tr>
<tr>
<td>mupA</td>
<td>Primers</td>
<td>GCGACGGTTTAGTTAATGCA/TGAACAATACCAGTTTCCTCTGA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>ATTACGTTTGTTGAGGAATTTGT</td>
</tr>
<tr>
<td>qacA/B</td>
<td>Primers</td>
<td>GTTGCACTGCTCTAATAATG/GGCTACCAAGTACTGCTA</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TCATACATAGACTCTTCAACACGCAGC</td>
</tr>
</tbody>
</table>
3.2 Media and Reagents

3.2.1 Agar plates
5% Columbia sheep blood agar and Mueller Hinton CLSI agar plates were purchased from Fort Richard (Auckland, New Zealand).

3.2.2 Glycerol broth for storage of isolates
15% Glycerol broths were prepared by dissolving 3 g of Bacto™ tryptic soy broth dehydrated culture media dissolved in 100 mL of distilled water over gentle heat. 15 mL of Prolabo™ bidistilled 99.5% glycerol was added to the tryptic soy broth mixture. The pH was adjusted to a consistent 7.0 using 1M NaOH or 1M HCl. 0.8 mL of the broth was dispensed into Nunc™ Cryotubes. Sterilisation was achieved by autoclaving at 10 PSI for 10 minutes.

3.2.3 Tryptone Soya Agar for transport of isolates
40 g of dehydrated Oxoid™ tryptone soya agar was added to 1 L of distilled water. Ingredients were dissolved on a hotplate and cooled to 50-55°C. The pH was adjusted to 7.1 - 7.5 using 1M NaOH or 1M HCl. Aliquots of 3 mL were dispensed into glass bijoux then autoclaved at 15 PSI for 15 minutes. Once sterilised, the bijoux were laid out to create a slope and left to set at room temperature.

3.2.4 UltraPure™ DNase/RNase-Free Distilled Water
UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen) was used for all molecular work.

3.2.5 Lysostaphin
1 mg/mL working dilution of lysostaphin was prepared by adding 10 mg of lysostaphin (Sigma-Aldrich™) to 10 mL of 10 mM Tris-HCl solution.

3.2.6 MgCl₂ used for PCR
MgCl₂ was prepared for molecular procedures. 50 mM working dilution of MgCl₂ was prepared using 225 mL 1M MgCl₂ (Sigma™) in 4275 mL of distilled water.
3.2.7 Master mixes used for PCR
AmpliTaq Gold® 360 Master Mix 2x (Applied Biosystems, Life Technologies) was used for method validation of PCR and real-time PCR set up. TaqMan® Universal Master Mix II, with UNG 2x (Applied Biosystems, Life Technologies) was used for real-time PCR.

3.2.8 Magnesium and Calcium stock solutions
Magnesium and calcium stock solutions were prepared for cation adjusted Mueller-Hinton broths. Calcium stock solution was prepared by adding 3.68 g of CaCl$_2$.2H$_2$O to 100 mL of distilled water to make up 10 mg/mL of Ca$^{2+}$. Magnesium stock solution was prepared by adding 8.36 MgCl$_2$.6H$_2$O to 100 mL of distilled water to make up 10 mg/mL of Mg$^{2+}$.

3.2.9 Cation Adjusted Mueller Hinton Broth (CAMHB)
21 g of Difco™ Mueller Hinton dehydrated media was added to 1 L of distilled water. The media was mixed thoroughly then boiled whilst stirring. Once cool, pH was adjusted to 7.2 using 1M NaOH or 1M HCl then the solution was autoclaved to reach a desired end product pH of 7.3 ± 0.1.

Cation concentration of the broth was obtained by accessing the certificate of analysis at http://regdocs.bd.com/regdocs/searchCOA.do using the product and lot number of the dehydrated media. Cation concentration was adjusted to 20 mg/L for Ca$^{2+}$ and 10 mg/L for Mg$^{2+}$ according to CLSI standards by adding calcium stock solution and magnesium stock solution to the broth. CAMHB was then autoclaved at 15 PSI for 15 minutes for sterilisation. The CAMHB was dispensed as required.

3.2.10 Chlorhexidine digluconate
20% (w/v) Chlorhexidine digluconate (Sigma-Aldrich) was used to prepare a working dilution. To determine the final concentration of the product, a certificate of analysis was obtained from http://www.sigmaaldrich.com/catalog/AdvancedSearchPage.do using the product and lot number. The working dilution of chlorhexidine digluconate was prepared by converting the final percentage into mg/L units. The final concentration of the chlorhexidine product used in this project was 20.2%, thus 1.27 mL was added to 98.73 mL of distilled water to make 100mL of 2560 mg/L of chlorhexidine digluconate. Aliquots of 2.5 mL of the working dilution were frozen at -20°C until ready to be used for MIC and MBC assay.
3.3 General methods

3.3.1 Growth conditions
*S. aureus* isolates, and other isolates used as controls were streaked onto Columbia sheep blood agar plate and incubated at 35°C in 5% CO\(_2\) for 18-24 hours.

3.3.2 Storage of isolates
All strains used in this study were suspended in glycerol broths using a sterile cotton swab and kept in a -80°C ultra-low freezer. When accessing isolates from storage, aseptic technique was used and broths were kept on ice to prevent defrosting to maintain the integrity of the frozen isolate. Cotton swabs were used to scrape the top layer of the frozen suspension and inoculate the Columbia sheep blood agar.

3.3.3 DNA extract storage
DNA extracts (templates) were kept in a -20°C freezer until ready for use. Extracts were defrosted at 4°C before addition to the PCR reaction.

3.3.4 Transport of isolates
*S. aureus* isolates were inoculated onto Tryptose Soya Agar then incubated overnight at 37°C. All bijoux were parafilmed and kept at ambient temperature for the duration of the transport.
3.4 Molecular Methods

3.4.1 DNA extraction using boil method
A rice grain sized of overnight *S. aureus* culture was suspended in 250 µL of Ultra-pure™ water in an Eppendorf Safe-lock 1.5 mL tube using a sterile 10 µL loop. Tubes were placed in a 100°C heating block for 30 minutes then removed from the heating block and left to cool at room temperature. Tubes were spun at 13,000 rpm for 3 minutes then stored ready for use.

3.4.2 DNA extraction using lysostaphin
A rice grain sized of overnight *S. aureus* culture was suspended in 250 µL of Ultra-pure™ water in an Eppendorf Safe-lock 1.5 mL tube using a sterile 10 µL loop. 3 µL of 1 mg/mL lysostaphin was added to the suspension and mixed by pipetting up and down then placed in a 37°C water bath for 30 minutes. Tubes were transferred to a 100°C heating block for 30 minutes then removed from the heating block and left to cool at room temperature. Tubes were spun at 13,000 rpm for 3 minutes then stored until ready for use.

3.4.3 PCR for primer validation
PCR was performed to validate primer performance for *qacA*, *mupA*, and *femA*. The primer concentration and PCR protocol for each reaction was adapted from McGann et al. (86). Each reaction was performed as a single 25 µL reaction. 1.5 µL of DNA extract was added per reaction.

Each reaction mixture used the AmpliTaq Gold® 360 Master Mix, 1.25 µL of MgCl₂ solution, primer mix as specified in Table 7, with the remaining volume made up with UltraPure™ DNase / RNase-Free Distilled (Invitrogen) water.

PCR was performed on the ProFlex™ 3 x 32-well PCR system. Protocol was set at 95°C for 2 minutes as an activation step, followed by 40 cycles of 95°C for 10 seconds and 56°C for 45 seconds, with a final extension of 56°C for 2 minutes then hold at 10°C until removed from the PCR system.
Table 7. Primer mix volume required and final concentration for primer validation PCR

<table>
<thead>
<tr>
<th>PCR reaction</th>
<th>Primer mix volume required (μL)</th>
<th>Final primer concentration in 25 μL reaction mixture (μM)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qacA</td>
<td>2.0</td>
<td>0.40</td>
<td>118</td>
</tr>
<tr>
<td>mupA</td>
<td>0.75</td>
<td>0.15</td>
<td>76</td>
</tr>
<tr>
<td>femA</td>
<td>1.0</td>
<td>0.20</td>
<td>116</td>
</tr>
</tbody>
</table>

3.4.4 Gel electrophoresis and documentation system

PCR products were visualised by presence or absence of the gene of interest using gel electrophoresis. Gel electrophoresis was performed on the E-Gel® Electrophoresis System, using the E-gel EX 2% single comb gels. Invitrogen 100 bp DNA Ladder (Life Technologies) was loaded into lane M. The gel was run on the iBase™ Power System for 15 minutes. Gel Doc XR Imager was used for documenting gel visualisation.

3.4.5 Reaction mixture preparation for real-time PCR to screen for qacA and mupA

Each reaction was prepared as a 20 μL, duplex reaction. The femA gene target was used as an internal control to validate each reaction. 2 μL of DNA extract was required for each reaction. The reaction mixture was prepared in bulk for the number of reactions required and prepared on ice or in a cold block.

For qacA, the primer concentration was optimised to have a femA and qacA primer concentration of 0.3 μM and a femA and qacA probe concentration of 0.125 μM. Universal Master Mix II with UNG was used for each reaction with the addition of 1 μL of MgCl₂. The remaining volume was made up with UltraPure™ DNase / RNase-Free Distilled (Invitrogen) water.

Similarly for mupA, the primer concentration for mupA and femA was 0.3 μM and mupA and femA had a probe concentration of 0.125 μM and was performed in a duplex reaction using the same master mix, MgCl₂ and water amounts as for the qacA real-time PCR.
3.4.6 Real-time PCR for \textit{qacA} and \textit{mupA}

Real-time PCR reactions were all performed in Bio-Rad Hard-Shell® PCR 96 well plates. Each run had a maximum of 93 test isolates from the survey, plus control strains NZRM 2205 \textit{S. epidermidis} as the negative control, ARS99/580 \textit{S. aureus} as positive control, and DNase / RNase free water as the negative template control.

DNA extracts were defrosted at 4°C and were spun down at 13,000 rpm for 1 minute. 2 µL of DNA extract was required for each reaction.

Real-time PCR was performed on the Bio-Rad CFX 96 Touch™. Fluorophores selected for \textit{qacA} screening were FAM and quasar 705 for \textit{femA} and \textit{qacA} reaction respectively. Fluorophores selected for \textit{mupA} screening were FAM and Cal Red 610 for \textit{femA} and \textit{mupA} respectively. The protocol used was 50°C for 2 minutes, 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 55°C for 45 seconds. Plate reading was programmed after each cycle.

A positive reaction for \textit{qacA} was indicated when the $C_q$ value was less than 38 for \textit{qacA} and \textit{femA}. A negative result for \textit{qacA} had a $C_q$ value of greater than 38 for \textit{qacA} and less than 38 for \textit{femA}. For a valid run, negative controls had values of greater than 38 for both \textit{qacA} and \textit{femA}, and positive controls had values of less than 38 for both \textit{femA} and \textit{qacA}. Any sample which had a \textit{femA} value of greater than 38 may indicate a degradation of the fluorophores or insufficient DNA within the extract, and was repeated using the DNA template from the lysostaphin extraction.

Similarly the \textit{mupA} real-time PCR used the same cut-off values for $C_q$ as that of the \textit{qacA} real-time PCR. A positive reaction for \textit{mupA} was indicated when the $C_q$ value was less than 38 for \textit{mupA} and \textit{femA}. A negative result for \textit{mupA} had a $C_q$ value of greater than 38 for \textit{mupA} and less than 38 for \textit{femA}. For a valid run, negative controls had $C_q$ values of greater than 38 for both \textit{mupA} and \textit{femA}, and positive controls had $C_q$ values of less than 38 for both \textit{mupA} and \textit{femA}.
3.5 Phenotypic susceptibility to Chlorhexidine

The method that was used for phenotypic susceptibility testing is as described in the CLSI M26-A document ‘Methods for Determining Bactericidal Activity of Antibacterial Agents; Approved Guidelines’ (96). The CLSI M26-A document outlines procedures for determining minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) in a broth microdilution method. This method is not restricted to any specific antimicrobial agent, however the primary use of this document in clinical microbiology laboratories is for antibacterial agents that are used for the treatment of infections at concentrations that are safe for use in humans.

3.5.1 Selection of organisms for phenotypic susceptibility testing

Isolates which screened positive for qacA were all tested for phenotypic susceptibility to chlorhexidine. For the control group, strains were matched on spa type. For spa types which had no qacA negative equivalent, the next qacA negative isolate was selected.

3.5.2 Preparation of suspensions

CLSI M26-A recommends that the number of organisms within each well in a 96 well plate using the broth microdilution method to be $5 \times 10^4$ colony forming units (cfu) (96). In order to achieve this, the following suspension preparation method was used.

Using a sterile loop, 3 or 4 colonies of an overnight culture of S. aureus were touched then inoculated into 2 mL of CAMHB. This inoculum was incubated at 37°C for 2 hours in order for organisms to reach logarithmic phase.

This inoculum was adjusted to 0.5 McFarland using the Siemens Turbidity Meter by addition of CAMHB if required. 150 μL of the 0.5 McFarland suspension was added to 15 mL of CAMHB. This suspension was considered the final working inoculum used to inoculate the 96 well plate.

3.5.3 Preparation of 96 well plate with chlorhexidine digluconate for broth microdilution

The broth microdilution method using a 96 well plate was set up to test four specimens in duplicate, with concentrations in doubling dilutions as recommended by the CLSI M26-A document (96).
Working dilutions of chlorhexidine digluconate were prepared and ranged from 0.25 mg/L to 256 mg/L. The final concentrations in the 96 well plate after the addition of the inoculum ranged from 0.12 mg/L to 128 mg/L. Dilutions were prepared in bulk with 50 μL of each dilution aliquoted into wells in each column as outlined in Table 8. Wells in column 12 were used as a growth control with 50 μL of CAMHB aliquoted into these wells.

This dilution method was prepared as suggested by CLSI for broth microdilution. The primary source for this dilution method uses 2560 mg/L chlorhexidine digluconate as described in section 2.4.10, with each following dilution using dilutions that were prepared in a previous step as specified in the table below. CAMHB were used as diluents for each step.

<table>
<thead>
<tr>
<th>Step</th>
<th>Source</th>
<th>Concentration of source (mg/L)</th>
<th>Volume of source (mL)</th>
<th>Volume of diluent (mL)</th>
<th>Dilution concentration (mg/L)</th>
<th>Final well concentration (mg/L)</th>
<th>Well column</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Working dilution</td>
<td>2560</td>
<td>1</td>
<td>9</td>
<td>256</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Step 1</td>
<td>256</td>
<td>2</td>
<td>2</td>
<td>128</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Step 1</td>
<td>256</td>
<td>1</td>
<td>3</td>
<td>64</td>
<td>32</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Step 1</td>
<td>256</td>
<td>1</td>
<td>7</td>
<td>32</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>Step 4</td>
<td>32</td>
<td>2</td>
<td>2</td>
<td>16</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>Step 4</td>
<td>32</td>
<td>1</td>
<td>3</td>
<td>8</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>Step 4</td>
<td>32</td>
<td>1</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>Step 7</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>Step 7</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0.5</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>Step 7</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>0.5</td>
<td>0.25</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>Step 10</td>
<td>0.5</td>
<td>2</td>
<td>2</td>
<td>0.25</td>
<td>0.12</td>
<td>11</td>
</tr>
</tbody>
</table>

### 3.5.4 Inoculation of the plate

Each isolate was tested in duplicate, therefore the working inoculum, as prepared in section 3.5.2, was added to two rows on a 96 well plate. 50 μL aliquots of the working
inoculum was added to the wells starting from the control, then working from the lowest concentration to the highest concentration. The 96 well plate was incubated at 37°C for 18 – 20 hours.

3.5.5 Determining MIC endpoints

The MIC is the concentration of the antimicrobial agent where no visible growth can be seen. Using a mirrored plate reader, the MIC for the isolate is determined to be the concentration of the well where no growth can be seen with the naked eye.

Due to the nature of this assay, MIC results can vary within 1 to 2 dilution steps for any antibiotic organism combination. Isolates were done in duplicate to address this issue to ensure that the results for MICs were valid. However, because the concentrations were in doubling dilutions, the concentration values were not calculated as an average of the two results. Instead the first result was taken, with the second value to validate the result by being within one dilution of the first.

MIC values that gave anomalous results, did not have consistent growth patterns within the 96 well plate, or were not within one dilution of each other were repeated.

3.5.6 Setting up MBC from the 96 well plate

The MBC is the concentration where 99.9% of colony forming units (cfu) are killed. MBC is performed following MIC determination.

Using the MIC broth microdilution plates, 10 µL of broth was mixed then aliquoted from wells with no visible growth and plated and spread onto Mueller Hinton plates for colony counts. Each well was done in duplicate. A rejection value of 20 cfu between the duplicate samples (the sum of the duplicates has less than 20 cfu) was used to determine that the antibiotic level was lethal in the well that the colony count was taken from. This rejection value is lower than our intended inoculum within the wells and MBC results would potentially be slightly elevated and more sensitive to account for any variability in the inoculation process.

Each MBC for the isolates was performed in duplicate alongside the MIC. Any which showed anomalous results or paradoxical effect had the MIC and MBC repeated. The first MBC value was taken due to the variable nature of this assay with the second used
as validation. The duplicate MBC values had to be within one doubling dilution factor from each other.
3.6 Statistics for analysis of results

3.6.1 Analysis for the prevalence of qacA
SPSS software was used for analysis for the prevalence of qacA. Categorical variables, namely antibiotic resistance profiles, clonal lineages and hospitalisation status used Chi-square ($\chi^2$) analysis to determine associations of these profiles with qacA carriage. A $p$ value of less than 0.05 was considered statistically significant.

3.6.2 Analysis of phenotypic resistance to chlorhexidine
SPSS software was used to determine the difference in phenotypic chlorhexidine resistance between qacA carrying isolates and the control group. The Mann-Whitney $U$-test was used for susceptibility data analysis as MIC and MBC values are non-parametric.
3.8 Whole Genome Sequencing (WGS)

3.8.1 Isolates selected for WGS

Five representative isolates were selected for whole genome sequencing in order to determine the location of the \( qacA \) gene and proximity to other associated resistance genes. Isolates were selected from throughout the dataset that proportionally represented the different phenotypes and \( spa \) types of \( qacA \) isolates within the survey. ARS14215 inclusion was based on mupirocin susceptibility and ARS1456 was chosen on the basis that it had a different \( spa \) type than the others but had the most common susceptibility pattern of being mupirocin- and fusidic acid-resistant.

Table 9. \( qacA \) carrying isolates selected for whole genome sequencing

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence accession no.</th>
<th>( spa ) type</th>
<th>Mupirocin MIC (Interpretation)</th>
<th>Fusidic acid MIC (Interpretation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARS1428</td>
<td>2015-09176</td>
<td>t127</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>ARS1444</td>
<td>2015-09177</td>
<td>t127</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>ARS1456</td>
<td>2015-09178</td>
<td>t128</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>ARS1498</td>
<td>2015-09179</td>
<td>t127</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>ARS14215</td>
<td>2015-09180</td>
<td>t127</td>
<td>S</td>
<td>R</td>
</tr>
</tbody>
</table>

3.8.2 Sequencing

Genomic DNA was extracted from isolates using the DNeasy Blood and Tissue Extraction Kit (Qiagen) according to the manufacturer’s instructions. The Nextera XT DNA Library Preparation Kit (Illumina) was used to prepare the genome for sequencing on the Illumina Next-seq with 2- by 150 bp paired end chemistry.

3.8.3 Comparative analysis of \( S. \) aureus isolates

Isolate similarity was assessed using the Nullarbor pipeline (https://github.com/tseemann/nullarbor). Raw paired end sequencing data for each isolate was used for the input into Nullarbor. The \( S. \) aureus MLST scheme was used for MLST analysis, MSSA 476 (Table 10) was used as a reference genome. The pipeline also included an identification step as well as providing a report on the quality of
sequences. Maximum likelihood tree and single nucleotide polymorphism distance matrix was taken from the report generated from this pipeline.

### 3.8.4 De novo assembly and annotation of the genome

Raw sequence data was quality trimmed using Trimmomatic (version 0.35) (http://www.usadellab.org/cms/?page=trimmomatic). Parameters were set to exclude bases with phred scores of less than 20 and to exclude reads that were less than 100 bp in length.

Raw sequence data was *de novo* assembled using SPAdes Genome Assembler v3.5.0 (http://bioinf.spbau.ru/spades). Quality of assemblies was assessed using QUAST (http://quast.bioinf.spbau.ru/). Assembled scaffolds were imported into, and annotated using the Geneious v8.0.5 software package (http://www.geneious.com/). Import sequences were annotated using the annotation function in Geneious. Annotations were primarily from reference *S. aureus* genomes downloaded from NCBI (http://ncbi.nlm.nih.gov) through Geneious. Reference *S. aureus* genomes were selected based on sequence types and susceptibility profiles. Additional genes of interest were also downloaded from NCBI through Geneious for annotations that were not found within the reference *S. aureus* genomes, these genes being *mupA*, *qacA* and *qacB* with the corresponding regulator gene *qacR*. Genomes and genes used for annotation are summarised in Table 10.

### 3.8.5 Determining genetic context of *qacA*

A BLAST (Basic local alignment search tool) search function was used to search for *qacA*-containing contigs using Geneious software (Biomatters). Co-location of the *rep* with *qacA* on the same contig was used to determine plasmidic location of *qacA*.

Contigs were aligned to genes in common to each contig. These genes included *rep*, *blaZ*, *mupA (IleS)*, *qacA*, *qacR*, and *qacB*. Consensus sequences from each of the alignments were consequently aligned with each other to assemble the consensus sequence into a closed plasmid.
<table>
<thead>
<tr>
<th>Reference genome / gene</th>
<th>Sequence type</th>
<th>Description / Resistance profile</th>
<th>Size (bp)</th>
<th>NCBI code</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> strain MSSA476, complete genome</td>
<td>ST1</td>
<td>Complete genome of common global methicillin-susceptible strain</td>
<td>2,799,802</td>
<td>NC_002953</td>
</tr>
<tr>
<td><em>S. aureus</em> plasmid pSAS, complete genome</td>
<td></td>
<td>Plasmid belonging to <em>S. aureus</em> strain MSSA 476 genome</td>
<td>20,652</td>
<td>NC_005951</td>
</tr>
<tr>
<td><em>S. aureus</em> subsp. aureus Mu50 DNA, complete genome</td>
<td>ST5</td>
<td>Complete genome of vancomycin- and methicillin- resistant strain</td>
<td>2,878,529</td>
<td>BA000017</td>
</tr>
<tr>
<td><em>S. aureus</em> subsp. aureus Mu50 plasmid VRSAp DNA, complete sequence</td>
<td></td>
<td>Plasmid belonging to <em>S. aureus</em> subsp. aureus Mu50 genome</td>
<td>25,107</td>
<td>AP003367</td>
</tr>
<tr>
<td><em>qacA</em> gene</td>
<td></td>
<td>Antiseptic resistance gene coding for efflux protein QacA</td>
<td>1545</td>
<td>NC_007931</td>
</tr>
<tr>
<td><em>qacR / qacB</em> gene</td>
<td></td>
<td>Antiseptic resistance and regulator gene for efflux protein QacB and QacR repressor protein</td>
<td>2421</td>
<td>NG_035045</td>
</tr>
<tr>
<td><em>mupA</em> gene (ileS2 gene)</td>
<td></td>
<td>Isoleucine tRNA ligase 2 encoding protein which encodes resistance to mupirocin</td>
<td>3553</td>
<td>HQ625435</td>
</tr>
</tbody>
</table>
Chapter 4. Results: Prevalence, clonality and co-resistance patterns of \textit{qacA}-containing \textit{S. aureus} in NZ

4.1 Introduction
As previously mentioned \textit{qacA} prevalence in \textit{S. aureus} has been widely studied due to the association of this gene to chlorhexidine resistance. Several of these studies targeted specific \textit{S. aureus} subgroups or populations and consequently, there are very few studies that looks at prevalence on a national level. The prevalence of \textit{qacA} in New Zealand \textit{S. aureus} isolates has not been previously studied. We set out to determine the prevalence of \textit{qacA} in New Zealand in addition to the 2014 \textit{S. aureus} survey conducted by ESR (102). Additional analysis for this section looks at correlations between clonal lineages, isolate acquisition status, and various antibiotic resistance patterns. Several studies have simultaneously screened for the presence of \textit{qacA} and \textit{mupA} in \textit{S. aureus} due to the use of chlorhexidine and mupirocin for decolonisation procedures. Thus, isolates from the 2014 \textit{S. aureus} survey were also screened for \textit{mupA}.

As numerous \textit{qacA} prevalence studies target MRSA isolates due to the association of both these elements with hospitalisation and decolonisation, we also screened the 2015 MRSA survey for \textit{qacA} to determine prevalence for New Zealand MRSA (107).

4.2 Results from \textit{qacA} studies in 2014 \textit{S. aureus} survey

4.2.1 Prevalence of \textit{qacA} in 2014 \textit{S. aureus} survey
Fifty-three out of 751 (7\%) isolates carried the \textit{qacA} gene. A summary of the prevalence and various associations is presented in Table 14.

4.2.2 Association of \textit{qacA} with MRSA / MSSA
Three out of 67 (4.4\%) MRSA strains had \textit{qacA}. Fifty out of 684 (7.3\%) MSSA strains had \textit{qacA} (Table 13). This result does not support the hypothesis that prevalence of the \textit{qacA} gene is higher among methicillin resistant strains of \textit{S. aureus}. This result may not be an accurate representation of the true prevalence of \textit{qacA} in New Zealand MRSA due to the small number of MRSA included in this survey.
4.2.3 Association of qacA with other antimicrobial resistance
Antimicrobial resistance patterns of qacA positive isolates from the 2014 S. aureus survey were evaluated to determine predominant resistance profiles. All qacA positive isolates were susceptible to cephalothin, clindamycin, co-trimoxazole, doxycycline, gentamicin, and vancomycin.

Only three out of 53 (6%) qacA positive strains were fully susceptible to all antimicrobials tested. Fusidic acid (Fus) resistance was found in 50 out of 53 (94%) qacA positive isolates. Mupirocin (Mup) resistance was found in 40 out of 53 (75%) isolates, all of which were also fusidic acid-resistant. This represented the most common antibiogram of the qacA positive isolates (Table 11).

Co-resistance of fusidic acid with ciprofloxacin (Cip) and / or erythromycin (Ery) was found in 3 out of 53 isolates, all of which were not resistant to mupirocin. None of the qacA positive isolates had mono-resistance to mupirocin.

Table 11. Summary of antimicrobial resistance profiles for qacA positive isolates

<table>
<thead>
<tr>
<th>Antimicrobial resistance profile</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant to:</td>
<td></td>
</tr>
<tr>
<td>Fus, Mup</td>
<td>40 (75)</td>
</tr>
<tr>
<td>Fus</td>
<td>7 (13)</td>
</tr>
<tr>
<td>none</td>
<td>3 (6)</td>
</tr>
<tr>
<td>Fus, Cip, Ery</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Fus, Cip</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
</tr>
</tbody>
</table>

4.2.4 Association of qacA with isolate acquisition – hospital vs community
Prevalence of qacA was calculated for each hospitalisation status of the 2014 S. aureus survey. Nine out of 178 (5%) hospital acquired isolates tested positive for qacA.
Forty-two out of 501 (8%) community-acquired isolates tested positive for \textit{qacA}. This result does not support the hypothesis that prevalence of \textit{qacA} is higher in hospital-acquired isolates (Table 12).

### Table 12. \textit{S. aureus} acquisition and \textit{qacA} carriage for 2014 \textit{S. aureus} survey

<table>
<thead>
<tr>
<th>Likely place of acquisition</th>
<th>No. of isolates with \textit{qacA} (Total n=53)</th>
<th>Proportion of isolates with \textit{qacA} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital (n=178)</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Community (n=501)</td>
<td>42</td>
<td>8</td>
</tr>
<tr>
<td>Unknown (n=72)</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

### 4.2.5 Association of \textit{qacA} with clonal lineages

The prevalence of \textit{qacA} was highly clonal, such that 41 out of 53 (77%) were \textit{spa} type t127. Three isolates with \textit{spa} type t127 were WR/AK1 MRSA strains. The remainder of isolates were shared among nine other \textit{spa} types as indicated in Table 13.

### Table 13. Distribution of \textit{spa} type for \textit{qacA} carrying \textit{S. aureus}

<table>
<thead>
<tr>
<th>\textit{spa} type</th>
<th>No. of isolates with \textit{qacA} (Total n=53)</th>
<th>Proportion of isolates with \textit{qacA} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t127</td>
<td>41</td>
<td>77</td>
</tr>
<tr>
<td>t1784</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>t2601</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Other(^1)</td>
<td>7</td>
<td>13</td>
</tr>
</tbody>
</table>

\(^1\)\textit{spa} types included t128, t160, t321, t386, t786, t2249 and t5837
Table 14. Summary of 2014 *S. aureus* isolates and associations with *qac*A

<table>
<thead>
<tr>
<th>Characteristic (n)</th>
<th>No. <em>qac</em>A positive (%)</th>
<th>No. <em>qac</em>A negative (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>All <em>S. aureus</em> isolates (751)</td>
<td>53 (7)</td>
<td>698 (93)</td>
<td>-</td>
</tr>
<tr>
<td>Hospital vs Community²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospital-acquired (178)</td>
<td>9 (5)</td>
<td>169 (95)</td>
<td>0.148</td>
</tr>
<tr>
<td>Community-associated (501)</td>
<td>42 (8)</td>
<td>459 (92)</td>
<td></td>
</tr>
<tr>
<td>MRSA vs MSSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRSA (67)</td>
<td>3 (4)</td>
<td>64 (96)</td>
<td>0.388</td>
</tr>
<tr>
<td>MSSA (684)</td>
<td>50 (7)</td>
<td>634 (93)</td>
<td></td>
</tr>
<tr>
<td>spa type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t127 (105)</td>
<td>41 (39)</td>
<td>64 (61)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>t189 (71)</td>
<td>0 (0)</td>
<td>71 (100)</td>
<td>0.015</td>
</tr>
<tr>
<td>t002 (67)</td>
<td>0 (0)</td>
<td>67 (100)</td>
<td>0.018</td>
</tr>
<tr>
<td>t1265 (28)</td>
<td>0 (0)</td>
<td>28 (100)</td>
<td>0.137</td>
</tr>
<tr>
<td>t084 (23)</td>
<td>0 (0)</td>
<td>23 (100)</td>
<td>0.179</td>
</tr>
<tr>
<td>t645 (23)</td>
<td>0 (0)</td>
<td>23 (100)</td>
<td>0.179</td>
</tr>
<tr>
<td>Antimicrobial resistance profiles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mupirocin-resistant³ (43)</td>
<td>32 (74)</td>
<td>11 (26)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fusidic acid-resistant (127)</td>
<td>39 (31)</td>
<td>88 (69)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mupirocin- and fusidic acid-resistant (51)</td>
<td>38 (75)</td>
<td>13 (25)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹p value of <0.05 was considered statistically significant.

²Hospitalisation criteria determined by hospitalisation three months prior to the date of sample collection. Community isolates were from patients who had not been to a hospital three months prior to sample collection. 72 isolates had undefined hospitalisation details and were not included for this data.

³High level mupirocin resistant (MIC >256 µg/mL) criteria used.
4.3 Results from qacA studies in Annual 2015 New Zealand MRSA survey

4.3.1 Prevalence of qacA

Ninety out of 1218 (7%) of the 2015 New Zealand MRSA isolates carried the qacA gene. This prevalence is similar to the prevalence of qacA in isolates from the 2014 New Zealand S. aureus survey.

4.3.2 Association of qacA with clonal lineages

The prevalence of qacA amongst MRSA was also highly clonal. Eighty-one out of 100 (81%) qacA positive MRSA were spa type t127. The clonal lineages for the remainder of qacA positive isolates not represented in Table 15 were made up of non-t127 WR/AK1 MRSA strain (n=4), EMRSA-15 strain (n=1) and spa types which are not associated with any currently recognized MRSA strain (n=2). These results have been summarised in Table 15.

Table 15. Summary 2015 MRSA and association with qacA carriage by spa and strain type

<table>
<thead>
<tr>
<th>Characteristic (n)</th>
<th>No. qacA positive (%)</th>
<th>No. qacA negative (%)</th>
<th>p^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>All MRSA isolates (1218)</td>
<td>90 (7)</td>
<td>1128 (93)</td>
<td>-</td>
</tr>
<tr>
<td><strong>spa type – MRSA strain type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t002 - AK3 (538)</td>
<td>2 (&lt;1)</td>
<td>533 (&gt;99)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>t127 - WR/AK1 (100)</td>
<td>81 (81)</td>
<td>19 (19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>t3949 - Queensland clone (86)</td>
<td>0 (0)</td>
<td>86 (100)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

^1 p value of <0.05 was considered statistically significant.
4.4 Association of \textit{mupA} with \textit{qacA}

4.4.1 Mupirocin resistance

Mupirocin susceptibility was tested and recorded for 751 \textit{S. aureus} isolates from the 2014 survey. Eighty-three out of 751 (11\%) isolates of \textit{S. aureus} isolates tested had elevated mupirocin MICs (> 2 mg/L). Sixty-one out of 83 (73\%) of these isolates with elevated MICs displayed high level mupirocin resistance.

4.4.2 Prevalence of \textit{mupA} in mupirocin resistant strains

All 83 strains with elevated mupirocin MICs were screened for \textit{mupA} gene as described in section 3.4.6. Sixty-one out of 83 (73\%) mupirocin resistant strains were \textit{mupA} positive and had a direct correlation with isolates that displayed high level mupirocin resistance. The \textit{mupA} gene was not detected in isolates which displayed low level mupirocin resistance.

4.4.3 Association of \textit{qacA} with \textit{mupA}

All \textit{qacA} positive isolates that were mupirocin-resistant tested positive for \textit{mupA}. This association is statistically significant and suggests that these genes may be closely linked within the \textit{S. aureus} genome. These results have been summarised in Table 16.
Table 16. Summary of *mupA* and associations with phenotypic mupirocin resistance and *qacA* carriage

<table>
<thead>
<tr>
<th>Characteristic (n)</th>
<th>No. <em>mupA</em> positive (%)</th>
<th>No. <em>mupA</em> negative (%)</th>
<th>( p^1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mupirocin resistant isolates (83)</td>
<td>61 (73)</td>
<td>22 (27)</td>
<td>-</td>
</tr>
<tr>
<td>Mupirocin resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High level resistance (61)</td>
<td>61 (100)</td>
<td>0 (0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low level resistance (22)</td>
<td>0 (0)</td>
<td>22 (100)</td>
<td></td>
</tr>
<tr>
<td><em>qacA</em> carriage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>qacA</em> positive (40)</td>
<td>40 (100)</td>
<td>0 (0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>qacA</em> negative (43)</td>
<td>21 (49)</td>
<td>22 (51)</td>
<td></td>
</tr>
</tbody>
</table>

\(^2\) \( p \) value of <0.05 was considered statistically significant.
4.5 Key findings

Screening for qacA provided previously unknown information about prevalence for this gene and the association of qacA with different phenotypic and genotypic characteristics. Namely, our findings were:

- Prevalence for qacA carriage in New Zealand S. aureus isolates in 2014 was 7%. Prevalence was also 7% in 2015 New Zealand MRSA isolates.
- Carriage of qacA was not to found to have an association with hospital acquisition or methicillin resistance.
- Carriage of qacA in MSSA was highly clonal and strongly associated with spa type t127; and fusidic acid and mupirocin resistance.
- Carriage of qacA in MRSA was also highly clonal and also strongly associated with spa type t127, WR/AK1 strain.
- Association of qacA with high level mupirocin resistance gene was supported by the finding that mupA gene carriage was associated with qacA carriage. This strongly suggests that these genes may be closely located in the S. aureus genome.
Chapter 5. Results: Phenotypic susceptibility to chlorhexidine

5.1 Introduction
The *qacA* gene has been the interest of numerous studies due to the association of this gene with increased chlorhexidine tolerance and the heavy use of this antiseptic in the hospital environment. Studies often looked for phenotypic differences in susceptibility alongside genotypic screening for chlorhexidine resistance. Results from these studies were inconclusive, and collectively used a variety of different methods to determine MICs and MBCs. For this study, we used the CLSI method which was used by the majority of similar studies to determine whether *qacA* carriage in New Zealand *S. aureus* results in increased phenotypic resistance to chlorhexidine.

5.2 Minimum inhibitory concentration to chlorhexidine
A total of 50 out of 53 MIC results to chlorhexidine were available for *qacA* positive strains (Figure 5). MIC results ranged from 2 – 4 mg/L. Fourteen isolates had an MIC of 2 mg/L, and 35 isolates had an MIC of 4 mg/L.

Forty-seven MIC results were available for the control group (*qacA* negative). MIC results also ranged from 2 – 4 mg/L with 36 isolates with an MIC of 2 mg/L, and 11 isolates with an MIC of 4 mg/L. MIC results are summarised in Table 17 and Figure 5.

Despite displaying similar ranges of MIC results in both groups, the MIC\textsubscript{50} in the *qacA* positive group was higher than the MIC\textsubscript{50} in the *qacA* negative group due to the frequency of a higher MIC among those that carried a *qacA* gene over the control group (Table 17). Although this may be a statistically significant difference (*U*=598.5, *p*=<0.001), a single doubling dilution factor can be the result of normal variation in this method of testing. The MIC distribution also does not show a natural “cut-off” value between the two groups due to the small range of results generated for the MIC.

5.3 Minimum bactericidal concentration to chlorhexidine
MBC results were available for 50 out of 53 *qacA* positive strains (Figure 6). MBC results ranged from 2 – 32 mg/L. Twenty-nine strains had an MBC of 4 mg/L and 19 strains had an MBC of 8 mg/L. The lowest and highest MBC values of 2 mg/L and 32 mg/L respectively were both displayed by a single isolate.
Forty-seven out of the 53 MBC results were available for the control group. MBC results ranged from 2 – 8 mg/L for this group. Thirty-two isolates had an MBC of 4 mg/L, followed by 8 isolates with an MBC of 8 mg/L. Seven isolates had an MBC of 2 mg/L. MBC results are summarised in Table 18 and Figure 6.

The MBC results are more relevant to the use of chlorhexidine as this determines the actual concentration to achieve a 99.9% reduction in colony forming units. Statistical analysis of the results does show that the \textit{qacA} positive group has a higher MBC than the control group ($U=815.5$, $p=0.002$).

This perceived difference in phenotypic resistance to chlorhexidine, although not discernible, may be due to lineage association with \textit{spa} type t127 rather than to \textit{qacA} carriage in \textit{S. aureus}.

\textbf{Table 17. Summary of MIC results for \textit{qacA} positive and \textit{qacA} negative isolates against chlorhexidine}

<table>
<thead>
<tr>
<th>MIC (mg/L)</th>
<th>Range</th>
<th>MIC$_{50}$</th>
<th>MIC$_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{qacA} positive</td>
<td>2 – 4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>\textit{qacA} negative</td>
<td>2 – 4</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

$^1$ MIC$_{50}$ and MIC$_{90}$ refers to the 50th and 90th percentile MIC result respectively
Figure 5. MIC distribution for *S. aureus* against chlorhexidine for *qacA* positive and *qacA* negative isolates

Table 18. Summary of MBC results for *qacA* positive and *qacA* negative isolates against chlorhexidine

<table>
<thead>
<tr>
<th></th>
<th>MBC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
</tr>
<tr>
<td><em>qacA</em> positive</td>
<td>2 – 32</td>
</tr>
<tr>
<td><em>qacA</em> negative</td>
<td>2 – 8</td>
</tr>
</tbody>
</table>

<sup>1</sup> MBC<sub>50</sub> and MBC<sub>90</sub> refers to the 50<sup>th</sup> and 90<sup>th</sup> percentile MBC result respectively
Figure 6. MBC distribution for *S. aureus* against chlorhexidine for *qacA* positive and *qacA* negative isolates
5.4 Key findings

Our key findings for \textit{qacA} carriage and concurrent phenotypic resistance in chlorhexidine resistance indicated the following:

- All strains had an MIC range of 2 – 4 mg/L, with no natural cut-off value separating the \textit{qacA} positive and control group.
- The \textit{qacA} positive group had a larger MBC range (2 – 32 mg/L) than the control group (2 - 8 mg/L).
- The carriage of \textit{qacA} gene does not contribute to a discernible difference in MIC and MBC in the \textit{spa t127} lineage.
- Carriage of the \textit{qacA} gene does not directly correlate to a higher phenotypic resistance using the broth microdilution method.
Chapter 6. Results: Genomic analysis of qacA isolates

6.1 Introduction
Studies which had a typing component often found that the clonal lineage associated with qacA is ST239. The qacA gene has also been identified on the pSK1-family of plasmids. As our prevalence studies have shown in Chapter 4, qacA in New Zealand S. aureus is strongly associated with spa type t127 and resistance to mupirocin and fusidic acid. In this chapter, we set out to determine the location of the qacA gene and the links with other resistance genes, paying particular interest to those that confer resistance to mupirocin and fusidic acid.

6.2 Quality of sequence data and assembly from whole genome sequencing
The sequence data and assembly quality was assessed using the .fastq files; and assembled scaffolds from SPAdes and annotation output from Nullarbor respectively. All isolates had passed overall quality checks in both sequence data and assembly.

6.2.1 Sequence data quality metrics
All isolates that were sent for sequencing had a similar GC% content to S. aureus (35.2 – 35.8%). The average phred quality score per base was a minimum of 33.5. The depth of read for each organism ranged from 82 to 99. Organisms were assessed as poor quality if the average phred quality score was less than 30 and depth was less than 50. This data is summarised in Table 19.

Table 19. Quality metrics for sequence data

<table>
<thead>
<tr>
<th></th>
<th>ARS1428</th>
<th>ARS1444</th>
<th>ARS1456</th>
<th>ARS1498</th>
<th>ARS14215</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC%</td>
<td>35.3</td>
<td>35.2</td>
<td>35.3</td>
<td>35.6</td>
<td>35.8</td>
</tr>
<tr>
<td>Phred average quality score</td>
<td>33.7</td>
<td>33.5</td>
<td>33.5</td>
<td>33.6</td>
<td>33.5</td>
</tr>
<tr>
<td>Depth</td>
<td>99</td>
<td>82</td>
<td>84</td>
<td>92</td>
<td>88</td>
</tr>
</tbody>
</table>
6.2.2 Assembly quality metrics

All isolates had a genome of ~2.8 Mbp which is the expected approximate genome size of *S. aureus*. N50 describes the shortest length of contig at which 50% of the genome mass is represented. This was used to assess the quality of assembly with a higher contig indicating a better quality of assembly. N50 for our group of organisms ranged from 22,400 to 43,034 bp in length. This data is summarised in Table 20.

Table 20. Quality metrics for assembly of sequences

<table>
<thead>
<tr>
<th></th>
<th>ARS1428</th>
<th>ARS1444</th>
<th>ARS1456</th>
<th>ARS1498</th>
<th>ARS14215</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of contigs</td>
<td>148</td>
<td>119</td>
<td>139</td>
<td>275</td>
<td>255</td>
</tr>
<tr>
<td>Total bp</td>
<td>2,868,177</td>
<td>2,798,481</td>
<td>2,835,458</td>
<td>2,819,134</td>
<td>2,820,959</td>
</tr>
<tr>
<td>Max contig (bp)</td>
<td>116,340</td>
<td>159,778</td>
<td>86,020</td>
<td>74,117</td>
<td>109,511</td>
</tr>
<tr>
<td>N50 length (bp)</td>
<td>42,509</td>
<td>41,650</td>
<td>43,034</td>
<td>22,400</td>
<td>24,562</td>
</tr>
</tbody>
</table>
6.3 Whole genome analysis of *S. aureus* strains

The Nullarbor output provided data to assess similarities between strains. Included in this analysis was sequence typing results using MLST, core SNP distance analysis, and pairwise core SNP distance matrix between the isolates.

6.3.1 Similarity of strains

Nullarbor analysis confirmed all the above isolates were ST1 as summarised in Table 21. Isolates shared an average of 94.18% similarity with results ranging from between 92.40 to 95.85% similarity to reference genome MSSA 476. A total of 2077 core SNPs were found across all isolates.

Table 21 MLST of isolates under the *S. aureus* scheme showing allelic profile and sequence types

<table>
<thead>
<tr>
<th>MLST Loci</th>
<th>Sequence Type (ST)</th>
</tr>
</thead>
<tbody>
<tr>
<td>arcC</td>
<td>aroE</td>
</tr>
<tr>
<td>ARS1428</td>
<td>1</td>
</tr>
<tr>
<td>ARS1444</td>
<td>1</td>
</tr>
<tr>
<td>ARS1456</td>
<td>1</td>
</tr>
<tr>
<td>ARS1498</td>
<td>1</td>
</tr>
<tr>
<td>ARS14215</td>
<td>1</td>
</tr>
</tbody>
</table>

All isolates that were submitted had a SNP distance which ranged from 147 SNPs to 600 SNPs, and all isolates had a SNP distance of at least 773 from reference genome MSSA 476. This suggests that our isolates are more closely related to one another than to the reference genome which is expected. These results are summarised in Table 22. Due to the small number of isolates that were submitted for sequencing, there is not enough supporting data to suggest a cluster of similarity between any of the isolates.
Table 22. Pairwise Core SNP distance matrix between isolates

<table>
<thead>
<tr>
<th>ID</th>
<th>ARS14215</th>
<th>ARS1428</th>
<th>ARS1444</th>
<th>ARS1456</th>
<th>ARS1498</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARS14215</td>
<td>0</td>
<td>300</td>
<td>249</td>
<td>430</td>
<td>381</td>
<td>1027</td>
</tr>
<tr>
<td>ARS1428</td>
<td>300</td>
<td>0</td>
<td>217</td>
<td>600</td>
<td>545</td>
<td>1044</td>
</tr>
<tr>
<td>ARS1444</td>
<td>249</td>
<td>217</td>
<td>0</td>
<td>545</td>
<td>502</td>
<td>1169</td>
</tr>
<tr>
<td>ARS1456</td>
<td>430</td>
<td>600</td>
<td>545</td>
<td>0</td>
<td>147</td>
<td>798</td>
</tr>
<tr>
<td>ARS1498</td>
<td>381</td>
<td>545</td>
<td>502</td>
<td>147</td>
<td>0</td>
<td>773</td>
</tr>
<tr>
<td>Reference</td>
<td>1027</td>
<td>1044</td>
<td>1169</td>
<td>798</td>
<td>773</td>
<td>0</td>
</tr>
</tbody>
</table>

6.3.2 Fusidic acid resistance gene

Fusidic acid resistance genes were found in all strains that were sequenced. The \textit{fusC} gene was found in all sequences except in isolate ARS14215 (2015-09180). Although \textit{fusC} was found within these genomes, they were not found on the same contig as the \textit{qacA} gene suggesting that these resistant determinants are not co-located. The \textit{fusC} gene was found on the Staphylococcal cassette chromosome (SCC) which shared \textgreater{}99\% gene sequence similarity within the SCC found in MSSA 476.

The \textit{fusC} gene in t127 ST1 strains were previously described by Baines et al. who identified this gene on SCC 476 (SCC\textsubscript{476}) of the dominant fusidic acid-resistant MSSA found in New Zealand (109). Fusidic acid resistance on the SCC\textsubscript{476} mobile element is thus considered to be the main mode for the horizontal gene transfer of the \textit{fusC} gene in New Zealand \textit{S. aureus} isolates. The \textasciitilde{}10.3 kb \textit{fusC} element on the SCC shares \textgreater{}99\% homology with the \textit{fusC} element in SCC\textsubscript{mecAK3} found in the predominant AK3 CC5 MRSA, and the SCC\textsubscript{mec}-SCC\textsubscript{476} element in the CC1 MRSA (Figure 7). In addition, the paper has also described the co-location of the \textit{fusC} with the \textit{tirS} gene which encodes for a virulence factor which gives a selective advantage for the survival of \textit{S. aureus} in the host (96). This, combined with use of the topical antibiotic fusidic acid may contribute to the predominance of the t127 strain in New Zealand.

Despite \textit{qacA} and \textit{fusC} not being closely located in the genome, both \textit{fusC} and \textit{qacA} are both associated with the clonal lineage t127.
Figure 7. Genetic organisation of the \textit{fusC} gene in SCC in CC5 MRSA, CC1 MSSA and CC1 MRSA

Schematic showing \textit{fusC} and \textit{mecA} (orange and green respectively) arrangement in the SCC. White genes represent hypothetical proteins within the SCC. The \textit{ccr} recombinase genes and the \textit{hsd} genes (encode proteins for the restriction modification system) are also found within the SCC\textsubscript{476} element. The grey areas represent regions with > 99\% DNA sequence homology between the sequences. CC1 MSSA: SCC\textsubscript{476} is the likely to be the location of the fusidic acid resistance gene within our t127 S. aureus genome (Figure provided by D. Williamson, personal communication February 8, 2017).
6.4 Analysis of the qacA contig

6.4.1 Co-located genes within qacA contigs

The qacA-containing contigs were investigated for co-location with other genes of interest. All qacA contigs had the presence of rep indicating that the qacA gene is found on a plasmid. The mupA (ileS2) and blaZ genes were also found within the qacA-containing contigs. The presence of mupA supports the initial findings of the screening results found in chapter 4.4 of this thesis. Table 23 summarises the presence or absence of the genes of interest found on qacA contigs.

Table 23. Genes of interest on qacA contigs

<table>
<thead>
<tr>
<th></th>
<th>ARS1428</th>
<th>ARS1444</th>
<th>ARS1456</th>
<th>ARS1498</th>
<th>ARS14215</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of contig (bp)</td>
<td>27,852</td>
<td>27,993</td>
<td>30,182</td>
<td>27,874</td>
<td>27,991</td>
</tr>
<tr>
<td>Presence of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qacA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>qacR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>rep</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>blaZ</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>mupA (ileS2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

6.4.2 Similarity of qacA contigs

Percentage identity similarity was calculated in order to determine pairwise distance of qacA contigs between different strains. Contig 9178_51 had the lowest percentage similarity (92.223% – 92.346%) with others due to a ~2250 bp insertion not present in the other strains. Other strains had percentage similarities of greater than 99.4% with less than 160 nucleotide differences found among contigs 9176_107, 9177_98, 9179_223, and 9180_279. Mauve genome alignment viewer was used to visualise relatedness of qacA contigs (Figure 8). These results have been summarised in Table 24.
<table>
<thead>
<tr>
<th></th>
<th>ARS1428 9176_107</th>
<th>ARS1444 9177_98</th>
<th>ARS1456 9178_51</th>
<th>ARS1498 9179_223</th>
<th>ARS14215 9180_279</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARS1428 9176_107</td>
<td>99.439</td>
<td>92.223</td>
<td>99.563</td>
<td>99.432</td>
<td></td>
</tr>
<tr>
<td>ARS1444 9177_98</td>
<td>92.250</td>
<td>99.993</td>
<td>99.575</td>
<td>99.993</td>
<td></td>
</tr>
<tr>
<td>ARS1456 9178_51</td>
<td>92.346</td>
<td>92.244</td>
<td>99.568</td>
<td>92.346</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8. Mauve alignment of *qacA* containing contigs.
Areas of similarities shared among the contigs are indicated by the same colour blocks with lines connecting areas of consensus sequences. White blocks indicate insertion sequences that were not found within the other contigs.
6.5 The *qacA* plasmid

6.5.1 Plasmid structure

In order to determine the plasmid structure from the four *qacA*-containing contigs, consensus sequences were extracted from the CLUSTALW alignments of these contigs. The resulting plasmid was compared to the PacBio plasmid of a representative ST1 *qacA*, *mupA*, and *fusC* carrying t127 strain (ARS14487). PacBio of ARS14487 sequence was provided by the Microbiological Diagnostic Unit Public Health Laboratory, The University of Melbourne. The Mauve alignment of the two plasmids confirmed that the *qacA*-containing Illumina contigs have plasmids that are similar to that of the PacBio assembled plasmid found in ARS14487.

![Mauve alignment of pNZAK1 and PacBio assembled plasmid of ARS14487](image)

**Figure 9. Mauve alignment of pNZAK1 and PacBio assembled plasmid of ARS14487**

The two red blocks indicate areas of 100% similarity between the two plasmids. The two lines found within the ARS14487 block at ~22,500 bp and 27,800 bp are short sequences which are not found in pNZAK1.

The PacBio assembled plasmid ARS14487 is longer than the assembled consensus pNZAK1 plasmid by 64 bp. The Mauve (Figure 9) and the CLUSTALW alignment (Figure 10) show that these extra bases are missing from the pNZAK1 plasmid at ~22,500 bp and at 27,800 bp.
Figure 10. CLUSTALW alignment of pNZAK1 with PacBio assembly of ARS14487 plasmid
The green areas within the horizontal bar represents consensus identity sequence of 100%. Breaks in the green lines indicate areas where short sequences that are not found in pNZAK1.

Figure 11. pNZAK1 containing qacA constructed from consensus sequences extracted from Illumina contigs
The qacA operon (blue) is co-located with other genes of interest including the penicillinase operon (yellow and orange containing blaR and blaZ respectively) and corresponding transposase genes (pink) and mupA (green).
The representative plasmid pNZAK1 (Figure 1) is ~28 kb in size. The qac operon consisting of qacA (blue) and corresponding regulatory gene qacR (light blue) are found ~17 kb downstream from rep (red). Upstream from qacR is the penicillinase gene blaZ (orange) and the penicillin regulator protein gene blaR (yellow). Downstream from qacA is mupA (green) which encodes the protein for mupirocin resistance. Other genes of interest include cadmium resistance genes cadD, cadC; cadX (purple) and transposase genes (pink) adjacent to the bla operon. These transposase genes share similarities to transposon 552 (Tn552), a mobile genetic element known to carry the penicillinase genes (36). Annotations in grey are genes associated with replication and recombination proteins.

6.5.2 BLAST search of qacA plasmid
A nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search was performed on the plasmid sequence. The pNZAK1 plasmid most closely resembled S. aureus plasmids pMW2 (AP004832) and pSAS (BX571858) sharing 73.9% similarity in identities. This has been visualised using the Mauve alignment viewer (Figure 12).

The similarity between these three plasmid suggests that the pNZAK1 with the qacA gene belongs to the major S. aureus plasmid family of pMW2-like plasmids. This plasmid is ubiquitous with a wide geographical distribution from the US, UK and Australia (54). The pMW2-like plasmids typically carry the cadmium resistance genes and the transposon Tn552 which carries the penicillinase gene blaZ.
Figure 12. Mauve alignment of pNZAK1 and the two most closely resembling plasmids from the BLAST search
Areas that are in red show areas of similarity among the three different plasmids. The white block within pNZAK1 indicates an insertion sequence not found in the other two plasmids.
Plasmid pNZAK1 has an insertion sequence of ~7 kb not found in the two most closely resembling plasmids pSAS and pMW2 (Figure 13a). This insertion sequence contains qacA, qacR, and mupA (Figure 13b). A nucleotide BLAST search was performed on the insertion sequence. Results from this search had top hits matching to the mupA portion due to the larger presence of this gene on the insertion sequence with 59% query coverage. This was followed by hits matching to the qacA portion in various S. aureus sequences within NCBI database with a 39% query coverage. This may indicate that the incorporation of these genes on to this plasmid may be from independent recombination events.

Figure 13. CLUSTALW alignment of pNZAK1 with pMW2 and pSAS plasmid with magnified insertion sequence found in pNZAK1
a. The green areas within the horizontal bar indicate consensus identity sequence of 100%. Observed insertion seen ~15 kb downstream from rep gene (red).
b. Magnified view of ~7 kb insertion in spa t127 plasmid.
6.6 Key findings

Our genomic analysis sheds new light on the genetic context of genes associated with topical antimicrobials and biocide resistance. Key findings are:

- The *qacA* gene was found on a ~28 kb plasmid and is closely co-located with high level mupirocin resistance gene *mupA* and penicillinase gene *blaZ*.
- The *fusC* gene was found on a staphylococcal cassette chromosome.
- Fusidic acid, mupirocin, and chlorhexidine associated resistance genes are all found on mobile genetic elements which are able to disseminate these genes via horizontal gene transfer.
- The similarity of the plasmids in all the analysed ST1 isolates suggests clonal expansion of ST1.
- The *qacA*-containing plasmid belongs to a major *S. aureus* plasmid family, namely the pMW2-like plasmids.
- Both *qacA* and *mupA* are found within a ~7kb insertion sequence in the pMW2-like backbone.
Chapter 7. Discussion

7.1 The prevalence of qacA carriage in New Zealand S. aureus is ~7%

Prevalence of qacA was found to be ~7% for S. aureus in New Zealand. This study is the first to describe prevalence of qacA in New Zealand S. aureus isolates. Due to the lack of historical data on qacA carriage, the prevalence rates cannot be perceived as increasing or decreasing. By establishing the current prevalence it lays the groundwork for future surveillance of qacA and associated chlorhexidine resistance. This will contribute to future studies about the significance of qacA carriage within the S. aureus genome, with particular interest in circulating strains in New Zealand.

The prevalence rates in this study are comparable to studies conducted in China and Korea with qacA carriage rates ranging from 4.4% to 11.8% in clinical isolates (Table 3) (69,74,77). The results of this study cannot be directly compared to other prevalence studies highlighted in Table 3 due to differences in isolate inclusion criteria. The majority of studies were unable to match the same parameters set out in this study, as often the studies (i) were targeted towards resistant phenotypes, (ii) were collected over an extended time-span, (iii) were collected from a single centre, or (iv) had small sample numbers. This is the first study to capture the prevalence of qacA in S. aureus on a national level with all New Zealand diagnostic laboratories taking part in the survey. This ensured that this was a multi-centred study which included both community and nosocomial isolates. The collection criteria for isolates for the 2014 survey used in this study enabled us to capture the true prevalence of qacA in clinical isolates at a specified three day window in New Zealand. This specified criteria makes this study an accurate snapshot representation of the current prevalence of qacA in New Zealand S. aureus.

Similarly, the prevalence was found to be 7% among MRSA from the New Zealand 2015 MRSA survey. Studies on qacA prevalence often focused on MRSA due to the importance of decolonisation particularly in wards with high colonisation pressures (44). The majority of these studies focused on nosocomial isolates, with one study using isolates from patients in a long term care facility. The MRSA survey was designed as a multi-centred study, incorporating both nosocomial and community isolates throughout New Zealand within a one month period, ensuring that this study was able to capture accurate prevalence rates of qacA on a national level. This makes the qacA prevalence
data from this study invaluable as community isolates of MRSA are a burgeoning reservoir of multi-resistant strains of *S. aureus*. This is particularly significant given the emergence of fusidic acid-resistant strains of MRSA, potentially driven by the heavy use of topical antibiotics within the community (21).

### 7.2 New Zealand *S. aureus qacA* carriage is highly clonal in community strains and is likely driven by topical antibiotic use

As alluded to earlier, *qacA* was thought to be related to decreased susceptibility to chlorhexidine. As chlorhexidine is used extensively in healthcare facilities, we hypothesised that the increased exposure to chlorhexidine in this environment would lead to higher prevalence of *qacA* in hospital-acquired *S. aureus*. Historically, methicillin resistance in *S. aureus* was also associated with hospital-acquired isolates, thus we hypothesised that these two characteristics would be associated with increased prevalence of *qacA* carriage. The results of our prevalence study revealed that there was no association between *qacA* carriage and hospital acquisition. We also found that there was no association between methicillin resistance and *qacA* carriage, with similar prevalence rates of ~7% among MRSA in the 2015 survey and ~7% prevalence among New Zealand *S. aureus* 2014 survey.

Although *qacA* was not found to be associated with the predominant AK3 MRSA, carriage of *qacA* was found in WR/AK1 MRSA. WR/AK1 is the second most common strain of MRSA in New Zealand and is commonly resistant to mupirocin and fusidic acid; and occasionally shows resistance to erythromycin (107,110). The proportion of WR/AK1 strains rose from 8.9% in 2014 to 11.4% in 2015, 68.2% of which are community-associated isolates (21,107). Concerningly, chlorhexidine and mupirocin decolonisation is often used to prevent HCAI, and routinely administered to newly admitted patients who have screened positive for MRSA carriage. This decolonisation strategy may become ineffective in eradicating MRSA from patients who carry this strain within the community. This could potentially lead to the co-selection and introduction of this strain into health care facilities.

Our prevalence carriage studies also showed that *qacA* carriage is highly clonal and most commonly associated with *spa* type t127, belonging to ST1. Strains with *spa* type t127 are the most common MSSA in New Zealand making up 15% of all MSSA collected in
the 2014 survey (102). Concerningly this *spa* type is typically associated with resistance to the commonly used topical anbiotics fusidic acid and mupirocin. As mentioned previously fusidic acid prescribing trends were associated with an increased prevalence of fusidic acid resistant *S. aureus* which were largely driven by the clones t127 and t002 (26). Prior studies to this have also indicated that increased mupirocin use drove an increase in mupirocin resistance (111). Unfortunately, it is not known whether the rise in mupirocin-resistant strains were due to the t127, ST1 clone.

The marked prevalence of chlorhexidine resistance associated with *qacA* in the predominant multi-resistant t127, ST1 lineage, presents a pressing challenge to healthcare providers to curb the expansion of this clone in the community. Strains belonging to the t127, ST1 lineage are likely to be resistant to the commonly used topical treatment options prescribed to treat, as well as decolonise, patients with *S. aureus*. Fusidic acid is currently prescribed as the preferred topical antibiotic in New Zealand for skin infections as this is subsidised by the Ministry of Health. In addition, eradication of *S. aureus* still uses a combination of mupirocin ointment and chlorhexidine. The co-carriage of *fusC*, *mupA*, and *qacA* mediated resistance in the t127, ST1 lineage is likely to thrive under current antibiotic prescribing habits, which has the potential to lead to increased prevalence of multiply resistant strains circulating in the community. Additionally, the predominance of t127, ST1 clones could also lead to a greater likelihood of exchange of resistance genes found on mobile genetic elements.

### 7.3 Arrangement of *qacA* on the pNZAK1 is unique

To date the arrangement of *qacA* with *mupA* in the plasmid of our isolates has not been found in its entirety by BLAST search of this sequence. Despite several studies looking for the co-existence of these genes in *S. aureus* (74,76,81–83,86,112), little is known about the organisation of these genes within the *S. aureus* genome or the organisation of these genes in different clonal lineages. The co-location of *qacR* and *qacA* adjacent to Tn552 carrying the *blaZ* of the pMW2-like plasmid is unsurprising as *qacA* is often found, and associated with, the penicillinase gene on other plasmids (113). To date the *qacA* on the pMW2-like plasmid has not been previously described, and this is the first study to find this gene within the ubiquitous ST1 clone.
Numerous papers with a clonal typing component in *qacA* prevalence studies found that sequence types ST239 (CC5) was often associated with *qacA* carriage (76,80,81). The pSK1-like plasmid is the likely location of the *qacA* gene in the ST239 clones. The pSK1 family of plasmids also carries genes that confers resistance to penicillin through carriage of Tn552, to aminoglycosides through carriage of Tn4001, and to trimethoprim through carriage of *dfrA* (114). In addition to pSK1, *qacA* has been associated with transmissible plasmids pSK105, pSK107, pSK4032, pSK4769, and pSK57 (99). Recombination events leading to the incorporation of *qacA* onto these plasmids have not been widely studied.

The distribution of resistance and virulence genes on plasmids of *S. aureus* has been found to be lineage associated (39). McCarthy and Lindsay’s study grouped together plasmids based on rep gene sequences and combinations of rep genes from *S. aureus* plasmid sequences that were available in the public domain. In their study, both *qacA* and *mupA* were found on plasmids, however neither of these genes are core genes of any of the plasmid groups defined in McCarthy and Lindsay’s study (39). Plasmid groups were also found to be lineage-associated due to clonal expansion and restriction modification systems that prevent incompatible transfers of genes between lineages. Dissemination of the pNZAK1 plasmid in the predominant NZ strain may be attributed to clonal expansion from a single clone from the ST1 lineage rather than independent recombination events leading to the incorporation of *qacA* into the plasmid. This theory is supported by the rare finding in this study showing that *qacA* is associated with the ST1 lineage; which has not been previously described despite the frequency of *qacA* and ST1 worldwide.

The *mupA* gene typically disseminates on mobile genetic elements. Insertion sequences flanking *mupA* indicate the likely transposable mechanism for the transfer of the mupirocin resistance gene between plasmids (115). Dissemination of *mupA* can also be through complete plasmid transfers. Transfers of these *mupA* carrying plasmids has been described among different pandemic lineages of *S. aureus* and in *S. epidermidis* (115). Our study found direct correlation of presence of *mupA* with phenotypic high level mupirocin resistance in New Zealand strains. Thus, mupirocin use is likely to be the selective factor for the maintenance of the *mupA* gene in pNZAK1.

As *qacA*, *mupA*, and *blaZ* are co-located on the pNZAK1 plasmid, co-selection for these genes may be linked to the use of antimicrobials in New Zealand. A 2016 report was
prepared for the Ministry of Health on the prescribing trends and antibiotic consumption in New Zealand by ESR. The report observed an increase in consumption of the extended spectrum penicillin, amoxicillin, in New Zealand between 2006 and 2014 (116). This trend of increased amoxicillin consumption, plus the use of the topical antimicrobials mupirocin and fusidic acid as mentioned earlier may be a contributing factor in the findings of a notably high prevalence of qacA in New Zealand S. aureus isolates. Essentially, the use of these antibiotics has potentially led to the selection for certain genes and gene networks resulting in the success of the predominant lineage; in this case, the multi-resistant, community-associated t127, ST1 lineage.

7.4 Carriage of qacA in S. aureus did not show reduced susceptibility to chlorhexidine

MIC and MBC findings to chlorhexidine in this study showed negligible differences between qacA positive and qacA negative isolates. Alternatively, qacA may not have been expressed under the conditions that were used for susceptibility testing. This outcome was similar to results found in other studies that addressed the phenotypic expression of the qacA gene. The methodology chosen for this study is one commonly used for organisms and antimicrobials for therapeutic use. Currently there is no standardised, reproducible method that is able to assess the effectiveness of antiseptics like chlorhexidine against specific organisms (90).

Our findings are supported by Grkovic et al. who were unable to detect an increased expression of QacA in S. aureus in their study of the repressor protein QacR with various substrates (59). The chlorhexidine digluconate MIC required to inhibit binding of QacR to the IR1 was too high for the survival of S. aureus. The negligible differences in MIC and MBC found between our two groups may be within the natural tolerance levels of S. aureus to chlorhexidine regardless of qacA carriage status. Resistance to chlorhexidine, if mediated by the expression of the qacA gene, may be due to simultaneous exposure to other compounds or substrates. Simultaneous exposure of chlorhexidine and other recognised substrates may perhaps overcome the binding of QacR to the operator region IR1 which then increases the expression of QacA. The Grkovic et al. study showed substrates that increased expression of QacA included compounds that have also been used for antisepsis, disinfection, and preservation (42).
Work on qacA carriage and association with failed decolonisation strategies continues to be a focus area due to the reliance on chlorhexidine for the prevention of nosocomial infections. Batra et al. conducted a study which showed qacA/B carrying strains were still isolated in patients post administration of decolonisation procedures that included the use of chlorhexidine (117). A similar result was found in a nested case control study conducted in Geneva following patients post decolonisation therapy. This study showed that decolonisation therapy with mupirocin and chlorhexidine was less effective in patients who were previously colonised with qacA/B carrying ST239 S. aureus than patients who were colonised with non-ST239 S. aureus without qacA/B (odds ratio, 10.2 95% confidence interval, 2.6 - 40.7 p<0.01) (112).

Interestingly, the Batra et al study did note a threefold increase in chlorhexidine MBC in ST239 S. aureus when compared to non-ST239 S. aureus (117). This could mean that the perceived tolerance to chlorhexidine might be associated with lineage ST239, rather than due to the carriage of qacA/B in the ST239 S. aureus. As our phenotypic chlorhexidine susceptibility study matched the control group on clonal type, we were unable to ascertain whether the ST1 lineage shows naturally higher levels of resistance to chlorhexidine than other lineages found in New Zealand. Namely, qacA carriage in the predominant New Zealand ST1 lineage may not display increased resistance to chlorhexidine, instead showing resistance levels that are alike to ST1 strains without qacA.

MICs and MBCs of S. aureus in phenotypic chlorhexidine resistance studies often yield values that are several magnitudes lower than the concentration of chlorhexidine solutions that are used in clinical applications (99). Thus, the argument may be made that increased tolerance to chlorhexidine is minimal and should not warrant concern from the health sector. However, an unintended consequence of widespread use of chlorhexidine in healthcare for decolonisation and hand antisepsis is the residual levels in the environment which potentially creates a selective advantage for isolates with increased tolerance to chlorhexidine. Additionally, studies have indicated that repeated exposure to sub-inhibitory levels of chlorhexidine can lead to increased MIC levels in S. aureus (99).

Concerningly, the predominant t127, ST1 strain in New Zealand is commonly resistant to fusidic acid and, as demonstrated in this study, also associated with mupirocin resistance. Resistance to these two antibiotics along with chlorhexidine resistance make
a trio of topical treatment options which potentially are unable to treat the common skin or soft tissue infection. Failure to treat simple *S. aureus* infections sufficiently will continue to contribute to the high rates of incidence observed in New Zealand. This will continue to be a significant burden to New Zealand public health, and highlights the need to find an alternative solution to reduce the use of these topical agents in the community.
Thesis summary and future work

The overarching aim of this work was to investigate the phenotypic and genotypic context of chlorhexidine resistance in New Zealand \textit{S. aureus} isolates. This included establishing the prevalence rates and associations of \textit{qacA} carriage with various characteristics including patient demographics, phenotypic susceptibility profiles and clonal lineages. Furthermore, this work also looked at the genetic setting of \textit{qacA} with other resistance genes commonly associated with this strain.

Our key findings were:

i. That the gene associated with chlorhexidine resistance, \textit{qacA}, has a prevalence of 7\% in New Zealand \textit{S. aureus} isolates.

ii. That the carriage of the \textit{qacA} gene is highly clonal and is associated with the predominant fusidic acid- and mupirocin-resistant t127, ST1 strain, making these strains genotypically resistant to currently prescribed topical antibiotics and antiseptics.

iii. That \textit{qacA} and \textit{mupA} are co-located often in \textit{fusC} harbouring \textit{S. aureus} strain suggesting the genetic potential for co-selection.

Taken together, the association of \textit{qacA} with reduced susceptibility to chlorhexidine alongside the high use of chlorhexidine in healthcare, may be inadvertently contributing to the success of the predominant t127 clone in New Zealand. This is particularly significant because of the high incidence rates of \textit{S. aureus} infections in New Zealand (15). Furthermore, the t127, ST1 clone is multi-resistant, with the strain being synonymous with fusidic acid resistance and in 45\% of cases co-resistant to mupirocin (100). The combination of resistance to the common topical antibiotics alongside chlorhexidine makes this predominant strain potentially untreatable using the first-line and preferred topical antibiotics and antiseptics. This makes it increasingly harder to execute successful decolonisation strategies to prevent nosocomial infections caused by \textit{S. aureus}. 
Future Work

Based on the key findings from this study, further work is required to address the issue of ongoing chlorhexidine use and \textit{qacA} carriage in \textit{S. aureus}. Further work should focus on the following questions:

\textbf{How can the expression of QacA and increased phenotypic resistance to chlorhexidine be tested in clinical \textit{S. aureus} isolates?}

This study was unable to show the association between \textit{qacA} carriage in \textit{S. aureus} and phenotypic resistance to chlorhexidine. The method described in this study is typically used for measuring MIC and MBC levels of organisms to antibiotics that are used for therapeutic purposes. Work on this aspect should develop an assay which is able to test the resistance to chlorhexidine under conditions which better represent the environment and application of chlorhexidine in a clinical setting. One factor to be considered is the activity of \textit{qacA}-containing \textit{S. aureus} when exposed to chlorhexidine on the skin of patients. Other compounds or substrates found within the environment that can lead to increased expression of QacA should also be considered when designing an assay for the detection of increased chlorhexidine resistance. Finding the link and establishing a successful method that can be used in a routine or reference laboratory can lead to better patient management for the eradication of \textit{S. aureus} and improve clinical outcomes.

\textbf{What are the external factors leading to the marked prevalence of \textit{qacA} in New Zealand \textit{S. aureus} isolates?}

Due to the multi-resistance associated with \textit{qacA} and the t127, ST1 clone of \textit{S. aureus} in New Zealand, future work should aim to look at factors, other than phenotypic chlorhexidine resistance, that have led to the preservation of this gene in the predominant strain (e.g. selection by fusidic acid or mupirocin). Ongoing surveillance of \textit{qacA} in New Zealand \textit{S. aureus} strains is recommended in order to determine whether prevalence is on the rise or decline.
What genetic factors are contributing to the spread or maintenance of the *qacA* gene?

The location of the *qacA* gene in the plasmid indicates that this gene can be easily transferred between organisms and found alongside multiple resistance genes. Work should be targeted towards why and how the genes were incorporated into the pMW2-like backbone, and the estimated introduction of the gene into New Zealand. Future studies should also determine the fitness cost of maintaining the gene within the predominant t127.

What alternative is there to chlorhexidine in healthcare?

Currently, studies are targeted towards the *qacA/B* carriage and the association with failed attempts at decolonisation strategies. Alternative options should be explored in future studies to find substrates, compounds, or decolonisation strategies that are able to be used for decolonisation of *S. aureus* for at-risk patients. Ideally work should focus on strategies that have minimal impact on gene selection so as to not lead to selection or development of resistance mechanisms.


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