The Molecular Epidemiology of Antimicrobial Resistant Staphylococci in Australian Animals

© Kate Alice Worthing

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DECLARATION

This thesis is submitted to The University of Sydney in fulfilment of the requirements for the Degree of Doctor of Philosophy.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Parts of this thesis have been published in the candidate's name (see List of Peer Reviewed Publications below).

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ABBREVIATIONS AND ACRONYMS

AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CA	Community-associated
CC	Clonal complex
CfA	Clumping factor A
CFU	Colony forming units
CLSI	Clinical and laboratory standards institute
CPS	Coagulase-positive staphylococci
Dru	Direct repeat unit
DSG1	Desmoglein-1
HA	Hospital-associated
hr	Hours
IEC	Immune evasion complex
IgG	Immunoglobulin G
MALDI-TOF MS	Matrix assisted laser desorption ionisation-time of flight mass spectrometry
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
min	Minutes
MLST	Multilocus sequence typing
MRS	Methicillin resistant staphylococci
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant Staphylococcus pseudintermedius
MSSA	Methicillin-susceptible *Staphylococcus aureus*

MSSP	Methicillin-susceptible Staphylococcus pseudintermedius
NSW	New South Wales
NT	Northern Territory
PBP2a	Penicillin binding protein 2a
PFGE	Pulsed field gel electrophoresis
PVL	Panton-Valentine Leukocidin
QAC	Quaternary ammonium compound
QLD	Queensland
SA	South Australia
SCCmec	Staphylococcal cassette chromosome *mec*
SEA	Staphylococcal enterotoxin A
SNP	Single nucleotide polymorphism
Sec	Seconds
SPA	Staphylococcal protein A
Spp.	Species (plural)
ST	Sequence type
TAS	Tasmania
UK	United Kingdom
USA	United States of America
VDL	Veterinary diagnostic laboratory
Vic	Victoria
VPDS	Veterinary pathology diagnostic service
WA	Western Australia
WGS	Whole genome sequencing
w/v	Weight per volume

ABSTRACT

Staphylococcus species (spp.) are among the most common bacterial pathogens of humans and animals. The rise of antimicrobial resistance amongst this genus has significantly added to the threat it poses to public health. This thesis examines the epidemiology of the *Staphylococcus* genus in veterinary medicine, with particular reference to methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP). Chapter 1 begins with an in-depth literature review detailing our current understanding of this fascinating genus. Due to its importance as a human pathogen, much research has already been conducted into the mechanisms of antimicrobial resistance, population structure, host adaptation markers and virulence factors found in *S. aureus*. Research into *S. aureus* can often be extrapolated to other staphylococcal species, but differences and knowledge gaps exist for staphylococci of veterinary significance such as *S. pseudintermedius* and *S. felis;* common pathogens of dogs and cats respectively. The literature review therefore compares what is known about *S. aureus* to that of other *Staphylococcus* spp. of veterinary importance. Chapter 2 describes how whole genome sequencing and *in silico* analysis were used to elucidate the molecular epidemiology of staphylococcal isolates examined in the subsequent chapters. MRSP and MRSA have been reported amongst animals from several countries overseas, but little is known about the epidemiology of these pathogens in the Australian veterinary setting. Chapters 3 and 4 consequently describe the first nationwide surveillance study that examined 1080 clinical *Staphylococcus* isolates collected from veterinary diagnostic laboratories across all Australian states and territories. Chapter 3 describes the molecular epidemiology of clinical MRSA from animals in Australia and examines the relationship between clinical MRSA from animals and that carried by Australian veterinarians. In contrast to Australian companion animals, MRSA was not found in the collection of 203 *S. aureus* bovine mastitis samples. Chapter 4 examines 77 MRSP isolates collected during the surveillance study and describes how novel sequence types and methicillin-resistance determinants are present amongst Australian MRSP. To more closely examine the transmission cycle of MRSA and MRSP in companion animal practice, Chapter 5 describes a prospective study that investigated MRSP and

MRSA carriage amongst veterinary personnel, canine hospital admissions and veterinary-owned pet dogs and cats. MRSA carriage was found in veterinary personnel at a higher rate than what is reported for the general public. However, MRSA found in veterinary personnel was not reflected in carriage by canine patients treated by the personnel or pets owned by personnel, suggesting there are additional risk factors for MRSA carriage amongst veterinary personnel besides contact with animals carrying resistant staphylococci. Returning to isolates collected in the surveillance study, Chapter 6 examines genomic and phenotypic virulence factors amongst 38 *S. felis* isolates collected from cats. *S. felis* has traditionally been viewed as a commensal of cats and could be discounted as a contaminant of clinical samples. However, Chapter 6 details how it has sufficient markers of virulence including newly detected coagulation of feline plasma to make it a potential pathogen of cats. A multilocus sequence typing scheme was developed which gives an initial framework for the population structure of *S. felis* in Australian cats. A common finding of the surveillance study conducted here, and previous similar studies conducted elsewhere, is the predominance of a particular clone of MRSP, ST71, amongst clinical isolates from animals. Chapter 7 describes how genomic screening established that ST71 isolates from Australia are significantly more likely to carry *qac* genes than other MRSP clones. *Qac* genes are associated with increased biocide tolerance in MRSA and prior to this study, had not been reported in MRSP. Chapter 7 investigates whether *qac* genes lead to the phenotypic expression of increased biocide tolerance in MRSP. Isolates with and without *qac* genes were tested to determine their tolerance to chlorhexidine and benzalkonium chloride, two common veterinary biocides. Surprisingly, the presence of *qac* genes in MRSP isolates did not affect the minimum inhibitory concentration (MBC) of either biocide. However, a consistent finding was that protein contamination significantly increased the MBC of both biocides which is a pertinent reminder that biocides may be ineffective against MRSA and MRSP if used in the absence of effective cleaning. Taken together, the studies of this thesis paint a compelling picture of the epidemiology of resistant staphylococci in the Australian veterinary setting: shedding light on questions that had previously been unanswered and raising questions that will pave the way for interesting studies in the future.

“Rivers know this: there is no hurry. We shall get there some day.”

— A.A. Milne

Chapter 1. Introduction

1.1 Antimicrobial resistance and the *Staphylococcus* genus

Alexander Fleming's 1929 report of an 'antibacterial substance' (1) marked the start of an arms race between manmade antimicrobial drugs and bacterial superbugs. Since Fleming first described how he serendipitously killed staphylococci with penicillin (1), the *Staphylococcus* genus, with its arsenal of virulence factors and refined host adaptation, has been a key target of antimicrobial drug development. Despite their close association with humans and animals as resident flora, *Staphylococcus* spp. are among the most common bacterial pathogens. They are adept at creating opportunistic infections that continually challenge clinicians, compounded by the ongoing emergence of antimicrobial resistance. The following thesis will explore the epidemiology of this important pathogen in veterinary medicine, with particular reference to antimicrobial-resistant staphylococci in Australian animals.

Staphylococcus spp. are Gram-positive, catalase-positive, facultatively anaerobic bacterial cocci that are generally viewed as commensals, with skin and mucous membrane carriage being common amongst healthy humans and animals (2-8). *Staphylococcus* spp. are typically adapted to one or two host species. Humans are naturally carriers of *S. aureus* (2), while *S. pseudintermedius* is considered a commensal in dogs (3, 5), and cats are the natural host of *S. felis* (9, 10). Molecular studies have suggested a genetic basis for this host-specificity (11) and host specific markers are thought to exist. These markers mostly pertain to evasion of the host immune system (11-15). The coagulase test, which detects the presence of clotting within plasma, has traditionally been used to delineate pathogenic from less pathogenic staphylococcal species (16). Although coagulase-negative staphylococci are regarded as less pathogenic, their role as significant opportunistic pathogens in

humans was clearly demonstrated when a large multi-centre study found coagulase-negative staphylococci to be the foremost cause (31%) of nosocomial bloodstream infections in the USA (17). Coagulase-negative staphylococci are also common pathogens in bovine mastitis (18) and they are accordingly now considered potential veterinary pathogens.

Over 50 *Staphylococcus* spp. have been identified (19) and while *Staphylococcus aureus* is considered the chief coagulase-positive spp. in human medicine, several other *Staphylococcus* spp. are potential pathogens for both humans and animals (6, 20-26). Staphylococci are generally found in one or two host species, but asymptomatic carriage and infection can occur across the species divide (27-35). In veterinary medicine, *Staphylococcus* spp. are responsible for a wide range of diseases including canine pyoderma, bovine mastitis and pyogenic infections in virtually every body system of any mammal, reflecting their role as opportunistic pathogens that capitalise on host compromise to allow tissue invasion (36, 37). While staphylococcal infections were once relatively simple to treat with the provision of systemic or topical antimicrobials, management of staphylococcal infections has been greatly complicated by the rise of antimicrobial resistance in both human and veterinary medicine (38-40).

This literature review introduces the key themes of this thesis, focusing on the veterinary staphylococcal species investigated within the thesis, the virulence factors that allow *Staphylococcus* spp. to create disease and the epidemiology of antimicrobial-resistant staphylococci in veterinary medicine.

1.2 *Staphylococcus* spp. of veterinary significance

1.2.1 *Staphylococcus aureus*

In 1881, Alexander Ogston (41) published a report with several elegant hand-drawn diagrams in which ‘micrococcus’, a round micro-organism, could be observed in samples from various purulent infection sites in humans. Ogston isolated this round micro-organism from 65 of 85 human abscesses and found

that when purulent discharge from these abscesses was injected into mice and guinea pigs, the animals developed similar abscesses from which the organism could also be isolated (41). Having fulfilled Koch's postulates, Ogston concluded that while 'micrococcus' (later renamed *Staphylococcus aureus*) could be an 'innocent organism', it could also 'under suitable conditions… give rise to blood-poisoning, to acute inflammation, and to suppuration' (41). *Staphylococcus aureus* was thus the first staphylococcal species to be described, and clinical reports have appeared in the literature since the late 19th century (41-43).

A defining feature of *S. aureus* is its gold pigmentation, which is due to carotenoid pigments that actually confer a survival advantage by acting as antioxidants against neutrophil oxidative burst (44). *S. aureus* is a commensal of the skin and mucous membranes of humans, with the anterior nares being the most common site of carriage (8). Carriage rates vary between study populations but a median nasal carriage rate of 37.2% amongst the general human population was reported in a meta-analysis that examined 18 studies (45). It is a common pathogen of both humans and animals and, true to the first report by Ogston (41), can cause suppurative disease in any organ (46).

Although *S. aureus* can potentially cause disease in any animal, it is a particularly prevalent and economically important pathogen in bovine mastitis (47-49). It is also a common cause of wound infections in companion animals (dogs, cats and horses) (35) and can be carried asymptomatically by pigs (32, 50). Due to its significance as a human pathogen, the scientific literature and general media often debate the extent to which animals are a reservoir for *S. aureus* infections in humans (32, 51-53). To aid rational debate about the subject, several studies have focussed on determining markers to differentiate human-adapted from livestock-adapted strains *S. aureus* (15, 54). To further investigate the epidemiology of *S. aureus* at the human-animal interface, Chapter 3 examines the phylogeny and variety of host adaptation markers amongst a collection of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from Australian animals and veterinarians and Chapter 5

describes a screening study of veterinarians and their pets for concurrent carriage of MRSA and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP).

1.2.2 *Staphylococcus pseudintermedius*

Hajek *et al.* (1976) (6) first described *S. intermedius* as a newly identified coagulase-positive species that had previously been misidentified as *S. aureus.* This new species could be differentiated from *S. aureus* phenotypically because it was unpigmented, did not produce acetoin and did not ferment maltose (6). Several surveys then reported that *S. intermedius* was the predominant *Staphylococcus* spp. in dogs and cats (3, 5, 36). Two decades later, Devriese *et al.* (20) found a cluster of four isolates from a dog, cat, horse and parrot that were distinct from other isolates previously described as *S. intermedius*. DNA-DNA hybridisation confirmed that the four isolates represented a novel species called *S. pseudintermedius* – so named because of its close phylogenetic relationship to *S. intermedius*. It was also closely related to *S. delphini*, a coagulase-positive species originally isolated from dolphins (55). Sasaki *et al.* (56) also used DNA-DNA hybridisation to confirm the status of *S. pseudintermedius* as a novel species, closely related to *S. intermedius* and *S. delphini,* and recommended that the three species be grouped as '*Staphylococcus intermedius* group (SIG)' because they could only be differentiated through advanced phenotypic tests and molecular tests such as DNA-DNA hybridisation. Sasaki *et* al. (56) analysed 117 isolates previously described as *S. intermedius* and found that all isolates from dogs, cats and humans were in fact *S. pseudintermedius*. Bannoehr *et al.* (57) then used multilocus sequence typing (MLST) to analyse 99 isolates previously described as *S. intermedius* and confirmed that *S. pseudintermedius*, not *S. intermedius,* was the most common cause of canine pyoderma. As a result, subsequent studies generally refer to SIG isolates from dogs as *S. pseudintermedius* and it can be assumed that most, if not all, older articles that refer to *S. intermedius* from dogs are in fact discussing *S. pseudintermedius*.

Staphylococcus pseudintermedius is normal flora of the skin and mucous membrane for most dogs and some cats (3-5, 58). The high frequency of *S. pseudintermedius* carriage by dogs is similar to carriage

of *S. aureus* in healthy humans (2), although *S. pseudintermedius* carriage in dogs is more frequent than *S. aureus* carriage in humans (58). As well as being the most common *Staphylococcus* spp. isolated from healthy dogs, *S. pseudintermedius* is also the most common cause of staphylococcal infections in dogs (59, 60). A wide range of canine diseases are caused by *S. pseudintermedius*, but most studies concur that the skin is the most common site of *S. pseudintermedius* infection (particularly pyoderma), followed in various frequencies by otitis externa, cystitis, surgical site infections, and infections in the skeletal, reproductive and respiratory systems (22, 59-64). It is also an occasional zoonotic pathogen (30, 65, 66).

Although it can cause disease in humans, *S. pseudintermedius* is generally thought to be canine host-adapted. Simou *et al.* (67) provided evidence of host-specificity towards dogs by showing that *S. pseudintermedius* bound to a significantly higher percentage of canine corneocytes than human corneocytes, whereas *S. aureus* bound to significantly more human than canine corneocytes. While *S. pseudintermedius* is a common and important pathogen of dogs in its own right, the occurrence of methicillin-resistance amongst *S. pseudintermedius* has increased in significance firstly due to increased complexity in treating such infections in animals and secondly as a potential public health threat for humans. Chapter 4 outlines the epidemiology of *S. pseudintermedius* in Australia, focussing on the molecular epidemiology of clinical MRSP from dogs and cats. Chapter 5 discusses carriage of MRSP by Australian dogs while Chapter 7 describes investigations into biocide tolerance amongst veterinary-derived *S. pseudintermedius* isolates.

1.2.3 *Staphylococcus felis*

While *S. pseudintermedius* is the most common staphylococcal pathogen of dogs and is occasionally found in cats, *S. felis* is the resident staphylococcal species of cats. It was first identified as a new species in 1989, when Igimi *et al.* (68) characterised a group of 39 previously unidentified isolates of coagulase-negative staphylococci from cats and concluded that *S. felis*, a newly identified species, was the most common species amongst cats. The isolates had come from a range of clinical syndromes in

cats including otitis externa, cystitis, abscesses and other skin infections. The isolates were coagulase-negative when tested on rabbit plasma, and were weakly haemolytic on sheep blood agar. Previously, Devriese *et al.* (69) had examined 112 specimens from both healthy and diseased cats and concluded that *S. sciuri* was the most common staphylococcal species in cats. In the paper that first described *S. felis,* Igimi *et al*. (68) noted that *S. felis* was phenotypically similar to another coagulase-negative species, *S. simulans*, but the two could be differentiated by DNA-DNA hybridisation. Igimi *et al.* (70) subsequently examined a larger collection of 93 clinical specimens and found that *S. felis* was indeed the most commonly isolated species of *Staphylococcus* from cats. More recent studies have confirmed that *S. felis*, not *S. simulans* or *S. sciuri*, is the most common *Staphylococcus* spp. carried on the skin (9) and in the saliva (10) of healthy cats. It is also becoming increasingly recognised as a common pathogen of the feline urinary tract (71, 72), ear canals (73) and skin (74). Apart from the handful of studies described above (71-74), the literature on *S. felis* is scant. Consequently, Chapter 6 examines the molecular epidemiology and potential virulence factors of a collection of clinical *S. felis* isolates from Australian cats.

1.3 **Virulence factors of *Staphylococcus* spp.**

Detailed knowledge of the pathogenesis of staphylococcal infections is somewhat restricted, but investigation into virulence factors has provided important links between *Staphylococcus* spp., host immune responses and disease states. Staphylococcal infections are initiated when the skin or mucosal barrier is physically breached, or local defences are otherwise overwhelmed and the bacteria are allowed to penetrate into deeper tissues. Infections are typically characterised by pus formation due to enzymatic tissue liquefaction (46) and a propensity towards chronicity which is aided by intracellular survival (75). The majority of research into specific staphylococcal virulence factors relates to *S. aureus*, due to its importance as a human pathogen. There is nevertheless a growing body of evidence that other *Staphylococcus* spp. have virulence factors that are either homologous to those described in *S. aureus*, or unique to the different staphylococcal species. Staphylococcal virulence factors are

generally divided into four categories: exoenzymes, exotoxins, adhesins and others (76, 77). This can be more broadly divided into cell wall and membrane components and secreted virulence factors.

1.3.1 Cell wall and membrane components

The Gram-positive cell wall of *Staphylococcus* is composed of a thick peptidoglycan layer interwoven with teichoic acids, transmembrane lipoteichoic acids and proteins (78, 79). Each component of the staphylococcal cell can play a dual role in cell functioning and virulence. The functions of cell wall components include evading host immune defences (80, 81), aiding adhesion to host tissues (82, 83), conferring antimicrobial resistance (84), surviving in adverse conditions (85) and modulating the host inflammatory response (86, 87). One example of a cell wall-associated protein with several functions is staphylococcal autolysin (*Atl*), which functions as an autolytic enzyme during bacterial cell division but can also facilitate binding to host tissues (88, 89). The staphylococcal protein A (SpA) is a cell wall-associated protein that is well conserved in the *Staphylococcus* genome, although it demonstrates sequence variation (90). SpA, encoded by the *spa* gene, is involved with non-host specific binding to IgG from a range of animal species (91, 92). Binding of SpA to host IgG helps *Staphylococcus* evade host defences by interfering with complement fixation and phagocytosis (81). The reliable presence of the *spa* gene in the *Staphylococcus* genome has proven useful to researchers, as *spa* typing is now a common way of classifying *S. aureus* (93) and *S. pseudintermedius* (90). Clumping factor is another cell wall-associated protein that plays a role in adhesion and host specificity by mediating binding to host fibrinogen (94, 95). Clumping factor A (*ClfA*) is thought to aid adherence to host cells (96) and may play a role in host specificity, as genetic analysis can divide *S. aureus* isolates into human and bovine groups based on *ClfA* gene expression (97). The presence or absence of clumping factor is the basis of the slide agglutination test, which is a common phenotypic method of differentiating *Staphylococcus* isolates.

Some *Staphylococcus* spp. isolates produce biofilm, a particularly important virulence factor for surgical implant infections. Biofilm is characterised by clumps of bacteria bound together by a matrix of peptides and polysaccharide adhesion molecules (98). Christensen *et al*. (99) found that *S.*

epidermidis isolates that made biofilm were significantly more likely to cause intravenous catheter infections in mice than those without biofilm production. Tojo *et al.* (100) further demonstrated that an integral part of biofilm is a capsular polysaccharide adhesin molecule. This adhesin facilitates cell to cell contact between bacteria, which allows formation of the large bacterial clusters that characterise a biofilm (101). In addition to aiding cell tocell adhesion in biofilms, capsular polysaccharides help evade host immunity by protecting against phagocytosis by neutrophils (102). Biofilm production appears to be an essential virulence factor for implant infections by coagulase-negative species such as *Staphylococcus epidermidis* (99, 103, 104), but studies have shown that *S. aureus* (103) and *S. pseudintermedius* also readily produce biofilm (105). Bacterial cells deep within the biofilm are less metabolically active, so staphylococcal biofilms can be intrinsically resistant to antimicrobials simply because the bacteria are not undergoing the cell division that is required for some antimicrobials to be effective (98). Given that biofilm production is a multifactorial process, it is not surprising that there are several genes and cell wall-associated proteins involved. Biofilm-associated surface protein, encoded by the *bap* gene, is one such protein that facilitates staphylococcal adhesion to inert surfaces and intercellular adhesion (106). The *ica* operon is also involved in several aspects of biofilm production, particularly in the production of the glucosaminoglycan-based matrix (107). In addition to biofilm production, many *Staphylococcus* spp. are resistant to lysozyme (108), which is a bactericidal enzyme intended to attack the peptidoglycan layer of the bacterial cell wall and is produced by polymorphonuclear cell granules, intestinal cells and bodily secretions of mammalian hosts (109-111). Bera *et al.* (80) found that O-acetylation of the peptidoglycan layer, aided by a membrane-bound protein called *OatA,* was the major determinant of lysozyme resistance in *S. aureus.*

Several of the aforementioned cell wall-associated proteins can be antigenic, but staphylococci can counter this by producing an extracellular polysaccharide capsule that masks surface antigens and thus aids in evasion of host immunity (112). The capsule of other bacterial species is thought to impede phagocytosis and this is likely true for *Staphylococcus* spp. too, although variation within and between studies has made this difficult to prove (113). Capsular serotyping was initially used as a potential

measure of virulence amongst *S. aureus* isolates (114, 115), but this is not a common procedure due to the variable effects of culture conditions on capsule production.

Studies investigating staphylococcal virulence factors give the best insight into staphylococcal disease when both phenotype and genotype are examined. Nevertheless, genome screening for genes that encode putative virulence factors is a useful place to start (76, 77, 116). In Chapter 6, a collection of *S. felis* isolates from cats were screened for genetic markers of biofilm formation, lysozyme resistance and capsule production.

1.3.2 Secreted virulence factors

Exotoxins are bacterial products (generally proteins) that damage or kill host cells while exoenzymes are extracellularly secreted enzymes that degrade host tissues for the purpose of bacterial digestion. Staphylococcal exotoxins include haemolysins, cytotoxins, exfoliative toxins and enterotoxins. Exfoliative toxins are generally involved in dermatoses such as impetigo and exudative dermatitis. One example of an exfoliative toxin is EXI in *S. pseudintermedius,* which is encoded by the *exi* gene. The EXI toxin of *S. pseudintermedius* causes host-specific degradation of canine desmoglein 1 (Dsg1), which is a desmosomal adhesion molecule of mammalian skin (117). Desmoglein breakdown causes epidermal splitting and this manifests as blister formation. Degradation of Dsg1 by homologous exfoliative toxins has been demonstrated in *S. pseudintermedius* bullous impetigo in dogs (117), *S. hyicus* exudative epidermitis in pigs (118) and *S. aureus* scalded skin syndrome in humans (119).

Compared to exfoliative toxins, staphylococcal enterotoxins are generally less host specific in their activity. Enterotoxins are so named because they induce emesis in humans after oral ingestion (120). Some staphylococcal enterotoxins are also described as superantigens because they can activate T-lymphocytes, causing massive cytokine release that is far in excess of what would be induced by a normal antigen (121-124). In addition to their role as superantigens in diseases such as toxic shock syndrome, enterotoxins are frequently associated with human food poisoning outbreaks caused by *S.*

aureus (125). Dairy products are a major source of staphylococcal enterotoxin in human food poisoning outbreaks (125, 126), so it is not surprising that most veterinary studies into enterotoxins relate to bovine mastitis. In contrast to their emetic effects in humans, enterotoxins are thought to play an immunomodulatory role in bovine mastitis by disrupting T lymphocytes (127). This T lymphocyte disruption may aid in the intracellular survival that is intrinsic to the pathogenesis of staphylococcal mastitis (127). Rall *et al.* (128) found that the gene encoding staphylococcal enterotoxin A (*sea*) was the most common enterotoxin gene in a herd that included clinically healthy cows and cows with mastitis in Brazil. Despite the *sea* gene being uniformly present in *Staphylococcus* isolates from all cows, isolates from cows with mastitis were more likely to produce the *sea* toxin than isolates from healthy cows, further indicating that *sea* expression and enterotoxin production play a role in mastitis pathogenesis. In Chapter 3, a human-derived *sea* gene sequence was used as one of the markers of host adaptation in a collection of MRSA from humans and animals.

Haemolysins are toxic to both leucocytes and erythrocytes while bicomponent leukocidins/leukotoxins such as Panton-Valentine leukocidin mainly target leucocytes and epithelial cells (129, 130). The presence of haemolysin can usually be detected by visible haemolysis on blood agar plates, making haemolysis detection a useful phenotypic test in differentiating haemolytic from non-haemolytic staphylococcal species. Leukocidins cause cell death by adhering to host cells and creating pores in their cell membrane (131). Panton-Valentine leukocidin (PVL) is produced by some *S. aureus* isolates from animals and humans but it does not appear to be produced by any other *Staphylococcus* spp. (56, 132, 133). While PVL is uncommon in *S. aureus* strains overall, it is very commonly found in those strains associated with primary necrotic skin lesions in adult humans and community-acquired necrotising pneumonia in children (129). PVL has been found in clinical MRSA isolates from companion animals and transmission of a PVL-positive MRSA strain has been demonstrated between a healthy dog and its owner who had recurrent skin infections (134). Due to its association with serious infections in immune-competent humans, PVL is thought to be an important virulence factor, so epidemiological investigations often include PVL screening in their categorization of *S. aureus* isolates

(56, 133). The MRSA isolates examined in Chapters 3 and 5 were consequently screened for PVL and other leukotoxins while *S. felis* isolates in Chapter 6 were screened for leukotoxin homologues.

Coagulase is a pro-inflammatory enzyme that activates host prothrombin and causes clotting of host plasma and is implicated in abscess formation by *S. aureus* (16, 135). Coagulase production is variable amongst staphylococcal species and is generally associated with heightened virulence (16, 136). Whether an isolate was positive for coagulase or not was once considered the major determinant of its pathogenicity but it is now widely accepted that both coagulase-positive and -negative species can be pathogenic (17, 68). *S. felis* is a coagulase-negative species of cats that has received very little attention in the literature despite its predominance in feline clinical disease. Chapter 6 investigates several virulence factors in *S. felis* including coagulase production, and found that feline host-specific coagulase activity can be present amongst *S. felis* isolates.

1.4 Methicillin-resistance in *Staphylococcus* spp.

The sheer speed at which bacteria can replicate and mutate has meant that the discovery of novel antimicrobials is inevitably met with a bacterial counterattack using mechanisms that confer antimicrobial resistance. Patients treated in early penicillin trials showed seemingly miraculous recoveries (137) and the use of penicillin to treat war wounds undoubtedly reduced mortalities in the second half of World War II (138). However, shortly after penicillin began to be used on a mass scale in 1944, penicillin-resistant strains of *S. aureus* were reported (139). As a β-lactam, penicillin kills bacteria by disrupting the layering of peptidoglycans during cell wall synthesis (140). Kirby (139) found that the mode of staphylococcal β-lactam resistance was due to a mobile genetic element which has since been characterised as the plasmid-encoded enzyme, β-lactamase (141).

To circumvent β-lactamase producing staphylococci, methicillin was introduced into human medicine around 1960 (142) as a novel β–lactam antimicrobial. Due to a modified structure compared to penicillin, methicillin is stable against enzymatic degradation by β-lactamase (142). Just as penicillin

resistance was reported shortly after it began to be used clinically, the introduction of methicillin into human hospitals was closely followed by reports of phenotypic methicillin-resistance amongst clinical *S. aureus* isolates (38). It was another two decades before Hartman and Tomasz (143) found that methicillin-resistance was conferred by a modified penicillin binding protein (penicillin binding protein 2a (PBP2a)) that had low binding affinity to methicillin and other β-lactams. PBP2a is a functional protein involved in crosslinking of the peptidoglycan cell wall, but has decreased affinity to all β-lactams so that it can continue to perform its function even in the presence of β-lactams (144, 145). PBP2a is encoded by the *mecA* gene and its homologues (146, 147). *Mec* genes are carried in a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*) (148, 149). SCC*mec* cassettes can harbour other resistance genes (150), which is one reason why methicillin-resistant isolates are often multidrug-resistant (151). The original source of the *mecA* gene was likely a naturally occurring homologous gene amongst the ubiquitous coagulase-negative species, *S. sciuri,* which moved into coagulase-positive species via horizontal gene transfer (152, 153). Antimicrobial use in human and veterinary medicine, particularly in food producing animals, then selected for *mecA* positive isolates, leading to a dramatic rise in the prevalence of methicillin-resistant staphylococci (152, 154).

Since the early reports of methicillin-resistance amongst *S. aureus* isolates, the frequency of MRSA has steadily increased in human and veterinary medicine. Prevalence rates of MRSA now vary between epidemiological settings, making it difficult to draw direct comparisons between studies. For example, one study reported that in 2016 the rate of MRSA amongst *S. aureus* bacteraemia in Australian human hospitals ranged from 11% in Tasmania to 47.4% in the Northern Territory (155) while a 2015 study reported a 38% MRSA prevalence amongst *S. aureus* bacteraemia cases in Taiwan (156). The difference in prevalence is multifactorial and is likely influenced by varying study methodologies, geographic variations in antimicrobial use, population density and the dominant strains of MRSA that are circulating within a community. Although prevalence rates of MRSA in human medicine vary, a common finding is that the presence of methicillin-resistance amongst *S. aureus* infections is always

associated with significantly higher mortality compared to methicillin-susceptible infections (155, 157). Unlike human medicine, it is not yet clear whether methicillin-resistance amongst *S. aureus* infections in animals has a negative impact upon their survival. Nevertheless, the public health significance of MRSA amongst companion and food producing animals is the source of much debate. MRSA was first reported in bovine mastitis in the 1970's (158) and it continues to be detected at low frequencies in surveillance studies overseas (18, 159, 160). Similar Australian surveillance studies are lacking, and the unique restrictions on antimicrobial usage in Australian food producing animals (161) means that the results of overseas studies cannot be applied to an Australian setting. In addition to being a bovine mastitis pathogen, MRSA is a common cause of wound infections in companion animals (35). To establish a baseline for future epidemiological studies into MRSA in the Australian veterinary setting, Chapter 3 describes the first nationwide surveillance study that examined the frequency of MRSA in clinical isolates from Australian animals. The prevalence of methicillin-resistant *S. pseudintermedius* (MRSP) was first reported in the late 1990's (38) and, like *S. aureus*, the prevalence of methicillin-resistance amongst *S. pseudintermedius* has risen steadily since that time. The frequency of MRSP similarly varies between reports (59, 162-165), but frequencies of up to 60% of canine *S. pseudintermedius* pyoderma cases have been reported outside Australia (166). Chapter 4 describes the first Australian surveillance study into MRSP amongst clinical veterinary isolates.

Today, methicillin is not used as a therapeutic because of its acid intolerance, instability in storage (167), and its parenteral-only administration (168). However, 'methicillin-resistance' has remained as a term used to describe bacteria with resistance to β–lactams conferred by *mecA* and its homologues. It was recognised soon after the discovery of methicillin-resistance that test conditions such as temperature and antimicrobial disk choice could affect the ability of phenotypic tests to detect methicillin-resistance (169-171). Oxacillin disks were found to be more stable than methicillin (172), and oxacillin is now used as a proxy to detect methicillin-resistant in *S. pseudintermedius* and several other staphylococcal species (173, 174). However, oxacillin is unreliable for *S. aureus* disk diffusion testing because is a poor inducer of *mecA* mediated resistance and it can fail to recognise phenotypic

resistance in heterogeneous MRSA populations that contain *mecA*-positive and -negative isolates (175, 176). Cefoxitin is consequently used to detect methicillin-resistance in *S. aureus* (173, 174, 176). Methicillin-resistance can still be heterogeneously expressed, so gold standard diagnosis requires identification of the *mecA* gene and homologues (40) or demonstration of the PBP2a protein by latex agglutination (171). Beyond clinical diagnosis, useful epidemiological information is gleaned by strain typing, as described in Section 1.5.

1.5 Diagnosis and characterisation of *Staphylococcus* spp.

Identification of *Staphylococcus* infections to a genus level is not difficult, as the organism grows well in a range of conditions. Genus identification is readily achieved with basic phenotypic tests such as Gram staining and the catalase test, as outlined in Section 2.2. Identification to the species level is more challenging and requires a range of more advanced phenotypic tests or more costly but more accurate molecular tests such as matrix assisted laser desorption ionisation- time of flight mass spectrometry (MALDI-TOF MS), outlined below.

1.5.1 Matrix-assisted laser desorption/ionisation- time of flight (MALDI-TOF MS) mass spectrometry

Matrix assisted laser desorption ionisation- time of flight mass spectrometry (MALDI-TOF MS) is a relatively simple molecular method of characterizing *Staphylococcus* isolates to the genus and species level (177-179). This technique analyses the protein constituents of the cell wall of each isolate and produces a unique dendrogram based on the size and 'time of flight' of protein particles when they are hit with a laser (177). Only a small amount of sample is needed (one colony) and samples can be applied directly from an agar plate. It is a quick and accurate method of differentiation between similar *Staphylococcus* spp. (for example, the SIG members) (178). Due to its speed, accuracy and relative cost effectiveness, MALDI-TOF MS was used to confirm the species of most of the isolates examined in this thesis.

1.5.2 Strain typing

As molecular technology and bioinformatics have advanced, so too has the degree of discrimination that can be achieved within staphylococcal species. Defining a staphylococcal isolate to its strain or 'clone' level provides useful information about the possible epidemiological origin of the isolate. This information is useful both on a broad scale, such as national and international surveillance studies which track movement of important pathogens around the world, and on smaller scales such as clinical settings where infection control may be improved if the origin of the isolate is known. Several methods of strain typing *Staphylococcus* have been developed, as outlined below.

1.5.2.1 Pulsed field gel electrophoresis

Gel electrophoresis is a common technique in molecular biology where fragments of DNA, RNA or protein are run across an electrically charged gel. The distance that each fragment moves through the gel is governed by its size and electric charge, so that smaller fragments typically move further than large fragments. Pulsed field gel electrophoresis (PFGE) was once a major tool in investigating outbreaks of staphylococcal related disease (180, 181). The high discriminatory ability of PFGE allowed researchers to detect minute changes in the *Staphylococcus* genome which allows tracking of strains within outbreak settings. The downside of PFGE is that it requires multiple technical steps that can be performed several different ways, making it difficult to directly compare results between laboratories and thus hindering efforts to track epidemic strains across different geographic areas. Furthermore, the lack of a standardised protocol for *S. pseudintermedius* makes it difficult to compare results between laboratories. Given its limited utility for epidemiological studies, particularly for *S. pseudintermedius*, PFGE was not used as a typing method for any of the studies in this thesis.

1.5.2.2 Multilocus sequence typing

Multilocus sequence typing (MLST) was first described in 1998 (182) as a more convenient and transferable mode of characterising bacterial isolates than PFGE. MLST uses gene sequencing to compare the alleles of short fragments of several highly conserved genes. A curated online database

is used to designate an isolate as a particular allelic profile or 'sequence type' based on its allele pattern for each gene (182). Suitable genes for MLST schemes are those that are highly conserved and therefore reliably present in every isolate but which demonstrate enough allelic variability that isolates may be differentiated based on their allelic profile for the set of genes (182, 183). The use of sequencing allows comparison between laboratories because sequence types can be grouped on computer, stored as a file and subsequently compared across the internet (182, 183). The output of MLST has been variably referred to as allelic profile, sequence type, strain or clone but from herein this thesis will refer to the output of MLST as sequence type or clone.

MLST provides investigators with useful epidemiological information because clusters of closely related strains can be identified and inferences made about which strains predominate in an outbreak situation or particular geographic area (57, 183, 184). The first MLST scheme for *S. aureus* was developed in 2000 (183) and schemes have since been developed for *S. pseudintermedius* (57, 185) and *S. epidermidis* (186). The epidemiological utility of MLST was exemplified when Perreten *et al.* (184) used MLST to analyse MRSP isolates from around the world and identified two major strains of MRSP: ST71 in Europe and ST68 in USA. The strains were geographically separate and genotypically different, yet the isolates all had methicillin-resistance conferred by *mecA* gene, indicating that *mecA* acquisition occurred simultaneously in at least two geographically separate clones. These clones then spread widely across each region. The changes in house keeper gene alleles as detected by MLST accumulate slower than the changes in protein expression detected by PFGE (183). Therefore PFGE remains useful to analyse microvariations in active outbreak investigation, whereas MLST is useful in assessing macrovariations across larger epidemiological investigations (57, 184, 187). MLST was used as a typing methods for several studies in this thesis, as outlined in Chapters 3, 4, 5 and 6.

1.5.2.2.1 Spa typing

Spa typing is a single locus typing method that compares alleles of the ubiquitous staphylococcal protein A gene. First developed for *S. aureus* in 2004 (187), *spa* typing has overtaken PFGE as a highly

discriminatory method of typing *S. aureus* (93). A separate *spa* typing protocol has also been developed for *S. pseudintermedius* (90). Both protocols analyse polymorphisms of region X of the *spa* gene. With only one gene analysed and a computerised output, *spa* typing is less time consuming and easier to interpret than PFGE. It has similar (90) or lower discriminatory ability than PFGE (184) but higher discriminatory ability than MLST, in that isolates from a single MLST sequence type can have different *spa* types (90). Like MLST, its computerised format makes it easier to compare results across studies than PFGE (62). Although many *S. pseudintermedius* isolates can be typed this way, not all *S. pseudintermedius* lineages contain a homologue of the *spa* gene and thus cannot be typed using this method (188). Consequently, in Chapters 3, 4 and 5, *spa* typing of isolates was complemented by a second highly discriminatory typing method, *dru* typing.

1.5.2.3 **Direct repeat unit (*dru*) typing**

Most MRSA and many MRSP isolates harbour a cluster of 40 base pair (bp) direct repeat sequences within the SCC*mec* cassette, termed the direct repeat unit (*dru*) (189). Analysis of *dru* sequence variation was the basis of a PCR-based typing method reported by Goering *et* al. (190) in 2008. The authors examined 104 closely related clinical MRSA isolates from human hospitals that had previously been divided into just two PFGE types. *Dru* typing further discriminated 25 *dru* types amongst the 104 isolates, highlighting the epidemiological utility of *dru* typing in a hospital setting where analysis of microvariation is integral to outbreak investigation (190). One advantage that *dru* typing has over *spa* typing is that *dru* types are carried within the SCC*mec* cassette, so the same *dru* typing method can be applied to any staphylococcal species that harbours a *mec* cassette. As a result, an online database exists that can be used to type any SCC*mec*-positive isolate, provided that researchers have obtained the *dru* sequence by PCR or whole genome sequencing. However, like the *spa* gene, not all isolates harbour a direct repeat unit (190). Consequently, Chapters 3, 4 and 5 used both *spa* and *dru* typing as discriminatory typing methods to more accurately characterise isolates that could not be typed by one of the two methods.

1.5.2.4 **SCC*mec* typing**

SCC*mec* typing provides information about the genetic origin of an isolate's methicillin-resistance and its ecological niche. Methicillin-resistance is conferred by an apparently unique mobile genetic element that carries the *mecA* gene, called the staphylococcal cassette chromosome (SCC*mec*) (148, 149). As well as carrying the *mecA* gene, SCC*mec* carries regulatory genes called cassette chromosome recombinase genes (*ccr*) that enable transfer of the element between bacteria (148), although their exact role in SCC*mec* transmission is yet to be determined. SCC*mec* typing uses DNA sequencing to analyse polymorphisms in the *mec* and *ccr* genes (191, 192). In 2009, the International Working Group on the Classification of the Staphylococcal Cassette Chromosome Elements (IWG-SCC) described eight recognised SCC*mec* types in MRSA (193). As typing and PCR methods develop, more SCC*mec* types are being discovered and there are currently at least 10 recognised SCC*mec* types (194).

Most MRSP isolates of animal origin can be SCC*mec* typed using the same methodology as described for MRSA of human origin, but occasionally the isolates cannot be typed because of unrecognised combinations of *mec* and *ccr* genes (184, 191, 192, 195). Similar to MLST, it appears that SCC*mec* types may vary according to geographic location. SCC*mec* type V was the most common MRSP in dogs in USA (195), while types II and III were most common in Europe (184). The utility of SCC*mec* typing in public health epidemiology was shown when researchers in Korea SCC*mec* typed MRSA from bovine mastitis isolates and compared these to the SCC*mec* types from an outbreak community acquired MRSA (CA-MRSA) in humans (160). They found the two populations had different SCC*mec* types, thereby showing that the mastitis milk was not likely to be the source of the CA-MRSA outbreak.

Although the SCC*mec* element was initially thought to be the only method of widespread *mecA* acquisition in staphylococci, Perreten *et al.* (188) found pseudo-SCC*mec* elements in several MRSP isolates. The pseudo-SCC*mec* element of MRSP ST45 described by Perreten *et al.* (2013) contains the *mecA* gene positioned adjacent to *orfX,* which is universally located close to SCC*mec* insertion sites, but does not contain any recombinase genes. This suggests that *ccr* genes are not an integral part in

all mobile genetic elements that confer methicillin-resistance(196). As recombinase genes comprise one of the key components of SCC*mec* typing outlined by the IWG-SCC (193), SCC*mec* elements without *ccr* genes cannot be typed using traditional SCC*mec* methods. As a result, several pseudo-*SCCmec* elements have now been reported (188, 197, 198) and it is clear that *mec* acquisition is not yet fully understood. Nevertheless, SCC*mec* typing can yield useful epidemiological information and was therefore used in several studies in this thesis. Chapter 4 outlines the diversity of SCC*mec* elements amongst Australian MRSP and describes a novel pseudo-SCC*mec* element in a cluster of MRSP from Melbourne, Victoria.

1.6 **Transmission of staphylococci in veterinary medicine**

With the rise in methicillin-resistance amongst human and veterinary-associated staphylococci, there has been increased focus on the role that animals play in the transmission of resistant bacteria. More research has been conducted into the epidemiology of *S. aureus* and MRSA in humans than *Staphylococcus* spp. in the veterinary setting, and it is unclear to what extent the epidemiology of veterinary staphylococci mirrors the situation in human medicine. Around 30% of humans carry *S. aureus* (45) and these patients are generally more likely to develop nosocomial staphylococcal bacteraemia than patients who are not *S. aureus* carriers (199). Furthermore, patients who carry MRSA are more likely to develop nosocomial infections such as MRSA bacteraemia and surgical site infections than patients who harbour methicillin-susceptible *S. aureus* (MSSA) (199, 200). Isolates from infections are generally identical to that carried by the patient, suggesting that at least some nosocomial *S. aureus* and MRSA infections are endogenous (201, 202).

Analogous to MRSA carriage being a risk factor for development of certain nosocomial infections in humans, MRSP carriage was recently identified as a risk factor for development of post-operative infections in 549 canine patients undergoing tibial plateau levelling osteotomy surgery (203). The *dru* types of MRSP from infection sites were almost always the same as those carried by each dog pre-operatively, again suggesting that the infections were endogenous (203). Other risk factors for MRSP

infections include a history of antibiotic use, chronic skin disease, prolonged hospitalisation and frequent vet visits (204-206). By far the most common pathogen in dogs is *S. pseudintermedius*, but infections with *S. aureus* do occur (60, 207). Soares Magalhaes *et al.* (207) found that prolonged hospitalisation and antibiotic use increased the likelihood of a *S. aureus* infection being methicillin-resistant. In addition, they found that contact with a human who had been unwell in the last two months also increased the risk of an MRSA infection. Dogs have been shown to share *S. aureus* clones with their owners, and are more likely to carry *S. aureus* if their owner is a health care worker (208). Most studies find that MRSA infections in companion animals are likely of human origin (209, 210), but there is also evidence to suggest that zoonotic transfer of MRSA and MRSP can also occur from animals to humans. A recurrent MRSA surgical site infection in a diabetic human patient was only eradicated once MRSA carriage in a pet dog was eliminated by treatment with vancomycin nasal ointment (52). Contact with pigs is a clear risk factor for MRSA carriage in piggery workers and pig veterinarians (211, 212). Veterinarians treating dogs, cats and horses are also at increased risk of MRSA and MRSP carriage compared to pet owners and veterinarians who don't treat these animals (29, 213-215). It is not yet known if contact with animals alone or additional risk factors are responsible for the increased rate of MRSA and MRSP carriage seen in companion animal veterinarians. In this context, Chapter 5 describes a pilot study that examined MRS carriage amongst 46 veterinary personnel in Sydney, Australia.

It is clear that staphylococci are shared between humans and animals, but much remains to be determined about staphylococcal transmission between humans and animals. Chapter 3 describes how MRSA carried by Australian veterinarians is related to MRSA infections in Australian animals, while Chapter 5 examines the transmission of MRSP and MRSA amongst the veterinary personnel, their pets and patients in two Sydney veterinary hospitals. In order for the true significance of MRSP carriage to be accurately assessed, establishing an estimate of local baseline prevalence was required. Therefore, Chapter 5 also describes a screening study that examined the frequency of MRSP amongst 151 canine hospital admissions in Sydney, Australia.

1.7 Biocide tolerance in antimicrobial-resistant *Staphylococcus* spp.

History has repeatedly demonstrated that the development and use of new antimicrobials creates selection pressure, resulting in the development of new modes of antimicrobial resistance amongst bacteria. The inevitable effect of selection pressure has led human and animal physicians to seek alternative methods of infectious disease management besides antibiotics, such as topical treatment and infection prevention measures (216). Topical treatments such as chlorhexidine-based shampoo can be effective primary or adjunct treatment for various types of pyoderma involving MRSP in dogs without the need for systemic antimicrobials (217, 218). In light of this, recently published international treatment guidelines recommend anti-staphylococcal topical therapies as the treatment of choice for superficial or localised pyoderma involving methicillin-resistant staphylococci (219). In addition to their role as topical therapeutics, biocides such as chlorhexidine are also useful in curbing the spread of resistant infections. This is exemplified by the success of chlorhexidine-based hand hygiene programs in human hospitals that have been shown to significantly reduce the incidence of nosocomial MRSA bacteraemia (220, 221). Chlorhexidine is also recommended as a pre-operative patient and surgeon cleaning agent in veterinary surgical textbooks and publications (222, 223).

Cationic biocides such as chlorhexidine and benzalkonium chloride exert their effect by disrupting the negatively-charged outer layers of the cell (224). Theoretically, such physical bombardment requires a complex counterattack from bacteria, making it unlikely that rapid genetic change such as a point mutation would result in phenotypic resistance to biocides. Biocides may consequently appear to be an attractive alternative to systemic antimicrobials because of the decreased selection pressure that they exert. However, newer research has questioned the overuse of biocides for a number of reasons including the possibility that their use may actually select for antimicrobial resistance (216, 225). Efflux pumps can extrude toxic substances such as biocides from the bacterial cell. The quaternary ammonium compound (QAC) family of proteins are a group of efflux pumps implicated in extrusion of cationic quaternary ammonium compounds such as benzalkonium chloride, a common veterinary

biocide (226, 227). QAC proteins are encoded by *qac* genes and these genes have been associated with increased *in vitro* tolerance to benzalkonium chloride and chlorhexidine (226, 227). The presence of *qac* genes appears to confer a selective advantage to certain MRSA clones within human hospitals (225), but the significance of *qac* genes amongst veterinary staphylococci such as MRSP was previously unknown. Consequently, Chapter 7 describes a study that screened 125 veterinary staphylococci for *qac* genes and investigated the *in vitro* efficacy of chlorhexidine and benzalkonium chlorhexidine.

1.8 **Scope and aims of this thesis**

The high profile of *Staphylococcus* as a human pathogen has meant that there is already a large body of research into the epidemiology of staphylococcal disease. It is also a significant veterinary pathogen, and veterinary literature has not lagged far behind human medicine in reports focussed on staphylococci. Despite this large body of work, much remains to investigate in the Australian veterinary setting. By conducting a surveillance study in 2013, the research in this thesis first sought to establish baseline data about the frequency and molecular epidemiology of MRSA and MRSP in Australian animals, the foremost methicillin-resistant species in veterinary medicine. Given that *Staphylococcus* spp. clearly circulate between humans and animals, the studies in this thesis also sought to shed light on staphylococcal epidemiology at the human-animal interface. The 2013 surveillance study yielded a convenient collection of *S. felis* isolates, which afforded the opportunity for a thorough investigation into this little-understood pathogen of cats. As methicillin-resistant and multidrug-resistant staphylococci continue to emerge in veterinary medicine, we will likely need to draw on additional infection control measures besides systemic antimicrobials. It was consequently investigated whether two common veterinary biocides were still effective against veterinary MRSP and MRSA *in vitro.* Whole genome sequencing and bioinformatics were used extensively throughout all studies, producing a volume of typing and gene-screening data that would not have been possible just a few years before this thesis commenced.

Together, the five studies contained within this thesis aim to significantly increase the overall understanding of the epidemiology of staphylococci in the Australian veterinary setting and provide a suitable platform for ongoing studies.

Chapter 2. General materials and methods

2.1 *Staphylococcus* isolate acquisition and storage

Sample collection for this thesis was undertaken in several stages and used several different methodologies, as outlined below.

2.1.1 Surveillance isolate acquisition

Veterinary diagnostic laboratories (VDLs) from all states and territories of Australia were asked to participate in a nationwide surveillance study into antimicrobial resistance in coagulase-positive *Staphylococcus* spp. Isolates were collected from January 2013 to January 2014 from all 22 Australian VDLs. VDLs were located across all Australian states and territories (Figure 2.1). Inclusion criteria for sample submission to the surveillance study were: a) coagulase-positive staphylococci as determined by the VDLs' standard diagnostic testing and b) a clinically significant sample. 'Clinical significance' was determined by VDL microbiologists, who were asked to use their judgement of culture results (heavy and pure cultures) and clinical information provided by the veterinarian (animal signalment and clinical signs) to decide whether the staphylococcal isolate was the likely causative agent of disease for each submission.

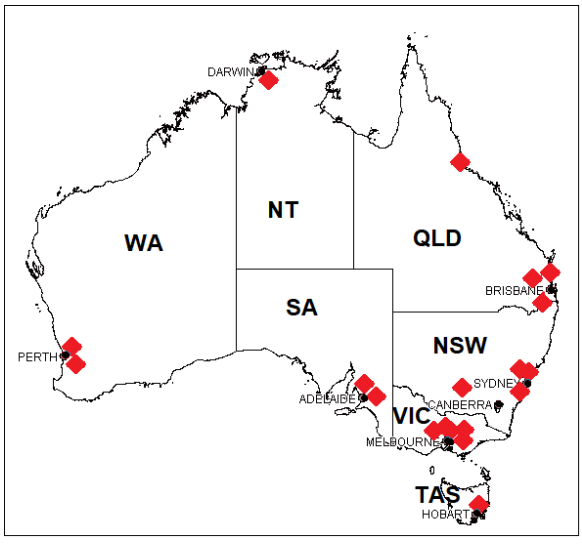


Figure 2-1. (pg 24) Distribution of Australian veterinary diagnostic laboratories (VDLs) that submitted staphylococcal isolates to this study. Red diamonds indicate VDLs.

Laboratories were asked to test the isolates as they normally would, but to also subculture and forward any coagulase-positive clinically significant *Staphylococcus* to the research team. Refrigerated isolates were sent on Amies™ Agar Gel transport swabs (Copan Diagnostics, USA) taken from 24hr culture plates. Apart from their post code of origin, the submitting veterinary clinics remained anonymous. Where available from the veterinarians' submission sheets, the following data was recorded for each isolate: the animal of origin (species, breed, sex, age), body system that the swab was taken from (skin/soft tissue, surgical site, respiratory, ear, ocular, bone/joint, genitourinary, mammary, other) and the disease process as recorded by the submitting veterinarian. When the research team received isolates from the laboratories, they were given a unique number based on their laboratory of origin. The isolates were then cryopreserved as outlined in 2.1.4.1**.**

2.1.2 Acquisition of methicillin-resistant *S. aureus* and *S. pseudintermedius* carriage isolates

From July 2015 to April 2017, healthy veterinary personnel, their respective pets and canine hospital admissions were screened for asymptomatic carriage of MRSA and MRSP as outlined below. Samples were taken from humans and animals associated with two veterinary hospitals in Sydney, NSW, Australia. The first hospital was a primary accession hospital located in an inner city suburb. The second hospital was a tertiary referral hospital located 10km west of the first hospital.

2.1.2.1 Isolation of *Staphylococcus* spp. from healthy veterinary personnel

Sampling of veterinary personnel and their pets (dogs and/or cats) was undertaken over a two week period in February 2017. Veterinary personnel included veterinarians and support staff (veterinary nurses, kennel hands and administrative staff). Personnel were invited to participate whether or not they had pets at home. Personnel were instructed to take samples from themselves in their own home by inserting a single sterile swab into the anterior nares. Personnel were provided with the necessary

sampling materials and were instructed to wear gloves during the procedure and to refrigerate samples after the procedure. Once collected, samples were forwarded to the researcher with 24hr for further processing. All swabs were collected using Amies™ Agar Gel swabs (Copan Diagnostics, USA). The study was approved by the University of Sydney Human Ethics Committee (Project 2016/837).

2.1.2.2 Isolation of Staphylococcus spp. from healthy dogs and cats owned by veterinary personnel

In addition to veterinary personnel collecting samples from themselves (see Section 2.1.2.1), they were also asked to collect samples from up to three of their pet dogs and/or cats. Personnel were instructed to take three separate swabs from their pet/s': 1) anterior nares (large dogs) or nasal planum (small dogs and cats); 2) oral cavity; and 3) perineum. Personnel were provided with the necessary sampling materials and were instructed to wear gloves during the procedure and to refrigerate samples immediately after the procedure. All swabs were again collected using Amies™ Agar Gel swabs (Copan Diagnostics, USA) and were sent to the researcher within 48hr of collection. This part of the study was approved by the University of Sydney Animal Ethics Committee (Project 2016/1072).

2.1.2.3 Isolation of *Staphylococcus* spp. from canine hospital admissions

Similar to veterinary-owned pet dogs, swabs were taken from the nares/nasal planum, oral cavity and perineum of canine hospital admissions to two veterinary hospitals in Sydney. Samples were taken within 10 min of admission to hospital and were taken with the owner's written consent. Swabs were refrigerated and forwarded for further processing by the researcher within 72hr of collection. Sample collection from hospital admissions was also approved by the University of Sydney Animal Ethics Committee (Project 2015/866).

2.1.3 Acquisition of *Staphylococcus* spp. from the veterinary hospital

Environmental sampling from a veterinary hospital in Sydney involved two sampling periods in October 2016 and February 2017. Both sampling expeditions occurred on a single day. Amies™ Gel agar swabs

that were pre-moistened with sterile saline were used for sampling. In order to detect bacteria with potential higher tolerance to low levels of veterinary biocides, residual disinfectants were not neutralised prior to environmental sampling. To take samples, pre-moistened swabs were gently rolled for five seconds across the following areas: 1) patient cages (one swab each for cage latch, cage floor, right cage wall and cage roof (where accessible without a ladder); 2) waiting room chairs (one pooled swab for the seat, back and right front leg of five chairs); 3) door handles (one pooled swab for each of the two door handles to the following areas: consultation room (n= two rooms), treatment room, radiology room, pharmacy and bathroom) 4) computer keyboards and mice (one pooled swab for the computer equipment in two consultation rooms, treatment room and radiology room) and one mobile telephone owned by a veterinary staff member. Samples were refrigerated prior to further processing.

2.1.4 Sample storage of prospectively collected samples

Samples collected in Sections 2.1.2 and 2.1.3 were refrigerated at 2-4°C on Amies™ Agar Gel swabs generally within 48 hr. If storage was required for greater than one week, they were subcultured onto 5% sheep blood agar (Oxoid, Hampshire) or tryptose agar (Difco, Franklin Lakes) and incubated at 37°C for 18-24 hr. Isolates were either processed directly after incubation or were refrigerated at 2-4°C for up to two months. Within two months of subculturing, all isolates underwent phenotypic testing as outlined in Section 2.2 and then underwent cryopreservation as follows.

2.1.4.1 Cryopreservation of *Staphylococcus* spp. isolates

Isolates from the aforementioned surveillance and carriage studies were prepared for storage at -80°C using a 2:1 mixture of brain heart infusion broth (BHIB):glycerol. BHIB was provided as a substrate and sterile 65% glycerol was used as the cryoprotectant. After overnight incubation at 37°C on sheep blood or tryptose agar, approximately 30-50 colonies were inoculated into the BHIB:glycerol mixture. The suspension was then thoroughly mixed using gentle pipetting. The isolates were prepared as duplicates and then transferred to two separate -80°C freezers. Sterile 1.8mL CryoPure tubes were used for storage (Sardsedt, Germany).

2.1.5 Freeze-dry storage and retrieval of archived *Staphylococcus* spp.

Chapter 7 examined biocide tolerance amongst a mixed collection of staphylococci that included an archival collection of MRSP. These isolates originated as submissions made to the Veterinary Pathology Diagnostic Service (VPDS) at the University of Sydney between 1952 and 2008 that had been freeze dried and stored by the Faculty of Veterinary Science. To prepare isolates for freeze drying, colonies of *Staphylococcus* isolates were grown on sloped plates of 5% sheep blood agar (Oxoid, Hampshire) or tryptose agar (Difco, Franklin Lakes) and incubated at 37°C for 18-24 hr. The agar slopes were then washed with sterile skim milk and the colonies were emulsified with the milk by gentle pipetting. Approximately 100µL of the mixture was placed into sterile glass ampoules before being snap frozen in dry ice and plugged with cotton wool. The plugged ampoules were freeze dried overnight and then vacuum sealed to create a small pellet of skim milk and bacteria at the base of the ampoule. Multiple identical vials were made for each bacterial isolate. The ampoules were stored at 4°C or at room temperature. To retrieve the isolates, the freeze dried plugs were resuspended in 100µL of sheep brain heart infusion broth and placed on tryptose agar. The isolates were incubated at 37°C for 18-24 hr. Following incubation, single colonies were subcultured onto 5% sheep blood agar and incubated at 37°C for 18-24 hr. The subcultured isolates underwent phenotypic tests to check for purity and contamination (Gram staining, catalase and coagulase (slide clumping factor and tube coagulase tests)). Several colonies of the subcultured isolates were then prepared for cryopreservation for future use, as described in 2.1.4.1.

2.2 Species determination of *Staphylococcus* isolates

2.2.1 Phenotypic tests

To confirm the species of staphylococci in all studies, isolates underwent phenotypic tests and matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS). All isolates were subcultured onto 5% sheep blood agar and incubated at 37°C for 18-24 hr prior to testing. Isolates were either inoculated directly from transport swabs, subcultured from storage agar plates or

retrieved from cryopreserved samples. Isolates underwent Gram staining, catalase testing and tube coagulase testing as outlined below. Gram-positive, catalase-positive isolates underwent MALDI-TOF MS as described in Section 2.2.2.

2.2.1.1 **Gram stain**

Hucker's modification of the Gram stain was performed as follows. A flamed loop was used to emulsify a small portion of a colony within a drop of water that had been placed on a glass slide. The emulsification was spread to a thin layer and allowed to air dry. Once dry, the slide was heat-fixed by briefly passing the slide through a Bunsen flame. The fixed slide was flooded with Hucker's Crystal Violet (Merck, Darmstadt) for 1 min, and then washed with water. The slide was flooded with Gram's iodine for 1 min and washed with water. Decolourisation with 95% ethanol was performed until the slide produced a clear run off. Safranin (Fluka, Castle Hill) was used to counterstain the slide for 10 sec. Safranin was washed off with water; the slide was blotted dry and examined under oil emersion at 1000X magnification.

2.2.1.2 **Catalase test**

The catalase test was used to differentiate *Staphylococcus* spp. from *Streptococcus* spp. A sterile platinum loop was used to emulsify a single colony into a droplet of 3% hydrogen peroxide on a glass slide. The presence of instant bubbling was indicated the presence of catalase (228).

2.2.1.3 **Tube coagulase test**

A sterile loop was used to colonies into a sterile test tube containing 0.3mL sterile EDTA rabbit plasma (Sigma Aldrich, USA). The tubes were then incubated at 37°C for 4 hrs, with visual checks at 1, 2 and 4 hrs. Formation of a clot in the rabbit plasma within the 4 hrs was deemed a positive result, and no further observation was undertaken if a positive result was received within 4 hrs. If the isolates had not produced a clot after 4 hrs of incubation, they were left at room temperature overnight. Samples were observed after 18-24 hr at room temperature. Samples that had produced a solid clot that did

not dislodge when the tube was inverted at 4 or 24 hr were deemed coagulase-positive, while those that produced no clot were coagulase-negative.

2.2.2 Matrix-assisted laser desorption/ionisation- time of flight (MALDI-TOF MS) mass spectrometry

The species of all Gram-positive, catalase-positive isolates was confirmed using matrix assisted laser desorption ionisation time of flight mass spectrometry, which was undertaken using the BD™ Bruker MALDI Biotyper in positive linear mode (m/z = 2000–20,000). Isolates were subcultured onto tryptose agar and incubated at 37°C overnight prior to testing. A small amount of a single colony of each pure culture was transferred to a FlexiMass™ target well using a disposable loop, overlaid with 0.5µL of 2,5-dihydroxybenzoic acid matrix solution (DHB; 10 mg/mL in acetonitrile/0.1% trifluoroacetic acid 1:1) and air-dehydrated within 1–2 min at 24–27C° before being placed in the spectrometer. If no dendrogram peaks were obtained in the first run, isolates were again transferred to the target but 1µL 20% formic acid was applied to the isolate prior to the matrix solution being applied. All isolates were run in duplicate and results with a confidence score above 1.8 were deemed acceptable for speciation. All *Staphylococcus* spp. underwent antimicrobial susceptibility testing as follows.

2.3 Antimicrobial susceptibility testing

All *Staphylococcus* spp. collected in the surveillance study (Chapters 3 and 4) underwent antimicrobial susceptibility testing by both manual broth microdilution and disk diffusion according to the Clinical Laboratory Standards Institute (CLSI) guidelines (173), as outlined in Sections 2.3.1 and 2.3.2. As previously described (229), when CLSI guidelines for animals were not available, human CLSI guidelines were used (174) or data was interpreted based on European Committee on Antimicrobial Susceptibility Testing guidelines and/or using ECOFFinder (230). Isolates were tested against 18 antimicrobials from nine drug categories. Rather than using antimicrobial 'classes' to classify organisms as multi-, extensive- or pan-drug resistant, we used the drug 'category' system previously described (231). This method of classification groups antimicrobials into broader 'categories' than the traditional system of

antimicrobial 'classes'. Magiorakos *et al.* (231) argued that the use of categories is more valid for epidemiological studies than the class system, as pathogens such as MRSA and MRSP are automatically multidrug-resistant when defined by the class system, due to their resistance to almost all β-lactam classes. The following antimicrobial categories (231) were tested, with the drugs from each category in parentheses: anti-staphylococcal β-lactams (oxacillin, cefoxitin, amoxicillin-clavulanic acid, cephalothin, cefovecin, ceftriaxone); aminoglycosides (gentamicin, amikacin); ansamycins (rifampicin); fluoroquinolones (enrofloxacin, ciprofloxacin, marbofloxacin, pradofloxacin); folate pathway inhibitors (trimethoprim-sulfamethoxazole); lincosamides (clindamycin); macrolides (erythromycin); phenicols (chloramphenicol) and tetracyclines (tetracycline). Most antimicrobials were tested by both disk diffusion and broth microdilution. However, for the macrolides, erythromycin (ERY) was tested by disk diffusion only. For the aminoglycosides, gentamicin (GEN) was tested by disk diffusion and amikacin (AMK) was tested by broth microdilution. Antimicrobials were obtained from Sigma Aldrich (Australia) and Zoetis (Australia). *S. aureus* ATCC 25923 and ATCC 29213 were used as quality control strains. Isolates defined as 'Intermediate' according to CLSI guidelines were defined as resistant in this study. *S. pseudintermedius* isolates were classified as phenotypically methicillin-resistant if their oxacillin MIC was ≥0.5mg/L and/or disk diffusion diameter ≤17mm. *S. aureus* isolates were classified as methicillin-resistant if cefoxitin resistant (MIC ≥ 8mg/L and/or disk diffusion diameter ≤21mm). Methicillin-resistance was confirmed by detection of the *mecA* or *mecC* gene (174) as determined by whole genome sequencing.

While Chapters 3 and 4 used the method described above, isolates in all other studies underwent automated broth microdilution antimicrobial susceptibility testing using Vitek 2^{TM} (bioMerieux, USA), according to the manufacturer's instructions.

2.3.1 Broth microdilution

The minimum inhibitory concentration (MIC) of antimicrobials was determined using the broth microdilution according to the CLSI guidelines (173, 174, 229). Antimicrobials were obtained from

Sigma Aldrich (USA) and Zoetis (Australia). *S. aureus* ATCC 25923 and ATCC 29213 were used as control strains. MICs were performed in cation-adjusted Mueller-Hinton broth (Becton, Dickinson and Company, USA) and serially diluted antimicrobial agents. MIC was defined as the lowest antimicrobial concentration that completely inhibited bacterial growth and was determined after 16-20 hr incubation at 35°C.

2.3.2 Disk diffusion

Antimicrobial disk diffusions was undertaken using the direct colony suspension method according to the CLSI guidelines for bacteria isolated from animals (173). Prior to testing, isolates were subcultured onto tryptose agar and incubated at 37°C overnight. Three to five colonies were then suspended in sterile saline and inoculum density was adjusted until suspension turbidity visually matched the 0.5 McFarland standard (approximately 1.5 x 10^8 CFU/mL). The suspension was inoculated onto 100mm Mueller-Hinton agar plates using sterile cotton swabs to streak the entire plate. Antimicrobial disks were then placed onto the Mueller-Hinton agar plates using a commercial disk applicator device (BD, Sparks). Three or four disks were placed on each plate. The plates were incubated at 37°C for 18-24 hr after which the zones of inhibition were viewed and measured. All disks were BD brand (BD BBL™ Sensi-Disk™, Sparks) except cefovecin (Oxoid, Basingstoke). All phenotypically methicillin-resistant *S. aureus* and *S. pseudintermedius* underwent whole genome sequencing and *in silico* analysis, as well as all *S. felis* isolates collected in the surveillance study.

2.4 Whole genome sequencing

Whole genome sequencing was used as an analytic tool in every study of this thesis. DNA was extracted from staphylococcal samples using the Qiagen DNA MiniAmp kit (Qiagen, Valencia, USA), with modifications. To prepare for DNA extraction, isolates were subcultured onto tryptose agar and incubated overnight at 37°C. Between four to eight colonies were then inoculated into 10mL sterile plastic tubes containing a 6mL lemcose broth and incubated for a further 18-36 hr at 37°C. Cell pellets were prepared from the broth by centrifuging the tubes at 8000xrpm for 10min. The supernatant was

removed except for 50µl liquid, then the cell pellet and liquid were transferred to 1.5mL microcentrifuge tubes before being centrifuged a second time at 8000xrpm for 5 min. Cell pellets were then frozen at -20°C. To achieve cell wall lysis, frozen cell pellets were resuspended in 180µL lysozyme 200mg/mL (Sigma Aldrich, USA) and incubated at 37°C for 1 hr. DNA extraction then continued following the Qiagen DNA MiniAmp protocol for Gram-positive bacteria. DNA yield and purity (A_{260}/A_{280}) were measured using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Delaware, USA). Only DNA samples with a A_{260}/A_{280} purity ratio over 1.8 were used for further analysis. DNA extractions with lower purity ratios underwent repeat extraction. DNA concentration was verified using the QuBit dsDNA kit (Thermo Scientific, Delaware, USA) and standardised to 0.2ng/µl by dilution with sterile distilled water. DNA library preparation was then performed according to the manufacturer's instructions of a commercial DNA library preparation kit (Nextera, Illumina, San Diego, USA). Whole genome sequencing was performed using the NextSeq 500 Desktop Sequencer, according to the manufacturer's instructions (Illumina, San Diego, USA). Whole genome sequencing yielded 250bp paired-end reads as fasta and fastq files.

2.5 *In silico* analysis

The molecular epidemiology of sequenced isolates was determined largely by *in silico* analysis. *De novo* assembly of Illumina sequence files was carried out using CLC Genomics Workbench computer software under default settings (Qiagen, Valencia, USA). When the following basic local alignment search tool (BLAST) analyses were performed, sequences required at least 90% homology to the reference sequence unless otherwise stated.

2.5.1 Genomic confirmation of species and methicillin-resistance

Confirmation of staphylococcal species was determined by identification of the species-specific thermonuclease (*nuc*) gene in assembled contigs. This was achieved by downloading the *nuc* genes of *S. aureus*, *S. pseudintermedius* and *S. felis* from the National Centre for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov; accession numbers: GU129659.1, AB327164.1 and

AB465335.1 respectively). A local BLAST database of *nuc* sequences was created and blasted against *de novo* contigs. BLAST results required >99% homology with the respective *nuc* sequence to be deemed a particular species. Detection of *mecA* and its homologues was similarly performed by downloading the relevant sequences and creating a local BLAST database of *mecA*, *mecB* and *mecC* (Accession numbers: CP003166.1, NG_047953.1 and FR821779.1 respectively).

2.5.2 Multilocus sequence typing

MecA or *mecC*-positive *S. aureus* and *S. pseudintermedius* isolates from the surveillance study underwent manual MLST, while isolates from all other studies underwent automated MLST using an online bioinformatics platform. For manual MLST, a local BLAST database was created in CLC Genomics Workbench by downloading the alleles listed from the MLST databases for *S. aureus* (http://saureus.mLst.net) and *S. pseudintermedius* (https://pubmLst.org/spseudintermedius/). As per the relevant databases, the seven loci used for MLST were *arcC, aroE, glpF, gmk, pta, tpi* and *yqiL* for *S. aureus* and *ack, cpn60, fdh, pta, purA, sar* and *tuf* for *S. pseudintermedius.* Downloaded MLST alleles were blasted against *de novo* assembled contigs. Isolates were assigned an MLST type by entering allelic profiles into the respective MLST websites for *S. aureus* and *S. pseudintermedius*. The previously described seven allele MLST schemes were used *S. aureus* (183) and *S. pseudintermedius* (185). In 2016, an online bioinformatics platform became available in Australia that was capable of providing the MLST of *S. aureus* and *S. pseudintermedius* (https://cge.cbs.dtu.dk/services/MLST/) (232). Consequently, MRSP and MRSA from all studies except the surveillance study underwent MLST using this platform. *De novo* contigs were loaded onto the respective MRSA and MRSP online MLST platforms, which provided the allelic profile and multilocus sequence type of each isolate.

2.5.3 SCC*mec* typing

SCC*mec* typing was performed by downloading the *mec* gene complex and *ccr* elements of SCC*mec* from the NCBI online database (http://www.ncbi.nlm.nih.gov/), according to the SCC*mec* elements described by the International Working Group on the Staphylococcal Cassette Chromosome (193). A

local BLAST database was created and SCC*mec* element sequences were then blasted against *de novo* contigs.

2.5.4 *Spa* typing

For MRSA isolates, *spa* types were obtained by uploading fasta files onto the online bioinformatics platform https://cge.cbs.dtu.dk/services/spatyper/ (233). For MRSP, no online *spa* typing platform currently exists. Consequently, manual *spa* typing was performed on fastq files using CLC Genomics Workbench. *Spa* repeat codes described for *S. pseudintermedius* (90) were downloaded from the *spa* typing website, http://www.pse-spa.org/. As contigs from *de novo* assembly tend to cleave at repeat regions, 250bp fastq raw read files were analysed. Fastq files were searched for *spa* repeat codes and neighbouring regions were searched for subsequent *spa* repeats. *Spa* types were assigned according to *spa* repeat sequence profiles described by Moodley and colleagues (90).

2.5.5 *Dru* typing

Similar to *spa* typing in *S. pseudintermedius,* no online *dru* typing platform for MRSA or MRSP currently exists. Consequently, *dru* typing was performed manually using CLC Genomics Workbench. *Dru* repeats were downloaded from the *dru* typing website, http://dru-typing.org/site/ (190) A local BLAST database of *dru* repeats was made and contigs were examined to determine order of *dru* repeats, starting with the 5a repeat sequence. Contig segments required 100% homology with a repeat sequence to be deemed positive for that *dru* repeat. *Dru* types were assigned by entering the *dru* repeat succession onto the *dru* typing website.

2.5.6 Resistance and virulence gene screening

Isolates were screened for virulence and/or resistance genes in several of the studies in this thesis. To achieve this, the nucleotide sequences of antimicrobial resistance and resistance genes were downloaded from the open-access bioinformatics websites, ResFinder (https://cge.cbs.dtu.dk//services/ResFinder/) (234) and VirulenceFinder

(https://cge.cbs.dtu.dk/services/VirulenceFinder/) (235) respectively. A BLAST database was created in CLC Genomics Workbench and the study sequences were then blasted against *de novo* contains. Most studies also screened for additional elements not contained within the ResFinder and VirulenceFinder databases, the details of which can be found in each of the following chapters.

Chapter 3. Molecular characterisation of methicillin-resistant *Staphylococcus aureus* isolated from Australian animals and veterinarians

The content of this Chapter is published in two journal articles, as follows:

Worthing KA, Coombs GW, Pang S, Abraham S, Saputra S, Trott DJ, Jordan D, Wong HS, Abraham RJ, Norris JM. **(2016)** Isolation of *mecC* MRSA in Australia. *Journal of Antimicrobial Chemotherapy* **71**, 2348-2349

Worthing KA, Abraham S, Pang S, Coombs GW, Saputra S, Jordan D, Wong HS, Abraham RJ, Trott DJ, Norris JM. **(2017)** Molecular characterisation of methicillin-resistant *Staphylococcus aureus* isolated from Australian animals and veterinarians. *Microbial drug resistance:* Doi: https://doi.org/10.1089/mdr.2017.0032 (accepted and online June 17th, 2017)

3.1 Abstract

This study aimed to determine the frequency and molecular epidemiology of methicillin-resistant *Staphylococcus aureus* (MRSA) from Australian animals and to determine whether animal-derived MRSA was similar to that from Australian veterinarians. A total of 1080 coagulase-positive clinical *Staphylococcus* isolates from Australian animals were collected during 2013. Sixteen (4%) of 360 *S. aureus* isolates were MRSA. Most MRSA came from companion animals, while none came from the 203 dairy cattle examined. MRSA isolates were characterised using whole genome sequencing. ST22-IV (EMRSA-15) was the most common clone in dogs and cats. CC8 was most common in horses. Most ST22-IV isolates were resistant to ciprofloxacin. Animal-derived MRSA genomes were interrogated for the presence of host-specific genetic markers (*scn, chp, sak, sea* and *vwb*). A subset of MRSA genomes previously collected from Australian veterinarians was also interrogated. There was no clear pattern in the distribution of host-specific markers amongst animal and veterinarian isolates. Animal and

veterinarian-derived MRSA were intermingled in the phylogenetic tree. The absence of MRSA in Australian dairy cattle is in contrast with its presence in dairy cattle from other countries. Possible explanations include Australia's geographic isolation, the absence of live animal importation into Australia and the restrictions placed on the use of antimicrobials of critical importance in Australian livestock.

3.2 Introduction

Methicillin-resistance has been detected in several staphylococcal species, but methicillin-resistant *S. aureus* (MRSA) is the foremost *Staphylococcus* species in human medicine. Since its first isolation in the 1960s (38), MRSA has become a major nosocomial pathogen in human hospitals around the world and is a common cause of community-acquired skin and soft tissue infections (39). MRSA is also a veterinary pathogen, with a recent study from Germany noting an MRSA frequency of 52% amongst canine, equine and feline *S. aureus* wound infections (35). In other European countries, Asia and the Americas, MRSA has also been identified in bovine mastitis samples, food-producing animals and companion animals (32-34, 236, 237). There is evidence that animals may act as a reservoir for MRSA in humans via contamination of the food chain (238) or via transmission between humans, pets (52) and farm animals (32). However, the epidemiology of MRSA in veterinary medicine is yet to be fully elucidated.

S. aureus is thought to readily adapt to various host species, and several studies have investigated specific markers for host adaptation (11-15). Five potential markers have been described, all located on mobile genetic elements. They include the staphylokinase gene (*scn*), staphylococcal complement inhibitor gene (*sak*), chemotaxis-inhibiting proteins gene *(chp)*, enteroxin A gene (*sea*) and Von Willebrand Factor binding protein gene (*vwb*). The presence of bacteriophage-mediated *scn, sak, chp* and *sea* are thought to be markers of adaptation towards humans because of their specificity to the human immune cascade (11, 12, 14, 239). *vwb* is located both in the core genome and on mobile *S.*

aureus pathogenicity islands (SaPIs) (15). Variation of SaPI *vwb* alleles is thought to enable *S. aureus* adaption towards ruminants or horses by facilitating host-specific coagulation activity (15).

MRSA in animals appears to have a defined clonal distribution, with certain MRSA clones found in particular populations of animals and in their human contacts. For example, the international epidemic healthcare-associated ST22-IV clone, colloquially known as EMRSA-15, is the most frequently isolated MRSA clone in dogs and cats (33-35, 210) and in their veterinarians (210, 214, 240). Australian equine veterinarians are more likely than other veterinarians to carry clonal complex 8 MRSA (240), an MRSA clonal complex (CC) which has also been reported in Australian horses (241). In the Australian porcine industry, CC398 MRSA, typically deemed a livestock-associated lineage associated with pigs (32), has recently been isolated at low frequency in Australian pigs (242) and from an Australian pig veterinarian (240). It appears that there is an association between human contact with certain animal species and the likelihood of not only carrying MRSA, but of carrying a particular clone of MRSA.

Several studies have documented the presence of MRSA in animals and veterinarians (210, 238) but only sporadic reports of MRSA in animals in Australia exist (241, 242), and the frequency and molecular epidemiology of MRSA in Australian animals is currently unknown. Australia is geographically isolated from the rest of the world, does not import livestock and has unique antimicrobial prescribing regulations for food producing animals. These differences make Australia an interesting comparison to previous surveillance reports from the rest of the world. Previous studies have used genetic markers of host specificity to infer the origin of staphylococcal isolates (11, 12, 14, 15, 239), but no study has specifically searched for these markers in veterinarian and animal isolates. Consequently, the aims of this study were to 1) determine the frequency of MRSA amongst clinical submissions of *Staphylococcus* spp. from animals in Australia; 2) determine the molecular epidemiology of MRSA from clinical disease in Australian animals; and 3) determine whether the presence or absence of host immune adaptation markers amongst isolates from Australian animals and veterinarians could elucidate patterns of host adaptation in MRSA isolates.

3.3 Materials and methods

3.3.1 Sample acquisition and speciation

Animal-derived *Staphylococcus aureus* isolates were collected during the first national Australian survey into antimicrobial-resistant coagulase-positive staphylococci (CPS) in animals (229). Samples were collected from January 2013 to January 2014 from all 22 Australian veterinary diagnostic laboratories (VDLs) as outlined in Section 2.1.1. *S. aureus* isolates were identified by routine phenotypic laboratory methods and by the BD™ Bruker MALDI Biotyper™, as per Section 2.2.2. The genomic sequences of nineteen human-derived MRSA were also examined, originating from a previously reported collection of carriage samples retrieved from the anterior nares of Australian veterinarians in 2009 (213, 240). The 19 veterinarian-derived MRSA isolates had previously been sequenced to determine their MLST type, and were deemed to be representative of a larger collection of 44 isolates, as described in the report by Grove and colleagues (240).

3.3.2 Phenotypic antimicrobial resistance profiling

The phenotypic antimicrobial resistance profile of each animal-derived isolate was determined by broth micro-dilution and disk diffusion, according to the CLSI guidelines as outlined in Section 2.3. For disk diffusion, erythromycin was additionally used and gentamicin was used instead of amikacin as an aminoglycoside. If there was discrepancy between broth micro-dilution and disk diffusion results, assignment was made based on the presence or absence relevant resistance genes. Genetic resistance markers were not used for rifampicin and ciprofloxacin, so the micro-dilution result was used in preference to disk diffusion to determine the phenotypic resistance status for these antimicrobials. An isolate was classified as methicillin-resistant if phenotypically cefoxitin-resistant and it harboured the *mecA* or *mecC* gene in whole genome sequenced data.

3.3.3 Whole genome sequencing

Whole genome sequencing was performed on all phenotypically methicillin-resistant CPS. DNA was extracted from overnight lemcose broth cultures using the Qiagen DNA MiniAmp kit, according to the manufacturer's instructions for Gram-positive bacteria (Qiagen, Valencia, USA) as described in Section 2.4. DNA library preparation was performed using the Nextera XT DNA genomic library preparation kit (Illumina, San Diego, USA). Whole genome sequencing was performed using the MiSeq System according to the manufacturer's instructions (Illumina, San Diego, USA). *De novo* and reference assembly was performed using CLC Genomics Workbench computer software (CLCbio, Qiagen, USA).

3.3.4 *In silico* analysis

Molecular typing and resistance/virulence gene detection was performed on whole genome sequence data using the bioinformatics program, CLC Genomics Workbench (CLCbio, Qiagen, USA). MLST, SCC*mec*-, *spa-,* and *dru* typing were undertaken as described in Section 2.5. Resistance and virulence gene screening was also undertaken as per Section 2.5.6. In addition to resistance and virulence genes, MRSA isolates were also screened for immune evasion genes. Human immune evasion genes were downloaded from the open-access bioinformatics websites, (https://cge.cbs.dtu.dk/services/VirulenceFinder/) (235). The nucleotide sequence for *chp*, an immune evasion cluster gene not included in the Virulence Finder database, was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov; accession number: AF285146.1). Similarly, the sequences of animal-specific variant *vwb* alleles (15) were downloaded from the NCBI database (Accession numbers: HM211303, HM228919 and HM228920 respectively.)

3.3.5 Phylogenetic tree construction

A phylogenetic tree was generated using the online bioinformatics program, CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) (243). Briefly, this program used a Maximum Likelihood algorithm to depict inferred phylogeny based on concatenated alignment of high quality single nucleotide polymorphisms. To construct the tree, fasta files of all genomes were upload to the

online platform, which aligned them to a reference genome (EMRSA-15, accession number: HE681097). FigTree 1.4.3 was used to optimise visualisation of the phylogenetic tree.

3.4 **Results**

3.4.1 Proportion of MRSA amongst clinical veterinary submissions

A total of 1080 unique CPS isolates were collected during the study. Consecutive samples from the same animal were initially received in the study, but only unique isolates from each animal were included in sample analysis. Isolates were identified as *S. pseudintermedius* (n= 669), *S. aureus* (n= 360), *S. schleiferi* (n= 44), *S. hyicus* (n= 3), *S. intermedius* (n= 2) and *S. delphini* (n= 2). Of the 360 *S. aureus*, 203 (56%) came from bovine mastitis samples. The remaining *S. aureus* were isolated from horses (n=53), dogs (n=48), cats (n=18), birds (n=5), pigs (n=3), rabbits (n=2), goats (n= 2), and one isolate each from a kangaroo, sheep, wallaby and guinea pig. Animal species was not recorded for 22 *S. aureus* isolates. Overall, 4% (16/360) of *S. aureus* were MRSA (Table 3.1).

Table 3-1. Methicillin-susceptible (MSSA) and resistant Staphylococcus aureus (MRSA) submissions as a proportion of 360 S. aureus isolated from animals in Australia, 2013-2014.

Staphylococcus aureus isolates (%)	Bovine	Equine	Canine	Feline	Porcine	Other	Total
MSSA	203 (100%)	47 (88%)	42 (87%)	15 (83%)	3 (100%)	34 (97%)	345 (96%)
MRSA	0	6 (12%)	6 (13%)	3 (17%)	0	1 (3%)	16 (4%)
Total	203	53	48	18	3	35	360

Fifteen of the MRSA isolates came from companion animals (six horses, six dogs and three cats). The remaining isolate came from a kangaroo. The proportion of methicillin-resistance amongst the 119 companion animal *S. aureus* isolates was 13%. None of the 203 bovine *S. aureus* were methicillin-

resistant. Fourteen of the 16 MRSA isolates came from different post codes, suggesting MRSA in Australian animals is geographically dispersed. Two equine isolates, both ST8-IV, came from a single postcode and thus likely originated from the same veterinary clinic. Amongst the remaining 720 CPS isolates, methicillin-resistance was only detected in *S. pseudintermedius* (Chapter 4).

3.4.2 Molecular epidemiology of animal-derived MRSA

The molecular epidemiology of the 16 MRSA isolates is shown in Table 3.2. The most frequently identified MRSA clone was ST22-IV (n=8), isolated from four dogs, two cats, a horse and a kangaroo. Apart from a single ST22-IV isolate, all equine MRSA were CC8 (ST612-IV and ST8-IV). Fifteen of the sixteen isolates harboured a type IV SCC*mec* element. The final isolate, ST425 from a cat, harboured a type XI SCC*mec* cassette, as detailed below (244). The most frequently identified *spa* types were four t379 (associated with ST22-IV) and four t064 (associated with ST612-IV/ST8-IV). A variety of *dru* types were identified. Two isolates, ST93-IV and ST30-IV both from dogs, were PVL positive. The phenotypic and genotypic antimicrobial resistance profile for the animal derived MRSA is shown in Table 3.2. The genomic sequences from all MRSA isolates have been deposited as biosamples at GenBank, under the accession numbers SAMN04537353 (KW2: ST425 *mec*C from a cat) and SAMN0654665 to SAMN06546698 (all other samples).

3.4.3 First detection of *mec*C MRSA in Australia

While 15 of the 16 MRSA isolates harboured the *mecA* gene, one harboured a *mecC* gene. The *mecC* isolate was collected in February 2013 from the mandible of a five year old domestic cat located in a semi-urban area of Melbourne, Victoria. The isolate had an oxacillin and cefoxitin MIC of 2 and 32 mg/L respectively. The isolate was tested using the PBP2a latex agglutination test according to manufacturer's instructions (Oxoid, Thermo Scientific, Basingstoke, UK) and was negative. Although resistant to β-lactams, the isolate was susceptible to all other antimicrobials tested including ciprofloxacin, marbofloxacin, pradofloxacin, enrofloxacin, tetracycline, chloramphenicol, rifampicin and trimethoprim-sulfamethoxazole. *MecA* was not detected in whole genome sequence data. The

isolate was characterised as MLST ST425 with an SCC*mec* type XI element. *De novo* contigs were blasted against a SCC*mec* type XI reference (*mecC* MRSA LGA251, GenBank accession number: FR821779.1) (147), which allowed identification of the *mecC* type 8 *ccr* (*ccrA1* and *ccrB3*) genes. The *mecC* gene had 96% and 100% sequence similarity to the *mecC* described in *S aureus* LGA251 (147) and *S. sciuri* GVGS2 (245) respectively.

3.4.4 Phylogenetic analysis

Phylogenetic tree construction showed clustering of MRSA isolates according to MLST type, with animal isolates sharing clustering with veterinarian isolates (Figure 3.1). All ST22-IV isolates, including those from both animals and veterinarians, clustered within a single clade. Equine- and human-derived ST8-IV and ST612-IV isolates also clustered within the CC8 same clade. ST425-XI, ST30-IV and ST93-IV were distantly related to each other and to the other isolates.

3.4.5 Host-specific adaptation markers amongst animal- and veterinarian-derived MRSA isolates

Eleven animal-derived MRSA isolates harboured at least one human immune evasion complex (IEC) gene. None of the animal-derived isolates possessed the ruminant or equine-associated variant of *vwb* (Table 3.3). Meanwhile, 15 of the 19 veterinarian-derived MRSA isolates harboured IEC-associated genes and one isolate (ST398) harboured the ruminant-associated variant of the *vwb* gene, *vwb-sb04*. The ST398 MRSA was isolated from a pig veterinarian (240), and did not harbour any human IEC-associated genes.

Table 3-2. Genotypic and phenotypic characterisation of 16 methicillin-resistant S*taphylococcus aureus* isolates from animals in Australia, 2013-2014

Isolate	Vet clinic region	Origin	MLST	SCC*mec*	*Spa* type	*Dru* type	Resistance phenotype	Resistance genotype
KW8	QLD	Cat, skin	ST22	IV	t379	dt8h	FOX, CIP	blaZ, mecA
KW1	Unknown	Cat, skin	ST22	IV	t3457	dt10j	FOX, CIP	blaZ, mecA
KW4	NT	Dog, surgical site	ST22	IV	t379	dt10a	FOX, CIP	blaZ, mecA
KW12	QLD	Dog, surgical site	ST22	IV	t790	dt9ah	FOX, CIP	blaZ, mecA
KW3	QLD	Dog, surgical site	ST22	IV	t845	dt9ah	FOX, CIP	blaZ, mecA
KW13	Vic	Dog, surgical site	ST22	IV	t2122	dt10e	FOX, CIP	blaZ, mecA
KW6	NSW	Kangaroo, surgical site	ST22	IV	t379	dt10a	FOX, CIP	blaZ, mecA
KW5	NSW	Horse, corneal ulcer	ST22	IV	t1302	dt10az	FOX, CIP, ERY	blaZ, mecA, ermC
KW10	Vic	Horse, skin	ST612	IV	t064	dt7d	FOX, ERY, TET, CHL, SXT, RIF, GEN	blaZ, mecA, ermC, tetK, cat(pC221,) dfrA, aac(6')-aph(2'')
KW11	NSW	Horse, joint	ST612	IV	t723	dt7d	FOX, TET, SXT, RIF, GEN	blaZ, mecA, tetK, dfrA, aac(6')-aph(2'')
KW16	Unknown	Horse, catheter site	ST612	IV	t064	dt9z	FOX, TET, SXT, RIF, GEN	blaZ, mecA, tetK, dfrA
KW7	NSW	Horse, catheter site	ST8	IV	t064	ND	FOX, ERY, TET, SXT, RIF, GEN	blaZ, mecA, ermC, tetK, dfrA, aac(6')-aph(2''), aadD
KW9	NSW	Horse, surgical site	ST8	IV	t064	dt10g	FOX, ERY, TET, SXT, RIF, GEN	blaZ, mecA,ermC, dfrA, aac(6')-aph(2''), aadD
KW14	NT	Dog, skin	ST93	IV	t10656	dt9ah	FOX	blaZ, mecA
KW15	Vic	Dog, surgical site	ST30	IV	t019	dt10m	FOX	mecA
KW2	Vic	Cat, mandible	ST425	XI	t6292	ND	FOX	blaZ, mecC

ND, determined. Vic= Victoria, NSW= New South Wales, QLD= Queensland, NT= Northern Territory. FOX= cefoxitin, ERY= erythromycin, TET= tetracycline, SXT= trimethoprim-sulfamethoxazole, RIF= rifampicin, GEN= gentamicin, CHL= chloramphenicol

Figure 3-1. (Page 47). Phylogenetic tree of animal- and veterinarian-derived methicillin-resistant Staphylococcus aureus isolates, generated using a Maximum Likelihood algorithm constructed by CSIPhylogeny 1.4 and optimised using FigTree v1.4.3. Length of branches indicates the approximate number of single nucleotide polymorphisms (SNPs). EMRSA-15 (ST22, accession number: HE681097) was used as the reference genome. Shapes indicate the species of animal from which the MRSA was isolated, or the species of animal treated by the veterinarian. Square= horse, circle= dog or cat, diamond= pig, star= kangaroo, triangle= mixed practice. Hollow shapes indicate isolates from veterinarians, black shapes indicate isolates from animals.

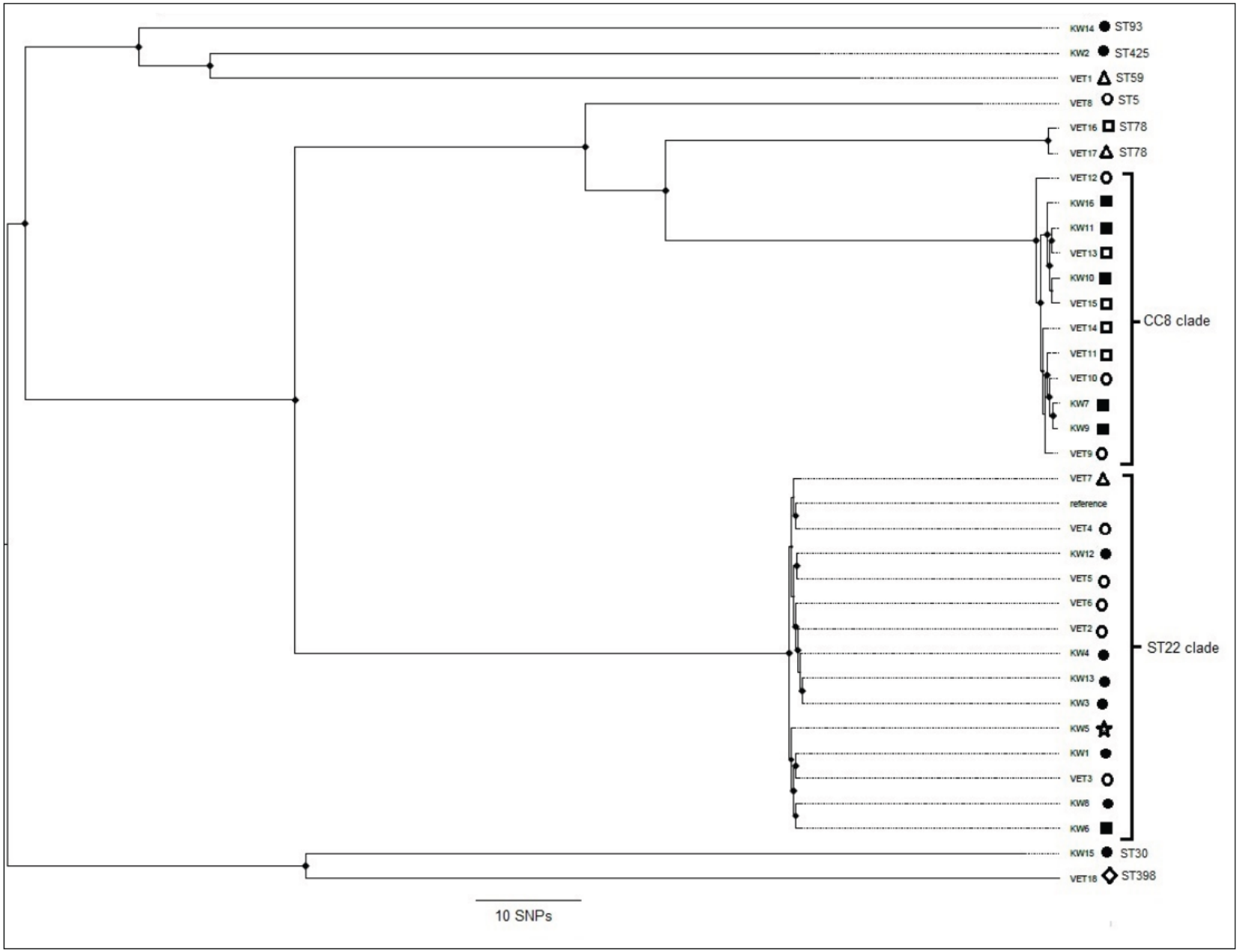

KW14 ST93
KW2 ST425
VET1 ST59
VET8 ST5
VET16 ST78
VET17 ST78
VET12
KW16
KW11
VET13
KW10
VET15
VET14
VET11
VET10
KW7
KW9
VET9
CC8 clade
VET7
reference
VET4
KW12
VET5
VET6
VET2
KW4
KW13
KW3
KW5
KW1
VET3
KW8
KW6
ST22 clade
KW15 ST30
VET18 ST398
10 SNPs

Table 3-3. Host-specificity markers in methicillin-resistant Staphylococcus aureus isolates from animals and veterinarians in Australia

Isolate	Origin	MLST	Human immune evasion cluster genes	Animal variant *vwb* gene
KW1	Cat	ST22	0	0
KW8	Cat	ST22	0	0
KW2	Cat	ST425	0	0
KW3	Dog	ST22	sak, chp	0
KW4	Dog	ST22	sak, chp, scn	0
KW13	Dog	ST22	0	0
KW12	Dog	ST22	0	0
KW14	Dog	ST93	sak, chp, scn	0
KW15	Dog	ST30	sak, chp, scn	0
KW6	Kangaroo	ST22	sak, chp, scn	0
KW5	Horse	ST22	sak, chp, scn	0
KW10	Horse	ST612	scn, sea	0
KW11	Horse	ST612	sak	0
KW16	Horse	ST612	scn	0
KW7	Horse	ST8	scn, sea	0
KW9	Horse	ST8	sak, scn, sea	0
VET1	Human	ST59	sak, chp, scn, sea	0
VET2	Human	ST22	sak, chp, scn	0
VET3	Human	ST22	sak, scn, sea	0
VET4	Human	ST22	0	0
VET5	Human	ST22	0	0
VET6	Human	ST22	sak, chp, scn	0
VET7	Human	ST22	sak, chp, scn	0
VET8	Human	ST5	0	0
VET9	Human	ST8	sak, scn, sea	0

VET10	Human	ST8	sak, scn, sea	0
VET11	Human	ST8	sak, chp, scn, sea	0
VET12	Human	ST8	sak, scn, sea	0
VET14	Human	ST8	sak, scn	0
VET13	Human	ST612	sak, scn, sea	0
VET15	Human	ST612	sak, scn, sea	0
VET16	Human	ST78	sak, scn	0
VET18	Human	ST398	0	vwb-sb04
VET17	Human	ST78	sak, scn	0

3.5 **Discussion**

This study found 4% of clinical veterinary *S. aureus* isolated in Australia in 2013 were methicillin-resistant. The frequency of MRSA varied, depending on animal species. No MRSA was identified in cattle, while 13% of *S. aureus* isolated from companion animals were methicillin-resistant. The frequency of MRSA amongst companion animals was lower in this study than in previous studies from Europe and the USA (35, 60), but due to differences in study design and sample population, the current results cannot be directly compared to previous reports. For example, a study from Germany reported 52% of *S. aureus* wound infections from dogs, cats and horses were methicillin-resistant (35). While this is a high rate of MRSA infection, bacterial wound infections in companion animals are more likely to be due to MRSA than other types of infections (246). Thus, the population of animals in the German wound infection study (35) would have an intrinsically higher proportion of MRSA than other populations.

As reported in previous studies of MRSA in companion animals, the most frequently isolated MRSA clone in this study was ST22-IV (EMRSA-15) (33-35, 210). In Australia and elsewhere, ST22-IV is also the most frequently carried clone amongst small animal veterinarians (214, 240), is the most common clone isolated from hospital-onset MRSA bacteraemia in Australia (247) and colonises Australian

human healthcare workers with a greater frequency than other MRSA clones (248). Conversely, ST22-IV carriage was not detected in two Australian studies that sampled healthy humans with no known association to hospitals (249, 250). Previous reports have suggested veterinary personnel may be the source of MRSA infections to their patients (209). However, a common population of MRSA can circulate between companion animals and humans (34), so pet owners should be considered an additional potential source of MRSA for their pets, particularly if the owner is a health care worker (208) or has a history of hospitalisation (134). Regardless of the directionality of transmission of MRSA between companion animals and humans, ST22-IV MRSA is over-represented in hospital settings, and this clearly extends to veterinary hospitals.

Just as isolates from dogs and cats in this study came from similar clonal MRSA types as Australian companion animal veterinarians, the predominance of CC8 MRSA lineages ST612-IV and ST8-IV amongst the equine isolates in this study mirrors previously reported carriage of these clones by Australian equine veterinarians (240). This is in contrast to the low incidence of CC8MRSA in the rest of the Australian population (247). All six of the MRSA from horses were multidrug-resistant, with resistance to at least three classes of antimicrobials. With the exception of one equine ST22 isolate, the equine isolates demonstrated a common pattern of resistance to cefoxitin, tetracycline, trimethoprim-sulfamethoxazole, gentamicin and rifampicin. A similar pattern of resistance amongst CC8 isolates was seen in MRSA from Australian equine veterinarians (240). Four equine isolates also harboured the *ermC* gene and were resistant to erythromycin. *ermC* was not detected in any other MRSA isolates. This could reflect a difference in selection pressure between horses and other companion animals, because erythromycin is commonly combined with rifampicin to treat *Rhodococcus equi*-associated pneumonia in foals but it is seldom used in dogs and cats. Alternatively, the *ermC*-positive isolates could have been associated with humans more recently than the *ermC*-negative isolates, as *ermC* has previously been flagged as marker of human-associated infections (34). As in-contact humans were not sampled in this study, the true relationship of animal and veterinarian-derived ST22-IV and ST612-IV MRSA isolates cannot be fully elucidated. Nevertheless, the mirroring of

MRSA clonal lineages in animals in this study with carriage by Australian veterinarians (240) adds to the mounting evidence that MRSA may be shared between humans and animals and that clonal niches may be present within different veterinary environments.

In contrast to MRSA in companion animals, there were no MRSA isolates amongst the 203 bovine mastitis submissions. To date, the only report of MRSA in Australian dairy cattle is of a single dairy cow where reversed zoonotic transfer of a human-derived clone was suspected (251). The paucity of bovine mastitis MRSA reports and the results of the present study strongly suggest that the frequency of MRSA in Australian dairy cattle is currently very low. MRSA has been isolated from bovine samples in Europe (237) and around the world including Australia's close neighbour, New Zealand (236), so the absence of MRSA in Australian dairy cattle is somewhat surprising. However, dairy farm management practices could have contributed to the apparent lack of MRSA in Australian dairy cattle. It is common for cows with refractory *S. aureus* mastitis to be culled from the herd so refractory mastitis cases, potentially caused by MRSA, may not have been sent to the laboratory or subsequently included in this study (252). The absence of MRSA amongst Australian bovine samples could also be due to the current low frequency of livestock-associated CC398 MRSA within Australia. Although CC398 was not detected in the current study, it has recently been identified in pigs (242) and a pig veterinarian in Australia (240). Pigs can act as a reservoir for CC398 MRSA carriage (238) and infection (32) in humans. CC398 is also becoming an increasingly common cause of bovine mastitis (237). A recent study determined that the CC398 isolates found in animals in the United Kingdom likely came from mainland Europe (253), so importation of animals may be a risk factor for the presence of CC398 LA-MRSA within a country. The strict quarantine of Australian pig farms aims to prevent contact between cattle and pigs. This lack of contact between cattle and pigs and the ban of live importation of pigs and cattle into Australia could also explain why CC398 MRSA has not yet been detected in the Australian dairy cattle population.

The absence of MRSA in dairy cattle in this study could also be influenced by the antimicrobial prescribing practices for food-producing animals in Australia. Unlike much of the rest of the world

(254), fluoroquinolones have never been registered for use in food-producing animals in Australia and third-generation cephalosporin use is restricted (255). Ceftiofur is the only third generation cephalosporin registered for cattle in Australia, and its labelled use is restricted to treatment of bacterial respiratory infections due to *Mannheimia haemolytica, Pasteurella multocida* and *Histophilus* spp. in individual cattle (Excenel^{TM}, Pfizer Animal Health, Australia). In contrast to food-producing animals, fluoroquinolones and third generation cephalosporins are registered and regularly used in dogs and cats in Australia (256). In horses, ceftiofur is registered for use in Australia while fluoroquinolones are used off label and are listed as a third line or 'last resort' antimicrobial for horses by the Australian Veterinary Association (256). In humans, treatment with fluoroquinolones has been shown to increase the risk of MRSA carriage and nosocomial MRSA infections (257, 258). In the absence of fluoroquinolone exposure amongst livestock, there may be less selection pressure for MRSA amongst Australian dairy cattle when compared to cattle exposed to fluoroquinolones. Meanwhile, selection pressure may be one reason why the ST22-IV MRSA isolates, which were primarily obtained from dogs and cats, were resistant to β-lactams and all fluoroquinolones tested but susceptible to other drugs. Holden *et al.*(259) suggested that the acquisition of fluoroquinolone resistance by ST22-IV was one of the main drivers in its success as a nosocomial pathogen. The patterns of antimicrobial resistance in this study appeared to divide along MRSA clonal lines as well as by animal species, likely reflecting a complex epidemiology driven by clonal adaptation towards particular hosts and differing antimicrobial usage patterns between animal species.

The distribution of host-specific genetic markers amongst the MRSA from animals and veterinarians was variable. Four of the veterinarian-derived isolates did not harbour human IEC genes, while none of the animal-derived isolates harboured the animal-associated *vwb* variant. The previously reported ST398 from a pig veterinarian (240) harboured the animal-associated *vwb* variant, *sb04-vwb* and was IEC-negative. This is in keeping with the reputation of ST398 as a livestock-associated MRSA lineage and provides further evidence that humans can carry such lineages (238). Three of the animal-derived and two human-derived ST22-IV isolates were IEC-negative, which is unusual because IEC genes are

highly conserved amongst ST22-IV isolates (259). The absence of IEC genes in a human-associated clone such as ST22-IV could indicate a move away from human adaptation in certain isolates, assuming that the phage-mediated IEC genes are lost when they no longer conferred a biological advantage to the isolate. Alternatively, the absence of IEC genes in human-derived ST22-IV isolates could suggest that *S. aureus* can still successfully colonise humans without harbouring these genes and thus, IEC genes may not be a reliable marker of human adaptation. Nevertheless, the finding that human- and animal-derived MRSA isolates had variably distributed host-adaptation markers is still noteworthy, because it shows 'human-associated' and 'livestock-associated' MRSA lineages do not necessarily stay within their assumed host species.

Despite the veterinarian- and animal-derived MRSA samples being collected four years apart (2009 and 2013 respectively), phylogenetic analysis showed that veterinarian and animal MRSA isolates shared the same clusters within the phylogenetic tree. Similar intermingling was found in phylogenetic analysis of ST22-IV from humans and companion animals in the UK (34). In this study, there was separation of isolates into a dog/cat-associated- ST22-IV cluster and an equine- associated CC8 cluster, further demonstrating how specific MRSA lineages may circulate within specific veterinary environments.

Two isolates, ST93-IV and ST30-IV each from dogs, were PVL-positive. PVL-positive ST93-IV is the major cause of community-acquired MRSA infections in Australia (260). This is the first report of ST93-IV in a dog. Isolation of ST425-XI *mecC* MRSA in Australia was also a novel finding (244). ST425-XI MRSA is typically thought of as a livestock-associated MRSA lineage previously found only in Europe, but in this study it was isolated from a cat in semi-rural area of Victoria. The ST425-XI-t6292 lineage and absence of human immune evasion genes suggest the isolate could be linked to the dairy industry in the UK. Unfortunately, we were not able to obtain further epidemiological information about the cat, its origin, or its human contacts. Nevertheless, the detection of ST425-XI is pertinent because it demonstrates the often unexpected manner in which MRSA lineages may spread around the globe. The observed

disparity between cefoxitin and oxacillin susceptibility in the *mecC* isolate is consistent with previous reports (261, 262) and could serve as a flag for laboratory technicians that *mecC*-mediated methicillin-resistance may be present. The negative result on PBP2a assay is also in keeping with other *mecC*-positive isolates (263), showing the superiority of MIC testing over PBP2a detection as a phenotypic test for *mecC*-mediated resistance. The presence of a *mecC* isolate in this study highlights the importance of including *mecA* homologues such as *mecC* when using molecular assays for methicillin-resistance amongst staphylococci. Finally, while human-associated methicillin-susceptible *S. aureus* clones have previously been reported in Australian macropods (264), this is the first report of MRSA in a kangaroo.

The isolates in this study originated from clinical samples sent by private and government veterinarians and as a result, isolate submission may have been subject to a number of sampling biases. Firstly, financial cost may have discouraged animal owners to permit the attending veterinarian to send a sample for culture and susceptibility testing when it would otherwise have been clinically indicated. Secondly, it is possible that this study included more complicated clinical infections that warranted a veterinarian to send a sample for further testing, so isolates may be more likely to come from resistant infections that are not responding to treatment. Thirdly, although livestock associated CC398 MRSA was not detected in this study, this could be due to the very low number of samples from pigs in this study (n= 3). It could also be due to the focus of this study on samples collected from animals with clinical disease, so carriage of CC398 MRSA in Australian livestock would not have been detected. Given that CC398 has been isolated in Australian pigs (242), a focused surveillance study into the prevalence of MRSA carriage amongst Australian commercial piggeries is now warranted. Although this study demonstrates that some associations exist between MRSA clonal type and host animal species, it is limited by the small number of MRSA cases, the lack of detailed epidemiological information about each case and the absence of concurrent sampling of in-contact humans. Additionally, the non-contemporaneous nature of veterinarian and animal sampling means that further associations between human and animal MRSA isolates may have been missed. A large-scale longitudinal study

investigating carriage and infection amongst animals and all in-contact humans is clearly required so that the epidemiology of MRSA in animals and humans in Australia can be more clearly elucidated.

3.6 **Conclusion**

In summary, sixteen out of 360 (4%) clinical *S. aureus* isolates in this study were MRSA. The presence of human-associated MRSA lineages in animal hosts, livestock-associated MRSA in humans, and the heterogeneity of host immune markers amongst the isolates in this study add to evidence that MRSA can circulate between humans and animals. The presence of MRSA in Australian companion animals and veterinarians should serve as a further reminder for human physicians and veterinarians to consider all human and animal contacts as potential sources of MRSA. The presence of MRSA in Australian companion animals starkly contrasts with its absence amongst cattle and this finding adds further weight to the important role that antimicrobial stewardship plays in curbing antimicrobial resistance.

Chapter 4. Molecular epidemiology of methicillin-resistant *Staphylococcus pseudintermedius* in Australian animals

The content of this Chapter is published in the following journal article:

Worthing KA, Abraham S, Pang S, Coombs GW, Saputra S, Jordan D, Trott DJ, Norris JM. **(2018)** Clonal diversity and geographic distribution of methicillin-resistant *Staphylococcus pseudintermedius* from Australian animals: discovery of novel sequence types. *Veterinary Microbiology*, **213**, 58-65

4.1 Abstract

This study examined the molecular epidemiology of clinical MRSP isolated from Australian animals. Clinical staphylococci submitted to all Australian veterinary diagnostic laboratories were collected during 2013 and identified using traditional phenotypic tests and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Phenotypic antimicrobial resistance was determined using broth microdilution and disk diffusion. MRSP isolates were characterised by whole genome sequencing which included identification of the *mecA* gene. Phylogenetic relationships were inferred by comparison of single nucleotide polymorphisms. Of the 669 *S. pseudintermedius* isolates collected from dogs, cats and cattle samples, 77 (11.5%) were MRSP. Nineteen multilocus sequence types (STs) were identified, with most isolates belonging to one of five STs (ST71, ST497, ST316, ST496 and ST45). Phylogenetic analysis revealed that Australian ST71 appears closely related to ST71 from overseas. ST497 and ST496 represented novel sequence types not previously reported outside Australia. Most other STs were novel and only distantly related to each other. Geographical clustering of STs was observed. All isolates belonging to the five main STs were multi- to extensively drug-resistant while isolates from singleton STs generally had lower levels of antimicrobial resistance. The

frequency of ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin, chloramphenicol and tetracycline resistance varied significantly between STs ($p<0.01$). Six SCC*mec* types were identified. Oxacillin MIC varied significantly between SCC*mec* types. We characterised ΨSCC*mec*KW21, a novel pseudo-SCC*mec* element found in nine geographically-clustered MRSP isolates. Australian MRSP isolates are phylogenetically diverse, with a mix of previously unreported and well known international MRSP clones that demonstrate geographic clustering and exhibit both multidrug-resistant and extensively drug-resistant phenotypes.

4.2 **Introduction**

Methicillin-resistance in *S. pseudintermedius* (265) is a more recent phenomenon than in *S. aureus* (38) but its frequency has increased remarkably, ranging from 8.7% to 28% of *S. pseudintermedius* infections in dogs overall (59, 162-165) and up to 60% of canine *S. pseudintermedius* pyoderma cases (166). Many methicillin-resistant *S. pseudintermedius* (MRSP) are also multidrug-resistant (266), leaving veterinary clinicians with few treatment options. MRSP can be carried by pet owners (27) and veterinarians (29) and is an occasional zoonotic pathogen (30). MRSP appears able to acquire and maintain multidrug-resistance (151) (resistant to three or more antimicrobial categories) and extensive drug-resistance (susceptibility to two or less antimicrobial categories) (231). Its propensity for horizontal transfer of genetic resistance determinants makes it a potential threat to public health (151, 266).

MLST has identified several dominant MRSP lineages around the world, including ST71 in Europe and Asia (59, 162, 163, 184, 267), ST68 in North America (184, 266) and ST45 in Asia (188, 266). This suggests that successful MRSP clones may circulate amongst the dogs in a particular geographic area. In addition to a growing body of literature researching the distribution of MLST types amongst MRSP populations, recent studies have found that SCC*mec* typing may provide useful information about the epidemiological origin of isolates. Echoing the epidemiological division of MRSA into hospital-associated (HA) and community-associated (CA) lineages (268, 269), Kasai *et al.* (270) (2016) found

that SCC*mec* II-III isolates may represent HA-MRSP lineages and SCC*mec* V isolates may represent CA-MRSP. Although early anecdotal reports of MRSP in Australia coincided with reports from other countries, MRSP was not reported in Australia in the scientific literature until 2014 (271, 272). A recent study conducted in parallel with this thesis examined 888 clinical *Staphylococcus* spp. isolates collected from Australian companion animals in 2013 and found that 11.8% of *S. pseudintermedius* submissions were MRSP (229). However, the molecular epidemiology of Australian MRSP has not yet been examined. Consequently, the current study aimed to determine the molecular epidemiology of 77 MRSP isolates collected from Australian animals in 2013.

4.3 Methods

4.3.1 Sample acquisition, speciation and antimicrobial susceptibility testing

S. pseudintermedius were collected in 2013 during the first Australian survey into antimicrobial resistance in veterinary clinical isolates and involved all 22 Australian veterinary diagnostic laboratories (VDLs) (See Section 2.1.1) (229). Preliminary staphylococcal speciation was determined by traditional phenotypic tests and confirmed by the BD™ Bruker MALDI Biotyper, as outlined in Section 2.2.2. While the previous study focused on the phenotypic antimicrobial resistance profiles of 629 *S. pseudintermedius* collected from dogs and cats in the surveillance study (229), the current study screened a larger collection of 669 *S. pseudintermedius* isolates collected from companion animal, food-producing animals and samples where no animal species was recorded. *S. pseudintermedius* samples came from dogs (n= 619), cats (n= 13) and cattle (n= 4). No species were recorded for the remaining 33 isolates. Repeat isolates from the same animal, indicated by repeated laboratory submission numbers, were removed from the study.

The phenotypic antimicrobial resistance profile of each isolate was determined by broth microdilution and disk diffusion according to the CLSI guidelines, as described in Section 2.3. Most antimicrobials were tested by both disk diffusion and broth microdilution. However, for the macrolides, erythromycin (ERY) was tested by disk diffusion only. For the aminoglycosides, gentamicin (GEN) was tested by disk

diffusion and amikacin (AMK) was tested by broth microdilution. Antimicrobials were obtained from Sigma Aldrich (Australia) and Zoetis (Australia). *S. aureus* ATCC 25923 and ATCC 29213 were used as quality control strains. If a discrepancy between broth microdilution and disk diffusion occurred, assignment was made based on the presence or absence of the relevant resistance genes. As genetic resistance markers were not used for rifampicin and ciprofloxacin, the microdilution result was used to determine the resistance status. Isolates defined as 'Intermediate' according to CLSI guidelines were defined as resistant in this study. An isolate was classified as methicillin-resistant if phenotypically oxacillin resistant (oxacillin MIC ≥0.5mg/L and/or disk diffusion diameter ≤17mm) and harboured the *mecA* or *mecC* gene as determined by whole genome sequencing.

4.3.2 Whole genome sequencing, *in silico* typing and resistance gene screening

Whole genome sequencing was performed on all phenotypically methicillin-resistant *S. pseudintermedius* using the MiSeq System (Illumina), as previously described (273) and outlined in Section 2.4. *De novo* assembly, molecular typing and resistance gene detection were performed using the bioinformatics program, CLC Genomics Workbench (CLCbio, Qiagen, USA). Determination of MLST *spa* typing, *dru* typing and resistance gene screening were undertaken as described in Section 2.5. Novel sequence types were assigned by the MLST database curator (vincent.perreten@vetsuisse.vbi.unibe.ch). For fluoroquinolone resistance, isolates were screened for mutations in the topoisomerase II (*gyrA*) and IV genes (*grlA*). Screening was performed by downloading *gyraA* and *grlA* sequences (Accession numbers: AM262968 and AM262971 respectively), creating an alignment in CLC Genomics Workbench and screening for nucleotide polymorphisms associated with fluoroquinolone resistance (274).

4.3.3 SCC*mec* typing

SCC*mec* typing was performed by downloading the sequences of the *mec* gene complex and *ccr* elements of the SCC*mec* elements described by the International Working Group on the Staphylococcal Cassette Chromosome (IWGSCC) (http://www.scc*mec*.org/Pages/SCC_TypesEN.htmL) from the NCBI

online database (http://www.ncbi.nlm.nih.gov/). SCC*mec* elements previously identified in *S. pseudintermedius* (ΨSCC*mec*$_{57385}$ (188), II-III$_{1381}$ (274), SCC*mec*$_{AL16}$ (197), *SCCmec*$_{NA45}$ (275) and VII$_{241}$ (274)) but not included on the *SCCmec* working group website were also downloaded. Downloaded SCC*mec* element sequences underwent BLAST against *de novo* contigs using CLC Genomics Workbench™. BLAST results required at least 90% homology to be assigned a particular *ccr* or *mec* gene complex. If an SCC*mec* type could not be assigned, contigs were mapped against a scaffold of reference SCC*mec* types (193) and the reference methicillin-susceptible *S. pseudintermedius* genome, ED99 (Accession: CP002478.1). Novel elements were then annotated manually using the BLASTn algorithm in CLC Genomics Workbench™, and compared to existing elements using EasyFig (Python, V. 5).

4.3.4 Phylogenetic analysis of MRSP isolates

A phylogenetic tree was generated to infer evolutionary history, by uploading Illumina paired-end sequencing reads to the Nullarbor bioinformatic pipeline software (https://github.com/tseemann/nullarbor). Single nucleotide polymorphisms (SNPs) in the core genome were identified by comparison with a reference genome (ST71 MRSP 081661, Accession number: CP16073.1). SNPs in recombination events were removed as previously described (276). A phylogenetic tree was constructed by MEGA (v7.0) (http://www.megasoftware.net/), using the Maximum Parsimony method (277). In order to infer the relationship of Australian MRSP with MRSP isolates from overseas, isolates were assigned to clonal clusters (CCs) using the BURST algorithm from the MLST database website, http://pubmLst.org/spseudintermedius/. The STs were compared to the entire *S. pseudintermedius* database, and were included within a CC if they were single- or double-locus variants of the predicted complex founder.

4.3.5 Geographic mapping of isolates

The postcode of the submitting veterinary hospital and ST of each isolate were mapped against an Australian postcode map using a geographic information system (http://www.aus-emaps.com/postcode_finder.php).

4.3.6 Statistical analysis

Two commercially available statistical programs were used to analyse the data (GraphPad Prism 6, 2015 and GenStat 16, 2016). Proportions of antimicrobial resistance in each multilocus sequence type were compared using the chi-squared test or Fisher's exact test as appropriate. Observed proportions of MRSP from each state were compared to expected MRSP proportions based on the total number of *S. pseudintermedius* submissions from each state. MLSTs and *SCCmec* types with more than eight isolates were compared as separate entities in analyses; other isolates were grouped together. The Kruskal-Wallis test was used to determine whether the median oxacillin MICs differed across SCC*mec* types. The Mann-Whitney U test was then used to assess differences in the median oxacillin MIC between SCC*mec* types. Differences were considered significant if $p<0.05$.

4.4 Results

4.4.1 Diversity of MRSP types

Seventy seven (11.5%) of the 669 *S. pseudintermedius* isolates were identified as phenotypically methicillin-resistant and harboured the *mecA* gene. No isolates carried the *mecC* gene. Although a further six isolates were phenotypically methicillin-resistant, no *mecA* gene or homologue was identified and consequently these isolates were excluded from further molecular analysis. Of the 77 genotypically-confirmed MRSP, 76 isolates were cultured from dogs and one isolated from a cat. Details of the molecular characteristics of the 77 MRSP are shown in Table 4.2. Nineteen STs were identified amongst the MRSP isolates. Five dominant STs (with more than five isolates) were present: ST71, ST497, ST316, ST496 and ST45. ST71 was the most frequently identified MRSP lineage (CC71: n=

26, 34% of MRSP isolates). Several sequences types (ST496, ST497, ST498, ST499, ST500, ST501, ST539, ST544 and ST547) had previously undescribed allelic profiles and were assigned new sequence types by the *S. pseudintermedius* MLST database curator. One isolate demonstrated a new *cpn* allele and was assigned a new sequence type, ST750. Six *spa* types were identified. The most frequent *spa* type was t05 (n= 20), followed by types t02 (n= 18), t06 (n = 8), t19 (n= 8), t09 (n = 3) and t15 (n= 1). Nineteen isolates did not harbour the *spa* variable repeat region and therefore could not be assigned a *spa* type. The most frequently identified *dru* types were dt9a (n= 25), dt11y (n= 10) and dt10t (n= 9). The genomic sequences from all MRSP isolates have been deposited as biosamples at GenBank, under the accession numbers SAMN08741554 to SAMN08741522.

4.4.2 Geographic distribution of MRSP lineages

MRSP was found in veterinary hospitals from six of the eight Australian states and territories. The highest number of total *S. pseudintermedius* isolates came from the states of New South Wales (n= 228/669; 34%) and Victoria (n= 159/669; 24%). Amongst the 77 MRSP isolates, 30 (39%) came from Victoria, which was significantly higher than the expected proportion of 24% based on total numbers of *S. pseudintermedius* from each state (OR= 2.1, CI= 1.1-4.0; p= 0.02). The frequency of MRSP from other states was proportional to the number of *S. pseudintermedius* submitted from that state. Figure 4.1 shows the distribution of the major Australian MRSP lineages varied with geography. Isolates from Victoria (VIC) tended to be ST71 or ST497, while ST316 and ST496 isolates were primarily identified in New South Wales (NSW), and ST45 isolates were from Western Australia (WA). Seven ST71 isolates and nine of the eleven ST497 isolates came from neighbouring post codes in inner Melbourne, indicating they originated from the same clinic or neighbouring veterinary clinics.

4.4.3 SCC*mec* types amongst Australian MRSP

Table 4.1 shows that six SCC*mec* types were identified amongst the 77 MRSP isolates and that isolates from the same STs tended to harbour the same SCC*mec* type. Several STs shared the same SCC*mec* type.

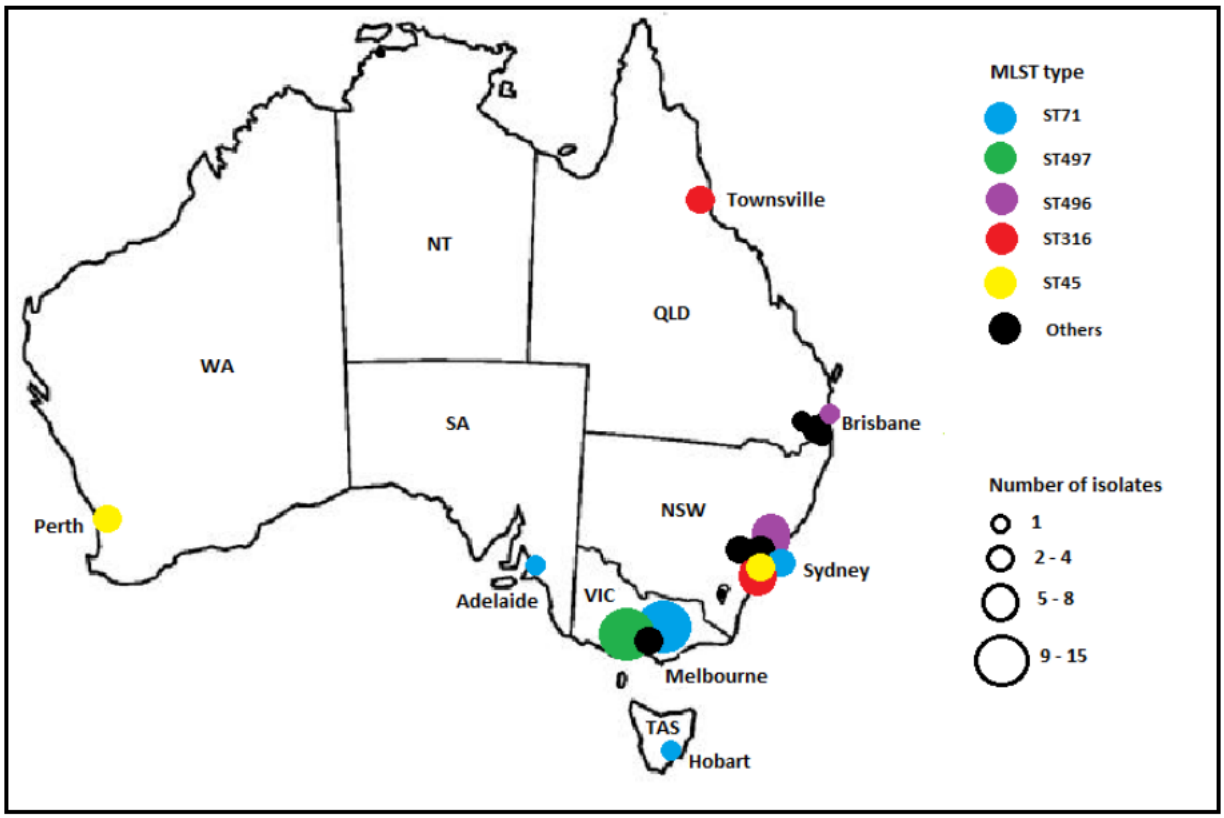


Figure 4-1. Geographic distribution of multilocus sequence types of methicillin-resistant Staphylococcus pseudintermedius in Australia, 2013-2014

Four of the SCC*mec* types have already been well characterised in MRSP or MRSA: II-III (n= 34), V (n= 10), ΨSCC*mec*$_{57395}$ (n= 7), and IVg (n= 4) (188, 193, 195, 274). The fifth SCC*mec* element was identified in nine isolates from eight different STs. These isolates harboured a class C1 *mec* complex and *ccrC6* element which had 99% homology to the SCC*mec* type X-like element recently identified in ST84 MRSP (275), ST398 MRSA (278) and methicillin-resistant *S. schleiferi* (279). This SCC*mec* element has not yet been described by the IWGSCC so for the purposes of analysis we have temporarily named the element C1/*ccrC6.* The final eleven MRSP isolates, all ST497, could not be mapped to previously described *SCCmec* elements. All ST497 isolates were from a geographic cluster in Melbourne, Victoria. ST497 contigs were mapped against SCC*mec* references and MSSP EDD99, then contigs were joined using the

Genome Finishing Module in CLC Genomic Workbench. Isolate KW21 yielded a 59,691bp contig containing a complete sequence of the novel SCC*mec* element, described below.

Table 4-1. SCCmec types and MLST of methicillin-resistant Staphylococcus pseudintermedius from animals in Australia, 2013-2014

SCCmec type	Multilocus sequence type
II-III (n= 34)	ST71 (n= 25)
	ST316 (n= 8)
	ST25 (n= 1)
V (n= 10)	ST496 (n= 8)
	ST64 (n= 1)
	Novel ST (n= 1)
C1/ccrC6 (n= 9)	ST64 (n= 2)
	ST84 (n= 1)
	ST283 (n= 1)
	ST499 (n= 1)
	ST500 (n= 1)
	ST501 (n= 1)
	ST525 (n= 1)
	ST547 (n= 1)
ΨSCCmecKW21 (n= 9)	ST497 (n= 9)
ψSCCmec57395 (n= 7)	ST45 (n= 6)
	ST544 (n= 1)
IVg (n= 5)	ST498 (n= 3)
	ST258 (n= 1)
	ST539 (n= 1)
Not typable (n= 3)	ST497 (n= 2)
	ST71 (n=1)

C1/ccrC6 harbours a C1 mec complex and ccrC6 element (275).

4.4.4 Characterisation of ΨSCC*mec*KW21, a novel SCC*mec* element from ST497 MRSP isolates

The *mec* gene complex of KW21 had 99% homology with several class A *mec* gene complex sequences from the NCBI database, including MRSA N315, methicillin-resistant *S. epidermidis* RP62A and MRSP KM1381 (Accession numbers: BA000018.3, CP000029.1 and AM904732.1, respectively). The complex contained a 1,680bp truncated *mecR1* gene that was interrupted by a truncated Tn552-like *blaZ*-locus found in MRSA252 (Accession number: 571856.1) and MSSP ED99 (Accession number: NC017568.1). No *mecI* gene or insertion sequences were found in the area flanking *mecR1.* Consequently, KW21 was deemed to have a class D *mec* gene complex, which has previously been described in methicillin-resistant *S. caprae* (280). The *mec* D gene complex adjacent to the Tn552-like *blaZ*-locus, which contained a contained a 633bp segment of the *blaR* gene that overlapped with the end of the truncated *mecR1* gene. A full copy of the *blaI* was situated adjacent to truncated *blaR/mecR* gene. No *ccr* genes were identified in KW21. *Ccr*-negative SCC*mec* elements have previously been reported in MRSP (188) and are common in coagulase-negative staphylococci (281). *BlaI* was flanked by a 12 nucleotide sequence, ACTAATTTA, that had an inverse repeat adjacent to the *orfX* gene at the other end of the complex. The inverse repeats were presumed to represent the element's approximate insertion sites. The SCC*mec* elements from the other eight isolates investigated also harboured *blaI* in lieu of a *mecI* gene, a truncated *mecR1*, and were void of *ccr* genes. Consequently, they were considered to have the same pseudo-element as KW21. We have designated the 7,802bp element 'ΨSCC*mec*KW21' (Figure 4.2).

4.4.5 Variation of oxacillin MIC amongst SCC*mec* types

Figure 4.3 shows the oxacillin MIC range for the major SCC*mec* types. The median oxacillin MIC differed significantly depending on SCC*mec* type (Figure 4.3; $p<0.001$). The median oxacillin MICs of C1*mec*/*ccr*C6 isolates (4mg/L) and "other" SCC*mec* types (1mg/L) were significantly lower than the median oxacillin MIC of SCC*mec* II-III, SCC*mec* V and ΨSCC*mec*KW21 isolates (64mg/L; $p<0.01$).

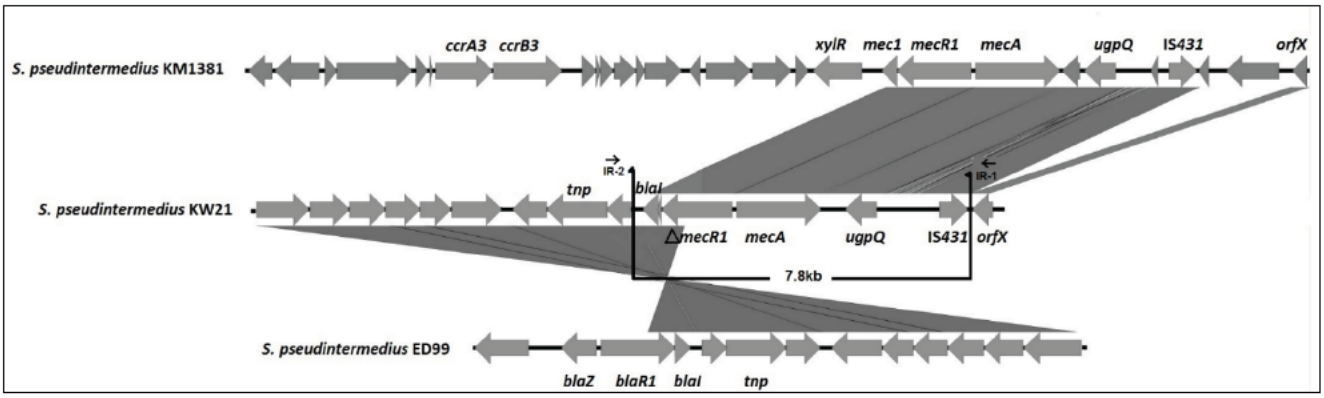


Figure 4-2. Alignment of *Staphylococcus pseudintermedius* ΨSCC*mec*KW21 with SCC*mec* II-III of *Staphylococcus pseudintermedius* KM1381 and the Tn552-like *blaZ*-locus of S. pseudintermedius ED99. The 7802bp ΨSCC*mec*KW21 element is delineated by inverted repeats IR1 and IR2. Alignment created in EasyFig (Python V. 5)

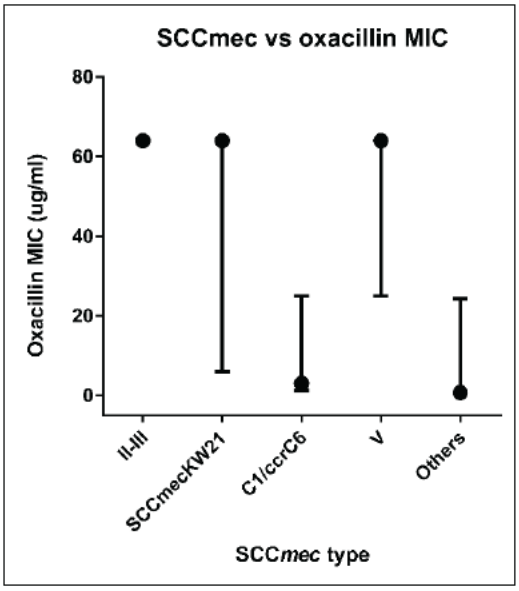


Figure 4-3. Oxacillin MIC for SCC*mec* types of methillin-resistant *Staphylococcus pseudintermedius* from Australia, 2013-2014. Black dots indicate the median MIC value, error bars indicate interquartile range

4.4.6 Variation of antimicrobial resistance amongst STs

The phenotypic and genotypic antimicrobial resistance profiles of the MRSP isolates are shown in Table 4.2. Except for ansamycins, where genetic markers were not searched for, the presence of relevant resistance genes generally corresponded to phenotype (Table 4.3). Mutations in *gyraA* and *grlA* generally, but not always, corresponded with the fluoroquinolone resistance phenotype. Amongst ciprofloxacin-resistant isolates, 98% and 44% were positive for the *gyrA* and *grlA* amino acid substitutions, Ser84Leu and Ser80Ile respectively. Amongst ciprofloxacin-susceptible isolates, 44% isolates (all ST316 isolates) had the aforementioned *gyrA* and *grlA* substitutions. Three ST496 isolates were phenotypically resistant to chloramphenicol but had no detectable *catp*C221 plasmid (Table 4.2). All isolates belonging to ST71, ST497, ST316, ST496 and ST45 were multi- to extensively drug-resistant, as defined in Section 2.3 (231). Forty isolates from the main STs were extensively drug-resistant, with resistance to seven to eight of the nine antimicrobial categories tested. Eight isolates, mostly from singleton STs and ST64, showed low levels of resistance (resistance to 2 or less drug categories). The frequency of ciprofloxacin, trimethoprim-sulfamethoxazole, gentamicin, chloramphenicol and tetracycline resistance varied significantly between STs (Figure 4.4; $p<0.01$). Six MRSP isolates were resistant to rifampicin and were characterised as ST497 (n= 3), ST71 (n= 2) and ST45 (n= 1). The frequency of erythromycin, rifampicin and clindamycin resistance was not significantly different between STs (data not shown). Notably, the frequency of ciprofloxacin resistance in ST71 and ST496 (100%) was significantly higher than in ST316, ST497 and "other" STs (63%, 54% and 32% respectively) while the frequency of chloramphenicol resistance in ST71 (8%) was significantly lower than all other STs ($p<0.01$). The frequency of tetracycline resistance in ST71 (32%) was also significantly lower than the 100% resistance seen in ST316, ST497 and ST496 ($p<0.05$).

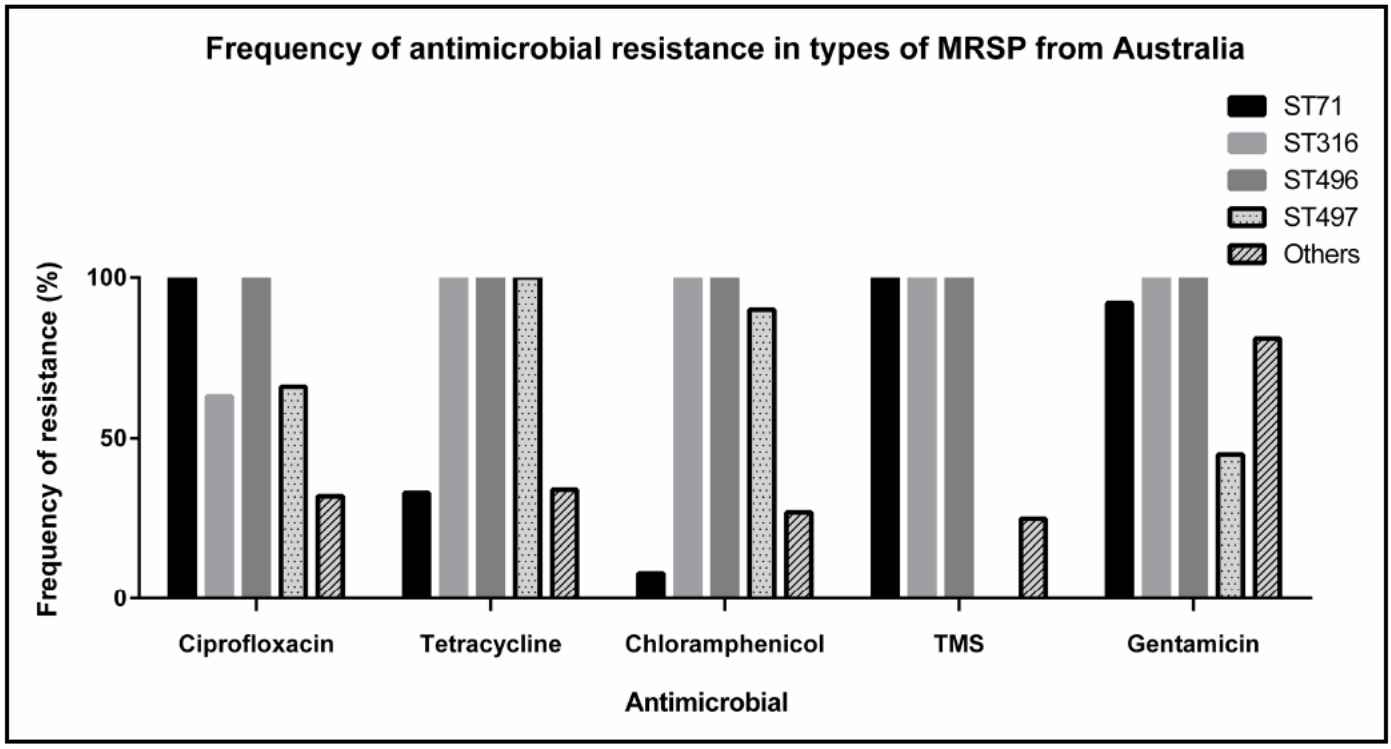


Figure 4-4. Frequency of antimicrobial resistance in major multilocus sequence types (STs) of methicillin-resistant *Staphylococcus pseudintermedius* from Australia, 2013-2014. Columns indicate the proportion of resistant isolates from each ST type, as determined by MIC. TMS= trimethoprim-sulfamethoxazole.

4.4.7 Phylogeny of Australian MRSP isolates

A total of 3,603 core genome SNPs were used to reconstruct phylogeny in a Maximum Parsimony tree (Figure 4.5). Isolates from the five major STs formed separate clades. Isolates also tended to form geographic clusters within MLST clades. For example, ST71 and ST497 isolates from the same postcode showed fewer SNP differences compared to isolates from different postcodes; and New South Wales-derived ST497 isolates had fewer SNP differences between each other when compared to Victorian ST497 isolates. The 26 ST71 isolates formed the most homogenous group, differing by only 39 SNPs.

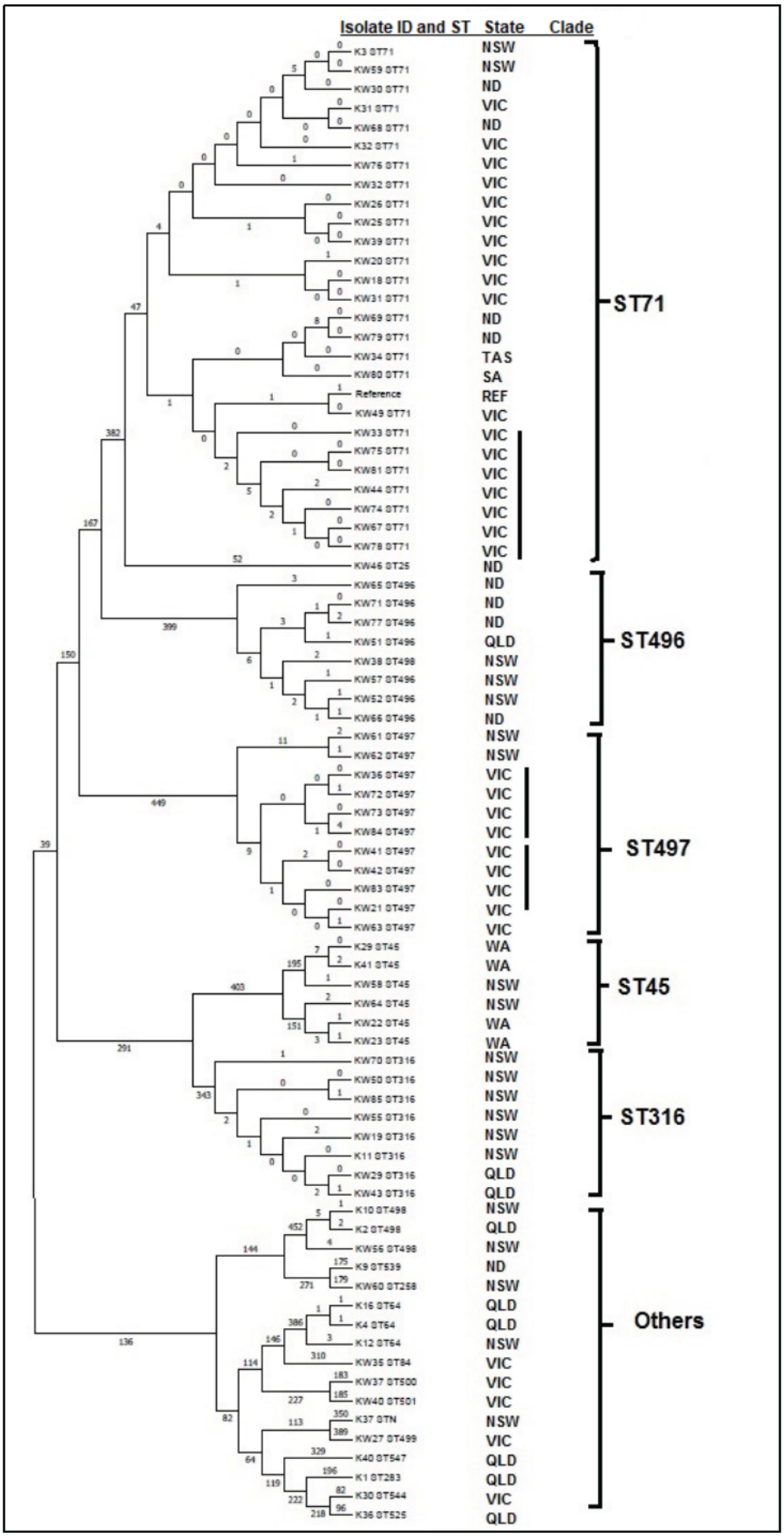
Table 4-2. Molecular epidemiology of methicillin-resistant *Staphylococcus pseudintermedius* from animals in Australia, 2013-2014

Clonal complex (CC)	MLST	Spa type	*Dru* type	Resistance phenotype	Resistance genotype
CC71	ST71 (n= 26)	t02 (n= 7)	dt9a (n=6), NT (n=1)	OXA-ERY-CLI- CIP-SXT (n= 7), GEN (n= 6), TET (n= 2), CHL (n= 2), RIF (n= 1)	mecA, erm(B), aac(6')-Ie–aph(2')-Ia, ant(6)-Ia, dfr(G), aph(3')-III (n= 6), tet(K) (n= 2), cat_{pC221} (n= 2)
		t05 (n= 13)	dt9a (n=12), dt8b (n=1)	OXA-ERY-CLI- CIP-SXT (n=13), GEN (n=12)	mecA, erm(B), aph(3')-III, dfr(G) (n= 13) aac(6')-Ie–aph(2')-Ia (n= 12), ant(6)-Ia (n= 12)
		t06 (n= 6)	dt9a	OXA-TET-ERY-CLI-GEN-CIP-SXT (n= 6), RIF (n= 1)	mecA. tet(K), aac(6')-Ie–aph(2')-Ia ,aph(3')-III, ant(6)-Ia, dfrG (n=6), erm(B) (n= 5)
CC309	ST497 (n= 11)	t02 (n=8)	dt10t	OXA-TET-ERY-CLI- CHL (n= 8)-GEN (n= 4), CIP (n= 4), RIF (n= 2)	mecA, tet(M), erm(B), aph(3')-III, aac(6')-Ie–aph(2')-Ia, ant(6)-Ia, cat_{pC221}
		t05 (n= 1)	dt10t	OXA-TET- CLI-GEN-CHL-CIP	
		t06 (n= 2)	dt11y	OXA-TET-ERY- CLI-CIP-CHL-RIF	
	ST525 (n= 1)	N/A	dt9a	OXA	mecA
CC84	ST84 (n= 1)	N/A	dt11af	OXA-ERY-CLI -GEN	mecA, erm(B), aph(3')-III, ant(6)-Ia
CC45	ST45 (n= 6)	t09 (n= 3), NT (n= 3)	dt11cb (n=1), NT (n=5)	OXA-TET-ERY-CLI-GEN-CIP (n= 6),CHL (n= 5), SXT (n= 3), RIF (n= 1)	mecA, tet(M), erm(B), aph(3')-III , aac(6')-Ie–aph(2')-Ia, ant(6)-Ia (n=6), cat_{pC221} (n= 5), dfr(G) (n= 3)
CC258	ST498 (n= 3)	N/A	dt10as	OXA-ERY-CLI-GEN	mecA, erm(B), aph(3')-III, ant(6)-Ia (n= 3), aac(6')-Ie–aph(2')-Ia (n= 1)
	ST258 (n= 1)	N/A	dt10h	OXA-TET-SXT	mecA, tet(M), dfr(G)

NA	ST316 (n= 8)	t19	dt11y	OXA-TET-ERY-CLI-GEN-CHL-SXT (n= 8), CIP (n= 5)	mecA ,tet(M), erm(B), aac(6')-Ie–aph(2')-Ia, ant(6)-Ia, aph(3')-III, dfr(G) (n= 8), cat_{pC221} (n= 7) erm(C) (n= 1)
	ST496 (n= 8)	t02 (n=2)	dt10a	OXA-TET- ERY-CLI -GEN- CIP-CHL-SXT	mecA , tetM, ermB,aph(3')-III, aac(6')-Ie–aph(2')-Ia, ant(6)-Ia, dfr(G) (n= 8), cat_{pC221} (n= 5)
		t05 (n= 6)	dt10a (n=2), dt4h (n= 2), NT (n=2)		
	ST64 (n= 3)	N/A	dt11af	OXA- GEN (n= 3) ERY (n= 1)	mecA, aac(6')-Ie–aph(2')-Ia, (n= 3), erm(C) (n= 1)
	ST25 (n= 1)	N/A	dt11a	OXA-TET-ERY-CLI-GEN-CIP-CHL-SXT	mecA, tet(M), erm(B), aph(3')-III , aac(6')-Ie–aph(2')-Ia , cat_{pC221}, dfr(G)
	ST499 (n= 1)	N/A	dt11bn	OXA	mecA
	ST500 (n= 1)	t15	dt11bn	OXA-ERY-CLI -GEN	mecA ,tet(M), erm(B), aph(3')-III, aac(6')-Ie–aph(2')-Ia, ant(6)-Ia
	ST501 (n= 1)	N/A	NT	OXA-ERY-CLI -GEN	mecA ,tet(M), erm(B), aph(3')-III, aac(6')-Ie–aph(2')-Ia, ant(6)-Ia
	ST539 (n= 1)	N/A	dt10h	OXA-GEN-SXT	mecA, aac(6')-Ie–aph(2')-Ia, dfr(G)
	ST547 (n= 1)	N/A	dt11a	OXA	mecA
	ST750 (n= 1)	N/A	dt11a	OXA-TET-ERY-CLI -GEN	mecA ,tet(M), erm(B), erm(C), apha(3), aac(6')-Ie–aph(2')-Ia
	ST283 (n= 1)	t02	dt11a	OXA-ERY-CLI -GEN	mecA , erm(B), apha(3), aac(6')-Ie–aph(2')-Ia, ant(6)-Ia
	ST544 (n= 1)	N/A	dt11a	OXA	mecA

Bold and underlined text denotes that not all isolates were phenotypically resistant to that antimicrobial or harboured that gene: the number of isolates in which resistance was present is indicated in parentheses. Plain texts denotes that all isolates were phenotypically resistant or harboured the resistant gene. OXA= oxacillin, ERY= erythromycin, CHL= chloramphenicol, CIP= ciprofloxacin, CLI= clindamycin, GEN= gentamicin, RIF= rifampicin, SXT= trimethoprim-sulfamethoxazole, TET= tetracycline. Resistance genes: *mecA*= alternate penicillin binding protein 2 gene; *tet*(K) and *tet*(M)= tetracycline resistance gene; *aph(3')-III* = aminoglycoside phosphotransferase (neomycin/kanamycin resistance) gene; *aac(6')-Ie–aph(2')-Ia* = aminoglycoside adenyl-/phosphotansferase (gentamicin/tobramycin resistance) gene; *ant(6)-Ia* = aminoglycoside nucleotidyltransferase (streptomycin resistance) gene; cat_{pC221}= chloramphenicol acetyltransferase gene; *dfr*(G)= dihydrofolate reductase gene mediating trimethoprim resistance; *erm*(B) and *erm*(C)= rRNA methyltransferase genes associated with macrolide/lincosamide resistance. NT= new *dru* type

Figure 4-5. (Page 72) Phylogenetic tree of methicillin-resistant *Staphylococcus pseudintermedius* from Australian animals, 2013-2014, derived from Maximum Parsimony analysis. Analyses were conducted in MEGA v7.0, from 3,603 SNPs in the core genome of 76 isolates. ST71 MRSP 081661 was used as the reference genome (Riley *et al.*, 2016). Tip annotations provide Isolate ID, State (region) isolated and clade. NSW= New South Wales, VIC= Victoria, QLD= Queensland, SA= South Australia, WA= Western Australia, TAS= Tasmania. Vertical lines next to State indicate that the isolates were from the same post code.


Isolate ID and ST
State
Clade
K3 ST71 NSW
KW59 ST71 NSW
KW30 ST71 ND
K31 ST71 VIC
KW68 ST71 ND
K32 ST71 VIC
KW76 ST71 VIC
KW32 ST71 VIC
KW26 ST71 VIC
KW25 ST71 VIC
KW39 ST71 VIC
KW20 ST71 VIC
KW18 ST71 VIC
KW31 ST71 VIC
KW69 ST71 ND
KW79 ST71 ND
KW34 ST71 TAS
KW80 ST71 SA
Reference REF
KW49 ST71 VIC
KW33 ST71 VIC
KW75 ST71 VIC
KW81 ST71 VIC
KW44 ST71 VIC
KW74 ST71 VIC
KW67 ST71 VIC
KW78 ST71 VIC
KW46 ST25 ND
KW65 ST496 ND
KW71 ST496 ND
KW77 ST496 ND
KW51 ST496 QLD
KW38 ST496 NSW
KW57 ST496 NSW
KW52 ST496 NSW
KW66 ST496 ND
KW61 ST497 NSW
KW62 ST497 NSW
KW36 ST497 VIC
KW72 ST497 VIC
KW73 ST497 VIC
KW84 ST497 VIC
KW41 ST497 VIC
KW42 ST497 VIC
KW83 ST497 VIC
KW21 ST497 VIC
KW63 ST497 VIC
K29 ST45 WA
K41 ST45 WA
KW58 ST45 NSW
KW64 ST45 NSW
KW22 ST45 WA
KW23 ST45 WA
KW70 ST316 NSW
KW50 ST316 NSW
KW85 ST316 NSW
KW55 ST316 NSW
KW19 ST316 NSW
K11 ST316 NSW
KW29 ST316 QLD
KW43 ST316 QLD
K10 ST498 NSW
K2 ST498 QLD
KW56 ST498 NSW
K9 ST539 ND
KW60 ST258 NSW
K16 ST64 QLD
K4 ST64 QLD
K12 ST64 NSW
KW35 ST84 VIC
KW37 ST500 VIC
KW40 ST501 VIC
K37 STN NSW
KW27 ST499 VIC
K40 ST547 QLD
K1 ST283 QLD
K30 ST544 VIC
K36 ST525 QLD
ST71
ST496
ST497
ST45
ST316
Others

4.5 Discussion

This study found that Australian MRSP are phylogenetically diverse and geographically clustered. The most frequently identified MRSP lineage was ST71, which is consistent with reports from other countries (59, 162, 163, 184, 267). In contrast to European ST71 isolates, where *spa* type t02 is most frequently identified, (184) the most frequent *spa* type in ST71 isolates from the present study was t05. The difference most likely reflects localised evolution of ST71 within Australia. Although isolates within ST71 and ST497 were delineated by t02, t05 and t06 *spa* types (Figure 1), all three *spa* types share a similar pattern of repeat sequences, suggesting they are closely related (90) and therefore these sequenced types (STs) are more homogenous than the varied *spa* types would suggest. Three major novel STs were identified that have not previously been reported: ST497 (n=11), ST316 (n=8) and ST496 (n=8). The fifth major lineage in this study, ST45 (n=6), is frequently reported in dogs Asia and Israel (165, 188, 266) and has been isolated from veterinary personnel in Thailand (215). One of the six ST45 isolates was *dru* type dt11cb, which has previously been associated with ST45 isolates from Thailand (188) and canine pyoderma patients in Western Australian (272). Four new *dru* types were found amongst the remaining five ST45 isolates which clustered into two closely related groups that varied by one to two *dru* repeats per group, suggesting microevolution of this clone.

Geographic clustering of MRSP clones was found in this study, particularly ST497 which mostly clustered in a small area in Victoria. A group of seven ST71 isolates also originated from the same postcode, indicating they came from the same veterinary clinic. Such clustering of ST497 and ST71 could suggest an outbreak occurred during the sample period; as has occurred with ST71 in a Finnish veterinary hospital (282) or could simply be that ST497 was established within clinics with a high sampling intensity during the study period. The nature of this surveillance study means that the collection was likely affected selection bias which must be taken into account. For example, the cluster of ST497 may have come from a referral practice where patients could have been drawn from a wider geographic range than one would expect for a primary accession clinic. Nevertheless, the potential for

such sample biases would have been present across all geographic areas in the study, so the finding of geographic clustering of MRSP clones is still noteworthy. Specific MRSP clonal lineages have previously been associated with particular regions, for instance ST71 first in Europe and subsequently much of the rest of the world, ST68 in North America and ST45 in Asia (184, 266). Geographic clustering within a country was also reported in MRSP isolates from Norway (283). Australia is a large country with an area exceeding Western Europe and with the majority of the population living on the coastal-periphery in cities typically separated by over 1000km. Therefore, it is not surprising that a geographically diverse clonal pattern was identified.

In addition to geographic clustering by STs, MRSP isolates also formed clades within the phylogenetic tree that aligned with their ST. This suggests that MLST is a useful method of inferring phylogenetic groupings. ST71 formed a particularly homogenous clade in the phylogenetic tree, with only 39 SNP differences between the 26 isolates. Similar phylogenetic homogeneity amongst ST71 isolates has previously been reported (151). Isolate KW33, (ST71-t02-II-III) demonstrated only one SNP difference to the ST71 reference isolate (275). This suggests that Australian ST71 MRSP is closely related to ST71 from overseas, rather than originating via convergent evolution. Introduction of ST71 into Australia could have occurred via importation of an infected or carrier dog, or via owner or veterinarian (29, 284). Similarly, travel of people and animals between South East Asia and Australia could explain why ST45 MRSP has been found in Thailand and Australia.

BURST analysis showed that Australian MRSP clones formed five clonal complexes (CC71, CC45, CC258, CC84 and CC309) and 12 singleton that did not belong to any clonal complex. The high genetic diversity seen in the current study echoes what has been reported in recent studies in Europe (162, 267, 285) and Asia (165). Dos Santos and colleagues analysed 503 STs from the MLST database and found the MRSP population was only weakly clonal (266), in contrast to the highly clonal methicillin-resistant *S. aureus* (MRSA) population (286). They hypothesised that this may be due to the greater diversity of *S. pseudintermedius* lineages that are capable of acquiring SCC*mec* in comparison to *S. aureus* (266);

which may reflect a higher capacity for horizontal gene transfer in *S. pseudintermedius* (151, 266). The variable presence of mobile genetic elements between staphylococcal species and *S. pseudintermedius* lineages could be due to species and lineage-specific restriction-modification systems that create a variable propensity for horizontal gene transfer (151, 287-289).

Several SCC*mec* types were identified in this study, including the novel pseudo-SCC*mec* element, ΨSCC*mec*KW21. This element harbours a class D *mec* complex, no *ccr* genes and *mec*I has been replaced by *bla*I as the putative repressor gene of *mec*A. To the authors' knowledge, this is the first time a *mec* D gene complex has been described in a coagulase-positive *Staphylococcus* species. The absence of a *mec*I gene but presence of a *mec*R gene could indicate that the ΨSCC*mec*KW21 element is an evolutionarily immature cassette, as it appears *mec*R gene arose in ancestral SCC*mec* elements before the occurrence of *mec*I genes (281). Although *mec*A can be repressed by either *mec*I or *bla*I, *mec*I is a stronger repressor of *mec*A (290). All ΨSCC*mec*KW21 isolates displayed high-level phenotypic oxacillin resistance, with a median oxacillin MIC of ≥64mg/L (interquartile range (IQR) = 8-64mg/L). The oxacillin resistance phenotype of ΨSCC*mec*KW21 isolates is in keeping with previous reports that MRSP isolates with the *bla*I gene demonstrate higher inducible *mec*A expression in isolates compared to isolates harbouring either *mec*I or *mec*I and *bla*I (291). ΨSCC*mec*KW21 was typed using locally assembled alignments in the bioinformatics program, CLC Genomics. While this method successfully revealed the structure of the element, a more streamlined approach would involve analysis of a single sequence rather than assembled contigs. Consequently, isolate KW31 is currently undergoing further analysis by long range PCR and future studies will determine whether the method described here is equal to long range PCR in its accuracy.

Oxacillin MIC varied significantly between *SCCmec* types. Recently, Kasai and colleagues (270) similarly reported differences in oxacillin MIC based on MRSP SCC*mec* types. Specifically, they found that isolates with SCC*mec* II-III generally had higher oxacillin MICs than isolates with SCC*mec* V and that SCC*mec* II-III isolates were more often associated with suspected hospital-acquired infections than

SCC*mec* V isolates. Analogous to MRSA in human medicine, they concluded that oxacillin MIC may give clues as to an isolate's epidemiological origin; where a high oxacillin MIC may indicate an MRSP isolate is from a successful 'healthcare-associated' clone, while isolates with lower MICs may represent 'community-associated' clones (270). Veterinary patients with 'healthcare-associated' MRSP SCC*mec* II-III lineages share some risk factors with humans patients that harbour healthcare-associate MRSA, such as a history of hospitalisation and surgery (270, 292). However, there are key differences between the healthcare systems of human and veterinary hospitals including longer hospitalisation times for humans, the presence of long term indwelling medical devices such as intravenous catheters being less common in animals and the fact that humans are more readily diagnosed with, and treated for, serious nosocomial infections such as bacteraemia (247, 292). Therefore, while there are similarities in the epidemiology of MRSA in humans and MRSP in veterinary medicine, more work is needed to truly define what is meant by 'healthcare' and 'community' environmental niches in veterinary medicine. There was insufficient epidemiological data available in the present study to infer whether epidemiological origin could predict the SCC*mec* type of an isolate. These results nevertheless show that oxacillin MIC varies significantly depending on SCC*mec* type and that isolates from the same STs almost always harbour the same SCC*mec* type. Thus, it follows that different MRSP lineages will demonstrate different oxacillin MICs.

Our broader research group previously reported that methicillin-resistance amongst Australian *S. pseudintermedius* was significantly associated with resistance to clindamycin, fluoroquinolones, tetracycline and trimethoprim-sulfamethoxazole (229). These findings can now at least be partly explained by the finding here that significant differences in antimicrobial resistance profiles exist between Australian MRSP multilocus sequence types. ST71 isolates showed a pattern of fluoroquinolone resistance with tetracycline and chloramphenicol susceptibility, which is often seen in ST71 (266), although the frequency of tetracycline resistance in ST71 varies with geography (293). The most common subgroup of ST71 isolates in this study was tetracycline-susceptible *spa* type t05. This is in contrast to Europe, where tetracycline-resistant *spa* type t02 isolates are most common (184).

Geographic differences in ST71 tetracycline-susceptibility are most likely attributable to lineage-specific acquisition of the *tetK* gene (294). Analogous to the most prevalent clones of MRSA in humans (259), widespread fluoroquinolone resistance identified in ST71 could in part explain its apparent dominance. However in epidemiological studies such as the present study, the effect of selection bias towards fluoroquinolone-resistant isolates cannot be discounted. Enrofloxacin, marbofloxacin and pradofloxacin are the three fluoroquinolones registered for systemic use in dogs and cats in Australia and may be used for a range of different infections, sometimes inappropriately, by clinicians as a second line treatment when empirical β-lactam therapy fails (295). Given sending a sample for culture and susceptibility testing is usually done at the expense of the pet owner, veterinarians rationalise their decision to perform microbiology culture on a cost-benefit basis. Consequently, second line therapy may be given in lieu of testing; creating a sample bias towards fluoroquinolone-resistant MRSP clones, because testing might only be performed when second line therapy has failed. Therefore, although fluoroquinolone resistance does appear more frequently in ST71 than in other clones, this observation may be affected by sample bias. It is nevertheless noteworthy that the major clonal groups in this study were all multi- to extensively drug-resistant, while singleton isolates generally had lower levels of resistance. This suggests that multidrug resistance adds to the competitive fitness of MRSP clones, at least in a clinical setting where antimicrobial selection pressure is present. Differing antimicrobial resistance patterns could also reflect different clonal propensities to maintain various resistance elements, or could reflect regional antimicrobial usage patterns (266). In contrast to fluoroquinolone resistance, rifampicin resistance is still at a low level amongst Australian MRSP. Nevertheless, widespread resistance to fluoroquinolones and sporadic resistance to critically important antimicrobials such as rifampicin is alarming as it suggests that, when faced with multidrug resistance, veterinarians may be contributing to selection pressure by usage of these important antimicrobials.

Like the previous study described in Chapter 3, this study is limited by the selection bias towards refractory conditions that is inherent in clinical submission surveys and the lack of more extensive

epidemiological data available for each isolate. Future studies into region-specific carriage of MRSP are warranted, as are studies investigating factors that may explain the predominance of particular clones such as ST71. Future studies are also warranted to determine what correlations exist between MLST and SCC*mec* types, oxacillin MIC and the epidemiological niches of MRSP. Furthermore, screening of healthy and diseased animals across Australia is indicated in the future, to determine if ST497-ΨSCC*mec*KW21 continues to be a geographically clustered clone or whether it has disseminated further into other states of Australia.

4.6 **Conclusion**

This study demonstrates the utility of surveillance studies in shedding light on the epidemiology of MRSP at a national level. International and locally evolved MRSP clones are present in Australia. Geographic variation in multilocus sequence types and varying antimicrobial resistance profiles according to MLST was observed. The discovery of a novel SCC*mec* type further shows that staphylococci possess a diverse range of methods for acquiring methicillin resistance. Echoing previous reports (266), a high frequency of multidrug resistance was found amongst the MRSP isolates. As the arsenal of effective, veterinary-licensed, anti-MRSP therapeutics continues to shrink, the bond between people and their pets continues to grow. It therefore seems likely that pet owners and veterinarians will increasingly reach for critically important human antimicrobials to treat multidrug-resistant MRSP infections. Consequently, the need for stewardship over the use of critically important antimicrobials in veterinary medicine has never been more pressing.

Chapter 5. Vets and pets: methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in Australian veterinary personnel, their pets, patients and the veterinary hospital environment

5.1 Abstract

This pilot study investigated the transmission cycle of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus pseudintermedius* (MRSP) in companion animal veterinary practice. Sampling was undertaken at two small animal veterinary hospitals in Sydney, Australia. Samples were collected from 46 veterinary personnel, 79 veterinary-owned dogs and cats, 151 healthy canine hospital admissions and 25 environmental sites in one of the hospitals. Nasal swabs were collected from veterinary personnel. Nasal, oral and perineal swabs were collected from animals. Methicillin resistance was detected by growth on Brilliance^{TM} MRSA 2 Agar and confirmed by cefoxitin and oxacillin broth microdilution for *S. aureus* and *S. pseudintermedius,* respectively. MRSA and MRSP isolates were characterised using whole genome sequencing including *mecA* gene screening and multilocus sequence typing. MRSA was isolated from four (8%) veterinary personnel but no animals. MRSP was isolated from 11/151 (7%) of canine hospital admissions and 4/53 (8%) of veterinary personnel-owned dogs but no veterinary personnel or cats. No MRSA or MRSP was isolated from the environment. The main MRSP clone carried by canine patients (ST496) was distinct to that carried by veterinary-owned dogs (ST64). One veterinary nurse, who carried Panton Valentine leucocidin-positive ST338 MRSA, also owned a ST749 MRSP-positive dog. Besides MRSP-positive dogs from the same household sharing the same clone of MRSP, MRSA and MRSP were not shared between humans,

animals or environment. Therefore, in the non-outbreak setting of this study, there was limited MRS transmission between veterinary personnel, their pets, patients or the veterinary environment.

5.2 **Introduction**

As outlined in Chapters 3 and 4, MRSA and MRSP can both be found in clinical infections of companion animals. Chapter 3 described how the clones of MRSA found in Australian animal species are similar to those found in Australian veterinarians treating these animal species (240, 273). It appears that MRSA can move between humans and animals in a bi-directional manner and veterinarians are a group particularly at risk of MRSA carriage (213, 214). It has been established that MRSA can be shared between humans and animals, but the epidemiology of *S. pseudintermedius* transmission between animals and humans is less understood. MRSP and multidrug-resistant *S. pseudintermedius* can certainly be carried by healthy pets (296), dog owners (297) and veterinarians (29), and it can cause infections in immune-compromised people (30). Although studies have documented concurrent carriage of MRSP and MRSA in veterinary dermatologists and their own pets (298), no studies have assessed *Staphylococcus* carriage by non-dermatologist veterinarians and their own pets. Veterinary personnel often bring their pets to their place of work, thereby potentially exposing them to environmental and patient-associated organisms. Given that veterinary hospital visits and having an owner who works in human healthcare are respective risk factors for MRSP and *S. aureus* carriage in dogs (206, 208), it follows that pets owned by veterinary personnel may also be at increased risk of MRSA and MRSP carriage. Consequently, the first aim of this study was to determine the concurrent carriage rates of MRSP and MRSA in a range of veterinary personnel and their pets and as well as subsequent determination of whether veterinarians carried similar clones to those carried by their pets.

The prevalence of methicillin-resistant *Staphylococcus* (MRS) carriage in veterinary patients is variably reported. The prevalence of MRSA and MRSP carriage in dogs sampled in their homes in USA was 8% and 1% respectively (299) while carriage of MRSP in canine hospital admissions in Portugal was 6.2%

(300). MRSP carriage rate is much higher in dogs with clinical pyoderma, with reports ranging from 33% in USA (60) to over 60% in reports from Japan (166). A 2006 Australian study found two of 252 surgical admissions were MRSA carriers while none were MRSP carriers (301). A second smaller study found no MRSP amongst 21 clinical *S. (pseud)intermedius* samples collected from Sydney dogs between 2004 and 2007 (302). Most recently, a 2016 study found two of 117 canine obedience school attendees were MRSP carriers but none were MRSA carriers (296). It is clear that the prevalence of resistant *Staphylococcus* carriage is dependent on study design and sample population, which makes comparison between studies difficult. Nevertheless, regardless of exact methodology, study population or country of origin, most veterinary studies show a steady upward trend of antimicrobial resistance in *S. pseudintermedius* as time progresses (40). Given that there was no MRSP amongst Australian veterinary clinical submissions in 2004 to 2007 and this frequency rose to 11.5% in 2013 (Chapter 4), a study is now indicated to determine whether there has been a similar increase in MRSP carriage. Consequently, this study's second aims were to estimate the current frequency of MRSP and MRSA carriage in Australian canine hospital admissions and to determine if MRS carried by hospital admissions was related to clones carried by veterinarians and their pets.

Previous studies have demonstrated that MRSA and MRSP can be found in the veterinary hospital environment (27, 210), and molecular typing has shown that MRSA found in the environment can be highly similar to that carried by veterinary personnel and patients (210). There is a growing body of literature investigating the transmission cycle of MRS in veterinary practice. However, no studies have concurrently examined MRSA and MRSP carriage in veterinary personnel, their pets, patients and the veterinary hospital environment. Therefore the third and final aim of this study was to screen the veterinary hospital environment for MRS contamination and to determine the relatedness of MRS isolated from veterinary personnel, their pets, patients and the veterinary hospital environment.

5.3 Materials and methods

5.3.1 Sampling

Sample collection involved two veterinary hospitals in Sydney, Australia: a primary accession clinic (Hospital A) and a multi-disciplined small animal referral hospital (Hospital B), located 10km apart from each other, as outlined in Sections 2.1.2 and 2.1.3. Samples were collected from veterinary personnel, veterinary-owned pets, canine hospital admissions and environmental sites within the veterinary hospital. All aspects of this study were approved by the Human and Animal Ethics Committees at the University of Sydney (Project numbers 2016/837, 2016/1072 and 2015/866, respectively). The details of sampling procedures are outlined below.

5.3.1.1 Veterinary personnel and their pets

Sampling of veterinary personnel and their pets (dogs and/or cats) was undertaken over a two-week period in February 2017 (Hospital A) and April 2017 (Hospital B). Veterinary personnel included veterinarians and support staff (veterinary nurses, kennel hands and administrative staff). Personnel were invited to participate whether or not they had pets at home. Personnel were given written instructions on how to take samples, then took samples from themselves and their pets, undertaking sampling in their own home. For veterinary personnel, a single sterile swab was used to sample the nares. For pet dogs and cats, personnel took three separate swabs: one each from the anterior nares (large dogs) or nasal planum (small dogs and cats), oral cavity and perineum. For multipet households, sampling was limited to three dogs and/or three cats. Personnel wore gloves during the procedure and refrigerated samples immediately after the procedure. All swabs were collected using Amies™ Agar Gel swabs (Copan Diagnostics, USA).

5.3.1.2 Canine hospital admissions

Swab were taken from the nares/nasal planum, oral cavity and perineum of canine hospital admissions from both veterinary hospitals. Convenience sampling of canine hospital admissions occurred in two sampling periods in August 2015 and August 2016 for Hospital A while sampling was sporadic from

April 2016 to April 2017 for Hospital B. To minimise sampling from dogs with known MRSP carriage risk factors such as recent hospitalisation or antimicrobial treatment (206), dogs had to meet the following selection criteria: a) they had not been in the hospital for more than 10 minutes at the time of sampling; b) they were admitted for an elective procedure (for example: non-emergency surgery, routine blood tests, imaging, dentistry, boarding); c) they were deemed systemically well by the attending clinician; and d) they had no visible skin lesions suggestive of pyoderma.

5.3.1.3 **Hospital environment**

Environmental sampling was undertaken at Hospital A only, on the same day in February 2017 that samples were collected from personnel and their pets. Twenty-five swabs were taken from the following areas: cage floors and walls, waiting room chair legs and seats, door handles into the consultation, treatment, pharmacy, bathroom, radiology and boarding rooms, and computer keyboards and computer mice in the consulting, treatment and radiology rooms. Samples were taken by pre-moistening Amies™ Gel agar swabs with sterile saline, then rolling the swab across the environmental surface for 10sec. Although MRSP-positive carriers and patients with MRSP-infected wounds were known to have been in the hospital within the last month, no known MRSP carriers or patients were present at the time environmental sampling. Therefore, it was assumed that environmental sampling was indicative of a non-outbreak setting for the hospital.

5.3.2 Phenotypic methicillin-resistance screening

Human, animal and environmental swabs were all processed as follows. Samples were initially enriched by inoculation onto Columbia sheep blood agar (Oxoid, UK) and overnight incubation at 37°C. After the enrichment step, a sterile microbiological loop was streaked across the blood agar plate to collect many colonies which were then subcultured onto the selective medium, Brilliance™ MRSA 2 Agar (Oxoid, UK) and incubated overnight at 37°C (Horstmann et al., 2012). Samples that grew as blue colonies on the Brilliance™ agar underwent catalase and tube coagulase testing as described in Section 2.2.1. Coagulase-positive Brilliance™-positive isolates underwent species confirmatory testing with

MALDI-TOF mass spectrometry (BD™ Bruker MALDI Biotyper™) as previously described (Worthing et al., 2018b). Phenotypic methicillin resistance was confirmed using the Vitek 2™ automated antimicrobial susceptibility testing (AST) system (bioMerieux, USA). Methicillin resistance in *S. aureus* was identified by cefoxitin screening and detection of the modified pbp2a protein. In *S. pseudintermedius*, phenotypic methicillin resistance was identified by an oxacillin MIC of ≥0.5mg/L by Vitek screening. Isolates also underwent testing by Vitek 2™ to the following antimicrobials: benzylpenicillin, enrofloxacin, erythromycin, gentamicin, clindamycin, tetracycline, chloramphenicol, rifampicin and trimethoprim-sulfamethoxazole. Clindamycin testing included screening for inducible clindamycin resistance and measurement of MIC by Vitek 2™.

5.3.3 Molecular characterisation of methicillin-resistant isolates

Methicillin-resistant *S. aureus* and *S. pseudintermedius* underwent whole genome sequencing (WGS) using the MiSeq system (Illumina, USA) as previously described (273). *In silico* characterisation of isolates was undertaken as outlined in Section 2.5. A rooted phylogenetic tree of MRSP isolates was generated using CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) (243). Briefly, this program used a Maximum Likelihood algorithm to depict inferred phylogeny based on concatenated alignment of high quality single nucleotide polymorphisms. To construct the tree, fasta files of all MRSP genomes were uploaded to the online platform which aligned them to a reference genome (ST71 MRSP 081661, Accession numbers: CP16073.1). FigTree 1.4.3 was used to optimise visualization of the phylogenetic tree.

5.3.4 Statistical analysis

Power analysis was conducted to determine the ideal sample size required to determine the frequency of MRSP carriage amongst Sydney canine hospital admissions. Based on previously reported MRS carriage rates amongst Australian dogs of around 1% (296, 301) and an anticipated frequency of 11.5% based on the frequency of clinical MRSP from dogs in NSW (303), power analysis suggested a sample size of at least 164 would be required to predict MRS carriage frequency with 95% confidence

(http://clincalc.com/stats/Sample Size.aspx). Categorical comparisons were undertaken by constructing contingency tables and performing Fishers exact test (GraphPad Prism 7, USA). Results were considered significant if $p<0.05$.

5.4 **Results**

5.4.1 MRSA and MRSP in humans, animals and environment

Samples were collected from 46 veterinary personnel (veterinarians, n= 19; support staff, n= 27 (nurses= 22, receptionists= 2, kennel hands= 3), 79 veterinary-owned pets, 151 canine hospital admissions and 25 environmental sites. This consisted of 191 convenience samples from Hospital A (118 canine hospital admissions, 21 veterinary personnel, 13 veterinary-owned pet dogs, 14 veterinary-owned pet cats and 25 environmental sites) and 110 samples from Hospital B (33 canine hospital admissions, 25 veterinary personnel, 40 veterinary-owned pet dogs and 12 veterinary-owned pet cats). MRS was isolated from 4/46 veterinary personnel (8%), 11/151 (7%) canine hospital admissions, and 4/53 veterinary-owned pet dogs (8%). MRS was not isolated from cats or the veterinary environment. The MRS carriage rate in veterinary-owned dogs (8%) was not significantly different to veterinary-owned cats (0%; p= 0.3). MRS frequency was proportional to sampling intensity from each hospital and was not significantly different between Hospital A and B (p= 0.91). Consequently, results from both hospitals were combined for analyses. Table 5.1 shows the proportions of MRSA and MRSP isolated from veterinary personnel, canine hospital admissions and veterinary-owned pets. No MRSA was isolated from animals nor MRSP from humans. The phenotypic antimicrobial resistance profile of all isolates is shown in Table 5.2. MRSP isolates were resistant to significantly more antimicrobial classes than MRSA ($p<0.001$). All personnel- and animal-derived MRS samples underwent WGS and *in silico* molecular characterisation (Table 5.3). Two Brilliance-positive isolates (one *S. aureus* (KW8) and one *S. pseudintermedius* (KWBH2)) were positive for the *mecA* gene but methicillin-susceptible on Vitek 2 screening. It was assumed that a methicillin-susceptible subpopulation had been inadvertently subcultured for AST testing and thus these two isolates were

still included in further analyses. No MRSP or MRSA was isolated from the 25 environmental sites sampled. Although one catalase-positive isolate grew on Brilliance^{TM} 2 agar, it was coagulase-negative and therefore not characterised further.

Table 5-1. Methicillin-resistant coagulase-positive staphylococci (MRS) isolated from veterinary personnel, canine hospital admissions, veterinary-owned pets and the veterinary hospital environment

Group	Number sampled	Number of carriers (%)	
		MRSA	MRSP
Veterinary personnel			
Veterinarians	19	3 (16%)	0
Support staff	27	1 (4%)	0
Total personnel	46	4 (8%)	0
Dogs			
Hospital admissions	151	0	11 (7%)
Personnel-owned	53	0	4 (8%)
Total dogs	204	0	15 (8%)
Cats			
Personnel-owned	26	0	0
Hospital environment			
Waiting room chairs	2	0	0
Door handles	8	0	0
Computer keyboards	3	0	0
Cage door handles	3	0	0
Cage interiors	9	0	0
Total environment	25	0	0
Total samples	301	4 (1%)	15 (5%)

Table 5-2. Frequency (%) of antimicrobial resistance in methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-resistant Staphylococcus pseudintermedius (MRSP) from veterinary hospitals

Group	PEN	ENR	ERY	CLI	TET	CHLOR	RIF	TMS
MRSP (n= 19)	100	37	32	16	58	42	0	37
MRSA (n= 4)	100	0	0	0*	25	0	0	0

PEN= benzylpenicillin, ENR= enrofloxacin, ERY= erythromycin, CLI= clindamycin, TET= tetracycline, CHLOR= chloramphenicol, RIF= rifampicin, TMS= trimethoprim sulfamethoxazole. *Susceptible to clindamycin by MIC testing and by screening for inducible clindamycin resistance using Vitek 2™.

5.4.2 Characterisation of MRSA from veterinary personnel

Of the four MRSA isolates from veterinary personnel, three were from veterinarians and one was from a veterinary nurse. The difference in MRSA carriage amongst veterinarians (16%) was not significantly different to support staff (4%; p= 0.29). Three of the four MRSA isolates were susceptible to all non-β-lactam antimicrobials. The fourth isolate, ST338 from a veterinary nurse (Table 3), was additionally resistant to tetracycline. One veterinarian from each of the hospitals carried ST59-IV MRSA. The two isolates were different *spa* types (t316 and t976). One MRSA isolate, ST338 SCC*mec* type V isolate from a veterinary nurse, harboured the Panton-Valentine leucocidin gene (*pvl*); the remaining isolates were *pvl*-negative. The final MRSA isolate, ST81 from a veterinarian, was phenotypically susceptible to oxacillin but harboured the *mecA* gene. *Post-hoc* power analysis found that with a sample size of 46, previously reported Australia-wide veterinarian MRSA carriage rate of 4.8% (213) and observed MRS frequency rate of 8%, the personnel-sampling component of the study was underpowered (power= 23%). A sample size of 409 would have been required to obtain 80% power in the veterinary personnel component of the study.

Table 5-3. Molecular epidemiology MRSA and MRSP isolated from veterinary personnel, personnel-owned dogs and canine hospital admissions in Sydney, Australia

Isolate	Year isolated	Source	Species	Isolation site	Phenotypic methicillin resistance §	MLST	SCC*mec*	*Spa* type	*Dru* type
HOSPITAL A									
KW12	2017	Veterinarian	MRSA	Nares	+	ST59	IV	t316	None
KW1606[a]	2015	Dog (H)	MRSP	Nares and perineum	+	ST496	Vt	t05	dt10a
KW1610[a]	2015	Dog (H)	MRSP	Nares, oral cavity and perineum	+	ST496	Vt	t05	dt10a
KW1608	2015	Dog (H)	MRSP	Perineum	+	ST496	Vt	t05	dt10a
KW1614 ±	2015	Dog (H)	MRSP	Perineum	+	ST525	Vt	t09	dt9bd
KW1613 ±	2015	Dog (H)	MRSP	Oral cavity	+	ST68	Vt	t23	New
KW1607	2015	Dog (H)	MRSP	Nares	+	ST64	C1/ccrC6	None	dt7ae
KWAH3	2016	Dog (H)	MRSP	Oral cavity	+	ST496	Vt	t05	New
KWAH4	2016	Dog (H)	MRSP	Oral cavity and perineum	+	ST496	Vt	t02	New
KWAH1	2016	Dog (H)	MRSP	Oral cavity	+	ST64	C1/ccrC6	None	dt11af
KWAH2	2016	Dog (H)	MRSP	Oral cavity	+	ST64	C1/ccrC6	None	dt10cj
KW5	2017	Dog (P)	MRSP	Nares	-	ST749	IV	None	NT
HOSPITAL B									
KW7[c]	2017	Veterinary nurse	MRSA	Nares	+	ST338	V	t441	NT
KW8	2017	Veterinarian	MRSA	Nares	-	ST81	IV	t177	dt7f
KW3	2017	Veterinarian	MRSA	Nares	+	ST59	IV	t976	dt7f
KWBH2 ¥	2016	Dog (H)	MRSP	Oral cavity and perineum	+	ST751	NT	None	NT
KW1	2017	Dog (H)	MRSP	Nares and oral cavity	+	ST496	Vt	t02	dt7f
KW10[b]	2017	Dog (P)	MRSP	Nares	+	ST64	C1/ccrC6	None	New
KW11[b]	2017	Dog (P)	MRSP	Oral cavity	+	ST64	C1/ccrC6	None	dt7f
KW6[c]	2017	Dog (P)	MRSP	Nares, oral cavity	+	ST64	C1/ccrC6	None	New

and perineum

§ Phenotypic methicillin resistance as determined by Vitek[2] testing: cefoxitin screening and PBP2a test for MRSA isolates; oxacillin MIC testing for MRSP isolates. ± Isolates 1613 and 1614 originated from the same dog but were different MLST types. ¥ Both isolates from dog KWBH2 underwent phenotypic and genotypic testing and both isolates were *mecA* positive but one isolate was resistant to oxacillin (MIC= 0.5mg/L) while the other was susceptible (MIC<0.25mg/L). [a, b, c] Isolates with the same superscript letters originated from the same household. Dog (H)= canine hospital admission; Dog (V)= personnel-owned dog. NT= not typable. Unless indicated, dogs that were positive at multiple sites carried the same MLST type at all sites.

5.4.3 Characterisation of MRSP from veterinary-owned pets and canine hospital admissions

The MRSP carriage rate between veterinary-owned pet dogs (8%) and canine hospital admissions (7%) was not significantly different (p= 0.77). Most dogs that carried MRSP at more than one site carried the same clone at each site. However, one dog had two different clones isolated; ST525 from the perineum and ST68 from the oral cavity. Two main MRSP clones were isolated: ST496 (n= 6) and ST64 (n= 6). ST496 was the most common clone amongst canine hospital admissions (6/12 dogs; 50%) but was not carried by any veterinary-owned dogs. ST64 was the most common clone amongst veterinary-owned dogs (three dogs from two households) and was carried by all three veterinary-owned dogs at Hospital B. ST496 was carried by two dogs from the same household who were hospital admissions to Hospital A and ST64 was carried by two veterinary-owned dogs from the same household at Hospital B. Within each of the hospitals, none of the veterinary-owned dogs carried the same clone as hospital admissions to that hospital. Figure 5.1 shows the phylogeny of MRSP isolated from canine hospital admissions and veterinary-owned dogs at both hospitals. Isolates from both hospitals were generally interspersed throughout the tree, suggesting a general lack of geographic clustering.

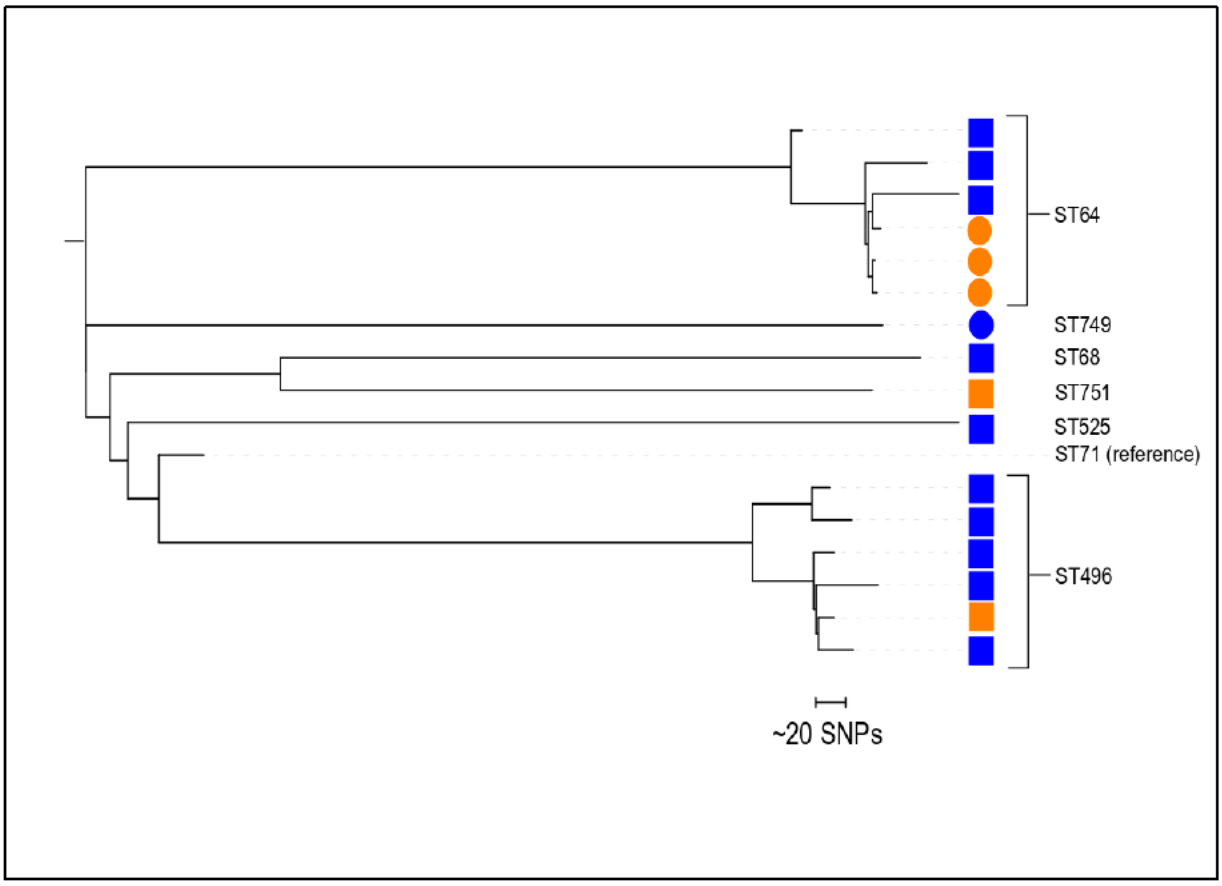


Figure 5-1. Phylogenetic tree of MRSP from veterinary hospital admissions and personnel-owned dogs, generated using a Maximum Likelihood algorithm based on a concatenated alignment of 1374 high-quality SNPs. The tree was constructed using CSIPhylogeny 1.4 and optimized using FigTree v1.4.3 and Interactive tree of life v3 (iTOL). ST71 MRSP 081661 (Accession: CP16073.1) was used as the reference genome. Circles indicate isolates from veterinary-owned dogs. Squares indicate isolates from canine hospital admissions. Blue shapes are from Hospital A, orange shapes are from Hospital B. The MLST of each isolate is shown on the right-hand margin of the tree.

5.4.4 Shared MRS carriage in veterinary personnel and their pets

Of the 46 veterinary personnel that were sampled, 38 also had their pets sampled. Of the 38 personnel-pet groupings, concurrent MRS carriage was identified in one veterinary nurse and one of the two dogs owned by that nurse. The isolate from the nurse was ST338 MRSA with SCC*mec* type V while the nurse's pet dog carried ST64 MRSP with a *mec* complex type C1/*ccr*C6 SCC*mec* element. Besides both SCC*mec* elements carrying a type C1 *mec* gene complex, the nurse- and pet- derived MRS samples did not appear related. Two pairs of dogs from the same household were both MRSP carriers. The first pair were personnel-owned dogs from Hospital B. Both dogs carried ST64 which clustered closely in the phylogenetic tree. The dog's owner, a veterinarian, was not a MRS carrier. The second MRSP-positive pair were hospital admissions at Hospital A, admitted on the same day for routine dental care. The ST496 MRSP isolated from this pair clustered closer to each other than other ST496 isolates.

5.5 **Discussion**

This study simultaneously sampled veterinary personnel, their pets and patients, and the veterinary hospital environment and detected MRSA from humans and MRSP from dogs but did not find MRSP carriage in humans, MRSA carriage in animals, or any MRS in the environment. The absence of MRSA in animals and MRSP in humans supports the notion that *S. aureus* is generally more host-adapted to humans and *S. pseudintermedius* to dogs (67). Four of 46 veterinary personnel (8%) were MRSA carriers, which is a higher rate of carriage than the previously reported rate of 4.9% in Australian companion animal veterinarians (213). These results add to evidence that the rate of MRSA carriage in veterinary personnel, like human healthcare workers, is higher than what is reported in the general population (213, 214, 304, 305). The veterinary-specific risk factors for MRSA carriage are not yet known, but it is possible that veterinary personnel have similar occupational risk factors to workers in healthcare such as caring for patients with MRSA-infected wounds (306). However, MRSA appears to

be much more prevalent amongst human hospital patients than veterinary hospital patients, so it is likely that additional risk factors exist for veterinarians such as the handling of antimicrobial drugs (298, 305), or environmental exposure to biocides and patient-excreted antimicrobials. It is clear that an extensive prospective cross-sectional study is warranted to better define the occupational risk factors for MRSA carriage in veterinary personnel.

The rate of MRSP carriage amongst veterinary-owned dogs (8%) was not significantly different to canine hospital admissions (7%), indicating that dogs of veterinary personnel are not at increased risk of MRSP or MRSA carriage compared to canine hospital admissions from the same geographic area. Previous studies have found that pets owned by human healthcare workers are at increased risk of carrying methicillin-susceptible *S. aureus* (MSSA) (208), but there are conflicting reports as to whether or not dogs owned by healthcare workers are at increased risk of MRSA carriage (208, 307). Future studies that directly compare the relative risk of MRSA and MRSP carriage amongst pets owned by veterinary personnel, human healthcare workers and non-healthcare workers are now warranted. All three MRSP-positive veterinary-owned dogs at Hospital B carried ST64 which closely clustered on the phylogenetic tree and differed by less than 10 SNPs, suggesting that ST64 had circulated amongst veterinary-owned pets in this hospital. Despite this, it was not isolated from any dogs admitted to the same hospital. Sampling of dogs upon discharge would have helped to determine whether canine hospital admissions subsequently acquired MRSP clones carried by veterinary-owned pets in the same hospital.

The rate of 7% MRSP carriage we found amongst canine hospital admissions is similar to some overseas studies (206), but higher than the 1% MRSP carriage rate in another Australian study that examined healthy dogs at an obedience school in regional Victoria (296). The difference could reflect increased local prevalence in Sydney compared to regional Victoria or increased prevalence amongst dogs attending veterinary hospitals compared to obedience school. ST496, a multidrug resistant MRSP strain, was the most common clone carried by canine hospital admissions in this study and was also

the most common clone amongst clinical MRSP from dogs in greater Sydney in 2013 (303). ST496 has not been reported outside Australia, but it appears that ST496 has become a common clone in Sydney that has evolved and diversified, evidenced by a greater diversity of *dru* types in this study compared to the 2013 surveillance study (303). Concurrent carriage of ST496 MRSP by two canine hospital admissions from the same household suggested intra-household transmission had occurred. Although neither patient showed signs of skin disease at the time of sampling, inspection of both patients' records revealed that one of the dogs had a long history of intermittent antimicrobial use associated pyoderma secondary to flea allergy dermatitis. It is likely that the dog with a history of skin disease acted as a source of MRSP for the asymptomatic dog in the same household (27, 308). While clear risk factors for MRSP carriage and infection such as previous antimicrobial use, frequent veterinary visits and a history of hospitalisation have already been identified (204, 206), veterinarians should be aware that apparently healthy dogs can also carry MRSP, particularly if they live with an MRSP carrier.

It is noteworthy that the MRSP isolates in this study displayed a significantly higher level of antimicrobial resistance than the MRSA isolates, with 37% resistance to fluoroquinolones and trimethoprim-sulfamethoxazole and 32% resistance to erythromycin amongst MRSP compared to no resistance to these classes amongst MRSA isolates. While MRSP is not a major zoonotic pathogen, the expansion of multidrug-resistant MRSP clones such as ST496 still presents a public health concern because such clones may act as a reservoir for genetic resistance determinants that could be horizontally-transferred to important human pathogens. Animal-derived staphylococci can be the source of resistance determinants in human *S. aureus* (281), but the extent to which *S. pseudintermedius* contributes to the resistance gene pool in human pathogens such as *S. aureus* is not fully determined (309) and could thus be examined in future genomic studies.

Besides concurrent MRSA carriage in a veterinary nurse and MRSP carriage in the nurse's pet dog, this study found no shared MRS carriage in veterinary personnel and their pets. The lack of MRSP isolation from veterinary personnel in this study likely reflects the general lack of human host tropism by MRSP

but could also reflect the small sample size and the fact that animals with overt skin disease were intentionally omitted from this study. Dogs with skin disease are more likely to carry MRSP than dogs with healthy skin (310), and transmission of MRSP from animals to owners is more commonly reported when the animal has clinical disease (27). Certain MRSP clones such as ST71 appear better able to colonize human corneocytes than MSSP or other MRSP clones and thus may have better zoonotic potential (311). It is therefore possible that the lack of human MRSP carriage in this study reflects the lack of ST71 in the animal population sampled. Although ST71 is a dominant MRSP clone in Europe (184) and represents 34% of clinical MRSP in Australia overall (303), it was not isolated from any dogs in this study and was isolated from only 1/24 (4%) clinical MRSP cases around Sydney in 2013 (303). Overall, it appears that MRS carriage by veterinary personnel is influenced by their role within the veterinary hospital and the local prevalence and clonal distribution of MRS in their respective patient population.

MRSP was not isolated from the veterinary hospital environment. This could be attributed to the low sample size, the lack of longitudinal sampling or the fact that Hospital A had developed a hospital protocol for the management of known MRS-infected patients which was initiated six months prior to this study. Successful reduction of environmental MRSA was reported in a human hospital that revised its hospital infection control protocols to address an increase in MRSA cases (312), but lack of longitudinal sampling pre- and post-cleaning protocol prevents us from assessing whether the absence of MRSP was truly due to a successful infection control program. Although the moistened swab method has been used in previous veterinary studies of hospital environmental sampling (210, 313), the wipe-rinse method of environmental sampling is now recommended for hospital infection control protocols (314). The larger surface area of wipes compared to swabs may have afforded greater sensitivity to the environmental sampling in this study, and thus the study method could have been improved by the use of the wipe-rinse method for environmental sampling.

5.6 **Conclusion**

In conclusion, this pilot study provides valuable insights into the molecular epidemiology of MRS within two veterinary hospitals. The clonal types of MRSP and MRSA found in veterinary personnel, veterinary-owned pets and hospital admissions were distinct from each other. This suggests that limited MRS transmission occurs between these groups, at least in a non-outbreak setting as was examined in this study. The rate of MRS isolation was not significantly different between the tertiary referral hospital and a primary accession hospital, nor between personnel-owned dogs and hospital admissions. MRSP carriage was not detected amongst veterinary personnel. However, it is apparent that clonal types of MRSP vary with geography, so sampling of dogs and veterinary-personnel in areas such as Victoria, where ST71 MRSP is common (303), would provide valuable comparative results for this study. Contrasting with the lack of MRSP carriage in the current study, MRSP carriage has been twice documented in veterinary dermatologists (29, 298). A study that simultaneously compares MRSP carriage amongst dermatologist and non-dermatologist veterinarians is warranted to evaluate whether specialty-specific risk for MRSP carriage exists amongst veterinarians. Lastly, like Chapter 3, the current study adds to existing literature in reporting that veterinarians have a higher rate of MRSA carriage than the general population. A large-scale case-control study is thus warranted to further investigate the occupational risk factors for MRSA carriage in veterinarians.

Chapter 6. Characterisation of clinical *Staphylococcus felis* from cats: detection of coagulase positive isolates and genetic virulence determinants

6.1 Abstract

This study used phenotypic tests and whole genome sequencing to characterise a collection of 38 clinical *Staphylococcus felis* isolates from cats. Samples were isolated from cats with a range of diseases including feline lower urinary tract disease (n = 15) and otitis externa (n= 14). Isolates were identified using MALDI-TOF MS and by identification of *S. felis*-specific *rpoB* and *nuc* genes in whole genome sequenced contigs. Phenotypic antimicrobial resistance was determined using disk diffusion and broth microdilution. Coagulase activity was assessed using feline and rabbit plasma. Contigs were screened for putative virulence and antimicrobial resistance genes using the sequences of known genes from other staphylococcal as homologous references. A preliminary multilocus sequence typing scheme was developed using five housekeeping genes. Phylogenetic relationships were further inferred by comparison of single nucleotide polymorphisms. Two isolates were coagulase-positive when tested against feline plasma while all isolates were coagulase-negative against rabbit plasma. Both coagulase-positive isolates harboured homologues of the *Staphylococcus aureus* coagulase gene while coagulase-negative isolates did not. A range of other putative virulence genes were found amongst isolates including genes associated with adhesion, toxin production and immune evasion. All isolates displayed low levels of phenotypic antimicrobial resistance which was reflected by a general absence of resistance genes. Fourteen sequence types were present in the collection. Isolates generally clustered in the phylogenetic tree according to their sequence type. The presence of

coagulase-positive *S. felis* isolates and widespread putative virulence genes suggests that *S. felis* is a potentially pathogenic species that warrants further research.

6.2 **Introduction**

The previous chapters of this thesis contain sporadic cases of MRSP and MRSA infections in cats, but perhaps the most significant disease causing staphylococcal species for cats is *Staphylococcus felis.* First identified as a new species in 1989 (68), *S. felis* is coagulase-negative when tested using rabbit plasma and produces incomplete haemolysis on sheep blood agar (68). Due to its phenotypic similarity to other coagulase-negative staphylococci such as *S. simulans* (68) and *S. sciuri* (69), the prevalence of *S. felis* may have been underestimated in studies prior to 1989 or in studies where advanced molecular methods were not utilised. Although it is generally agreed that *S. felis* is a common part of the normal flora of cats, its role as a feline pathogen is more contentious (71-74, 315). *S. felis*-positive urine samples have significantly higher pH than other culture-positive urine and are significantly more likely to contain urine crystals (71). Like many staphylococcal species, *S. felis* produces urease (68), which is the likely cause of the alkalinised urine seen in *S. felis*-positive urine. As a skin commensal, *S. felis* could be dismissed as a contaminant of urine samples, but Lister *et al.* (71) concluded that its urease production and resultant alterations in urine pH were sufficient virulence factors to deem it a potential urinary pathogen of cats. A subsequent study examined a single *S. felis* isolated from a cat's subcutaneous wound and found virulence factors such as biofilm and proteolytic enzyme production, demonstrating its potential as a skin pathogen (315). Besides a handful of studies (68, 71, 315), there is a scarcity of literature regarding the epidemiology and potential virulence factors of *S. felis* in cats.

There are divergent reports of the antimicrobial susceptibility profile of *S. felis* in the peer reviewed literature. As antimicrobial resistance continues to rise in other *Staphylococcus* spp. such as *S. aureus* and *S. pseudintermedius*, one would expect resistance to be increasing in *S. felis* as well. However, while methicillin-resistance and multidrug-resistance has been found amongst *S. felis* isolates (9, 316), other studies have identified low levels of antimicrobial resistance amongst *S. felis* (71, 72). With the

rise of antimicrobial resistance in *S. aureus* and *S. pseudintermedius*, it is not surprising that much research has been directed at further characterising these two species. MLST has already shed light on the population structure of *S. aureus* (183) and *S. pseudintermedius* (185) but no such scheme exists for *S. felis,* so its population structure is currently unknown. Given the current scarcity of research characterising *S. felis*, the three aims of this study were to 1) investigate potential virulence factors that may aid *S. felis* in its colonisation and formation of opportunistic infections; 2) investigate the phenotypic and genotypic antimicrobial resistance profiles of a range of clinical isolates of *S. felis* and 3) use MLST to infer the population structure of *S. felis* among Australian cats.

6.3 Methods

6.3.1 Collection and identification of *S. felis* isolates

Isolates were collected as part of the first Australian survey into antimicrobial resistance in veterinary staphylococcal clinical isolates, as described in Chapters 2, 3 and 4 (229). Isolates were collected from veterinary diagnostic laboratories located in all Australian states and mainland territories. Although laboratories were instructed to forward only coagulase-positive *Staphylococcus* spp. to the researchers, 172 isolates from coagulase-negative species were received. Samples were isolated from a range of animal species, including 74 isolates from cats. For each isolate, the geographic postcode of origin, clinical syndrome (as recorded by the submitting veterinarian) and site of isolation were recorded. Samples from the urinary bladder, urinary catheters or free catch urine samples were all recorded as feline lower urinary tract samples. Preliminary staphylococcal speciation was determined by traditional phenotypic tests (Gram stain, catalase and coagulase testing) and confirmed by the BD™ Bruker MALDI Biotyper™ with all isolates run in duplicate and results with a confidence score above 1.8 deemed acceptable for speciation.

All presumptive *S. felis* isolates underwent whole genome sequencing as previously described (Section 2.4) (273). DNA was extracted from staphylococcal samples using the Qiagen DNA MiniAmp kit according to the manufacturer's instructions for Gram positive bacteria, with lysozyme used as the

cell lysis agent (Qiagen, Valencia, USA). DNA yield and purity (A_{260}/A_{280}) were measured using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Delaware, USA). DNA concentration was verified using the QuBit dsDNA kit (Thermo Scientific, Delaware, USA). DNA library preparation was performed according to the manufacturer's instructions (Nextera, Illumina, San Diego, USA). Whole genome sequencing was performed using the NextSeq 500 Desktop Sequencer, according to the manufacturer's instructions (Illumina, San Diego, USA). *De novo* assembly of sequenced data was undertaken using a commercial bioinformatics program (CLC Genomics Workbench 10, Qiagen, USA). The identity of presumptive *S. felis* isolates was confirmed by identification of the species-specific genes, thermonuclease (*nuc;* Accession number: AB465335.1) (317) and RNA polymerase subunit-B (*rpoB;* Accession number: AF325878.1) (318) in whole genome sequenced contigs. Contigs underwent *BLA*STn analysis in CLC Genomics Workbench and had to display homology of >98% with the *S. felis nuc* gene and *rpoB* reference sequences to be deemed a true *S. felis* isolates.

6.3.2 Coagulase testing

The tube coagulase test was conducted using sterile EDTA-treated rabbit plasma (Sigma Aldrich, USA) and EDTA-treated feline plasma collected from residual blood samples sent to the University of Sydney Veterinary School's diagnostic laboratory for routine haematology, using the technique outlined in Section 2.2.1. The slide agglutination test was used to assess the presence of clumping factor using rabbit and feline plasma. *S. aureus* ATCC 29213 was used as a positive control for all coagulase and clumping factor tests.

6.3.3 Antimicrobial susceptibility testing

Two methods of phenotypic antimicrobial susceptibility testing (AST) were performed. Antimicrobial disk diffusion was undertaken using the direct colony suspension method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for bacteria isolated from animals, as outlined in Section 2.3. Isolates were deemed susceptible or resistant based on CLSI guidelines for veterinary coagulase-negative staphylococci, where available (173). If animal-derived data was not available,

human data from the CLSI guidelines were used (174). The following antimicrobials were tested by disk diffusion: amoxicillin-clavulanic acid, cephalothin, cefoxitin, cefovecin, oxacillin, penicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, enrofloxacin, marbofloxacin, pradofloxacin, clindamycin, gentamicin, chloramphenicol, rifampicin, azithromycin and tetracycline. All disks were BD brand (BD BBL™ Sensi-Disk™, Sparks) except cefovecin (Oxoid, Basingstoke). Automated AST was carried out by broth microdilution using the Vitek 2™ system (bioMerieux, USA), according to the manufacturer's instructions. The following antimicrobials were tested: benzylpenicillin, oxacillin, enrofloxacin, erythromycin, gentamicin, clindamycin, tetracycline, chloramphenicol, rifampicin and trimethoprim sulfamethoxazole. Minimum inhibitory breakpoints for coagulase-negative staphylococci were used, as outlined in CLSI guidelines for humans (174).

6.3.4 Resistance gene screening

Isolates were screened for resistance genes by uploading their fasta files onto the open-access bioinformatics website, ResFinder (https://cge.cbs.dtu.dk//services/ResFinder/) (234). Sequences required >90% homology do be deemed positive for a particular gene.

6.3.5 Virulence gene screening

Putative *S. felis* virulence genes were searched for using BLASTn in CLC Genomics. A local *bla*st database was created by downloading the sequences of virulence genes for *S. aureus* from the open access-bioinformatics website, VirulenceFinder (https://cge.cbs.dtu.dk/services/VirulenceFinder/) (235). *S. felis* sequences with >60% homology to the Virulence Finder gene sequences were then screened for homology to sequences from other staphylococcal species using the *BLA*STn function of the NCBI website. The following additional sequences that are described as staphylococcal virulence factors but not available from VirulenceFinder were downloaded from the NCBI website: biofilm-associated surface protein, *bap* (106), lysozyme-resistance determinant, peptidoglycan *O*-acetyltransferase, *oatA* (80), *S. (pseud)intermedius* exfoliative toxin, *siet* (319), autolysin/adhesion, *atl*

(89), capsular polysaccharide synthesis locus, *cap* (320), and coagulase, *coa* (321), (Accession numbers: AY220730.1, CP009046.1, AB099710.1, D17366.1, U81973.1 and X17679.1 respectively).

6.3.6 Development of a multilocus sequence typing scheme

Five genes were chosen as loci for the multilocus scheme, based on the housekeeper genes previously described in the MLST schemefor *S. pseudintermedius* (57, 185). The sequences of the *S. felis* heat shock protein gene, *cpn60*, and elongation factor Tu gene, *tuf,* were downloaded from the National Center for Biotechnology Information (NCBI) website (Table 6.1). As no *S. felis* sequences were available for the remaining five housekeeper genes from the NCBI website, the other loci were downloaded from the MLST website for *S. pseudintermedius* (Table 6.1). All downloaded sequences were placed in a local BLASTn database in CLC Genomics Workbench. Each sequence was then used as a template and blasted against *de novo S. felis* contigs to find the *S. felis* sequences. BLASTn homology was used to determine allele number: blast results that shared the same homology with the template sequence were given the same allele number.

Table 6-1. Details of loci and alleles used in multilocus sequence typing of *Staphylococcus felis*

Locus	Origin of template sequence	Location of template sequence/accession number	Template sequence length (bp)	S. felis sequence length (bp)	Alleles present in this study
cpn60	S. felis	AF242282.1	553	553	5
tuf	S. felis	HM352941.1	615	615	3
ack	S. pseudintermedius	S. pseudintermedius MLST website	564	564	6
fdh	S. pseudintermedius	S. pseudintermedius MLST website	259	258	6
pta	S. pseudintermedius	S. pseudintermedius MLST website	492	497	9

6.3.7 Phylogenetic analysis

A phylogenetic tree was generated using the online bioinformatics program, CSI Phylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) (243). This program used a Maximum Likelihood

algorithm to depict inferred phylogeny based on concatenated alignment of high quality single nucleotide polymorphisms. To construct the tree, fasta files of all genomes were upload to the online platform, which aligned them to a reference genome. As no reference genome is currently available for *S. felis*, both *S. aureus* NCTC8325 (Accession number: NC_007795.1) and *S. pseudintermedius* ED99 (Accession number: NC_017568.1) were trialled as reference genomes for construction of the tree. A higher percentage of the *S. pseudintermedius* genome (5.9%) was covered by all *S. felis* isolates than the *S. aureus* genome (2.3%), so *S. pseudintermedius* ED99 was chosen as the final reference genome for phylogenetic tree construction. FigTree 1.4.3 was used to optimise visualisation of the phylogenetic tree.

6.4 **Results**

6.4.1 Identification of *S. felis* isolates

Of the 74 isolates received from cats, 18 were coagulase positive staphylococci (CPS) and 56 were coagulase negative (CNS). Thirty-eight isolates were presumptively identified as *S. felis* using MALDI-TOF MS, which accounted for 51% of the total 74 staphylococcal isolates received from cats in the surveillance study. The remaining isolates were *S. aureus* (n= 18), *S. pseudintermedius* (n= 10), *S. epidermidis* (n= 3), *S. haemolyticus* (n= 2) and *S. warneri* (n= 1) No species could be identified in two coagulase-negative isolates, despite the isolates undergoing MALDI-TOF MS in triplicate. These isolates were removed from the study. *S. felis* were isolated from cats with a range of diseases including feline lower urinary tract disease (FLUTD; n= 15), otitis externa (n= 14), ocular disease (n= 2), and pyoderma (n= 1). For the remaining six cats, one had acute renal failure, two had neurological signs, and a clinical diagnosis was not recorded for three cats (Table 6.3).

Despite all 38 *S. felis* isolates undergoing DNA extraction, sufficient DNA purity (A_{260}/A_{280} >1.8) could not be obtained from nine isolates despite repeated attempts at extraction. Therefore, 29 of the 38 isolates underwent successful DNA extraction and whole genome sequencing. All isolates had their identity confirmed by demonstrating >98% homology with the *S. felis*-specific *nuc* gene in whole

genome sequence data. All isolates except F11 displayed 99.81% homology with the *rpoB* gene from *S. felis* (Accession: AF325878.1). F11 displayed 100% homology to a 91% long fragment of the *S. felis rpoB* gene but also displayed a second copy of *rpoB* that had 100% homology to a 91% length sequence of *S. aureus.*

6.4.2 Multilocus sequence typing of *S. felis* isolates

Fourteen sequence types were present amongst the 29 *S. felis* isolates analysed (Table 6.2). The most common sequence types were ST6 (n= 7), ST4 (n=3) and ST10 (n= 3). The allelic profiles of each ST are shown in Table 6.2. Sequence types did not cluster according to clinical syndrome or geographic origin (Table 6.3).

6.4.3 Antimicrobial resistance amongst *S. felis* isolates

Table 6.3 shows that isolates had a very low level of antimicrobial resistance. Automated Vitek AST detected tetracycline resistance (MIC>1mg/L) in one isolate while all other isolates were susceptible to all antimicrobials tested. Meanwhile, disk diffusion detected tetracycline resistance (zone diameter <14mm) in a further isolate and penicillin resistance (zone diameter <28mm) in three isolates. Resistance genes were rare, with *blaZ* being present in 2 of 29 isolates screened (7%) isolates and *tetK* in 1 (3%) isolate. Table 6.3 shows that disk diffusion results showed greater agreement with gene screening: both *blaZ*-positive isolates were also phenotypically penicillin-resistant on disk diffusion but susceptible on Vitek screening. Unfortunately, whole genome sequencing was not performed on one penicillin-resistant isolate and one tetracycline-resistant isolate, so the phenotype and genotype could not be compared for these two isolates.

6.4.4 Virulence factors

Two isolates (7%) (F11 and F33) were coagulase-positive when tested using feline plasma; the remaining isolates were negative (Table 6.4). The two coagulase-positive isolates were negative at 4 hr but positive at 24 hr. No isolates were coagulase-positive when tested with rabbit plasma.

Table 6-2. Allelic profiles and sequence types of clinical Staphylococcus felis isolates from cats in Australia

Sequence type	Loci and corresponding alleles				
	ack	pta	tuf	fdh	cpn60
1	1	4	2	1	3
2	1	4	2	2	1
3	1	4	2	6	4
5	2	3	1	1	3
6	2	8	1	1	3
7	3	5	2	4	6
8	3	7	1	5	3
9	3	9	1	1	3
10	4	7	1	5	3
11	5	6	3	5	3
12	5	1	1	5	1
13	2	4	1	1	3
14	3	7	1	5	2
15	6	7	1	5	2
16	1	4	1	5	1

All isolates were negative for the clumping factor test using rabbit and feline plasma. Both isolates that were coagulase-positive on feline plasma were positive for a homologue of the coagulase gene, *coa* (Table 6.3). Isolate F11 harboured a contig with a 1428bp sequence with 95% homology to the reference *coa* sequence, while isolate F33 harboured several smaller contigs that displayed between 81 to 95% homology with *S. aureus coa*. All isolates harboured putative homologues of the gene sequences for β-haemolysin (*hlb*), biofilm-associated protein (*bap*), the lysozyme-resistance

determinant, O-acetyltransferase (*oatA*), autolysin/adhesion (*atl*), exfoliative toxin (*siet*) and capsular polysaccharide synthesis protein, *capO* (Table 6.4). Additionally, 20/29 (69%) of isolates including all ST4, ST6 and ST8 isolates possessed a putative gene with 74% sequence homology to the second *S. pseudintermedius* exfoliative toxin gene, *exi*. None of the isolates harboured homologues of the enterotoxins from the VirulenceFinder database.

6.4.5 Phylogenetic analysis

In the phylogenetic tree, isolates generally clustered with other isolates from their sequence type (Figure 1). Isolates did not cluster according to disease syndrome or geographic origin (Data not shown). Isolate F11, a coagulase-positive isolate, formed a branch that was separate to all other isolates in the tree. F11 was more closely related to other *S. felis* isolates than *S. pseudintermedius* or *S. aureus*.

Table 6-3. Characterisation of clinical Staphylococcus felis isolates from cats in Australia, 2013-2014

Isolate	Site of isolation	Clinical syndrome	Geographic origin	Sequence Type	Resistance phenotype: VITEK MIC	Resistance phenotype: disk diffusion	Resistance genes
F2	Ear	Otitis externa	SA	1	0	0	0
F11	Ear	Otitis externa	SA	3	0	PEN	blaZ
F8	Ear	Otitis externa	NSW	4	0	0	0
F10	Ear	Otitis externa	SA	5	0	0	0
F4	Ear	Otitis externa	Unknown	6	0	0	0
F15	Ear	Otitis externa	NSW	6	0	0	0
F12	Ear	Otitis externa	NSW	9	0	0	0
F13	Ear	Otitis externa	Victoria	10	0	0	0
F30	Ear	Otitis externa	NSW	12	0	0	0
F27	Ear	Otitis externa	NSW	15	0	0	0
F23	Ear	Otitis externa	SA	ND	0	PEN	ND
F28	Ear	Otitis externa	NSW	ND	0	0	ND
F36	Ear	Otitis externa	NSW	ND	0	0	ND
F38	Ear	Otitis externa	NSW	ND	0	0	ND
F14	Ear	Neurological signs	NSW	8	0	0	0
F18	Ear	Neurological signs	NSW	11	0	0	0
F25	Ear	Not recorded	NSW	6	0	0	0

F1	Eye	Corneal stromal abscess	NSW	12	0	0	0
F17	Eye	Corneal ulcer	NSW	8	0	0	0
F35	Skin pustule	Pyoderma	QLD	ND	0	0	ND
F5	Urine	Acute renal failure	Victoria	6	0	0	0
F21	Urine	FLUTD	NSW	2	0	0	0
F6	Urine	FLUTD	Victoria	4	0	0	0
F22	Urine	FLUTD	NSW	6	0	0	0
F24	Urine	FLUTD	NSW	6	0	0	0
F20	Urine	FLUTD	NSW	7	TET	PEN, TET	blaZ, tetK
F16	Urine	FLUTD	NSW	10	0	0	0
F19	Urine	FLUTD	NSW	10	0	0	0
F29	Urine	FLUTD	NSW	13	0	0	0
F34	Urine	FLUTD	NSW	14	0	0	0
F33	Urine	FLUTD	NSW	16	0	0	0
F3	Urine	FLUTD	SA	ND	0	0	ND
F31	Urine	FLUTD	QLD	ND	0	0	ND
F32	Urine	FLUTD	Victoria	ND	0	0	ND
F37	Urine	FLUTD	NSW	ND	0	TET	ND
F7	Urine	FLUTD	QLD	6	0	0	0
F9	Urine	Not recorded	Unknown	4	0	0	0
F26	Urine	Not recorded	Victoria	14	0	0	0

FLUTD= feline lower urinary tract disease; QLD= Queensland, NSW= New South Wales; SA= South Australia; ND= not determined

Table 6-4. Putative virulence genes in clinical *S. felis* isolates from cats in Australia, 2013-2014

Gene name	Product name	Putative function	Reference sequence and S. felis homology to reference (%)	Number S. felis (%) isolates with gene
Exoenzymes				
Coa	Staphylcoagulase	Coagulation of host tissues	*S. aureus* 8325-4 (81-95%)	2 (7%)
Sak	Staphylokinase	Proteolysis of host tissues	*S. aureus* Mu50 (70%)	1 (3%)
Exotoxins				
Exi/expA	Exfoliative toxin	Proteolysis of host epidermis	S. pseudintermedius AB489850.1 (74%)	20 (69%)
Siet	Exfoliative toxin	Proteolysis of host epidermis	S. pseudintermedius ED99 (70%)	29 (100%)
Hlb	Beta-haemolysin	Destruction of red blood cells	*S. epidermidis* ATCC 12228 (67%)	29 (100%)
LukD/LukE	Leukotoxin	Destruction of white blood cells	*S. aureus* Mu50 (72%)	2 (7%)
Adhesins				
IcaA	Intercellular adhesion protein A	Biofilm production	*S. aureus* Mu50 (100%)	1 (3%)
Bap	Biofilm -associated surface protein	Biofilm formation	*S. aureus* V329 (61-64%)	29 (100%)
Atl	Autolysin/adhesin	Autolysis, adhesion and erythrocyte agglutination	S. pseudintermedius ED99 (76%)	29 (100%)
Others				
CapO	Capsular polysaccharide synthesis protein O	Capsule production	S. pseudintermedius ED99 (76%)	29 (100%)
OatA	O-acetyltransferase	Lysozyme resistance	S. epidermidis 12228 (68%)	29 (100%)
Scn	Staphylococcal complement inhibitor	Host immune evasion	S. aureus Mu50 (69%)	29 (100%)

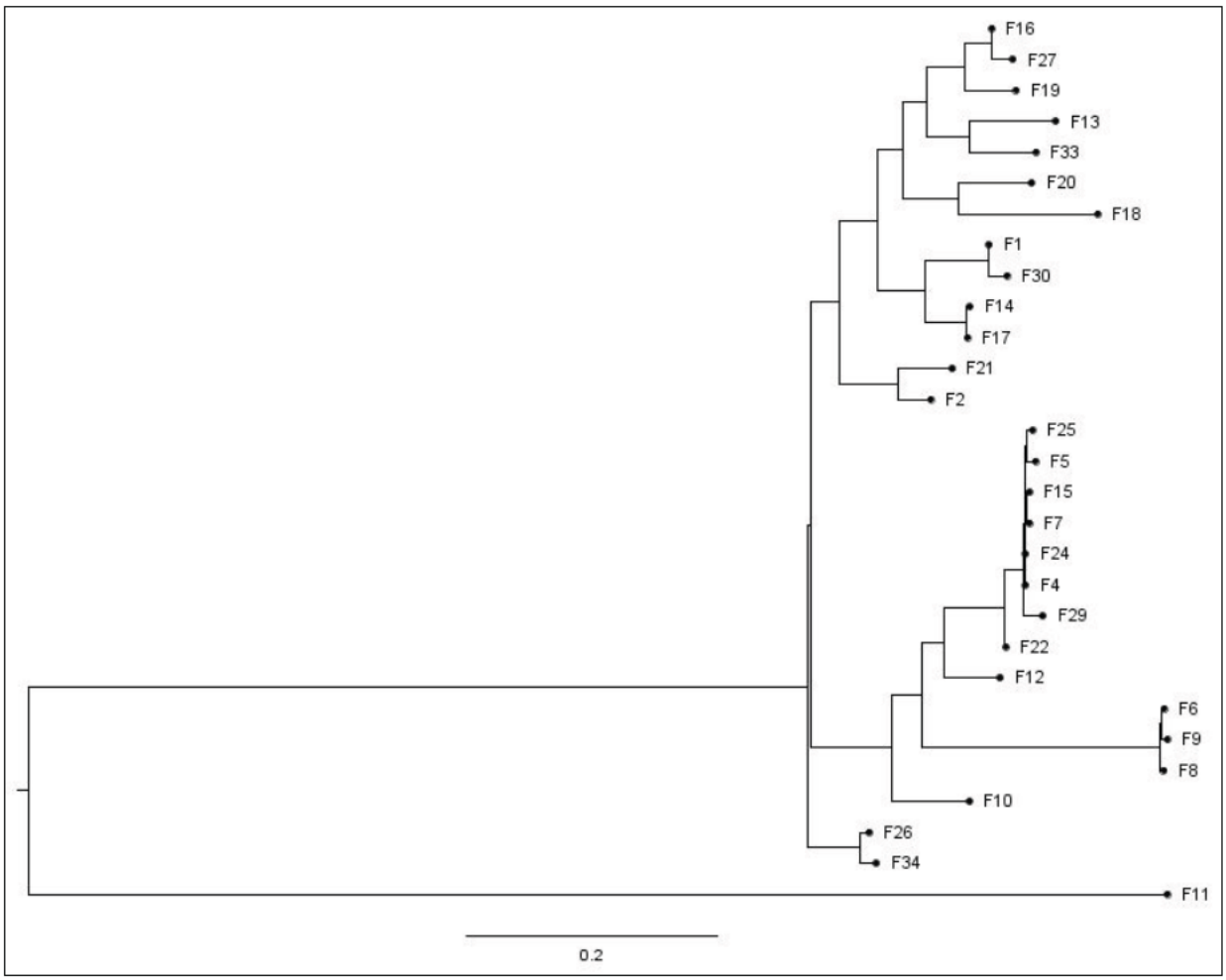


Figure 6-1. Phylogenetic tree of 29 *Staphylococcus felis* isolates from Australian cats. A Maximum Likelihood algorithm to depict inferred phylogeny based on concatenated alignment of high quality single nucleotide polymorphisms. *S. pseudintermedius* ED99 was used as a reference genome.

6.5 Discussion

This is the first study to use whole genome sequencing to identify putative virulence factors in *Staphylococcus felis* and the first to report coagulase-positive strains. The two coagulase-positive isolates harboured homologues of the coagulase gene *coa*, which each showed differing homology to the reference gene from *S. aureus* (321). The isolates were unrelated based on MLST and phylogenetic analysis. Detailed analysis of the coagulase gene homologues was not undertaken, but the distant relationship between the two coagulase-positive isolates and the lack of this gene in most *S. felis* isolates suggests that the two isolates acquired the gene by horizontal gene transfer. Staphylococcal species with variable coagulase phenotypes and genotypes have already been reported, such as *S.*

schleiferi in dogs (322, 323) and *S. agnetis* from production animals (324, 325). It is interesting that both coagulase-positive isolates were only positive when tested with feline plasma. Thus, they would still be deemed coagulase-negative if tested using the rabbit plasma that is traditionally used in diagnostic laboratories. Variation in coagulation activity against different host species' plasma has previously been observed in staphylococcal von Willebrand factor-binding protein (vWbp), which is one of the two coagulases produced by *S. aureus* (326). The finding of a novel coagulase homologue in two phenotypically coagulase-positive *S. felis* isolates shows that it can possess host-specific virulence determinants towards their main host species, cats.

Staphylococcal virulence factors are generally divided into four categories: exoenzymes, exotoxins, adhesins and others (76, 77). The *S. felis* isolates in this study demonstrated two or more gene homologues from each of these categories. Consistent with previous reports, the most common infections amongst *S. felis* isolates were urinary tract infections and otitis externa (68, 72, 73). Genomic analysis showed that most *S. felis* isolates harboured homologues of putative virulence genes that have been shown to play a role in cystitis and skin infections caused by other staphylococcal species. Specifically, 100% and 69% of isolates harboured homologues of *siet* and *exi* respectively, which are exfoliative toxin genes implicated in blister formation in staphylococcal dermatoses (117). Additionally, all isolates harboured a homologue of the *atl* gene which, in addition to conferring host blood cell agglutination in *S. aureus* isolates (327), is implicated in adhesion to uroepithelium by the human urinary pathogen, *S. saprophyticus* (328, 329). These results add further evidence to the notion that *S. felis* is a true urinary pathogen of cats, as suggested by Litster *et al.* (71).

Lysozyme is a host-produced enzyme that causes bacterial cell lysis (80). Bera *et al*. (80) examined a group of staphylococci from several different species and noted that those species with phenotypic lysozyme resistance were also those that are demonstrated pathogens in humans, while those that were sensitive to lysozyme were not known pathogens of humans. Thus, the authors concluded that lysozyme resistance is a marker of pathogenicity amongst *Staphylococcus* spp.. All *S. felis* isolates in

this study had a homologue of the *OatA* gene, which has been postulated to be the main determinant of lysozyme resistance in staphylococci (80). *S. felis* is a commensal of feline skin and is found in saliva (9, 10), both of which produce lysozyme (109). Any observer of cats will note their tendency for fastidious grooming, characterised by repetitive licking of their fur with lysozyme-laden saliva. It is therefore unsurprising that *S. felis* would require lysozyme-resistance to survive constant exposure to host lysozyme. Although phenotypic assays of lysozyme-resistance were not taken in this study, the fact that adequate DNA purity could not be obtained in 9/38 isolates could potentially be explained by the use of lysozyme as the cell lysis agent in the DNA extraction protocol, and lysozyme-resistance may have hampered cell lysis in these nine isolates. Future studies are required to determine whether the putative virulence gene homologues identified in the current study confer phenotypic virulence *in vitro*.

The isolates in this study had low levels of phenotypic antimicrobial resistance and very few harboured antimicrobial resistance genes. This is in contrast to other staphylococcal species collected from cats in the same surveillance study (229). Namely, 10% of the *S. pseudintermedius* and *S. aureus* collected from cats in the same surveillance study were methicillin-resistant and 12% of feline *S. aureus* isolates were resistant to fluoroquinolones (229). When methicillin-resistant *S. felis* has been reported previously, it has been from carriage rather than clinical isolates (10, 316). The current findings are in keeping with other studies that have found low levels of antimicrobial resistance amongst clinical *S. felis* isolates (71, 72). Possible reasons for lack of antimicrobial resistance amongst clinical *S. felis* isolates include: a fitness cost for resistant isolates allowing them to be outcompeted by more virulent pathogens, or the presence of other phenotypic virulence determinants that, at the site of an infection, result in failure of *in vivo* antimicrobial therapy despite *in vitro* susceptibility. Such determinants could include biofilm (106) and capsule formation (320), both of which the presence of putative gene homologues amongst the isolates in this study would suggest that *S. felis* is capable of producing. The practice of screening for occult urinary tract infections in cats, which entails sending a pre-antimicrobial sample of cystocentesed urine for culture and susceptibility as part of a diagnostic

work-up (330), may also create a selection bias that is in reverse to other clinical samples from surveillance studies, where samples are generally more likely to be submitted if refractory to antimicrobial treatment. A larger study that compares the antimicrobial susceptibility profiles of pre- and post-treatment feline urine samples would help to determine if *S. felis* truly has lower levels of resistance than other bacterial species.

MLST and analysis of single nucleotide polymorphisms were used to infer phylogeny amongst this group of *S. felis* isolates because both techniques are sequence based and therefore repeatable. Consequently, although the sample size is too small to give a detailed picture of the population structure of *S. felis*, clustering in the phylogenetic tree and the presence of several isolates from the same sequence types (e.g. ST6) suggest that *S. felis* may exhibit a clonal population structure similar to *S. aureus*, where successful strains arise from variation attributable to point mutations rather than recombination (286). The MLST scheme is admittedly preliminary, and will be benefited by analysis of a broader population of phenotypically characterised *S. felis* isolates. If validated, it is hoped that future studies can use this MLST scheme to examine carriage and infection isolates from wild and domesticated cats, allowing a deeper understanding of the *S. felis* population structure. Of note is the coagulase-positive isolate, F11, which formed its own branch in the phylogenetic tree and showed *rpoB* sequence homology to both *S. felis* and *S. aureus*. *Staphylococcus pseudintermedius* ED99 was used as a reference genome for the phylogenetic tree. However, as only 5.9% of the core genome was covered by the analysis, there may be some SNPs that were missed in the analysis. The closest phylogenetic relation of *S. felis* appears to be a coagulase-positive species isolated from otters, *Staphylococcus lutrae* (331). No whole genome sequence for this species was available at the time of analysis, but it has recently become available (332). Therefore, phylogenetic tree construction using this genome as a reference would be useful to confirm the phylogenetic structure of *S. felis*. Further, analysis of the 16s rRNA gene homology (331) may help to determine whether the outlying isolate F11 represents a novel coagulase-positive species or subspecies.

This study is limited by the fact that details regarding the initial culture conditions of each isolate were not recorded by the referring laboratory. We therefore cannot determine whether isolates came from heavy, pure cultures which would indicate that *S. felis* was truly the causative agent of the infections seen in this study. There is a chance that isolates came from mixed or light growth cultures, which would make it difficult to determine whether *S. felis* was the causative agent or merely a contaminant. Future studies would benefit from recording the quality of cultures received from infectious samples to provide a stronger aetiologic link between *S. felis* and the diseases seen. Secondly, besides phenotypic testing of the presence of coagulase, we did not confirm the putative functions of virulence genes homologues by undertaking phenotypic testing. Consequently, subsequent studies should compare genotype with phenotype to show whether *S. felis* does indeed possess the necessary virulence factors to be considered a true pathogen of cats. Lastly, the *S. felis* isolates in this study were collected as part of a surveillance study that requested veterinary diagnostic laboratories to submit coagulase-positive staphylococci to our research team. It is therefore possible that we selected for an atypical collection of *S. felis* that may have either appeared coagulase-positive during initial testing or eluded accurate speciation by the submitting laboratories. Nevertheless, these results again show the utility of surveillance studies in unearthing unexpected information about rarely-studied species such as *S. felis*.

6.6 **Conclusion**

In conclusion, this study examined a population of 38 *S. felis* clinical isolates from Australian cats and found that two were coagulase-positive when tested against feline-plasma, suggesting that host-specific coagulation can occur in this species. A preliminary five allele multilocus sequence typing scheme was developed, which found 14 sequence types amongst 28 typed isolates. Isolates mostly came from urinary tract infections and otitis, and all isolates demonstrated sufficient putative virulence genes to indicate they are capable of causing disease in these body systems. Overall, the findings of this study suggest that *S. felis* has the capacity to be a pathogen of cats.

Chapter 7. Biocide tolerance in methicillin-resistant *Staphylococcus aureus and Staphylococcus pseudintermedius* from animals

The content of this Chapter is published as a journal article, as follows:

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7.1 Abstract

Quaternary ammonium compound genes (*qac* genes) are associated with tolerance to certain veterinary biocides such as chlorhexidine and benzalkonium chloride. This study aimed to evaluate whether *qac* genes were present in a collection of 125 clinical veterinary-derived methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant and susceptible *S. pseudintermedius* (MRSP and MSSP), and to determine whether the isolates displayed phenotypic biocide tolerance. All isolates underwent whole genome sequencing, multilocus sequence typing and were screened for *qac* genes. A subset of 31 isolates underwent minimum bactericidal concentration (MBC) testing using chlorhexidine gluconate and benzalkonium chloride. MBC testing was conducted with and without the addition of bovine serum albumin (BSA) as an *in vitro* substitute for organic matter contamination. Two of 17 MRSA isolates (12%) harboured *qacA/B* genes; both isolates were ST8 from horses. *QacJ, qacG* and *smr* genes were identified in 28/90 (31%) MRSP and 1/18 (6%) MSSP isolates. ST71 isolates were significantly more likely to harbour *qac* genes than other MRSP clones ($p<0.05$). The presence of *qac* genes was not associated with increased phenotypic biocide tolerance. In the absence of bovine serum albumin, the MBC values for all isolates were well below the recommended usage concentration for each biocide. However, the presence of BSA significantly increased the median MBC

values of both chlorhexidine and benzalkonium chloride. Given that ST71 is the most common clone of MRSP in the world, further research is required to investigate why there is high *qac* gene prevalence amongst Australian ST71 isolates.

7.2 **Introduction**

Chapter 4 described how MRSP from Australia, like much of the rest of the world (184, 266), are generally multi- to extensively drug-resistant. The rise of multidrug and methicillin-resistant MRSP has led to heightened interest in the use of topical biocides as treatment for canine skin conditions (219, 333-335). *In vivo* (217, 218, 334) and *in vitro* (333, 335) studies have shown promising results for topical treatment of methicillin-susceptible and -resistant *S. pseudintermedius* infections with biocides such as chlorhexidine. However, there is growing concern in the human medical literature about the presence of genetic determinants of biocide tolerance in *Staphylococcus* species. One such genetic determinant is the quaternary ammonium compound (*qac*) resistance gene group (336, 337). Quaternary ammonium compound (QAC) resistance proteins are inducible efflux pumps encoded by plasmid-borne genes (227). QAC proteins appear to aid extraction of toxic cationic compounds and can protect against certain host-derived antimicrobial peptides (336, 338-341). QAC proteins are found in several bacterial genera including *Staphylococcus* spp. and can be divided into two broad groups: the Major Facilitator Family, which includes *qacA* and *qacB*, and the Small Multidrug Resistance protein family, which includes *qacG, qacH, qacJ* and *qacC/smr* (338). The prevalence and distribution of *qac* genes varies with geography, *Staphylococcus* species, and the clinical origin of the isolate (338).

In vitro studies have shown that *qac* genes can increase biocide tolerance amongst *Staphylococcus* isolates, but efflux capability varies depending on their specific *qac* gene and the compound being tested (226, 227). *QacA*-positive isolates have higher tolerance for biocides than *qacB* while isolates housing *qacJ* show increased biocide tolerance compared to *qacG* and *smr*-positive isolates (227). The

quaternary ammonium compound, benzalkonium chloride, and the bisbiguanide, chlorhexidine, are two cationic biocides that are commonly used in human and veterinary medicine. Several studies have found that *S. aureus* isolates that house *qac* genes demonstrate higher tolerance to benzalkonium chloride and chlorhexidine, evidenced by a significantly higher minimum bactericidal concentration (MBC) in *qac*-positive isolates compared to *qac*-negative isolates (342, 343). Although *qac* genes have historically been termed biocide 'resistance' genes, most studies have found that while *qac*-positive isolates tend to have a higher MBC than *qac*-negative isolates, the MBC for all isolates is still much lower than the recommended concentrations for practical biocide disinfectant use in hospitals (343, 346). It is therefore more appropriate to speak in terms of biocide 'tolerance' rather than resistance because if used at their correct concentration, biocides are generally effective at killing even *qac*-positive isolates.

Biocide tolerance has important implications for infection control, particularly for management of difficult to treat organisms like methicillin-resistant *Staphylococcus* spp in hospitals that use cationic biocides such as benzalkonium chloride or chlorhexidine. Several studies in human medicine have examined *qac* genes in MRSA and demonstrated their correlation with *in vitro* biocide tolerance (225, 342, 343), but similar studies in companion veterinary medicine are lacking. MRSA with increased tolerance to benzalkonium chloride has been found in *qac*-positive MRSA from pigs (344) and a meta-analysis of 400 pig herds found that heavy disinfectant use in fattening herds was associated with a higher prevalence of MRSA carriage amongst pigs than herds that did not use disinfection (345). *Qac* genes have been found in low numbers of methicillin-susceptible *S. pseudintermedius* (MSSP) from dogs (347) and a range of *Staphylococcus* species from horses (227, 348, 349) but they have not yet been reported in methicillin-resistant *S. pseudintermedius* (MRSP). Given the rising prevalence of MRSP in veterinary medicine (40) and its growing profile as a potential zoonotic pathogen (66), the possible presence of *qac* genes in MRSP needs to be addressed. Consequently, this study searched for *qac* genes in 125 *S. pseudintermedius* and *S. aureus* clinical veterinary isolates and examined phenotypic benzalkonium chloride and chlorhexidine tolerance in a subset of 31 isolates.

7.3 Materials and methods

7.3.1 Bacterial isolates

One hundred and eight clinical isolates of *S. pseudintermedius* (90 MRSP, 18 MSSP) and 17 methicillin-resistant *S. aureus* (MRSA) were included in the study. Bacterial isolates were obtained from three collections housed at the University of Sydney. Collection A (n= 113) came from a nation-wide surveillance study which collected all clinical veterinary isolates of coagulase-positive *Staphylococcus* from January 2013 to January 2014 (Chapters 3 and 4). Collection B (n= 7) were clinical *Staphylococcus* isolates from canine pyoderma cases in Sydney, NSW, that were collected as part of an undergraduate research project in 2013. Collection C (n= 5) were freeze-dried archived clinical *Staphylococcus* isolates that had been collected by the Veterinary Pathology Diagnostic Services (VPDS) at the University of Sydney. The isolates from Collection C dated from 1999 to 2002. The MRSP originated from dogs (n= 89) and cats (n= 1) while the MRSA isolates came from dogs (n=7), cats (n=3), horses (n= 6) and a kangaroo (n= 1). The MSSP originated from dogs (n= 16) and cats (n= 2). Isolates from Collection C were retrieved from freeze-dry storage as outlined in Section 2.1.5. The speciation of all isolates was determined by standard phenotypic tests and MALDI-TOF MS as outlined in Section 2.2, and were confirmed via identification of the species-specific thermonuclease gene, *nuc,* in sequenced data.

7.3.2 *In silico* analysis and typing

All isolates underwent whole genome sequencing and MLST as outlined in Section 2.5 and previously described (273). To screen isolates for *qac* genes, the following nucleotide sequences were downloaded from the NCBI database: *qacA/B, qacJ, qacG, qacH,* and *qacC/smr* (Accession numbers: NC_007931.1, NG_048046.1, NG_051904.1, NC_019081.1, GQ900464.1 respectively). Isolates were screened for *qac* genes using CLC Genomics Workbench (Qiagen, USA). A local BLAST database was created and the sequences were *bla*sted against *de novo* contigs. Isolates required 90% homology with the reference nucleotide to be deemed to be positive for that gene. Isolates that were positive for *qacA/B* will be referred to as '*qacA*-positive' from hereon in.

7.3.3 Biocide tolerance testing

MBC values were determined for two veterinary biocides: 5.4% w/v benzalkonium chloride (F10SC, batch number: 170922, Health and Hygiene Pty Ltd, South Africa) and 5% w/v chlorhexidine gluconate (Hexacon, batch number: 12355, Apex Laboratories Australia). MBC values were determined as previously described (339, 343, 346, 347), with the following modifications. Isolates were subcultured onto tryptose soy agar and incubated at 37°C overnight. They were then inoculated into 0.9% saline to obtain 0.5 McFarland standard, yielding a suspension with estimated $1.5x10^8$ CFU/mL. Two-fold dilutions of chlorhexidine and benzalkonium chloride were prepared in sterile water. The range of dilutions tested was 1:50 to 1:25600, which equated to chlorhexidine concentrations of 0.5 – 1000mg/L and benzalkonium chloride concentrations of 0.5 – 1080mg/L. To expose the bacteria to each biocide, 100µl of suspension colony was inoculated into 900µl of each diluted biocide and left at room temperature for 5 min. To inactivate the biocide, 100µl of the biocide/bacteria mix was then transferred to 900µl sterile neutralizer (3g/L lecithin, 30g/L tween 80 in phosphate buffered saline pH 7.4 +-0.4) and left at room temperature for 5min. Two 25ul drops of neutralized sample were then plated onto sheep blood agar (Oxoid, Basingstoke) and incubated for 24hr at 37°C. Survivors were enumerated using the drop plate method as previously described (346). Negative controls used sterile saline instead of biocide. MBC was determined by the concentration of biocide that yielded a 5 logarithmic reduction in bacterial survivors when compared to saline controls. Samples were run in duplicate. If duplicates returned a different MBC value, the higher value was designated as the MBC for that isolate. Duplicate results that were more than two-fold different from each other were repeated in triplicate; the median triplicate result was then recorded. Samples were prepared with and without the addition of bovine serum albumin (BSA in 0.9% sodium chloride, final concentration= 30g/L (3%)) to the biocide/bacteria mix. BSA was used to replicate the effect of protein contamination *in vitro* (343). Therefore, isolates were tested against four biocide preparations: chlorhexidine with 3% BSA (chlorhex + BSA); chlorhexidine without 3% BSA (chlorhex – BSA); benzalkonium chloride with 3%

BSA (benzalkonium + BSA) and benzalkonium chloride without 3% BSA (benzalkonium – BSA). ATCC *S. aureus* 29213 was used as an internal control strain.

7.3.4 Statistical analyses

For comparisons between groups of more than 10, the Mann-Whitney U test was used to assess differences in median MBC values (GraphPad Prism 7, USA). Categorical comparisons were undertaken by constructing contingency tables and performing Fishers exact test. Results were considered significant if $p<0.05$.

7.4 Results

7.4.1 Frequency of *qac* genes amongst *Staphylococcus* isolates

A total of 31/125 (25%) *Staphylococcus* isolates harboured *qac* genes, which consisted of 2/17 (12%) MRSA, 28/90 (31%) MRSP and 1/18 (6%) MSSP isolates (Table 7.1). The range of sequence types examined, and the *qac* genes that they harboured, are shown in Table 7.1. MRSA were the only isolates that housed *qacA/B* genes. Both *qacA/B*-positive isolates were ST8 MRSA isolates from horses. The most common *qac* gene amongst MRSP was *qacJ* (n= 15, 54%) followed by *qacG* (n= 8, 29%) and *smr* (n= 2, 7%). MRSP isolates from the same sequence type generally harboured the same *qac* gene, but ST71 MRSP isolates harboured either *qacJ* (n= 13), *qacG* (n= 2) or *smr* (n= 1). ST71 isolates were significantly more likely to harbour *qac* genes than other MRSP sequence types (OR= 6.9, CI= 2.5-19.0, $p<0.01$). Three MRSP isolates (one ST496, two ST45) housed a putative novel *qac* gene with only 83% homology to *qacJ*. This 324bp *qac* sequence had 100% homology to the sequence of an unnamed *qac* gene from the *S. aureus* plasmid, pKH4 (Accession number: U81980.1).

Table 7-1. *Qac* genes and multilocus sequence types (MLST) of coagulase-positive staphylococci from Australian animals

MLST	Number of *Qac*-positive isolates/total number of isolates	*Qac* genes present
MRSA		
ST8	2/2	qacA/B
Others	0/15	None
MRSP		
ST71	16/26	qacG, qacJ, smr
ST64	4/4	qacG
ST45	2/6	Unnamed putative *qac* gene#
ST496	1/8	Unnamed putative *qac* gene#
ST525	1/5	qacG
ST498	1/3	smr
ST25	1/1	qacJ
ST544	1/1	qacJ
ST537	1/1	qacG
ST544	1/1	qacG
Others	0/34	None
MSSP		
ST538	1/1	qacJ
Others	0/17	None

#Accession number: U81980.1

7.4.2 Biocide tolerance

The MBC values for benzalkonium chloride and chlorhexidine were determined for a randomly selected subset of 31 *qac*-positive and *qac*-negative isolates (Table 7.2). Fourteen of the tested isolates were *qac*-positive (n= 12 MRSP; n= 2 MRSA) while 17 were *qac*-negative (n= 11 MRSP; n= 6 MRSA). Of the *qac*-positive MRSP isolates that underwent MBC testing, most harboured *qacJ* (9/12; Table 7.2).

Table 7-2. Minimum bactericidal concentration (MBC) values of benzalkonium chloride and chlorhexidine for methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) isolates with and without *qac* genes

Isolate ID	Species	MLST	*Qac* gene present	MBC (mg/L)			
				Benzalkonium chloride		Chlorhexidine	
				With BSA*	Without BSA	With BSA	Without BSA
N13/1/408	MRSA	ST8	qacA/B	67.5	16.87	500	125
N13/1/396	MRSA	ST8	qacA/B	135	8.43	500	31.25
N13/1/17	MRSA	ST22	None	135	4.21	250	31.25
Q13/1/145	MRSA	ST22	None	135	2.1	250	62.5
N13/1/715	MRSA	ST22	None	67.5	2.1	250	31.25
N13/1/648	MRSA	ST612	None	135	16.87	250	250
V13/2/458	MRSA	ST612	None	67.5	4.21	500	31.25
N13/4/96	MRSA	ST612	None	33.75	4.21	250	62.5
ATCC29213	MSSA	Control	None	135	16.87	500	125
N13/4/25	MRSP	ST25	qacJ	67.5	2.1	125	15.63
Q13/1/190	MRSP	ST496	Unnamed *qac#*	135	8.43	250	15.63
V13/2/470	MRSP	ST71	qacG	135	8.43	250	7.81
V13/2/18	MRSP	ST71	qacJ	135	16.87	250	31.25
V13/2/18	MRSP	ST71	qacJ	67.5	4.21	500	31.25
V13/6/4	MRSP	ST71	qacJ	135	4.21	250	31.25
V13/6/5	MRSP	ST71	qacJ	67.5	4.21	250	31.25
N13/1/103	MRSP	ST71	qacJ	33.75	2.1	125	15.63
V13/2/133	MRSP	ST71	qacJ	16.87	4.21	250	15.63
V13/2/152	MRSP	ST71	qacJ	135	2.1	125	15.63
N13/1/480	MRSP	ST71	qacJ	67.5	2.1	250	15.63
Q13/1/311	MRSP	ST71	smr	33.75	2.1	250	15.63
N13/4/115	MRSP	ST496	None	135	4.21	250	15.63

V13/2/83	MRSP	ST497	None	67.5	4.21	500	15.63
V13/2/173	MRSP	ST497	None	67.5	67.5	250	15.63
V13/2/242	MRSP	ST497	None	33.75	1.05	125	31.25
V13/2/92	MRSP	ST497	None	16.87	1.05	125	15.63
V13/2/393	MRSP	ST71	None	67.5	2.1	125	7.81
V13/2/475	MRSP	ST71	None	135	4.21	125	7.81
V13/2/413	MRSP	ST71	None	135	4.21	250	7.81
V13/2/440	MRSP	ST71	None	135	4.21	250	7.81
V13/2/441	MRSP	ST71	None	16.87	2.1	250	15.93
V13/2/488	MRSP	ST71	None	135	2.1	125	7.81

*BSA= 3% bovine serum albumin; #Accession number: U81980.1

The range of benzalkonium chloride MBC values for MRSP isolates is shown in Figure 7.1. The median benzalkonium-BSA MBC of both *qac*-positive and *qac* negative MRSP isolates was 4.21mg/L. The median benzalkonium+BSA MBC of both groups was 67.5mg/L. Regardless of whether isolates were *qac*-positive or *qac*-negative, the median benzalkonium+BSA MBC (67.5mg/L) was significantly higher than the median benzalkonium-BSA MBC (4.21mg/L; $p<0.0001$). The range of chlorhexidine MBC values for MRSP isolates is shown in Figure 7.2. Like benzalkonium chloride, the median MBC of chlorhexidine was not significantly different between *qac*-positive and *qac*-negative isolates ($p=0.12$). The median chlorhex+BSA MBC of *qac*-positive isolates was 250mg/L, which was not significantly higher than *qac*-negative isolates (125mg/L; $p= 0.4$). The median chlorhex-BSA MBC of both groups was 16mg/L. Regardless of whether the isolates had *qac* genes, the median MBC of chlorhex+BSA (250mg/L) was significantly higher than the chlorhex-BSA MBC (15.63mg/L; $p<0.0001$).

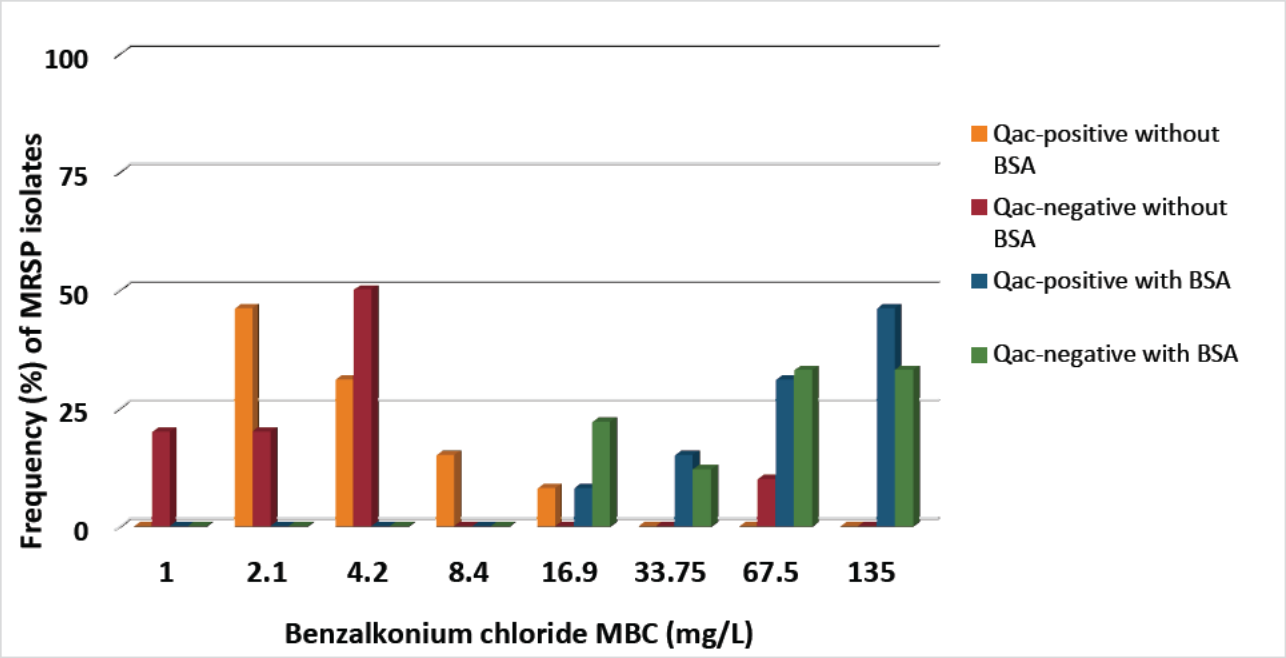

Figure 7-1. Minimum bactericidal concentration (MBC) of benzalkonium chloride tested on qac-positive and qac-negative methicillin-resistant Staphylococcus pseudintermedius isolates in the presence and absence of 3% bovine serum albumin (BSA). Minimum recommended in-used concentration= 108mg/L (Health and Hygiene Pty Ltd).

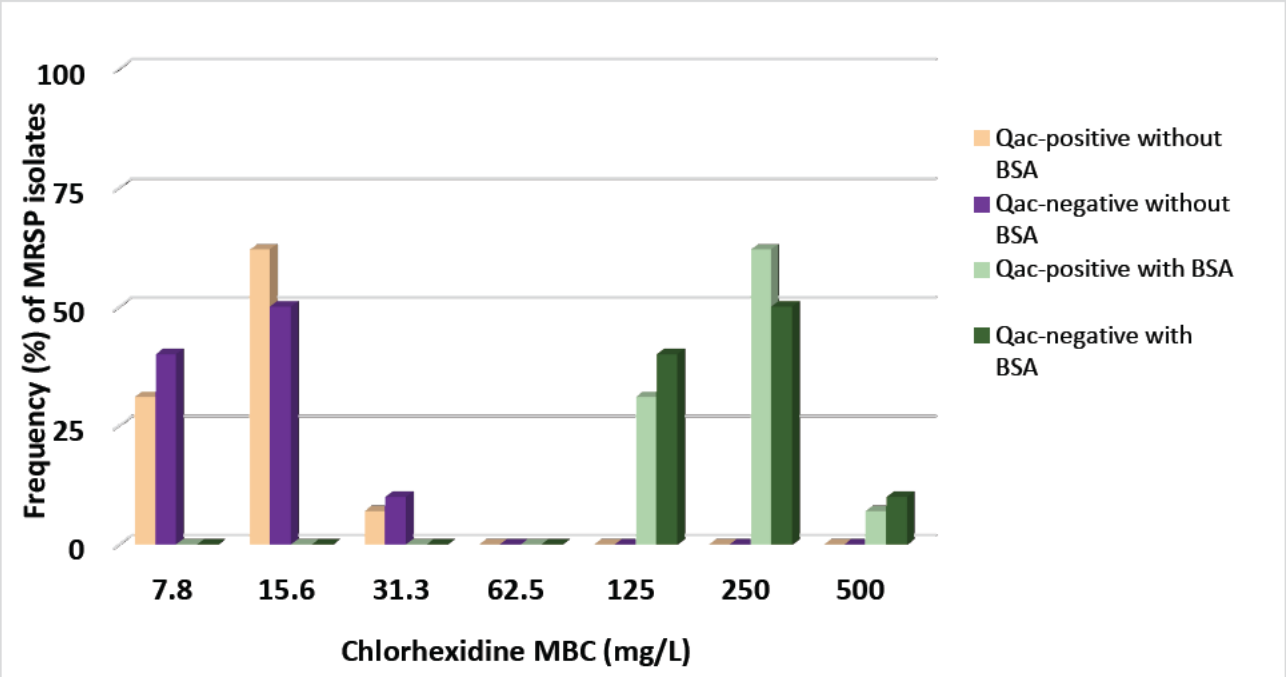

Figure 7-2. Minimum bactericidal concentration (MBC) of chlorhexidine tested on *qac*-positive and *qac*-negative methicillin-resistant *Staphylococcus pseudintermedius* isolates in the presence and absence of 3% bovine serum albumin (BSA). Recommended in-use concentration= 1000-5000mg/L (Apex Laboratories, Australia).

For MRSA, the benzalkonium+BSA MBC was 67.5mg/L for one *qac*-positive isolate and 135mg/L for the other, while the median MBC was 135mg/L for the *qac*-negative isolates (range= 33.75mg/L – 135mg/L). The benzalkonium-BSA MBC was 8.43mg/L for the first *qac*-positive MRSA isolate and 16.87mg/L for the other while the median MBC for *qac*-negative isolates was 4.21mg/L (range= 2.1mg/L – 16.87mg/L). The chlorhex+BSA MBC was 500mg/L for both *qac*-positive MRSA isolates while the median MBC for *qac*-negative isolates was 250mg/L (range= 125mg/L – 500mg/L). The chlorhex-BSA MBC for both *qac*-positive isolates was 62.5mg/L while for *qac*-negative isolates, the median MBC was 31mg/L (range= 16.87mg/L – 62.5mg/L).

7.5 **Discussion**

Biocide tolerance genes have previously been reported in MRSA from humans (225, 339, 342, 343) and MSSP from dogs (347), but this study documents the first instance of *qac* genes in clinical MRSP from dogs and MRSA from horses. Of the 90 MRSP isolates surveyed, 31% harboured either *qacJ, qacG* or *smr* while 2/17 (12%) of MRSA isolates harboured *qacA* genes. *In vitro* testing found no difference in the MBC of chlorhexidine or benzalkonium chloride between *qac*-positive and *qac*-negative MRSP isolates. Like previous reports (225, 342, 343), the MBC values for *qac*-positive MRSA isolates in the current study were generally higher than that of *qac*-negative isolates, but the sample size was too small to assess the statistical significance of this finding (n= 8). Both *qacA*-positive MRSA isolates were ST8 from horses. The ST8 MRSA lineage is more commonly associated with horses than most other MRSA lineages (350, 351) although equine-specific markers have not yet been detected. Screening of a larger sample of equine-derived MRSA will help to determine whether ST8 is more likely to carry *qac* genes than other lineages and if so, whether *qac* genes have phenotypic effects which might explain ST8's dominance amongst equine isolates.

ST71 MRSP isolates were significantly more likely to harbour *qac* genes than other MRSP clones. ST71 was the most common MRSP lineage in the current study and the surveillance study described in Chapter 4, which is consistent with previous reports (162, 184). It would be tempting to suggest that

the presence of *qac* genes has conferred biocide tolerance and a subsequent fitness advantage to the ST71 clone, as has been observed in an epidemic clone of *qac*-positive ST22 MRSA that outcompeted other MRSA clones in a human hospital setting (225). However, no difference was observed in MBC values between *qac*-positive and *qac*-negative isolates suggesting that the presence of *qac* genes does not confer increased biocide tolerance to MRSP isolates *in vitro*, or possibly that an unidentified mechanism exists in all isolates that overrides the effects of *qac* genes. A previous study found that *qacJ*-positive *S. aureus* isolates showed a higher benzalkonium chloride MIC than *qacJ*-negative isolates and those with other SMR protein family genes (*qacG* or *smr*) (227), but biocide tolerance was not assessed by MBC, so it is difficult to compare those results to the current study or to assess the practical implications of the previous study. The current sample size precluded comparing the MBC values of *qacJ*-positive isolates with other types of *qac* genes, so it is possible that significant differences exist that could not be detected. Alternatively, *qac* genes may confer a biological advantage to ST71 by mechanisms other than biocide resistance such as conferring resistance to host-derived antimicrobial peptides (340). *QacA*-positive *S. aureus* isolates survived exposure to thrombin-induced platelet microbicidal protein 1a (tPMP-1), a cationic peptide release by rabbit platelets. In acting as cationic compounds that disrupt the bacterial cell membrane (352), host antimicrobial peptides may act in a similar manner to cationic biocides like benzalkonium chloride or chlorhexidine (224). Staphylococci have been in contact with their hosts' antimicrobial peptides for many thousands of years more than manmade cationic biocides, so it is quite probable that any bacterial efflux effect originally evolved in response to exposure to antimicrobial peptides. If the function of *qac* genes was indeed originally to defend against mammalian cationic proteins, it makes sense that their coincidental efflux capacity for cationic biocides is variable, depending on the original mammalian protein targeted. This could explain why different *qac* genes appear to vary in their efflux capacity against various biocides (226, 227). Future studies could examine whether *qac*-positive MRSP lineages such as ST71 display higher tolerance to canine-derived antimicrobial peptides when compared to *qac*-negative isolates. Screening of archival veterinary staphylococcal isolates could also help to

determine whether any evolutionary correlations exist between the occurrence of *qac* genes and the use of biocides in veterinary practice.

Without the addition of bovine serum albumin (BSA), the benzalkonium chloride and chlorhexidine MBC values for MRSP isolates ranged from 1.05-4.2mg/L and 7.8-31.25mg/L respectively, which is similar to some previous studies (353) but higher than others (335, 347). However, detailed comparisons between studies cannot be made for a number of reasons. Firstly, the current study is the first to examine MRSP isolates with *qac* genes, so direct comparisons cannot be made. Secondly, most papers use different methodologies for MBC testing, with variations including time of exposure (353), formulation of chlorhexidine (335, 347), whether or not neutralisation occurred (353) and whether or not BSA was added as a study condition (343). The importance of neutralisation was exemplified in a recent study that showed without a neutralization step, the killing efficacy of a chlorhexidine-based hand scrub was estimated up to one logarithmic difference (354). Although standard recommendations exist for the testing of biocides, there are different recommendations given by the European and North American guidelines (355), which could explain some of the disparate methods used across studies. A single standardised method of MBC testing for staphylococci is clearly needed to obtain meaningful comparisons between studies.

A consistent finding in this study was that the presence of protein increased the MBC of chlorhexidine and benzalkonium chloride, regardless of whether isolates had *qac* genes or not. This study used the addition of 3% bovine serum albumin as an *in vitro* indicator of organic matter contamination that would be present on mammalian skin and would likely also be present in the veterinary hospital environment, where chlorhexidine and benzalkonium chloride are used as disinfectants. Liu *et al.* (343) compared chlorhexidine MBC values for MRSA isolates with and without 3% BSA, and found that the presence of BSA caused a four-fold increase in the chlorhexidine MBC of *qacA*-positive MRSA isolates. The current study also found that the addition of BSA yielded a statistically significant four-fold increase in the median chlorhexidine MBC for MRSP. A similar pattern of elevated MBC values in

the presence of 3% BSA was seen in the results of benzalkonium chloride tests. The labelled recommended concentration for Hexacon™ 5% chlorhexidine gluconate is 1000mg/L for general antisepsis and 5000mg/L for surgical skin preparation (Apex Laboratories, Australia). The chlorhexidine gluconate MBC values for MRSP and MRSA reported in this study are clearly below its recommended usage concentration and thus all isolates would still be killed if the product is used appropriately. Meanwhile, the recommended concentration of F10SC™ benzalkonium chloride is labelled as 1:500 (~108mg/L) for general disinfection, 1:250 (~432mg/L) for high level disinfection and 1:125 (~432mg/L) for resistant viruses such as parvovirus. In the presence of 3% BSA, 50% of MRSA isolates and 39% of MRSP isolates had an MBC of 135mg/L, which is above the recommended 108mg/L concentration for general disinfection. It is well known that the efficacy of biocides can be reduced by the presence of organic matter (343). Bovine serum albumin is merely an *in vitro* substitute for protein contamination, and it is likely that the real organic contamination found in a veterinary environment would have more of an effect on biocide efficacy than what was found in the current study. Overall, these results reinforce the importance of removing gross contamination and organic matter prior to disinfection in order to allow biocidal killing of MRSP and MRSA, particularly with disinfectants such as benzalkonium chloride.

This study used a biocide exposure time of 5 min to replicate the approximate time that a topical treatment such as a chlorhexidine-based shampoo may be applied to a dog (218). However, for several reasons the results of *in vitro* biocide testing undertaken here likely do not reflect what happens *in vivo* and thus the significance of these results must not be exaggerated. Firstly, BSA is a poor surrogate for the hair, skin cells and debris that would be on the skin of a dog with staphylococcal pyoderma. Secondly, compliance of animal owners and even veterinarians is an aspect that affects infection control and treatment measures which cannot be tested in *in vitro* studies such as this. An observational study found that veterinarians used a contact time of as low as seven seconds for a chlorhexidine-based surgical cleaning product during pre-operative hand scrubbing, despite the labelled recommendation being at least two minutes (222). Future studies must take into account the

high likelihood that pet owners and even veterinarians may not adhere to scientifically-proven biocide contact times, which could explain why topical therapy may fail in anecdotal accounts. More *in vivo* and prospective studies are required before the true efficacy of biocides against MRSP and MRSA can be truly known.

7.6 Conclusion

This study found that 31% of MRSP and 12% of MRSA isolates harboured *qac* genes. ST71 MRSP was significantly more likely than other lineages to harbour *qac* genes, raising interesting questions about the possible fitness advantages that *qac* genes may confer to this dominant MRSP clone. Although the sample size was larger than other previous studies (335, 347) this study was still limited by the relatively small sample size of isolates that underwent MBC testing. Future studies could be strengthened by undertaking MBC testing on a large sample size and ideally compare MBC values between as well as within clonal types, for example: a comparison of ST71 isolates with *qacJ, qacG* and *smr* against *qac*-negative ST71. Now that *qac*-MRSP isolates have been detected, ongoing surveillance studies will no doubt procure more *qac*-positive MRSP isolates that provide a larger sample pool for future studies. These *in vitro* results suggest that chlorhexidine and benzalkonium chloride are effective at killing MRSP and MRSA, provided protein contamination is limited. While *in vitro* studies are unarguably useful in obtaining baseline data about biocides, the clinical relevance of such studies must always be taken in the context of the veterinary practice. In such environments, biological parameters and human behaviour are equally important in determining the clinical efficacy of a biocide.

Chapter 8. Conclusions and future directions

This thesis contains five studies which expand our understanding of the epidemiology of veterinary staphylococci in Australia. The first Australian nationwide surveillance study generated a comprehensive collection of 1080 clinical *Staphylococcus* spp. isolates which allowed detailed examination of MRSA, MRSP and *S. felis* in Chapters 3, 4 and 6 respectively. Amongst companion animals, the frequency of MRSA and MRSP was 4% and 11.5% respectively. The frequency of resistant staphylococci in Australian dairy cattle appears lower than other countries, a key finding that could suggest Australia's strict antimicrobial prescribing practices for food producing animals may decrease selection pressure for resistant staphylococci. Chapter 3 also found that human- and ruminant-associated host adaptation markers can be present in MRSA isolates from both veterinarians and animals, highlighting how MRSA can cross the host species divide. Chapter 4 found that the globally successful ST71 lineage is the most common MRSP clone in Australia. Although universal fluoroquinolone resistance amongst ST71 could be one explanation for its dominance, Chapter 7 provided unexpected insights into another possibility. *Qac* genes, which are associated with increased biocide tolerance in MRSA, were discovered in MRSP isolates for the first time. ST71 was significantly more likely to harbour these genes than other MRSP clones, which raises fascinating questions regarding the potential fitness advantage that the presence of *qac* genes in ST71 may confer. Chapter 5 contained an interesting pilot study that examined a population of veterinary personnel and their patients simultaneously, in order to determine the extent to which transmission of MRSA and possibly MRSP occurred between humans and animals in this healthcare setting. A rate of 8% MRSA carriage was detected amongst veterinary personnel, but no shared carriage of MRSA or MRSP was found between veterinary personnel and their patients or their own pets. Chapter 6 detailed the unprecedented result of phenotypic coagulation of feline plasma by two *S. felis* isolates, a finding with significant diagnostic and clinical implications for both microbiologists and veterinarians. Although a

PhD is undoubtedly a magnum opus for the researcher who creates it, perhaps what is more interesting than the 200 pages of research initially generated are the questions raised and the future horizons made visible by the research. Consequently, it is now time to examine the findings of each study more thoroughly so that their clinical implications can be addressed and future studies envisioned.

The significance of MRSA as a public health pathogen is highlighted by its listing as one of nine bacteria of 'international concern' by the World Health Organisation (356). Research into the epidemiology of this pathogen is therefore of utmost importance, and consequently this thesis devoted two studies to the examination of MRSA in Australian animals and veterinarians. Chapter 3 found that human host-adaptation markers were present in MRSA isolates from animals, suggesting either that the isolates were associated with humans or that these markers are not accurate flags of host adaptation. From a clinical perspective, this finding serves to remind veterinarians that MRSA in companion animals, particularly wound infections, could well be of human origin (209). The role of veterinary personnel and pet owners in this cycle of transmission must consequently be considered in disease management plans. Knowledge regarding zoonotic diseases and infection control amongst veterinary personnel is generally quite poor (357-359), as is the level of communication about zoonoses between small animal veterinarians and their clients (358, 360). In showing that MRSA is most likely shared between veterinarians and the patients that they treat, Chapter 3 helps to provide sound evidence that will contribute to MRSA infection control guidelines for veterinary personnel. If veterinarians are aware that humans are a potential source of MRSA for veterinary patients, they may be more likely to heed recommendations for effective MRSA infection control such as hand hygiene (220) and may accordingly correctly advise pet owners of such practices.

To determine the extent to which humans are the source of MRSA wound infections in veterinary patients, a future study could undertake longitudinal concurrent sampling and molecular analysis of MRSA-infected wounds and carriage isolates from all in-contact humans and their environment. A

similar study was recently conducted into MRSP-infected dogs and in-contact dogs from the same household, which found that MRSP carriage was shared amongst household members until the index case became negative for MRSP carriage (361). An equivalent study in MRSA that includes sampling of human contacts might help to identify hotspots of household and hospital MRSA transmission. The study could draw on the sample processing and typing methods in Chapter 5. It would also be useful to establish whether correlations exist between veterinarian hand hygiene and the rate of nosocomial infections seen in practice. This could be achieved via a study that combines recently described direct surveillance methods to observe veterinarian hand hygiene behaviour (222) with a prospective assessment of the clinical outcomes of patients treated during the observation period. Such studies would ultimately enhance the appreciation for infection control measures and hand hygiene compliance in veterinary medicine.

Since the ground breaking Swann Report of 1969 (362), stakeholders at every jurisdiction level have been warned about the role that antimicrobial use in food producing animals may play in the global spread of antimicrobial resistance. Accordingly, Australia has taken a conservative stance when it comes to antimicrobial prescribing practices in veterinary medicine. Australia's antimicrobial control measures include the classification of antimicrobials as a 'restricted substance' that can only be supplied via prescription by a veterinarian or other health professional (363), and the prohibition of fluoroquinolone use in food producing animals (161, 255). The difference in MRSA frequency between companion canimals and cattle seen in Chapter 3, taken with the differences in antimicrobial use in Australian companion and food producing animals, provide evidence that antimicrobial usage patterns affect the frequency of antimicrobial resistance seen in particular regions and animal groups. Future investigations including ongoing surveillance studies can further strengthen the evidence regarding the link between Australian antimicrobial prescribing practices and the occurrence of antimicrobial resistance in particular animal groups. In particular, a study examining the prevalence of MRSA carriage in Australian pigs and cattle is strongly warranted, given that lineages such as CC398 may well have been missed in the current studies due to their focus on clinical submissions. *In vitro*

and retrospective human studies have already shown that fluoroquinolone use can select for methicillin-resistance amongst *S. aureus* populations (257, 258, 364). A prospective cross-over study that examined fluoroquinolone use and its effect on the microbiome as well as MRSA and MRSP carriage and infection in companion animals would be invaluable in providing evidence for the theory that fluoroquinolone use correlates with methicillin-resistance in veterinary medicine. Using methods featured in this thesis, such a study could involve serial longitudinal sampling of carriage and infection sites before and after the administration of fluoroquinolones, with subsequent *in silico* analysis of bacterial isolates comparing the sequence types, resistance genes and genetic mutations present at each sampling instance.

Australian legislation means that antimicrobials can only be prescribed by doctors, dentists and veterinarians under a patient-practitioner relationship (363), a practice that seeks to minimise inappropriate antimicrobial use in the community. In addition to legislation at a national level such as the prohibition of fluoroquinolones in food producing animals (363), antimicrobial usage at the hospital and patient level undoubtedly has a role in the selection for methicillin-resistant staphylococci in veterinary medicine. Recent studies have examined the antimicrobial prescribing practices of Australian veterinarians and found that veterinarians in companion animal and bovine sectors commonly demonstrate appropriate drug choice but inappropriate dosage intervals and treatment duration (295, 365-367). Thus, future studies must consider not only the effect of drug choice on the advent of antimicrobial resistance, but also the effects of inappropriate drug dosing. Given that pet owners and farmers are the main administrators of prescribed antimicrobials to outpatient animals, it follows that client compliance and the details of outpatient drug dosing are additional pieces of the puzzle that should be further investigated to understand the full effects of antimicrobial use on the selection of resistant pathogen such as MRSA and MRSP. As has already occurred in studies of Australian veterinarians (295, 365-367), a descriptive survey would be a useful start in gauging the knowledge and antimicrobial usage practices of Australian pet owners and farmers.

The pilot study in Chapter 5 found that 8% of veterinary personnel were MRSA carriers, a rate which is higher than that reported for the general population (210, 213, 214, 304, 305). There is now substantial evidence that veterinary professionals are at increased risk of MRSA carriage, but the occupation-specific risk factors are not yet known. A large multidisciplinary study is indicated to determine why veterinarians are at increased risk of MRSA carriage. It seems probable that veterinarians are exposed to risk factors that place selection pressure on their nasal microbiome. Potential risk factors that could be examined include patient handling techniques, the use of personal protective equipment, physical handling of antimicrobials, duration and frequency of exposure to biocides and concurrent burden of MRSA in the veterinarians' work and home environment. In the meantime, veterinarians should be aware of their increased occupational risk of MRSA carriage, both in terms of their own personal health and the potential risk of transfer to their patients.

Chapter 6 contained the surprising finding of phenotypic coagulation of feline plasma by two *S. felis* isolates. Given that the initial surveillance study aimed to collect only coagulase-positive staphylococci, the presence of 38 *S. felis* isolates amongst the resultant collection was unexpected. This serendipitously led to a study into *S. felis,* a species that may previously have been viewed by microbiologists and veterinarians as a lesser pathogen due to its status as a coagulase negative *Staphylococcus*. Variable coagulase activity has been reported in certain veterinary staphylococcal species (322-325), and such variable coagulase production draws into question whether the coagulase test is firstly reliable at differentiating staphylococci and secondly whether it truly does delineate pathogenic from non-pathogenic species. The finding that some *S. felis* isolates are able to produce host specific coagulase should make veterinarians and microbiologists view this species as a potential pathogen rather than discounting it as a contaminant. One coagulase-positive isolate formed a separate branch in the phylogenetic tree that was distinct from the other *S. felis* isolates. Future studies involving a larger collection of feline samples could investigate whether other isolates exist that are phylogenetically similar to the aberrant coagulase-positive isolate, which could then further use single nucleotide polymorphism analysis and DNA-DNA hybridisation to determine if this isolate

actually represents a novel species or subspecies. Molecular and phenotypic comparison of asymptomatic carriage samples with clinical isolates would also help to determine whether there is a difference between commensal *S. felis* isolates and those that create disease.

All *S. felis* isolates in this study originated as clinical submissions made by veterinarians presumably investigating a clinically overt disease process in a feline patient. It is therefore intriguing that, in stark contrast to the *S. pseudintermedius* and *S. aureus* isolates collected in the same surveillance study (229), all *S. felis* isolates displayed a very low level of antimicrobial resistance. It the absence of resistance which would cause predictable antimicrobial treatment failure, it seems likely that at least some of the isolates displayed sufficient clinical virulence to warrant sample submission by the veterinarian. The finding of putative virulence factors in coagulase-positive and negative *S. felis* is timely, given the current paradigm shift that is occurring regarding significance of CNS in veterinary medicine. Recently published guidelines for the management of staphylococcal skin and soft tissue infections in animals assert CNS can be considered the causative agents of disease as long as consistent pyogenic clinical signs are present and correct sample collection technique has been used (219).

As has been done with other similar studies examining the staphylococcal genome for virulence homologues (77, 116), the *S. felis* genomic study should be followed by an investigation into phenotypic expression of putative virulence factors. Such a study could involve isolation of *S. felis* from infectious samples from animals observed to have clinical disease and *in vitro* studies on these isolates, examining phenotypic expression of virulence factors such as lysozyme resistance and biofilm formation. The absence of antimicrobial resistance amongst *S. felis* also warrants further attention. Differing staphylococcal species appear to have varying capacity for horizontal gene transfer (151, 287-289) so comparison of *S. felis* genomes with other staphylococcal species such as *S. pseudintermedius* would be useful to see if there are differences in systems such as restriction modification. The first step in any such a study would be to complete the whole genome of *S. felis*, a feat which will be greatly facilitated by the framework generated from the genomic data in this thesis.

A novel finding of Chapter 7 was the predominance of *qac* genes amongst ST71 MRSP isolates in Australia. This is the first time that *qac* genes have been reported in MRSP. It initially appeared as though, in addition to universal fluoroquinolone resistance amongst ST71, the presence of *qac* genes might explain why ST71 is so dominant amongst clinical MRSP isolates. Theoretically, the presence of *qac* genes could elicit a survival advantage to ST71 by conferring biocide tolerance to *qac*-positive isolates. This hypothesis was not realised in the *in vitro* study of Chapter 7, which did not detect significantly increased biocide tolerance amongst ST71 or *qac*-positive isolates. The sample size for biocide testing was admittedly small, so a larger scale study is now warranted to further assess the effect of *qac* genes on biocide in veterinary-derived staphylococci. In addition to testing benzalkonium chloride and chlorhexidine, future studies could include newer veterinary products such as hypochlorous acid, which has shown promising *in vitro* results against veterinary staphylococci but has not yet been tested against *qac*-positive MRSP or challenged with protein contaminated test conditions (335). A study is currently underway that has screened the carriage isolates collected from dogs at Hospital B in Chapter 5 for the presence of *qac* genes and will now prospectively assess the clinical outcomes for *qac*-positive MRSP carriers and non-carriers undergoing surgery. This will help to determine the clinical relevance of *qac* genes amongst MRSP. As mentioned in Chapter 7, if the study finds that *qac* genes in MRSP do not in fact confer biocide tolerance, they may still confer a fitness advantage by yielding resistance to host-derived antimicrobial peptides (340). A future *in vitro* study that examines the bactericidal effect of canine-derived antimicrobial peptides against *qac*-positive and -negative MRSP would be useful to determine if this is the case. Meanwhile, it is heartening to remember that chlorhexidine effectively killed all *qac*-positive and –negative MRSP *in vitro,* which adds to existing *in vivo* evidence (218) that it remains an effective topical treatment option for methicillin-resistant skin infections.

Chapter 4 found clear geographic clustering of MRSP sequence types amongst clinical isolates from Australian animals, adding further weight to the notion that MRSP clones are shared between the dogs within a particular population (184, 266, 283). This concept was further supported by the findings

of Chapter 5, which found that dogs in the Sydney region mostly carried ST496 and ST64 MRSP. The finding that dogs within geographic regions share MRSP has interesting potential implications for infection control. While it is appreciated in human and veterinary medicine that many staphylococcal infections can be endogenous (201-203), it is also known that they can be exogenous. Indirect evidence for exogenous infection was found firstly in Chapter 3, where human-associated ST22 clones were the cause of most of the MRSA infections in companion animals. Further evidence for exogenous infection was found in Chapter 4, where the close geographic clustering of ST497 and ST71 isolates from the same postcode in Melbourne, Victoria, suggested a common external source of infection. It cannot be determined whether the MRSP isolates in Chapter 4 were endogenous or exogenous, because samples were only taken from clinical lesions and the dogs were not sampled for asymptomatic carriage before they developed their infection. To more thoroughly understand the epidemiology of exogenous and endogenous staphylococcal infections in companion animals, a large scale prospective study including repeated sampling at carriage and infection sites would be required. Such a study would need to consider several confounding factors such as sample population, host factors, disparate diagnostic criteria for the various staphylococcal diseases, and indeed the definition of what constitutes an endogenous or exogenous infection. Until the effects of each of these confounders can be extrapolated, such a study be would be logistically complex. A simple preliminary study could start by comparing MRSP carriage amongst dogs visiting veterinary hospitals with those at dog parks or grooming parlours, as this would help to determine the effects of sample population on the rate and molecular epidemiology of MRSP carriage amongst specific dog populations.

Taken together, Chapters 4 and 5 add to our knowledge about MRSP transmission by showing that dogs within the same environment can share the same clone of MRSP, both in carriage and infection samples. Dogs with known risk factors for MRSP carriage such as a history of antimicrobial use or skin disease (204-206) should thus be treated as potential sources of MRSP for other dogs, including those in their own household. Analogous to MRSA in human hospitals (220), appropriate infection control protocols and hand hygiene practices would likely play a key role in reducing the transmission of

MRSP. Although limited by its small sample size, Chapter 5 contains a very useful pilot study that was cost effective and efficient to undertake. Therefore, it is hoped that future studies might now use a similar study design to conduct larger scale studies to generate more robust results regarding the transmission of MRSP in veterinary practice.

A clinically relevant finding from Chapter 7 was that the minimum bactericidal concentration (MBC) of both chlorhexidine and benzalkonium chloride increased four-fold when bovine serum albumin (BSA) was added as an *in vitro* substitute for organic matter contamination. The addition of BSA caused the MBC of benzalkonium chloride to rise above the minimum recommended usage concentration for some isolates, indicating that these isolates would not be killed in the clinic environment if the site was not first cleared of organic matter. BSA was used as an *in vitro* substitute for the organic matter that is abundant in veterinary practice such as hair, skin, protein and bodily fluids. Although BSA is the best available *in vitro* analogue of organic contamination, it is admittedly a poor substitute for the complexity of contamination that would be present in reality. It is therefore likely that organic matter contamination would have an even greater detrimental effect on biocide efficacy than was evident in this *in vitro* study. Such a finding is useful to remember when designing guidelines for infection control measures and is also pertinent to future studies designed to assess the efficacy of veterinary biocides.

The design of future biocide studies is complicated by the fact that there is no standardised international protocol for biocide testing, making it difficult to compare between studies or to choose the best method to use. As noted by Kampf *et al.* (354), inappropriate methodology when biocide testing can cause inaccurate results being reported, leading to the production of misinformed infection control protocols and ultimately suboptimal patient care. For this reason, care was taken in the biocide study design to include factors such as neutralisation of the biocide and a five minute contact time, both of which have been shown to affect the results of MBC testing (339, 354). Short contact times might mimic or even exaggerate the time of biocide exposure used in veterinary practice (222), but it is unlikely that any *in vitro* model would ever be able to simulate the organic conditions

and underlying patient diseases that would influence veterinary biocide efficacy *in vivo*, not to mention the additional confounds introduced by veterinarian and owner compliance. Nevertheless, a standard *in vitro* methodology would at least permit direct comparisons between studies, something which would allow broader conclusions to be drawn and more evidence-based infection control guidelines to be created.

This thesis made extensive use of whole genome sequencing (WGS) to obtain typing and gene screening data. When the first MRSP isolate was sequenced in 2014, no online bioinformatics websites had been developed and all typing was undertaken using manually curated BLAST databases. Consequently, it took approximately one day per isolate to obtain MLST, SCC*mec, dru* and *spa* typing data. Since that time, several freely accessible online bioinformatics tools have become available such as the Center for Genomic Epidemiology website, https://cge.cbs.dtu.dk, which has separate automated tools for MLST, *spa* typing, resistance and virulence gene screening and phylogeny; reducing the time of analysis to one to two hours per isolate. Most recently, advances in bioinformatics and internet speed have enabled the development of 'all-in-one' pipelines such as www.goseqit.com that can provide MLST, *spa*, virulence and resistance gene screening within ten minutes. The goseqit website was developed after all bioinformatics analysis in this thesis had been completed so it was not used in any studies. Future studies will undoubtedly benefit from having such cost effective and rapid typing tools. Such rapidity means that WGS is almost at the stage of being a clinically useful tool because sample analysis time could now be as short as 48 to 72 hours. While genomic analysis provides invaluable epidemiological information about a bacterial isolate's likely ancestry and virulence potential, it must be remembered that it cannot shed light on two other crucial elements of staphylococcal pathogenesis: host factors and phenotypic expression of the bacterial genome. If rapid bioinformatics, time and resources had been available, this thesis could have benefited from deeper analysis of phenotype and host interactions, such as examining potential phenotypic virulence differences between ST71 and other clones of MRSP. Future studies will now benefit from rapid, automated, and freely available bioinformatics tools which will allow more time

and resources to be focussed on the concomitant examination of host factors and bacterial phenotype involved in staphylococcal disease. In the meantime, the studies contained in this thesis have provided an invaluable frame work upon which future investigations into veterinary staphylococci can be built.

To date, the findings of this thesis have been communicated with veterinarians and microbiologists by oral presentations at eight peer reviewed conferences, many of which have been followed by flurries of interest and offers of collaboration. It is heartening to know that veterinarians 'at the coal face' are interested and motivated to learn more about the epidemiology of resistant staphylococci in veterinary practice. Such interest can only serve to foster better veterinary medicine and ultimately better results for patients. Results have been further disseminated by publication of three peer-reviewed papers, with a further four likely to follow. The findings within this thesis will be most useful when shared with other stakeholders in veterinary medicine, such as pet owners and primary producers. MRSA clearly creates disease in humans as well as animals and MRSP isolates are a potential source of several resistance determinants that could be transferred to human pathogens. *Staphylococcus felis* has not been reported as a human pathogen, but its genome may hold clues that could mitigate the acquisition of antimicrobial resistance by other staphylococcal species. While this thesis has focussed on staphylococci of veterinary significance, its results are also pertinent to human medical physicians and public health experts. In order to effectively understand and manage staphylococcal disease, a One Health approach that combines the expertise of human and veterinary medicine is therefore truly indicated.

In the war against resistant bacteria, there is still much to learn before the remarkable *Staphylococcus* genus is fully understood. While we must concede that *Staphylococcus* is a formidable opponent, this thesis makes a valuable contribution to our arsenal in the fight against antimicrobial resistance in staphylococci.

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