PREVALENCE OF COLONIZATION AND ANTIMICROBIAL RESISTANCE AMONG COAGULASE POSITIVE STAPHYLOCOCCI IN DOGS, AND THE RELATEDNESS OF CANINE AND HUMAN *STAPHYLOCOCCUS AUREUS*

A Thesis Submitted to the College of
Graduate Studies and Research
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy
In the Department of Veterinary Microbiology
In the College of Graduate Studies and Research
University of Saskatchewan
Saskatoon, Saskatchewan

By

Joseph Elliot Rubin

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Abstract

Coagulase positive staphylococci, *Staphylococcus aureus* and *Staphylococcus pseudintermedius*, are important causes of infection in human beings and dogs respectively. The rapid increase in the incidence of methicillin resistant *S. aureus* (MRSA) in people and its emergence in dogs has raised the profile of this organism in the veterinary community. Similarly, human *S. pseudintermedius* infections have also been recognized as the awareness of bidirectional human-dog transmission increases.

Antimicrobial resistance has been complicating the treatment of *S. aureus* infections since the first penicillin resistance was observed in the 1940s. Methicillin resistance (resistance to the majority of β-lactams), is particularly troublesome as the β-lactams are a safe and effective class of antimicrobials for treating susceptible staphylococcal infections in both human beings and dogs. Additionally, resistance to other antimicrobial classes such as the macrolides, tetracyclines, sulfonamides and chloramphenicol, further complicates the treatment of staphylococcal infections. Particularly in small animal private practice, infections are often treated empirically, requiring knowledge of locally prevalent susceptibility patterns. The emergence of resistance to commonly used drugs necessitates surveillance to monitor the dissemination of resistance, and to guide antimicrobial therapy.

In the last decade there have been many studies attempting to address gaps in our knowledge of the ecology of *S. aureus* and *S. pseudintermedius* in dogs. In particular, the prevalence of colonization with methicillin resistant staphylococci has been documented in different dog populations. However, failing to sample all relevant sites of colonization, may have decreased the sensitivity of these studies. The sites where coagulase positive staphylococci colonize dogs have not been systematically evaluated.

The clinical and infection control implications of *S. aureus* infections, or colonization in the case of MRSA, requires timely laboratory identification. The tube coagulase test is arguably the most important tool used for identifying of staphylococcal species. Studies dating from the 1970s and 1980s suggested that the use of rabbit plasma, which is the current standard, may not be the ideal media for all situations and that different plasmas may need to be considered in different diagnostic situations.

In this thesis, the ecology of coagulase positive staphylococci in dogs was studied from start to finish including sample collection, bacterial identification, antimicrobial susceptibility testing and molecular epidemiological investigations. This thesis will serve as a template to be used for follow up studies or by investigators setting up a surveillance program in their region.

We found that multiple sites of colonization (nares, pharynx and rectum), are involved in both *S. aureus* and *S. pseudintermedius* carriage in dogs. Single site colonized dogs were identified, suggesting that maximal screening sensitivity requires sampling multiple body sites. When canine and rabbit plasma were compared, the time until clot formation was found to be significantly shorter with canine plasma. Although, the availability of canine plasma may limit its use in the diagnostic laboratory, investigators should be aware that rabbit plasma may not be ideal for all applications of the tube coagulase test. Antimicrobial susceptibility testing of canine *S. aureus* and *S. pseudintermedius* and human *S. aureus* isolates was done. Consistent with previous reports from Saskatoon, the *S. pseudintermedius* isolates were found to be overwhelmingly susceptible: pan-susceptibility was the most common phenotype identified. Antimicrobial resistance was more common among *S. aureus* than *S. pseudintermedius* including resistance to drugs which all *S. pseudintermedius* were susceptible to. No resistance to vancomycin, linezolid, daptomycin or quinupristin/dalfopristin was found. All isolates remained

susceptible to at least one of tetracycline, clindamycin, chloramphenicol or trimethoprim/sulfamethoxazole which are often used for treating infections caused by multidrug resistant staphylococci. Finally, DNA fingerprinting revealed that the canine and human *S. aureus* isolates tested did not belong to mutually exclusive populations. Using AFLP, IS-typing and *spa* typing, many human and canine isolates were indistinguishable suggesting a common population, supporting the hypothesis that interspecies transmission occurs.

The complex and under-characterized ecology of *S. aureus* and *S. pseudintermedius* requires more study so that risk factors for infection can be defined and effective infection control measures implemented. Because multiple species are involved, collaboration between veterinarians and human health professionals is imperative, and will no doubt yield the most success in our efforts to understand these potential pathogens.

Acknowledgments

The last four years has been a transformative experience and I owe thanks to a long list of people who prepared me for beginning this journey, helped me along the way and celebrated each accomplishment no matter how small.

I would like to thank my parents for always encouraging my curiosity, and supporting me in pursuing my wildest dreams – thanks Mom and Dad. No less I would like to thank Casey Gaunt, the love of my life whose endless support and companionship not only motivated and inspired me, but made this whole journey a lot more fun!

Of course this project would not have been possible without the tremendous support of my supervisor, Manuel Chirino-Trejo. I will be eternally grateful to Dr. Chirino not only for his mentorship and teaching me all the tricks that "aren't in the books", but also his encouragement in exploring intriguing side projects only tangentially related to my thesis. Thanks also to my entire graduate committee: Chris Clark, Janet Hill, Vikram Misra, Steve Sanche and Alice Wong for their guidance over the last few years – it was most appreciated. Special thanks to Scott Weese for taking the time to be my external examiner and travel to Saskatoon for my defense.

I would like to thank the staff, students, administration and clinicians of the Western College of Veterinary Medicine (WCVM), Veterinary Teaching Hospital for helping me to collect samples. Thanks also to everyone in GMP, particularly Brian Osler and Vivian Pulga for their meticulous preparation of thousands of plates and biochemical tests and Fei Huang for teaching me how to interpret those tests! Thanks to Bonnie Chaban and Janet Hill for teaching me the basics of PCR and never tiring of my endless questions about molecular microbiology. Thanks to Joe Blondeau at Royal University Hospital in Saskatoon, for generously providing human *S. aureus* isolates for my project.

Thank you to Trish Dowling for sparking my interest in antimicrobials/resistance all those years back and pushing me to pursue an area that I've grown fascinated with! Thanks to Katherine Ball and Frannie Sampieri for always being there to work through problems, brainstorm, go for coffee, collaborate and being great friends!

I would like to thank Dr. Paul Savelkoul at Vrije Universiteit medisch centrum (VUmc) in Amsterdam, the Netherlands for graciously hosting me in his lab and teaching me about AFLP. I would also like to thank Martine Rijnsburger, Madelone van der Bijl, Bianca Blok-Schuur, Dries Budding and Ascelijn Reuland at VUmc and Jaap Wagenaar at Utrecht University for helping me with my project in the Netherlands and making me feel at home in a new place.

Finally, I would like to thank the Interprovincial Graduate Student Fund and the Companion Animal Health Fund for financially supporting this work.

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1.1 *Staphylococcus*

1.1.1 Taxonomy

Staphylococcus is a genus of Eubacteria in the phylum *Firmicutes*, class *Bacilli*, order *Bacillales*, family *Staphylococcaceae* (NCBI 2011). These organisms are Gram positive coccoid bacteria, typically 0.5 – 1.5 μm in diameter (Winn, Allen et al. 2006).

The genus *Staphylococcus* is presently divided into 38 species, which are commonly divided by their ability to coagulate plasma (the coagulase test) (Van Hoovels, Vankeerberghen et al. 2006; Winn, Allen et al. 2006). The coagulase negative staphylococci are further subdivided by their susceptibility to novobiocin (Winn, Allen et al. 2006).

As the most clinically important staphylococcal species in humans, *S. aureus* has been extensively studied (Winn, Allen et al. 2006). In dogs, *S. pseudintermedius* is the most common coagulase positive species, playing an analogous role in dogs to *S. aureus* in people. Other coagulase positive staphylococci include *S. intermedius* and *S. delphini* (of the *Staphylococcus intermedius* group, SIG, along with *S. pseudintermedius*), *S. hyicus* and *S. lutrae* (Foster, Ross et al. 1997; Winn, Allen et al. 2006).

1.1.2 Identification

1.1.2.1 Biochemical identification

While *S. aureus* and *S. pseudinteremdius* are the most important etiological agents identified from skin and soft tissue infection in human beings and canine pyoderma, the lack of a pathognomonic presentation necessitates laboratory diagnostics (Moet, Jones et al. 2007; Fitzgerald 2009). Staphylococci are non-fastidious, facultative anaerobic bacteria which readily

grow on minimal media and sheep blood agar (Winn, Allen et al. 2006). *Staphylococcus aureus* can easily be biochemically differentiated from SIG species, including *S. pseudintermedius* (Table 1.1).

On sheep blood agar *S. aureus* and *S. pseudintermedius* grow as round 1-3 mm colonies which are creamy white-yellow, low convex and with few exceptions β-hemolytic (Van Hoovels, Vankeerberghen et al. 2006; Winn, Allen et al. 2006). *S. aureus* often produces two zones of hemolysis; this phenotype is less frequent with *S. pseudintermedius* (Cole 1990). Microscopically, staphylococci form characteristic "grape like clusters" (Anderson 2003; Winn, Allen et al. 2006).

The catalase test can be used to differentiate staphylococci (positive) from other Gram-positive cocci (negative) (Winn, Allen et al. 2006). A positive catalase test results from the presence of cytochrome oxidase enzymes found in staphylococci and micrococci, but not streptococci or enterococci (Winn, Allen et al. 2006).

Coagulase positive and negative staphylococci are differentiated using the tube coagulase test (Winn, Allen et al. 2006). Tubes containing plasma are inoculated with test organism, incubated at 35°C and observed for visible clot formation which indicates a positive test (Winn, Allen et al. 2006). The slide agglutination test can also be used, although the lower sensitivity of this test for *S. pseudintermedius* than *S. aureus* may result in false negatives in veterinary diagnostic labs (Cox, Newman et al. 1985; Winn, Allen et al. 2006). Commercially available rabbit plasma is most commonly used for the coagulase test (Winn, Allen et al. 2006). Although the availability of high quality rabbit plasma is convenient, it is not necessarily the best medium, the sensitivity of this test may be increased by using other plasmas in some scenarios (Orth, Chugg et al. 1971; Live 1972; Adesiyun and Shehu 1985). *Staphylococcus aureus* isolated from

Table 1.1 Biochemical differentiation of *S. aureus* and *S. pseudintermedius*

Biochemical Test	***S. aureus***	***S. pseudintermedius***	**Reference**
Hemolysis	Double zone	β-hemolytic	(Cole 1990)
Catalase	+	+	(Cole 1990; Van Hoovels, Vankeerberghen et al. 2006)
Coagulase	+	+	(Cole 1990; Van Hoovels, Vankeerberghen et al. 2006)
DNase	+	+	(Cole 1990; Van Hoovels, Vankeerberghen et al. 2006)
Mannitol Fermentation	+	+	(Talan, Staatz et al. 1989; Cole 1990)
Acetoin	+	-	(Talan, Staatz et al. 1989; Pottumarthy, Schapiro et al. 2004)
Hyaluronidase	+	-	(Devriese, Nzuambe et al. 1984)
Polymyxin B Susceptibility	Resistant	Susceptible	(Winn, Allen et al. 2006)

different host species have been shown to coagulate different plasmas with varying efficiency (Live 1972; Adesiyun and Shehu 1985). *Staphylococcus pseudintermedius* coagulase activity may be similarly fickle in its reaction to different plasmas, although the limited evidence available suggests that rabbit plasma may be adequate (Cox, Newman et al. 1985). This variability has been attributed to factors intrinsic to the plasma or characteristics of the test isolate. The amount of coagulase reacting factor among plasmas is intrinsic to each plasma, while the interaction between an organism's coagulase and different plasmas is organism specific (Orth, Chugg et al. 1971). Inconsistencies in the time to clot formation and fibrinolytic activity between isolates can complicate the identification of positive samples (Orth, Chugg et al. 1971). Coagulation times of isolates collected during this study ranged from 1-24 hours. Some fibrinolytic isolates, which were also fast coagulators, produced and dissolved a clot within eight hours. Without careful observation, this variability in coagulase/fibrinolysin activity between isolates could result in false negative results.

Coagulase negative staphylococci are often categorized as novobiocin susceptible or resistant. *Staphylococcus saprophyticus* a common cause of urinary tract infections in people and the most clinically relevant novobiocin-resistant species may be presumptively identified with that test (Winn, Allen et al. 2006). As these organisms are not within the scope of this thesis, their identification will not be discussed further.

Coagulase positive staphylococci can be further divided based on polymyxin B susceptibility. Polymyxin B resistance, defined as a Kirby-Bauer zone of inhibition less than 10mm using a 30µg disc, is characteristic of *S. aureus* (Winn, Allen et al. 2006). *Staphylococcus intermedius* group species are polymyxin B susceptible (Winn, Allen et al. 2006).

Production of acetoin, detected with the Voges-Proskauer test, aids in differentiation of *S. aureus* from *S. pseudintermedius* since *S. aureus* produces acetoin while SIG species do not (Van Hoovels, Vankeerberghen et al. 2006; Winn, Allen et al. 2006; Sasaki, Kikuchi et al. 2007).

The production of hyaluronidase is assessed by the interaction of a test isolate with a pure culture of *Pasteurella multocida* (*S. equi* has been previously reported) (Skalka 1985). A *P. multocida* streak is made on blood agar and a line of the test organism is then inoculated adjacent to the *P. multocida* streak at 90° without touching it (Carter and Rundell 1975). When a mucoid *P. multocida* isolate is used, *S. aureus* will result in a deviation of normal *P. multocida* colony morphology (flattened, non-mucoid growth) (Carter and Rundell 1975). Hyaluronidase production is useful for differentiating *S. aureus* (positive), from SIG species (negative) (Devriese, Nzuambe et al. 1984). There is a paucity of data in the literature regarding this test for the identification of staphylococci; but it has been useful in the diagnostic setting at the Western College of Veterinary Medicine (Chirino-Trejo 2011). In this study, hyaluronidase test results have been corroborated by other biochemical tests and sequence based techniques.

The production of DNase, a characteristic of coagulase positive staphylococci, can be detected with DNase solid agar (Winn, Allen et al. 2006). *Staphylococcus aureus* and *S. pseudintermedius* are DNase positive, distinguishing them from coagulase negative species (Van Hoovels, Vankeerberghen et al. 2006).

Bacterial species vary in their capacity to ferment carbohydrates, and fermentation of a given sugar is a commonly defined biochemical characteristic. When added to a minimal medium as the sole carbohydrate source, fermentation results in acidification which can be colourmetically detected with a pH indicator (Winn, Allen et al. 2006).

Selective and differential media allow rapid presumptive identification of organisms of clinical importance: CHROMagar MRSA, mannitol salt agar + cefoxitin, MRSA select and MRSA screen are several examples (Bischof, Lapsley et al. 2009; Graveland, van Duijkeren et al. 2009). Furthermore, antimicrobials such as oxacillin or cefoxitin can be incorporated into media to allow selective culture of methicillin resistant isolates (CLSI 2008).

While *S. aureus* can be readily identified by biochemical means, species within the *S. intermedius* group (*S. intermedius, S. pseudintermedius* and *S. delphinii*) are not biochemically distinct from one another (Sasaki, Kikuchi et al. 2007; Bannoehr, Franco et al. 2009; Devriese, Hermans et al. 2009; Weese and van Duijkeren 2010). Biochemical identification of *S. intermedius* group species combined with knowledge of host species allows a presumptive species identification. For example canine SIG isolates are assumed to be *S. pseudintermedius* (Devriese, Hermans et al. 2009; Weese and van Duijkeren 2010). Molecular testing is required to differentiate definitively between species within the *S. intermedius* group (Devriese, Vancanneyt et al. 2005; Bannoehr, Franco et al. 2009; Weese and van Duijkeren 2010).

1.1.2.2 Molecular identification

Sequence based techniques are objective, sensitive, specific and increasingly cost effective tools for bacterial identification. Classical identification of bacteria requires maintaining an extensive inventory of test media or commercially prepared biochemical test panels. Commercially prepared media packages such as API were developed for use in human clinical microbiology laboratories and are not designed to identify all organisms of interest to the veterinary or research microbiologist (Winn, Allen et al. 2006). Differentiation of coagulase positive staphylococci (*S. intermedius* group) and the many closely related coagulase negative

species is difficult-to-impossible and expensive using phenotypic techniques (Weese and van Duijkeren 2010). For example API Staph, a kit costing approximately $9 produced by BioMerieux (Marce l'Etoile, France) is useful for identifying common human pathogens, but is of little value for many coagulase negative staphylococci and closely related taxa, or the SIG organisms (Van Hoovels, Vankeerberghen et al. 2006; Winn, Allen et al. 2006; Rubin and Chirino-Trejo 2010). In the last decade, the cost of DNA sequencing has dramatically decreased making sequence based methods an attractive alternative to commercially available kits for identifying organisms.

The gene encoding the small subunit ribosomal RNA (16S rRNA) is universal among bacteria (Harmsen, Rothganger et al. 2002). The 16S rRNA gene is slow to evolve, resulting in little variation between isolates related at the species/genus level (Harmsen, Rothganger et al. 2002). Sequencing of the 16S rRNA gene yields a sequence which when compared with a database allows identification of the isolate to a clinically relevant level for many organisms, although not for *Staphylococcus* species (Harmsen, Rothganger et al. 2002). Historically, 16S rRNA has been the gene of choice for phylogenetic studies and has become entrenched as a benchmark method despite the discovery of higher resolution target sequences such as the gene encoding the Hsp60 chaperonin (*cpn*60) (Goh, Potter et al. 1996; Kwok and Chow 2003).

The *cpn*60 sequence has superior resolution for differentiating closely related coagulase negative staphylococci and SIG species (Goh, Santucci et al. 1997; Devriese, Hermans et al. 2009). In the experiments comprising this thesis, an organism grown on CHROMagar + 4μg/mL oxacillin that was morphologically indistinguishable from *S. aureus* but biochemically unidentifiable was investigated. Using the API system, this isolate was identified as *Staphylococcus lugdunensis* while 16S rRNA gene sequencing revealed 99% sequence identity

to *Micrococcus caseolyticus* (Rubin and Chirino-Trejo 2010). Sequencing of the *cpn*60 gene revealed that, while the most closely related organism is ***M. caseolyticus***, with only 89% sequence identity it is most likely a novel species (Rubin and Chirino-Trejo 2010).

Other techniques involve sequencing genus specific genes such as *sod*A and *fem* for species identification (Vannuffel, Gigi et al. 1995; Sasaki, Kikuchi et al. 2007; Weese and van Duijkeren 2010).

1.2 *Staphylococcus aureus*

1.2.1 Colonization

1.2.1.1 Human colonization

Staphylococcus aureus commonly colonizes mucosal surfaces of human beings (Kluytmans-Vandenbergh and Kluytmans 2006; Winn, Allen et al. 2006). The point prevalence of nasal colonization is frequently reported to be approximately 30% (Kuehnert, Kruszon-Moran et al. 2006; Gorwitz, Kruszon-Moran et al. 2008). However, a binary definition is inadequate to describe the complexity of colonization dynamics. Three general populations (of human beings) are recognized: those who are persistently colonized, intermittently colonized and non-colonized (Kluytmans and Wertheim 2005). Approximately 10-35% of human beings are persistently colonized, 20-70% are intermittently colonized and a further 5-50% are non-colonized (Kluytmans and Wertheim 2005; Wertheim, Melles et al. 2005; Hamdan-Partida, Sainz-Espunes et al. 2010).

Persistently colonized individuals tend to have their "own" strain; which is particularly adapted to colonize that individual (Hamdan-Partida, Sainz-Espunes et al. 2010). Since an individual's resident population of *S. aureus* has easy access to susceptible body sites,

colonization is a risk factor for infection (Wertheim, Vos et al. 2004). This risk factor necessitates screening for colonization so that preventive measures can be implemented to reduce the risk of infection following high risk medical procedures (Coates, Bax et al. 2009; de Smet, Kluytmans et al. 2009; Kluytmans and Harbarth 2009). Despite an increased risk of infection, colonized individuals are more likely to survive a systemic *S. aureus* infection than non-colonized persons (Wertheim, Vos et al. 2004; Wertheim, Melles et al. 2005). This protective effect is hypothesized to be due to the immunological priming effect of colonization (Wertheim, Vos et al. 2004).

While the nares have historically been recognized as an important site of *S. aureus* colonization, the role of other sites has recently been quantified (Mody, Kauffman et al. 2008). The pharynx, intestinal tract, perineum and axilla are recognized as potential sites of colonization, including single site colonized individuals (Mertz, Frei et al. 2007; Batra, Eziefula et al. 2008; Mody, Kauffman et al. 2008; Yang, Tan et al. 2009). Because the nares are not the primary site of colonization in every individual, successful elimination of *S. aureus* carriage may require decolonization of extra-nasal sites (Ammerlaan, Kluytmans et al. 2009; McConeghy, Mikolich et al. 2009). Single site *S. aureus* or MRSA colonization of the pharynx among colonized individuals has been reported in 9.3-21.8% of patients (Mertz, Frei et al. 2007; Mody, Kauffman et al. 2008; Ide, Lootens et al. 2009). Similarly, 8-31% of people have been shown to be enterally colonized, and among those colonized with MRSA, 8% were exclusively enterally colonized (Acton, Tempelmans Plat-Sinnige et al. 2008; Batra, Eziefula et al. 2008).

1.2.1.2 Canine colonization

Compared to our knowledge of colonization of human beings, much less is known about canine *S. aureus* colonization. Although most studies have focused on MRSA, the few studies investigating overall *S. aureus* colonization have found prevalences of 8.8-12% (Boost, O'Donoghue M et al. 2007; Griffeth, Morris et al. 2008). Recognizing the potential importance of multiple colonization sites, the skin, nares, pharynx and rectum have been included in prevalence studies; however a systematic comparison of these or other sites has not been done (Boost, O'Donoghue M et al. 2007; Griffeth, Morris et al. 2008; Nienhoff, Kadlec et al. 2009; Weese and van Duijkeren 2010).

1.2.2 *S. aureus* as a human pathogen

1.2.2.1 Common syndromes

Staphylococcus aureus is a common cause of both community and healthcare (hospital, long-term care facility, etc.) associated infections. As an opportunistic pathogen, *S. aureus* enters normally protected body sites that become exposed, or when host defenses are compromised (Gordon and Lowy 2008).

Skin and soft tissue infection (SSTI) is the most common infectious syndrome produced by *S. aureus,* and *S. aureus* is the most common cause of SSTI (Moet, Jones et al. 2007)**.** When these infections are localized and superficial treatment often requires only wound management including incision and drainage and topical antimicrobials (Barton, Hawkes et al. 2006). Lesions with a "spider-bite" or "pimple" appearance, a reddened area with a necrotic centre containing purulent material, are the archetypal community associated MRSA (CA-MRSA) SSTI and are more frequently caused by MRSA than other bacteria (Moran, Krishnadasan et al. 2006; Gorwitz 2008)**.**

Staphylococcus aureus is one of the most common causes of hospital acquired infections, and in recent decades has become the most common cause of septicemia (Rice 2006; Klein, Smith et al. 2007).

1.2.2.2 *S. aureus* virulence factors

Staphylococcus aureus carries a formidable array of virulence factors allowing it to cause a wide variety of infectious syndromes and intoxications (Table 1.2) (Archer 1998; Gordon and Lowy 2008).

1.2.2.2.1 Panton Valentine Leukocidin

Panton Valentine leukocidin (PVL), a toxin associated with community-associated MRSA (CA-MRSA), has been studied intensively as the incidence of CA-MRSA has increased (Boyle-Vavra and Daum 2007). While the role of PVL in pathogenesis has been somewhat controversial and recent evidence suggests that its contribution to disease was overstated, it is at least epidemiologically related to the archetypal CA-MRSA clone, USA300 (Kennedy, Otto et al. 2008; Lalani, Federspiel et al. 2008; Otto 2011).

Panton Valentine leukocidin is a heteromultimer encoded by the LukS-PV and LukF-PV genes (Lina, Piemont et al. 1999; Labandeira-Rey, Couzon et al. 2007). Like other toxins, the acquisition of PVL by *S. aureus* has been associated with phage transduction (Wolter, Tenover et al. 2007). The expression of PVL is not constitutive and may be affected by cell density (via quorum sensing) or phagocytosis by neutrophils (Loffler, Hussain et al. 2010). Quorum sensing is a process by which bacteria detect and respond to their density in their environment (Dancer 2008; Boyen, Eeckhaut et al. 2009). Quorum sensing aids in regulating gene expression via the

Table 1.2 Summary of *S. aureus* virulence factors

Toxins	**Effect and Syndromes**	**Reference**
Staphylococcal Enterotoxins (SEA-E, SEG-R and SEU	Super-antigen -Staphylococcal enterocolitis -Food poisoning -Toxic shock syndrome	(Winn, Allen et al. 2006; Baba-Moussa, Anani et al. 2008; Vaishnani 2009; Lin, Kotler et al. 2010; Stow, Douglas et al. 2010)
Toxic Shock Syndrome Toxin íTSST-1)	Super-antigen -Toxic shock syndrome	(Winn, Allen et al. 2006; Baba-Moussa, Anani et al. 2008; Vaishnani 2009)
Epidermolysins (ETA-D)	Proteolytic Enzymes -Bullous impetigo -Staphylococcal scalded skin syndrome -Blistering	(Prevost, Couppie et al. 2003; Winn, Allen et al. 2006; Baba-Moussa, Anani et al. 2008)
Leukotoxins (LukE-LukD, PVL)	Neutrophil toxin -Necrotic processes -Necrotizing pneumonia -CA-MRSA infections	(Labandeira-Rey, Couzon et al. 2007; Baba-Moussa, Anani et al. 2008; Loffler, Hussain et al. 2010)
Staphylococcal protein A (SpA)	Super-antigen Interfering with immune response	(Archer 1998; Winn, Allen et al. 2006; Vaishnani 2009)

accessory gene regulator (*agr*), which coordinates the cells transcriptional activity and can be turned on or off as appropriate (Dancer 2008; Boyen, Eeckhaut et al. 2009).

While *in vitro* experiments have demonstrated that PVL is active against human neutrophils, the species specificity of its activity is ill defined (Loffler, Hussain et al. 2010). *In vitro* experiments that aimed to determine the role of PVL in different infectious syndromes were confounded by the variable activity of PVL against neutrophils from different animal species (Loffler, Hussain et al. 2010). This specificity became apparent when *in vivo* models and *in vitro* experiments using non-human tissues yielded conflicting results (Loffler, Hussain et al. 2010). While PVL was found to potentiate osteomyelitis in a rabbit model it was ineffective against mouse and Java monkey neutrophils (Labandeira-Rey, Couzon et al. 2007; Cremieux, Dumitrescu et al. 2009; Loffler, Hussain et al. 2010). Although the activity of PVL against canine neutrophils has not been experimentally determined, the scarsity of PVL positive *S. aureus* isolated from dogs suggests that it does not play a role in the pathogenesis of canine infections (Weese, Faires et al. 2007).

The role of PVL is best understood in human beings, where it is believed to be involved in the pathogenesis of necrotizing pneumonia and other necrotic processes (Lina, Piemont et al. 1999; Labandeira-Rey, Couzon et al. 2007). *Staphylococcus aureus* necrotizing pneumonia is most frequently caused by the hypervirulent (PVL positive) USA300 MRSA strain (Labandeira-Rey, Couzon et al. 2007; Larsen, Stegger et al. 2007; Lalani, Federspiel et al. 2008; Simor, Gilbert et al. 2010). Panton Valentine leukocidin positive MRSA are also implicated in necrotic furunculosis (Lina, Piemont et al. 1999). Outbreaks (transmission) of SSTI caused by PVL positive MRSA have been reported following direct skin-to-skin contact or contact with contaminated fomites such as towels and sports equipment (Barton, Hawkes et al. 2006; Gorwitz

2008; Huijsdens, Janssen et al. 2008). Some have speculated that PVL may indirectly promote transmission of MRSA by upregulating the expression of adhesive proteins or increasing bacterial shedding (Lina, Piemont et al. 1999; Boyle-Vavra and Daum 2007). Interestingly, new evidence suggests that the importance of PVL, even in human infections, has been overestimated (Otto 2011).

1.2.3 *S. aureus* as a veterinary pathogen

1.2.3.1 Canine *S. aureus* infections

Staphylococcus aureus is likely under-recognized as a cause of infections in dogs. Based on colony morphology it is difficult to differentiate *S. aureus* and *S. pseudintermedius* on both non-selective and some differential media. Because *S. pseudintermedius* is the most common coagulase positive species isolated from dogs, presumptive identification based on a positive coagulase test could result in misidentifying *S. aureus* as *S. pseudintermedius* (Van Hoovels, Vankeerberghen et al. 2006).

The emergence of MRSA in dogs has raised awareness of non-*pseudintermedius* coagulase positive species in dogs and focused attention on accurate species specific identification (Weese and van Duijkeren 2010). *Staphylococcus aureus* seems to easily substitute for *S. pseudintermedius*; given the opportunity it will cause otitis externa, pyoderma, post-surgical and other hospital acquired infections (Kwon, Park et al. 2006; Jones, Kania et al. 2007; Leonard and Markey 2008; Faires, Traverse et al. 2010).

1.2.3.2 *S. aureus* infections in other species

Staphylococcus aureus is promiscuous in its ability to colonize and cause infections in a wide range of hosts (Table 1.3). *Staphylococcus aureus* mastitis, infection of the mammary gland leading to increased somatic cell counts in milk, is the single largest cause of economic loss to the North American dairy industry (Erskine 2001; Morin 2009). Sub-clinical mastitis caused by *S. aureus* is an insidious condition resulting in decreased milk production, that without active surveillance may go un-noticed (Morin 2009). Lapses in hygiene can facilitate transmission of *S. aureus* between cows via shared milking equipment (Morin 2009). Other species including horses and avian species are also commonly infected or colonized with *S. aureus* (Rubin, Ball et al. 2011). In chickens, foot and leg infections (bumblefoot) are commonly caused by *S. aureus* (White, Ayers et al. 2003; Lowder, Guinane et al. 2009). Equine *S. aureus* colonization and hospital associated infections (following arthroscopy or intravenous catheterization), and superficial skin infections are recognized (Devriese, Nzuambe et al. 1985; Weese, Rousseau et al. 2006; Sung, Lloyd et al. 2008).

1.2.4 The history and development of antimicrobial resistance in *S. aureus*

1.2.4.1 The early years – *S. aureus* before antibiotics

With the development of germ theory, preventive measures were recognized to be the best defense against hospital acquired infections following surgery. Early treatments for severe *S. aureus* infections required creativity and included the transfusion of "immune blood", a procedure which with our current knowledge of bloodborne pathogens would best be avoided (Hooker 1917). In the pre-antibiotic era, infections carried a much higher risk of mortality than they do today (McDermott and Rogers 1982). The introduction of penicillin was associated with

Table 1.3 Animals in which *S. aureus* has been identified

Animal	Reference
Bat	(Walther, Wieler et al. 2007)
Bison	(Rubin, Ball et al. 2011)
Cat	(Rankin, Roberts et al. 2005; Leonard and Markey 2008)
Cow	(Leonard and Markey 2008)
Caribou	(Rubin, Ball et al. 2011)
Chicken	(Leonard and Markey 2008)
Dog	(Rankin, Roberts et al. 2005; Walther, Wieler et al. 2007; Leonard and Markey 2008)
Dolphin	(Faires, Gehring et al. 2009; Schaefer, Goldstein et al. 2009)
Elephant	(CDC 2009)
Goat	(Rubin, Ball et al. 2011)
Guinea Pig	(Walther, Wieler et al. 2007)
Horse	(Leonard and Markey 2008)
Iguana	(Rubin, Ball et al. 2011)
Meercat	(Rubin, Ball et al. 2011)
Mouse	(Rubin, Ball et al. 2011)
Parrot	(Rankin, Roberts et al. 2005; Walther, Wieler et al. 2007)
Pigeon	(Losito, Vergara et al. 2005)
Pig	(Leonard and Markey 2008)
Rabbit	(Rankin, Roberts et al. 2005; Walther, Wieler et al. 2007; Leonard and Markey 2008)
Rat	(Rubin, Ball et al. 2011)
Seal	(Leonard and Markey 2008)
Sea Otter	(Rubin, Ball et al. 2011)
Sheep	(Leonard and Markey 2008; Rubin, Ball et al. 2011)
Tilapia	(Atyah, Zamri-Saad et al. 2011)
Turtle	(Walther, Wieler et al. 2007)
Walrus	(Faires, Gehring et al. 2009)
Wapiti	(Rubin, Ball et al. 2011)

a decrease in the mortality rate associated with staphylococcal bacteremeia from 70% to 25% in the early 1940s (Dancer 2008). Some have credited antimicrobials for a 10 year increase in the average life expectancy (McDermott and Rogers 1982).

1.2.4.2 The introduction of penicillin, and the first resistance

Penicillin was discovered in 1928 after Sir Alexander Fleming observed the inhibitory action of *Penicillium notatum* on bacterial cultures and hypothesized the presence of an inhibitory substance (Aronson 1992; Bryskier 2005). This work, along with that of Ernst Borris and Howard Walter Florey began the antibiotic age, revolutionizing modern medicine. Their contributions were recognized with a Nobel Prize in 1945 (NobelMedia 2011). When it was first used clinically in the United States in 1942, penicillin was touted as a wonder drug whose use was limited only by manufacturing capacity (Grossman 2008). Penicillin was used to treat gonorrhea, septicemia, pneumonia, infections following accidents and wounds in soldiers in WWII (Fraser 1974; Garrod 1974; Hey 1974; Fraser 1984). Unfortunately, the evolutionary power of "the enemy" was not anticipated and the bacteria were already mounting a formidable defense.

In hospitals, where the selection pressure was most intense, *S. aureus* (known as *S. pyogenes* at the time) were quickly becoming resistant (Barber 1947). In a 1947 report, Dr. Mary Barber, a bacteriologist in London, England, reported rapidly increasing penicillin resistance, describing it as "alarming" (Barber 1947). Eleven years later she reported "*S. pyogenes*" resistant to penicillin, streptomycin, tetracycline, chloramphenicol and erythromycin (Barber and Dutton 1958). Insightfully, Dr. Barber wrote in 1955:

> "It is a neck-and-neck race in which many of us tend to underestimate the opponent. Staphylococci will not be defeated by the haphazard use of each new antibiotic. As new antibacterial agents are discovered, let us use them with discrimination." (Barber and Burston 1955)

The overwhelming success of penicillin resistant *S. aureus* led to their dominance over penicillin susceptible *S. aureus* in the community where they predominate today; in one study 94% of methicillin-susceptible *S. aureus* (MSSA) were penicillin resistant (Sa-Leao, Sanches et al. 2001).

By the 1940s and 50s the antimicrobial arms race was in full swing; soil samples from every corner of the world were screened for new organisms and their potential antimicrobial products (Routien and Finlay 1952). Tetracycline, streptomycin (which was the subject of the 1952 Nobel Prize), chloramphenicol, erythromycin and vancomycin were introduced to counter the rapidly emerging resistance to penicillin and to treat those infections caused by organisms intrinsically resistant to penicillin, such as tuberculosis (Powers 2004; NobelMedia 2011).

1.2.4.3 The introduction of methicillin and resistance emergence, 1960 – present

Methicillin, introduced as celbenin in 1960 was the first semi-synthetic penicillin developed (Woodford and Livermore 2009). Semi-synthetic group M penicillins including methicillin, oxacillin and cloxacillin were developed to resist the hydrolytic activity of the *S. aureus* "penicillinase" which readily degrades penicillin (Barber 1961; Bryskier 2005). The first report of MRSA, including three isolates, was published in the British Medical Journal in 1961 (Jevons 1961).

1.2.4.3.1 What is Methicillin Resistance?

Methicillin resistance is not due to the production of a hydrolytic enzyme, unlike β-lactamase mediated penicillin resistance (Woodford and Livermore 2009). β-lactams (penicillin and methicillin) are cell wall synthesis inhibitors and act by binding to penicillin binding proteins (PBP) in the cell wall inhibiting peptidoglycan cross linking and leading to a defective cell wall (Bryskier 2005). In MRSA, the *mec*A gene encodes an altered PBP2 (PBP2a) that has low β-lactam affinity preventing the drug from binding to its target and bactericidal activity (Woodford and Livermore 2009). As all β-lactams bind to PBP, MRSA are resistant to all currently available drugs in this class including the penicillins, potentiated penicillins (those with β-lactamase inhibitors such as clavulanic acid), cephalosporins and carbapenems (Palavecino 2007). Methicillin resistance is a historical designation related to the first recognition of this resistance (resistance to methicillin) rather than the spectrum of resistance it confers (all β-lactams).

The *mec*A gene is carried by the staphylococcal chromosomal cassette (SCC), which with *mec*A is known as SCC*mec*A (Chambers and Deleo 2009). Among MRSA, the SCC is not a single distinct structure. By 2009 at least eight SCC allotypes denoted by roman numerals and many subtypes indicated by lowercase letters had been recognized (Chambers and Deleo 2009). The widespread use of DNA sequencing has shed light on SCC*mec* diversity, leading to a rapidly evolving nomenclature system for this element.

Table 1.4 Characteristics associated with CA-MRSA and HA-MRSA

Characteristic	CA-MRSA	HA-MRSA	Reference
PVL	Common	Uncommon	(Barton, Hawkes et al. 2006; Boyle-Vavra and Daum 2007)
SCC*mec* types	IV, V	I, II, III	(Barton, Hawkes et al. 2006)
Associated clones	USA 300 USA 400	USA 100 USA 200	(Barton, Hawkes et al. 2006)
Resistance to other drugs	Rare	Common	(Barton, Hawkes et al. 2006; Millar, Loughrey et al. 2007)
Patient characteristics	Young Otherwise healthy	Elderly Neonatal Immunocompromized	(Barton, Hawkes et al. 2006; Millar, Loughrey et al. 2007)
Associated syndromes	Skin and soft tissue infections Necrotizing pneumonia	Bacteremia Respiratory and urinary tract infections	(Millar, Loughrey et al. 2007; Kluytmans and Struelens 2009)
"Fitness" (Generation time)	Generally shorter	Generally longer	(Kluytmans-Vandenbergh and Kluytmans 2006)
Epidemiological factors	No health-care associated risk factors Occur within 48 hours of hospitalization	Previous hospitalization Occur after more than 48 hours of hospitalization Long term care facility residents	(Kreisel, Roghmann et al. 2010)

1.2.4.3.2 Emergence of MRSA: its 50 year history

1.2.4.3.3 Community versus hospital associated MRSA

MRSA infections are commonly classified as either community associated (CA-MRSA) or healthcare associated (HA) (Table 1.4). Recently, highly successful CA-MRSA clones, (including USA300) which now cause the majority of CA-MRSA infections, have become established in hospitals and are an important cause of hospital acquired infections (Kluytmans-Vandenbergh and Kluytmans 2006; Bartlett 2008; Arias and Murray 2009). The presence of historically CA-MRSA lineages in healthcare facilities in some cases replacing HA-MRSA strains is blurring the classical CA vs. HA definition (Millar, Loughrey et al. 2007).

1.2.4.3.4 Emerging resistance and new therapies

Vancomycin, a glycopeptide discovered in 1956, has long been the mainstay of anti-MRSA therapy (Bryskier and Veyssier 2005). Because vancomycin has low oral bioavailability, intravenous access is required for administration (Bryskier and Veyssier 2005). Glycopeptides, inhibit cell wall synthesis by a mechanism independent of PBP, and are therefore active against MRSA (Bryskier and Veyssier 2005; Palavecino 2007). While glycopeptide resistance is not uncommon among *Enterococcus faecalis* (VRE), until recently even multidrug resistant MRSA remained susceptible (Bryskier and Veyssier 2005). In 2002 the first vancomycin resistant *S. aureus* (VRSA) was isolated from a patient in Michigan and at least eight other cases have been recognized subsequently in the United States (CDC 2002; Finks, Wells et al. 2009).

Among VRE, vancomycin resistance is mediated by the van class of genes and *van*A, conferring high level resistance, is the most common (Woodford and Livermore 2009). The first VRSA was *van*A positive, and is thought to have acquired *van*A through conjugation with co-

colonizing VRE (Whitener, Park et al. 2004; Sievert, Rudrik et al. 2008). Conjugative transfer of *van*A from VRE to *S. aureus* has been achieved *in vitro*, supporting this hypothesis (Noble, Virani et al. 1992; Whitener, Park et al. 2004; Sievert, Rudrik et al. 2008). More widespread than VRSA, vancomycin intermediate (VISA) isolates are also presenting clinical challenges. Elevated vancomycin MICs have been associated with increased resistance to other drugs, a higher rate of post surgical infections, longer duration of bacteremia and treatment failure (Soriano, Marco et al. 2008; Deresinski 2009; Maor, Hagin et al. 2009).

For MRSA infections in patients not requiring hospitalization, without IV access, or when the use of vancomycin is contraindicated, other treatment options are required. Alternative therapies may include linezolid, the fluoroquinolones, macrolides/lincosamides, tetracyclines, sulfonamides or topically applied drugs such as mupirocin or fusidic acid (Walker, Dresser et al. 2006; Drekonja, Traynor et al. 2008; Enoch, Karas et al. 2009; Tattevin, Basuino et al. 2009).

The fluoroquinolones are commonly prescribed by both veterinarians and physicians, and offer many advantages including infrequent dosing, good tissue penetration and rapid bactericidal activity (Walker and Dowling 2006). Unfortunately, fluoroquinolone resistance has become common among MRSA, and at least in part due to this resistance, the use of these drugs has been identified as a risk factor for MRSA acquisition (Schneider-Lindner, Delaney et al. 2007; Dancer 2009; Tattevin, Basuino et al. 2009). The fluoroquinolones have also been shown to up-regulate the expression of adhesion proteins potentiating virulence, indicating that selection of MRSA is more complicated than simply advantaging a resistant population (Dancer 2008).

Macrolides and lincosamides have also historically been important in the treatment of *S. aureus* and *S. pseudintermedius* infections in people and dogs (Littlewood, Lakhani et al. 1999; Lewis and Jorgensen 2005; Giguere 2006). Unfortunately, resistance to these drugs is also

increasing and in the case of clindamycin, can be much more insidious. Macrolide/lincosamide resistance is not uniformly expressed in *S. aureus* and *S. pseudintermedius* and *in vitro* susceptibility test results may not be predictive of genotype or *in vivo* resistance expression (CLSI 2008; Rubin, Ball et al. 2011). Inducible clindamcyin resistance (iCR) is a phenomenon where the weak resistance inducing power of clindamycin results in an isolate appearing susceptible *in vitro* despite possessing the requisite resistance genes (CLSI 2008). Clinically, *in vivo* induction of resistance by clindamycin has led to treatment failure, highlighting the importance of iCR (Levin, Suh et al. 2005). In erythromycin resistant, clindamycin susceptible isolates, iCR can be confirmed using the "D-test" (CLSI 2008). In the D-test, erythromycin and clindamycin discs are placed 15 mm apart under standard antimicrobial susceptibility testing conditions (CLSI 2008). Blunting of the clindamycin inhibitory zone such that it resembles a "D" indicates induction of clindamycin resistance by erythromycin, confirming iCR (Figure 1.1) (CLSI 2008). Inducible clindamcyin resistance seems to be more common among *S. aureus* than *S. pseudintermedius*. One study found iCR in 17.7% of canine MRSA and 0% of MRSP, while another found it in only 1.7% of canine methicillin susceptible *S. pseudintermedius* (Faires, Gard et al. 2009; Rubin, Ball et al. 2011). Inducible clindamcyin resistance has been found in both MSSA (14.8% – 68%) and MRSA (4.8-24.4%) isolated from human beings (Levin, Suh et al. 2005; Yilmaz, Aydin et al. 2007).

Mupirocin, a pseudomonic acid antimicrobial, is topically applied for treating superficial, localized infections and for nasal decolonization (Bryskier 2005; McConeghy, Mikolich et al. 2009). Compared to other antimicrobials, relatively little is known about the prevalence of mupirocin resistance. Additionally standardized methods for measuring and interpreting mupirocin susceptibility are not available making comparison between studies difficult. In

Saskatchewan, previous studies have documented higher rates of mupirocin resistance (>50%) than elsewhere in Canada (4-7%) (Mulvey, MacDougall et al. 2005; Simor, Stuart et al. 2007). Whether the high prevalence of resistance is due to unusually high mupirocin use in this area, or some other factor is unknown. Mupirocin resistance is uncommon among canine isolates, one study including *S. aureus* and *S. pseudintermedius* found only a single mupirocin resistant MRSA, while another examining only MRSA found no mupirocin resistance (Fulham, Lemarie et al. 2010; Coelho, Torres et al. 2011).

Resistance to other classes of antimicrobials including the aminoglycosides, sulfonamides and tetracyclines further complicates the treatment of MRSA infections (Wulf and Voss 2008; Enoch, Karas et al. 2009). The increasing incidence of infections caused by multidrug resistant Gram-positive organisms (MRSA and VRE) has led to intensive drug discovery/development research which, unlike the case with Gram-negatives, has yielded several new antimicrobial classes (Loeffler, Linek et al. 2007; Livermore 2009). Fortunately, pan-resistant isolates are still uncommon, but the trend of increasing resistance foreshadows a future resembling the "pre-antibiotic era" (Arias and Murray 2009; Livermore 2009).

Linezolid, the first drug in the newly developed oxazolidinone class, is the first new antimicrobial class introduced since the fluoroquinolones in the 1980s (Fernandes 2006; Woodford and Livermore 2009). Unfortunately, linezolid resistance is already emerging, and although more common among coagulase negative staphylococci, linezolid resistant *S. aureus* has also been reported (Anderegg, Sader et al. 2005; Woodford 2005; Trevino, Martinez-Lamas et al. 2008). Daptomycin, a lipopeptide antimicrobial, was added to our antimicrobial armamentarium in 2003. It has proven useful in treating refractory infections, although few studies have been done (Vernadakis, Saner et al. 2009; Woodford and Livermore 2009). Other

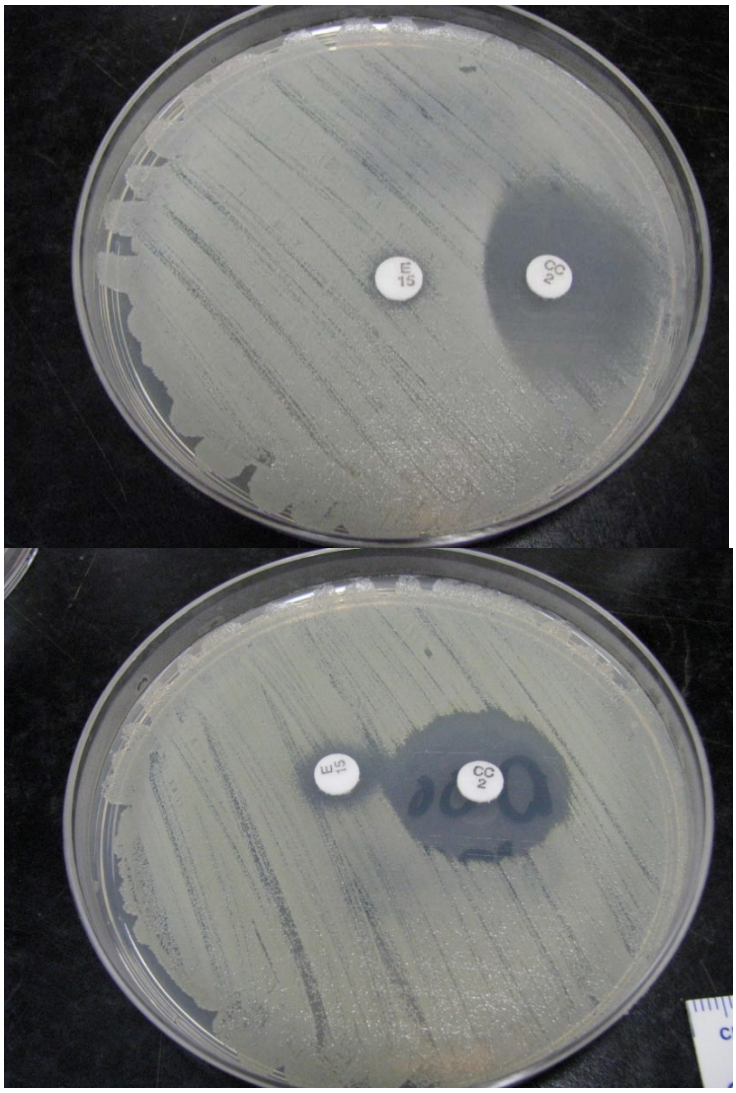


Figure 1.1 Inducible clindamycin resistance
Staphylococcus aureus displaying typical "D-zone" of inhibition associated with inducible clindamycin resistance (top), and clindamycin susceptibility with erythromycin resistance (bottom)

agents including quinupristin/dalfopristin (streptogramins) and tigecycline (glycylcycline) have also been recently become available (Livermore 2009). These drugs are derivatives of existing compounds and resistance may therefore be quicker to develop than to a drug with a novel mechanism of action (Giguere 2006; Woodford and Livermore 2009). Currently, novel PBP2a binding cephalosporins (ceftobiprole and ceftaroline), folate synthesis inhibitors (icalprim), glycopeptide derivatives (dalbavancin, telavancin and oritavancin) and carbapenems (razopenem) are under investigation (Bogdanovich, Ednie et al. 2005; Scheinfeld 2007; Peppard and Schuenke 2008; Livermore 2009). While the drugs currently in the development pipeline for Gram-positive bacteria are more promising than for Gram-negatives, the emergence of resistance will no doubt continue to challenge the treatment of these infections (Fischbach and Walsh 2009; Livermore 2009).

1.2.5 The implications of MRSA

1.2.5.1 Increased financial burden and mortality rate

The incidence of systemic *S. aureus* infection (sepsis) has been increasing compared to Gram-negative infections, and MRSA is comprising an increasing proportion of these *S. aureus* infections (Klein, Smith et al. 2007; Hodgin and Moss 2008; Laupland, Ross et al. 2008; Simor, Gilbert et al. 2010). The healthcare costs associated with septicemia are immense since these patients require intensive monitoring, intravenous access and often the administration of multiple drugs or other medical products such as plasma. In addition, the costs of supplies, facilities and staff increase with each day of admission. The costs of managing MRSA bloodstream and other nosocomial infections are up to three times higher than those for MSSA infections (Abramson and Sexton 1999; Ott, Bange et al. 2010). The length of hospitalization is a contributing factor: patients with MRSA septicemia spend 12 days in the hospital compared to four days for those

with MSSA septicemia (Abramson and Sexton 1999). While the financial burden of MRSA is obvious, the increased mortality rate associated with MRSA vs. MSSA septicemia or pneumonia clearly illustrates the human costs (Laupland, Ross et al. 2008; Ott, Bange et al. 2010). Hospital acquired infections (which are increasingly caused by MRSA) affect 1/9 patients and result in the deaths of 8,000 people annually in Canada (Backman, Zoutman et al. 2008; Simor, Gilbert et al. 2010).

1.2.5.2 Lack of information for dogs

In contrast to people, relatively little is known about the implications of MRSA for canine health or the costs of treatment. In dogs, MRSA infections most frequently present as pyoderma or otitis externa (Faires, Traverse et al. 2010). Perhaps due to the superficial nature of canine MRSA infections, or the small number of published cases available for analysis, Faires et al., did not find a difference in mortality or the requirement for surgery for dogs infected with MRSA versus MSSA (Faires, Traverse et al. 2010). Few studies have examined the incidence of canine MRSA infections, however one study found that 22-36% of clinical *S. aureus* isolates from dogs were methicillin resistant (Jones, Kania et al. 2007). Among clinically healthy dogs in the community, or upon entering veterinary clinics, between 0-1% of dogs were MRSA colonized, while 9% of hospitalized dogs were reported to carry MRSA (Loeffler, Boag et al. 2005; Boost, O'Donoghue M et al. 2007; Hanselman, Kruth et al. 2007; Murphy, Reid-Smith et al. 2009).

1.2.6 The situation in Saskatoon, Canada

1.2.6.1 In human beings

The epidemiology and incidence of MRSA is relatively ill defined in North America compared to the Netherlands and Denmark (Larsen, Stegger et al. 2007; van Rijen, Van Keulen et al. 2008; Nulens, Stobberingh et al. 2009; Hasman, Moodley et al. 2010; Simor, Gilbert et al. 2010). In Canada, current antimicrobial resistance surveillance programs including MRSA are targeted at specific patient populations, and may not be representative of *S. aureus* infections in the general public (Canada 2008; Zhanel, Decorby et al. 2008). Further confounding our understanding of MRSA in Saskatoon is the way in which resistance surveillance data is presented; the Canadian Nosocomial Infection Surveillance Program combines Saskatchewan data with the rest of western Canada (British Columbia, Alberta and Manitoba) (Canada 2008).

1.2.6.2 In dogs

Because very little attention was paid to *S. aureus* in dogs in Saskatoon before 2006 when the first canine case of MRSA was recognized, it is difficult to speculate how its epidemiology has changed. Although canine *S. aureus* infections have only recently gained attention, its true importance may have been underestimated in the past. Misidentification of *S. aureus* due clinical bias and morphological similarities with *S. pseudintermedius* should not be discounted. At the WCVM there have been relatively few (nine) canine MRSA infections associated with three outbreaks in 2006 and 2008 (Rubin and Chirino-Trejo In Press). Outside of Ontario, where a number of outbreaks have been reported, there is no other published data on canine MRSA in Canada (Weese, Dick et al. 2006; Weese, Faires et al. 2007).

1.2.7 Control strategies

1.2.7.1 Decolonization – control in the individual

In human beings, decolonization of the anterior nares to eliminate MRSA is commonly done prophylacticly before high risk medical procedures (McConeghy, Mikolich et al. 2009). Topically applied mupirocin is the most commonly used drug, and the only licensed product for nasal decolonization (McConeghy, Mikolich et al. 2009). Resistance to mupirocin necessitates the use of other drugs; fusidic acid as well as topically applied vancomycin, bacitracin/neomycin/polymyxin B and chlorhexidine gel have also been used (Manian 2003; McConeghy, Mikolich et al. 2009). Nasal colonization refractory to topical therapy may require systemic treatment for which rifampin and trimethoprim/sulfamethoxazole, clindamycin, clarithromycin, fusidic acid, doxycycline or ciprofloxacin have been used (van Duijkeren, Wolfhagen et al. 2005; Barton, Hawkes et al. 2006; Wertheim, Nouwen et al. 2007). Antimicrobial soaps and bathing solutions including chlorhexidine, triclosan and Dakin's solution (dilute hypochlorite) are also used in parallel with nasal decolonization (Elston 2009; McConeghy, Mikolich et al. 2009).

With the recognition of multiple sites of colonization, decolonization regimens for extra-nasal sites are being investigated (Ammerlaan, Kluytmans et al. 2009). Enteral decolonization presents unique challenges since the intestines are not accessible for topical therapy and the toxicity of potential decolonization agents must be carefully considered (Batra, Eziefula et al. 2008; Ammerlaan, Kluytmans et al. 2009). Some authors have suggested that oral vancomycin, due to its poor oral bioavailability, might be an effective agent for enteral decolonization although the risk of selecting vancomycin resistant enterococci must be considered (Batra, Eziefula et al. 2008; Huckabee, Huskins et al. 2009).

Pharmacologic decolonization of dogs is a controversial issue and there is no data to support the use of nasal mupirocin in dogs. It would be difficult to apply as an ointment to the

anterior nares, and it seems reasonable to assume that effective mupirocin concentrations would be quickly obliterated by licking and sneezing (Batra, Eziefula et al. 2008; Weese and van Duijkeren 2010). Successful decolonization of a dog using intranasal vancomycin ointment, or systemic ciprofloxacin + rifampin has been reported (Manian 2003; van Duijkeren, Wolfhagen et al. 2005). Unfortunately the drugs in these regemins may be associated with serious adverse effects (rifampin induced hepatitis) or uncertain and potentially serious resistance implications (vancomycin) (Dowling 2006; Weese 2008). Furthermore, the efficacy of canine decolonization regimens has not been investigated and prospective studies are required before evidence based interventions can be implemented. Currently, experts in the field have suggested that most dogs spontaneously decolonize and that pharmacological interventions are unlikely to be successful (Barton, Hawkes et al. 2006; Weese and van Duijkeren 2010).

1.2.7.2 Search and destroy – control in the population

Unlike other countries, the Netherlands has been very successful in minimizing the incidence of MRSA infection (Wertheim, Vos et al. 2004). The Dutch success story can be largely attributed to the national "search and destroy" strategy. Search and destroy encompasses four main components: 1. Isolating high risk individuals with known risk factors on admission to hospital, 2. Screening hospital patients for MRSA colonization, 3. Decolonization, 4. Molecular characterization of positive cultures (Infection.Prevention.Working.Party 2011).

In the Netherlands, previous MRSA diagnosis, working or living with pigs or veal calves and foreign hospitalization are considered important risk factors under search and destroy. High-risk individuals are physically isolated within the hospital including personal protective equipment for hospital staff treating the patient. Surveillance cultures are taken from both at risk

patients and the general hospital population. All MRSA positive individuals are decolonized (Infection.Prevention.Working.Party 2011). Depending on the clinical situation of the patient intra-nasal mupirocin, chlorhexidine soap for showering and frequent changing of towels and bed linens and daily underwear changes are prescribed (Wertheim, Nouwen et al. 2007). For complicated cases, systemic therapy may also be required (Wertheim, Nouwen et al. 2007).

Molecular epidemiological surveillance is a key component of the Dutch strategy. The first MRSA isolate from each patient is typed at the Dutch national lab (RIVM) (Wertheim, Nouwen et al. 2007; Infection.Prevention.Working.Party 2011). The characterization of isolates allows them to be compared to a database, facilitating detection of outbreaks and newly emerging strains.

There are high up front costs associated with search and destroy, however the prevention of MRSA infections has been demonstrated to result in overall savings (Vriens, Blok et al. 2002). In one hospital the annual cost of search and destroy was calculated to be €215,559 while the healthcare savings were estimated to be €427,356 (van Rijen and Kluytmans 2009). The vast resources needed for search and destroy limit its application to regions with a low incidence of MRSA. The strict MRSA policies in the Netherlands have resulted in one of the lowest incidences of MRSA infections in the world <1% of *S. aureus* bacteremia caused by MRSA compared to 50% in other areas of Europe (Kluytmans and Struelens 2009; van Rijen and Kluytmans 2009). In Canada, 24.4% of *S. aureus* bloodstream infections are caused by MRSA, while methicillin resistance is found in 19-54.8% of community associated *S. aureus* infections (Adam, Allen et al. 2009; Stenstrom, Grafstein et al. 2009; Adam, Decorby et al. 2011).

1.3 *Staphylococcus pseudintermedius*

1.3.1 Colonization

In dogs, *S. pseudintermedius* is the most commonly encountered coagulase positive staphylococcal species, as both a colonizer and cause of infections (Ball, Rubin et al. 2008; Griffeth, Morris et al. 2008). The prevalence of *S. pseudintermedius* colonization in dogs is ill defined. *Staphylococcus pseudintermedius* has been found in 58.5-81% of dogs with otitis and pyoderma, and up to 68% of healthy dogs although few studies have been done (Lyskova, Vydrzalova et al. 2007; Griffeth, Morris et al. 2008). Colonization studies have examined different body sites including the nares, pharynx, gastrointestinal tract, axilla and skin although a systematic comparison of these sites has not been done (Hanselman, Kruth et al. 2007; Griffeth, Morris et al. 2008). Knowledge of colonization sites will allow future investigations to achieve maximal screening sensitivity while avoiding sites of little diagnostic value.

1.3.2 *Staphylococcus pseudintermedius* as a Canine Pathogen

Staphylococcus pseudintermedius is commonly implicated in canine infections, accounting for the majority of otitis externa and pyoderma isolates, and 16.5% of urinary tract infection isolates (Werckenthin, Cardoso et al. 2001; Ball, Rubin et al. 2008). Like *S. aureus, S. pseudintermedius* is an opportunistic pathogen causing infections when host defenses are compromised (Weese and van Duijkeren 2010).

1.3.3 Antimicrobial resistance among *S. pseudintermedius*

In Saskatoon, *S. pseudintermedius* has historically remained remarkably susceptible to antimicrobials (Ball, Rubin et al. 2008; Rubin, Ball et al. 2011). *Staphylococcus*

pseudintermedius infections have been successfully treated with first-line drugs including the amino-penicillins and first generation cephalosporins (amoxicillin, cephalexin). For infections requiring a drug with increased tissue penetration, such as osteomyelitis and deep pyoderma, clindamycin has been the treatment of choice (Littlewood, Lakhani et al. 1999; Giguere 2006). Clinical *S. pseudintermedius* cultures isolated between 1986 and 2000 at the Western College of Veterinary Medicine were reported to be overwhelmingly susceptible; resistance to penicillin was found in only 7% of isolates, erythromycin in 13% and tetracycline in 34% and no methicillin resistance was found (Rubin, Ball et al. 2011).

The incidence of methicillin resistant *S. pseudintermedius* (MRSP) is increasing rapidly, challenging clinicians in treating their patients (Epstein, Yam et al. 2009; Ruscher, Lubke-Becker et al. 2010). While the only MRSP reports in Canada originate in Ontario and Saskatchewan, anecdotally it has also occurred in at least Alberta and British Columbia (Hanselman, Kruth et al. 2007; Rubin and Gaunt 2011). In 2009, the first canine MRSP recognized at the WCVM was isolated from a urinary tract infection (Rubin and Gaunt 2011). In stark contrast to the *S. pseudintermedius* typically encountered in Saskatoon, this isolate was resistant to the β-lactams, fluoroquinolones, macrolides, aminoglycosides, sulfonamides, chloramphenicol and rifampin (Rubin and Gaunt 2011). This urinary tract infection was community associated: no identifiable risk factors for a multidrug resistant organism were identified (Rubin and Gaunt 2011). The explosive increases in MRSP prevalence in Europe and North America are further complicated by multidrug resistance, which in contrast to CA-MRSA is common among MRSP (Wettstein, Descloux et al. 2008; Ruscher, Lubke-Becker et al. 2010; Weese and van Duijkeren 2010).

1.4 Antimicrobial susceptibility testing

Whether done to direct therapy or for resistance surveillance, antimicrobial susceptibility testing requires stringent quality control and standardized methods to yield valid results. Standard methods of dilutional susceptibility testing (broth and agar), disc diffusion and epsilometer testing have been developed (CLSI 2006; CLSI 2006; Biomerieux 2008). These guidelines describe all aspects of the testing procedure including media specifications, bacterial inoculum, solvents and diluents for each antimicrobial, incubation time and temperature and interpretive criteria for categorical analyses (CLSI 2006; CLSI 2006; CLSI 2008; CLSI 2008).

The validity of results can be objectively evaluated by testing quality control organisms along with the isolates under investigation; these QC organisms have defined susceptibility profiles allowing comparison to published QC ranges (CLSI 2008). The selection of QC organisms should be based on the drugs tested and each drug should have a corresponding organism with a QC range including the concentrations tested. Commonly used QC organisms for testing non-fastidious aerobic bacteria include *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (CLSI 2008).

While it is always tempting to classify an isolate as susceptible or resistant, this classification must be avoided for drugs without recognized interpretive criteria. Resistance breakpoints are designed to be clinically predictive and are established based on the intended use of the drug (licensed indication), dosing regimen (dose, route and frequency), pharmacokinetic properties of the drug in the host species and the pharmacodynamic interactions between the organism and the drug (Walker 2006; Andrews 2008; Dalhoff, Ambrose et al. 2009). Valid interpretive criteria are published by the Clinical and Laboratory Standards Institute (CLSI) and

other national and international standards organizations' but these criteria are only valid when testing is done according to the respective organizations guidelines. Epidemiological cut-off values differentiate organisms with acquired resistance from the wild type and are invaluable for detecting the emergence of "resistance" but they must be recognized as distinct from clinical breakpoints as they are not designed to be clinically predictive (Dalhoff, Ambrose et al. 2009).

1.5 Molecular epidemiology of *S. aureus*

The epidemiology of *S. aureus* is complex, with a distinct ecological niche in each host species, and geographic variation between the community and health care facilities within a region (Wertheim, van Leeuwen et al. 2005; Abbott, Leonard et al. 2010; Grundmann, Aanensen et al. 2010; Hasman, Moodley et al. 2010). This complexity is compounded when distinct *S. aureus* populations meet. A plethora of molecular tools have been developed for conducting evolutionary studies, documenting the population within a single niche or those moving between reservoirs and tracking outbreaks.

1.5.1 Techniques

There are many techniques available which are variably suited to particular research questions, and associated with their own set of technical advantages and disadvantages (Savelkoul, Aarts et al. 1999). The investigator must be aware of a technique's limitations, discriminatory power and reproducibility so that genetic fingerprints can be interpreted in a meaningful way; the level of discrimination relevant for the investigation must be considered.

When defining isolates as related or indistinguishable using a particular technique, it is important to specifically and consistently define the relationship. The term "strain" is ambiguous

in the literature and is imprecisely used to refer to a grouping such as a sequence type (MLST), pulsotype (PFGE) or isolates which are presumed to be related or "clonal". According to Webster's Third New International Dictionary, a strain is "a line descended or derived from a particular ancestral individual", and example would be a type strain such as those catalogued by the American Type Culture Collection (ATCC) (Grove 1993). The application of this definition to isolates defined using methods with varying discriminatory power is imprecise, a sequence type may not be phylogenitcally equivalent to an MLVA profile. Ambiguity is also associated with the term "clone", which when discussing *S. aureus* is often used to describe isolates belonging to the same pulsotype, the "USA300 clone" for example (Schwartz, Graber et al. 2009). Strictly speaking clones are derived from a common ancestor and are genetically identical, the level of similarity attributed to two given isolates is more reflective of the discriminatory power of the technique than the true homology of the organisms (Tibayrenc 2009).

The ambiguousness of the widely employed nomenclature used to describe the genetic relationships between isolates should be avoided and replaced with precise terminology specific to the method used. For example, instead of referring to to isolates as the "same strain", it would be clearer to say that they are: the same sequence type, *spa* type, have indistinguishable PFGE profiles or the same binary IS profile. A high degree of precision and accuracy in definitions are required to communicate scientific findings in an efficient and useful way.

1.5.1.1 Pulsed-field gel electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE), a technique popularized by the US Centers for Disease Control and Prevention (PulseNet) for molecular investigations of Gram negative enteric

bacteria, has been considered the gold standard technique for discriminating bacterial strains of many clinically relevant species (Mulvey, Chui et al. 2001; CDC 2011). In PFGE, whole genomic DNA is digested using a restriction enzyme, the resulting fragments are then resolved on a gel in a specialized apparatus and banding patterns are analyzed (CDC 2011). In addition to being technically demanding and time consuming, it has been difficult to achieve a high degree of inter-laboratory reproducibility despite the development of standardized protocols (Mulvey, Chui et al. 2001; Melles, van Leeuwen et al. 2007).

The resolving power of PFGE has made it a benchmark for comparison for new techniques (Malachowa, Sabat et al. 2005; Petersson, Olsson-Liljequist et al. 2009; Schouls, Spalburg et al. 2009). Until recently, PFGE was the method of choice for reference labs around the world and distinct typing nomenclatures were developed in different regions. Consequently, one system may not be uniformly described by another and the same "strain" may have different designations in each typing system (Mulvey, Chui et al. 2001; Christianson, Golding et al. 2007; Cookson, Robinson et al. 2007; Kim, Ferrato et al. 2010). In Canada, isolates are designated CMRSA 1 – CMRSA 10 (Christianson, Golding et al. 2007). The technical demands of PFGE combined with the decreasing costs of sequencing and the need for globally comparable data is giving rise to a new generation of sequence and PCR based techniques.

1.5.1.2 Multilocus sequence typing (MLST)

As a relatively low-resolution, high-fidelity technique, MLST is best suited to big picture evolutionary studies (Maiden, Bygraves et al. 1998; Robinson and Enright 2004; Aanensen and Spratt 2005; Melles, van Leeuwen et al. 2007; Palavecino 2007; Turner and Feil 2007). The online *S. aureus* database as well as protocols for other species can be found at www.mlst.net

(Aanensen and Spratt 2005). MLST is based on the sequences of seven housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*) (Enright, Day et al. 2000). Each unique sequence for each gene is assigned a number in the MLST database and the numeric profile from all seven genes defines the sequence type (Enright, Day et al. 2000). Unique sequence types are also assigned numerical identities in the database. New sequence types are numbered sequentially as they are describeded. The relatedness of ST97 and ST98 for example must be based on the seven gene numeric profile 3-1-1-1-1-5-3 and 1-4-1-35-12-1-10 respectively, rather than the sequence type number itself (Aanensen 2011). While MLST won't detect divergence among closely related *S. aureus* lineages, it is valuable for evolutionary studies and for broadly clustering the finely resolved types identified with other techniques.

1.5.1.3 *spa* typing

Sequencing the hyper-variable x-region of the staphylococcal protein A (*spa*) gene is another objective, sequenced based molecular tool for characterizing *S. aureus* (Harmsen, Claus et al. 2003). Unlike MLST, *spa* typing discriminates between closely related isolates; its resolving power is somewhere between PFGE and MLST (Harmsen, Claus et al. 2003; Hallin, Friedrich et al. 2009; Petersson, Olsson-Liljequist et al. 2009). Within a sequence type there may be many *spa* types (SpaServer 2011). Like MLST, *spa* types are cataloged in a central, web-based database allowing easy access to the details of each *spa* type and global epidemiological information (Harmsen, Claus et al. 2003). As an objective fast and inexpensive technique, *spa* typing is quickly gaining favour, and it is now routinely used in diagnostic, reference and research labs around the world. The hyper-variable x-region contains a variable number of short (21-30 base pair) sequences (Hallin, Friedrich et al. 2009; SpaServer 2011).

Presently , 462 unique, short sequences termed "repeats" have been described and assigned a numerical identifier (SpaServer 2011). For example the 24 base pair sequence 5' AAA GAA GAC AAC AAA AAA CCT GGT-3' was the thirty fourth repeat deposited in the database and is therefore identified as "r34" (SpaServer 2011). An isolate is assigned a *spa* type according to which and how many repeats are present (2-16) and their order (Hallin, Friedrich et al. 2009). *spa* types are identified numerically in the order of their discovery. Because *spa* types are numbered in the order of their discovery no genetic relationship can be inferred from sequentially numbered types; relatedness is dictated by the repeat sequence of each type. For example t034 (r08-r16-r02-r25-r02-r25-r34-r24-r25) is more closely related to t4652 (r08-r16-r02-r25-r02-r25-r34-r24) than t035 (r26-r17-r13-r12-r17-r17-r16) (SpaServer 2011).

1.5.1.4 Amplified fragment length polymorphism (AFLP)

Like PFGE, AFLP is a band-based technique which detects polymorphisms throughout the whole genome (Savelkoul, Aarts et al. 1999; Melles, Gorkink et al. 2004; Fry, Savelkoul et al. 2009). In AFLP, genomic DNA is digested using restriction enzymes, site specific adaptors are then ligated to restriction fragments, fragments are amplified by PCR and the amplicons are resolved by electrophoresis (Fry, Savelkoul et al. 2009). The resolving power of AFLP is similar to PFGE, allowing discrimination of closely related isolates without some of the technical challenges associated with PFGE (Melles, van Leeuwen et al. 2007). Despite its advantages, AFLP suffers from a number of disadvantages including a lack of inter-laboratory standardization and the development of more objective, sequence based techniques (Melles, van Leeuwen et al. 2007).

1.5.1.5 16S – 23S interspace typing (IS-typing)

Although the costs of sequencing are rapidly decreasing, the simplicity and objectivity of PCR based techniques has led to the development of IS-typing techniques. There are five or six copies of the 16S-23S interspace region in the *S. aureus* chromosome (Budding, Vandenbroucke-Grauls et al. 2010). The length of this region varies within and between individual isolates (Budding, Vandenbroucke-Grauls et al. 2010). While the length of this region is variable, this variation is limited to combinations of 16 defined region lengths (Budding, Vandenbroucke-Grauls et al. 2010). The IS-type is based on a binary profile of the isolates resulting from the presence of absence of each of the 16 defined region lengths (Budding, Vandenbroucke-Grauls et al. 2010). Resolution of amplicons on either agarose slab gels or high resolution capillary gels allows easy application of this technique in labs with only basic equipment (Budding, Vandenbroucke-Grauls et al. 2010). The discriminatory power of IS-typing is similar to MLST and a previous study demonstrated good agreement between these two techniques (Budding, Vandenbroucke-Grauls et al. 2010).

1.5.1.6 Multiple locus variable number tandem repeat analysis (MLVA)

Multiple locus variable number tandem repeat (VNTR) analysis (MLVA) is a new, very high resolution technique for differentiating closely related *S. aureus* isolates (Moser, Box et al. 2009; Schouls, Spalburg et al. 2009). A VNTR locus is one where a defined DNA sequence, between 9 – 560 base pairs for *S. aureus* depending on the loci used, repeats a variable number of times (Vergnaud and Pourcel 2009). Variation between isolates in the number of repeats at a given locus is the basis of the technique. Like IS-typing, MLVA is a PCR based technique relying on the lengths of VNTR regions throughout the genome rather than their sequence

(Malachowa, Sabat et al. 2005; Vergnaud and Pourcel 2009). The number of repeats determines the length of the locus and this length may vary diagnostically between individual isolates (Ikawaty, Willems et al. 2008; Vergnaud and Pourcel 2009). For typing, the repeat length at a given VNTR locus must first be determined using DNA sequencing, or by using a previously published method. Products from VNTR region PCR are resolved on a gel, and the size of the amplion calculated by comparison with a known DNA ladder run in parallel (Vergnaud and Pourcel 2009). The number of repeats is then calculated using the following equation:

$$n = (a - c) / r$$

Where: n = number of repeats
c = product constant of the locus
a = amplicon length
r = repeat length

The product constant "c" must be included to correct for non-repeat regions flanking the VNTR in the amplified product including primer binding sites (Vergnaud and Pourcel 2009)

When multiple VNTR loci are combined, the number of repeats at each locus are combined yielding a string of numbers which defines the strain (Schouls, Spalburg et al. 2009).

The hyper-variability of some VNTR loci allows changes in closely related isolates to be detected (Ikawaty, Willems et al. 2008). There is concern that MLVA may actually be too discriminatory, detecting temporal drift between clonal isolates highlighting differences not reflective of epidemiological relationships (Tanner, Hardy et al. 2010). In the future, MLVA with carefully calibrated cut offs used to differentiate isolates (or identify them as indistinguishable), may prove to be a highly valuable, high resolution, inexpensive, objective and internationally comparable epidemiological tool for *S. aureus*.

1.5.2 MRSA "strains" of particular interest

The dissemination of MRSA from the hospital into the community has, since the late 1990s, been associated with the emergence of a number of MRSA lineages (Oliveira, Tomasz et al. 2002; Adam, Allen et al. 2009; Chambers and Deleo 2009; Li, Diep et al. 2009).

In Canada there has been a 17-fold increase in the incidence of CA-MRSA infection since the mid 1990s, although this increase has not been geographically uniform in magnitude or "strain" type (Simor, Gilbert et al. 2010). In 2004, the first outbreak of USA 300 (CMRSA10) was recognized in Calgary, Alberta (Gilbert, MacDonald et al. 2006; Christianson, Golding et al. 2007). Between 2005 and 2008 the incidence of MRSA infection in Alberta nearly doubled and much of this increase was attributed to the rise of USA 300 (CMRSA10) which by 2008 comprised 53% of MRSA isolates (Christianson, Golding et al. 2007; Kim, Ferrato et al. 2010). In contrast, the USA 400 (CMRSA7) lineage predominates in Manitoba, northern Saskatchewan and Nunavut (Christianson, Golding et al. 2007; Simor, Gilbert et al. 2010). The most prevalent lineages in Saskatoon are unknown since recent, locally specific reports have not been published. However CMRSA10 is thought to be spreading rapidly and is likely increasing in Saskatchewan (Simor, Gilbert et al. 2010).

MRSA ST398 was first identified in the Netherlands in 2004 and was initially identified as non-typable by PFGE (Voss, Loeffen et al. 2005). This strain was first isolated from a girl with an "unexpected" MRSA infection (no defined risk factors) in July 2004 (Voss, Loeffen et al. 2005). Epidemiological trace-backs found that MRSA ST398 was highly significantly associated with livestock; Dutch residents in contact with pigs and veal calves are up to 1,000 times as likely as the general population to be colonized with MRSA (van Rijen, Van Keulen et al. 2008). MRSA ST398 is highly prevalent among commercially raised pigs. It has been found

on 11-70% of pig farms, in 11-49% of pigs on those farms and 20-45% of those working with them (Smith, Male et al. 2008; van Duijkeren, Ikawaty et al. 2008; Wulf and Voss 2008; Kock, Harlizius et al. 2009).

Diversity within the ST398 lineage, multiple *spa* types and heterogeneous PFGE profiles, indicates that different MRSA ST398 isolates may occupy unique epidemiological (host, farm or geographical) niches (Bosch, de Neeling et al. 2010; Fessler, Scott et al. 2010). On closer inspection, diversity in the resistance gene and virulence factor profile, and SCC*mec* types have also been reported among MRSA ST398 isolates (Laurent, Jouy et al. 2009; Skov, Li et al. 2009; Stegger, Lindsay et al. 2009).

In pigs, MSSA ST398 is common, and it is thought that acquisition of *mec*A by members of this already successful lineage may be responsible for the rise of livestock associated MRSA (Hasman, Moodley et al. 2010). There has been a lot of speculation regarding potential factors that could have selected for MRSA ST398 in pigs, particularly antimicrobial usage, however solid evidence has not been published (Wulf and Voss 2008). Authors of early reports hypothesized that the high frequency of tetracycline resistance among MRSA ST398 suggested that tetracycline use could select for this strain (Wulf and Voss 2008). However, these investigators failed to recognize that tetracycline resistance is common among porcine MSSA, and that tetracycline usage would therefore not likely select for MRSA over MSSA (Hasman, Moodley et al. 2010; Rubin, Ball et al. 2011).

Recently evidence implicating tiamulin, a pleuromutilin antimicrobial used exclusively in animal agriculture, as a potential selection pressure for MRSA ST398 was published (Rubin, Ball et al. In Press). Retapamulin, the only pleuromutilin antimicrobial available for human use, is a topical preparation for the treatment of MRSA infections and was not introduced until 2007,

several years after the emergence of MRSA ST398 and several decades after the introduction of tiamulin (FDA 1987; Daum, Kar et al. 2007). MRSA ST398 were reported to have tiamulin MICs significantly higher than human MSSA and non-ST398 MRSA isolates and porcine MSSA (Rubin, Ball et al. In Press). While further study is required, these data suggest that the use of tiamulin in pigs may impart a potent selective pressure in favour of MRSA ST398.

1.6 Interspecies transmission

1.6.1 *S. aureus*

Staphylococcus aureus is a promiscuous colonizer/cause of infections, which has been identified in many species (Weese and van Duijkeren 2010). While some *S. aureus* lineages tend to be species-specific only occasionally venturing into different hosts, others have little regard for such barriers (Sung, Lloyd et al. 2008; Weese and van Duijkeren 2010). For example *S. aureus* ST5 is an internationally prevalent lineage that crossed from people into chickens in the mid 1900s while CMRSA5 is a lineage disproportionally associated with horses and people with equine contact (Weese, Archambault et al. 2005; Lowder, Guinane et al. 2009; Abbott, Leonard et al. 2010; Weese 2010). In contrast to ST5, *S. aureus* isolated from bovine mastitis are genetically distinct from human isolates, indicating that transmission in either direction is infrequent (Smyth, Feil et al. 2009)

1.6.1.1 Dogs and people

With the emergence of MRSA in dogs, more attention has been paid to canine *S. aureus* infections and the origin of these organisms. As *S. pseudintermedius* colonization predominates in dogs, infections caused by *S. aureus* may indicate transmission from a reservoir of *S. aureus*

in close contact with the dogs (Weese and van Duijkeren 2010). The complex ecology of *S. aureus* including canine-human transmission dynamics, remains ill defined. Although transmission has been documented, and there is evidence of a shared *S. aureus* population, these interactions have not been adequately defined (Simoons-Smit, Savelkoul et al. 2000; Manian 2003).

In one report, a diabetic man with a non-healing wound infected with MRSA was unsuccessfully treated (Manian 2003). Repeated treatment and decolonization attempts were made. His wife was also colonized, although decolonization attempts of both failed to eradicate MRSA from the household. Eventually the couple's dog was sampled and found to be colonized. A decolonization regimen for the dog (intra-nasal vancomycin ointment) was administered and the man's infection was finally cleared (Manian 2003).

Transmission in veterinary hospitals is also recognized, and companion animal veterinarians (small animal and equine) are known to be at elevated risk of colonization (Hanselman, Kruth et al. 2006; Loeffler, Pfeiffer et al. 2010). Bidirectional transmission between companion animals (cats and dogs) and humans including veterinary staff has been described (Weese, Dick et al. 2006).

1.6.1.2 MRSA ST398 (livestock associated MRSA)

While MRSA ST398 has been reported in association with horses, chickens and dogs, swine contact is the most important risk factor for human colonization/infection (van Belkum, Melles et al. 2008; Kock, Harlizius et al. 2009; Nienhoff, Kadlec et al. 2009; Tokateloff, Manning et al. 2009; Mulders, Haenen et al. 2010; Weese and van Duijkeren 2010). However, the household contacts of those individuals (spouses and children) do not have the same level of

risk (van Rijen, Van Keulen et al. 2008; Cuny, Nathaus et al. 2009). Human to human transmission is rare even in the hospital environment; non-ST398 were recently shown to transmit 5.9 times as efficiently as ST398 between patients (Bootsma, Wassenberg et al. 2010).

1.6.2 *S. pseudintermedius*

In contrast to *S. aureus*, *S. pseudintermedius* is the most common coagulase positive staphylococcal species in dogs and is infrequently identified from people (Griffeth, Morris et al. 2008; Kempker, Mangalat et al. 2009). Like *S. aureus* in dogs, *S. pseudintermedius* may be under-recognized as a cause of human infections (Pottumarthy, Schapiro et al. 2004; van Duijkeren, Ikawaty et al. 2008). Depending on the methods used to identify *S. aureus, S. pseudintermedius* could easily be mistaken for *S. aureus* (van Duijkeren, Ikawaty et al. 2008). In human beings, *S. pseudintermedius* has been most commonly identified from canine bite wounds, a situation where canine flora would be expected, although transmission without traumatic contact has also been reported (Talan, Staatz et al. 1989). Among the small number of reported cases of human *S. pseudintermedius* infection, one was a patient who had undergone surgery for a pituitary neoplasia who subsequently developed a pituitary abscess (Tanner, Everett et al. 2000; van Duijkeren, Houwers et al. 2008; Kempker, Mangalat et al. 2009). A recent study provided indirect evidence of zoonotic *S. pseudintermedius* transmission by demonstrating MRSP colonization of veterinarians (Paul, Moodley et al. In Press). Similarly, transmission between dogs with pyoderma and their owners has also been reported (Guardabassi, Loeber et al. 2004).

Chapter 2 - Hypotheses, Rationale and Objectives

2.1 Hypotheses

The ability of *S. aureus* isolated from different species to coagulate canine vs. rabbit plasma will vary, and that canine *S. aureus* will coagulate canine plasma faster than rabbit plasma.

Dogs are nasally, pharyngeally and rectally colonized with *S. aureus* and *S. pseudintermedius* including individuals that are single site colonized.

Resistance to commonly used antimicrobials in canine medicine will be found among *S. pseudintermedius* isolated from healthy dogs in Saskatoon, Canada.

Resistance to commonly used antimicrobials in canine and human medicine will be found among *S. aureus* isolated from healthy dogs, clinical canine MRSA isolates and clinical *S. aureus* isolated from people in Saskatoon, Canada.

Human beings and dogs share a common population of *S. aureus* and bidirectional transmission occurs.

2.2 Rationale

In the clinical microbiological laboratory, rapid and sensitive tests are desirable for identifying organisms causing infections. The identification of *S. aureus* in dogs has clinical (for the individual patient) and infection control (for other patients, staff and owners) implications. As the coagulase test is integral to the identification of *S. aureus*, a test which rapidly yields highly sensitive and specific results is desirable. The utility of different plasmas for isolates of varying origin should therefore be assessed since previous studies have reported that some plasmas are coagulated faster than others.

Recent investigations in human clinical microbiology have revealed that multiple sites of colonization play a role in the ecology of *S. aureus* in humans. In dogs, the sites of colonization have been largely undefined. For studies defining the prevalence of *S. aureus* as well as for investigating outbreaks, maximal screening sensitivity (without excessive sampling), necessitates knowledge of sites of colonization.

The antimicrobial susceptibility profiles of *S. pseudintermedius* vary geographically. As canine *S. pseudintermedius* infections are often treated empirically, local definition of antibiograms is required to guide therapy. Additionally, local resistance surveillance is required to detect changes in antimicrobial susceptibility. The antimicrobial susceptibility profiles of *S. aureus* also vary geographically therefore local resistance surveillance is required to detect changes in antimicrobial susceptibility.

The current literature suggests that people and dogs share a common population of *S. aureus,* and that bidirectional transmission occurs. Most reports describe outbreak situations (in veterinary hospitals) or specific instances of transmission (in an individual household). Very few data have been published concerning both canine and human isolates related only by geographic

origin. To better define the ecology of *S. aureus* as it relates to people and dogs, the relatedness of canine and human *S. aureus* from the same region sould be assessed.

2.3 Objectives

Compare rabbit plasma (the current standard) to canine plasma in the tube coagulase test for canine, human and bovine *S. aureus* isolates.

Sample the nares, pharynx and rectum of healthy dogs and determine the prevalence of *S. aureus* and *S. pseudintermedius* colonization at each site. In addition to defining the sites of colonization overall, the sites colonized in an individual dog will be recorded, including single site colonization.

Test the antimicrobial susceptibility of *S. pseudintermedius* isolated from healthy colonized dogs to an extensive panel of antimicrobials. Compounds used systemically in both human and veterinary medicine will be evaluated as well and the topical agents mupirocin and fusidic acid.

Test the antimicrobial susceptibility of clinical MRSA isolated from dogs at the WCVM, *S. aureus* isolated from healthy colonized dogs and clinical MRSA and MSSA isolated from people in Saskatoon, Canada to an extensive panel of antimicrobials. Compounds used systemically in both human and veterinary medicine will be evaluated as well and the topical agents mupirocin and fusidic acid.

Determine the relatedness of canine and human *S. aureus* using a variety of molecular techniques. This investigation will allow isolates to be compared to each other and to a global database.

Chapter 3

Comparison of Dog and Rabbit Plasmas in the Tube Coagulase Test for *Staphylococcus aureus*

J.E. Rubin, M.K. Bayly and M. Chirino-Trejo

Journal of Veterinary Diagnostic Investigation, 2010; Vol 22 (5), 770-771

3.1 Abstract

The tube coagulase test, an invaluable laboratory tool for identifying *Staphylococcus aureus,* is most often done using rabbit plasma. However, there is evidence that depending on the origin of the isolates, other plasmas may be superior. In this study, we sought to compare the utility of dog and rabbit plasma in the coagulase test for *S. aureus* isolated from canine (n=28), bovine (n=29), and human (n=30) hosts. Overall, coagulation times were significantly faster for dog (2.38 hours) than rabbit (3.19 hours) plasma. When coagulation times were compared by isolate origin, no significant differences were found for rabbit plasma, whereas bovine isolates clotted dog plasma significantly faster (1.86 hours) than canine (2.79 hours) or human (2.38 hours) isolates. Investigators should be aware that rabbit plasma may not be the ideal coagulase testing medium for *S. aureus* from all sources.

3.2 The study

Staphylococcus aureus is one of the most common causes of infections in people worldwide, and is increasingly recognized in companion animals including dogs (Moet, Jones et al. 2007; Weese and van Duijkeren 2010). Recent reports of the increasing prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in people and animals have raised awareness in the veterinary community about this potential pathogen (Rubin and Chirino-Trejo 2010; Simor, Gilbert et al. 2010). *S. aureus* is identified using a number of standard biochemical tests including the tube coagulase test, typically using rabbit plasma (Winn, Allen et al. 2006).

Previous studies have documented variability in the coagulability of different plasmas for *S. aureus* isolated from different hosts (Orth, Chugg et al. 1971; Live 1972; Adesiyun and Shehu 1985; Dickson and Marples 1986). This variability may be intrinsic to the plasma or due to the

presence of *S. aureus* biotypes which preferentially clot certain plasmas (Orth, Chugg et al. 1971; Adesiyun and Shehu 1985). The importance of identifying *S. aureus* necessitates sensitive and timely coagulase test procedures, particularly in the case of MRSA, where therapeutic and infection control measures are markedly different than for susceptible organisms. The purpose of this study was to evaluate the relative utility of dog and rabbit plasmas in the tube coagulase test for *S. aureus* isolated from canine, bovine and human hosts.

Eighty seven archived *S. aureus* isolates were tested. A total of 30 were of human origin, and 29 and 28 were from bovine and canine hosts, respectively. Commercially prepared rabbit plasma[a] and dog blood bank plasma[b] were used. A single well-isolated colony was inoculated into 0.5 ml of plasma and incubated at 35°C for 4 hr, followed by room temperature incubation (Winn, Allen et al. 2006). Samples were evaluated hourly unitil clot formation was first detected by gently tilting the test tube.

Statistical analyses were done using SPSS Version 17.0[c]. Dog and rabbit plasma coagulase times for all samples were compared using a univariate analysis of variance (ANOVA) with Bonferroni correction. Differences in the time to clot formation for dog and rabbit plasma between isolates from each host were compared using one way ANOVAs. Differences in the time to clot formation for dog and rabbit plasma between isolates from the same host species were compared using *t*-tests. $P \leq 0.05$ was considered significant for all analyses.

Overall, the time to clot formation was significantly faster for dog (mean: 2.38 hours) than rabbit (mean: 3.19 hours) plasma ($P < 0.001$; Table 3.1). No significant differences were found in time to coagulate rabbit plasma between isolates from different hosts; however, bovine isolates coagulated dog plasma faster (mean: 1.86 hours) than either canine (mean: 2.79 hours, P

= 0.001) or human (2.50 hours, P = 0.029) isolates (Table 3.1). Bovine and human isolates coagulated dog plasma

Table 3.1. Coagulation times of canine and rabbit plasma for *S. aureus* isolated from different species

Host Species	Plasma	Mean time (hours)	Minimum time (hours)	Maximum time (hours)
Canine	Dog†	2.79	2	6
	Rabbit	3.07	2	5
Bovine*	Dog†,‡	1.86	1	6
	Rabbit	3.38	1	7
Human*	Dog‡	2.38	1	5
	Rabbit	3.19	1	7
Overall*	Dog	2.38	1	6
	Rabbit	3.19	1	7

Significant differences ($p \leq 0.05$) in the mean coagulation time between plasmas for a group of isolates are denoted by the superscript "*". Significant differences ($p \leq 0.05$) in the mean coagulation time with dog plasma between isolates from different hosts are denoted by the superscripts "†" and "‡".

significantly faster than rabbit plasma, whereas no significant differences were found among canine isolates (Table 3.1).

Previous studies have shown that *S. aureus* isolates coagulate plasmas of different origins to varying degrees, including false negatives, suggesting that multiple plasmas may need to be used (Live 1972; Adesiyun and Shehu 1985). However, because these data were collected in the 1970s and 1980s before some staphylococcal species (e.g, *S. pseudintermedius*) were recognized, the identity of those collections and the specificity of the data to *S. aureus* are uncertain. Additionally, because direct comparisons between dog and rabbit plasma have not been reported, this study fills an important gap in the literature.

The current study demonstrates that there are significant differences in the coagulase test using dog and rabbit plasmas. Although the time to clot dog plasma was significantly faster overall, suggesting an intrinsic superiority to rabbit plasma, there was also variability between isolates from different host species, giving credence to the biotype hypothesis. Although no false-negatives are described in the present report, the archived samples tested were initially identified using rabbit plasma and may not be representative of all clinical isolates, therefore potentially overestimating the utility of rabbit plasma.

Significant differences were not found between canine and human isolates, possibly reflecting the previously described genetic similarity between canine and human *S. aureus* collected from a single geographic location (Weese and van Duijkeren 2010). Additionally, the significant differences found in the time to clot dog plasma between bovine and human isolates is consistent with previously reported genetic dissimilarities between bovine and human strains (Smyth, Feil et al. 2009). In the future, it would be useful to compare these data to established

techniques for evaluating the relatedness of *S. aureus* such as *spa* typing and multilocus sequence typing.

Investigators should be aware of the potential limitations of only using rabbit plasma, particularly when testing samples from different hosts. Future studies including plasmas and prospectively collected isolates from a greater number of hosts should be done to define the utility of various plasmas for different diagnostic applications.

3.3 Acknowledgements, Sources and Manufacturers

The authors would like to acknowledge the Companion Animal Health Fund for funding this study.

Sources and Manufacturers

a. Becton, Dickinson and Company, Sparks MD

b. Canadian Animal Blood Bank, Winnipeg, Canada

c. SPSS Inc, Chicago, IL

Chapter 4

Pharyngeal, Rectal and Nasal Colonization of Clinically Healthy Dogs with *Staphylococcus aureus*

J.E. Rubin and M. Chirino-Trejo

Veterinary Microbiology, 2010; Vol 143 (2-4), 440-441

4.1 The study

Staphylococcus aureus is a promiscuous colonizer/pathogen, it lacks host specificity and colonizes various anatomical sites. To better understand the complex epidemiology of *S. aureus* in dogs, sensitive screening protocols, including which body sites to sample, are required. Extra-nasal colonization of humans is well recognized; 27% of carriers are not nasally colonized including 12.8% - 20% and 10% - 17.1% who are exclusively pharyngeally and enterally colonized respectively (Eveillard, de Lassence et al. 2006; Mertz, Frei et al. 2007; Batra, Eziefula et al. 2008; Mody, Kauffman et al. 2008). The relative importance of colonization at these sites has not been evaluated in dogs (Weese and van Duijkeren 2010). In this study, the prevalence of pharyngeal, nasal and rectal *S. aureus* carriage among healthy dogs was determined. One hundred sixty seven clinically healthy dogs presenting for vaccinations and annual physical examinations to the Western College of Veterinary Medicine, Veterinary Teaching Hospital were sampled. Using sterile swabs (BBL CultureSwab, Sparks, MD) the pharynx, nares and rectum were sampled. Sterile saline from single use 5 ml vials (Hospira Healthcare Corporation, Montreal, Canada) was used to moisten swabs prior to nasal sampling.

All samples were processed within five hours of collection. Each swab was plated on CHROMagar Staph aureus (CHROMagar, Paris, France) and mannitol salt agar (Becton, Dickenson and Company, Sparks, MD), both with and without 4µg/ml oxacillin (Sigma-Aldrich, St. Louis, MO), and Trypticase Soy Agar with 5% sheep's blood (Becton, Dickenson and Company, Sparks, MD) and incubated at 35°C overnight. Each swab was then broken off into a tube containing 2 ml of enrichment broth with 75g/L NaCl which, after overnight incubation at 35°C, was plated onto the same five media (Weese 2007). Growth on solid media was evaluated at 24, 48 and 96 hours. *S. aureus* was identified based on colony morphology, standard

biochemical tests and analysis of the *cpn*60 and 16S rRNA gene sequences; previously published primers were used (Dorsch and Stackebrandt 1992; Hill, Paccagnella et al. 2006). All isolates were confirmed to be methicillin susceptible or resistant by both oxacillin broth micro-dilution susceptibility testing (Sensititre, Trek Diagnostic Systems, Cleveland, OH) according to CLSI guidelines, and by screening for the *mec*A gene using published primers (de Neeling, van Leeuwen et al. 1998; CLSI 2008).

Of the 167 dogs sampled, 17 (10.2%) were positive for *S. aureus*, one of which was methicillin resistant. Among these dogs, 10 were pharyngeally colonized while 13 and seven were nasal and rectal carriers respectively (Table 4.1). From the MRSA colonized dog, only a single colony was isolated from CHROMagar without oxacillin. The MRSA was only detected after susceptibility testing; false negatives on selective media have previously been attributed to low bacterial numbers (Tande, Garo et al. 2008). The single phenotypically identified MRSA was *mec*A positive while all other isolates were negative.

Among *S. aureus* carriers, 5.9%, 17.6% and 17.6% were exclusively pharyngeally, rectally and nasally colonized respectively (Table 4.1). Using all three sites as the gold standard, the sensitivity of sampling the nares + rectum was 94.1%, nares + pharnx 82.4% and pharynx + rectum 82.4%. There is increasing interest in canine *S. aureus* colonization therefore sensitive screening protocols including multiple anatomic sites are necessary to accurately define the ecology of this potential pathogen.

Table 4.1. Sites of colonization in *Staphylococcus aureus* positive dogs

Subject ID (Dog) (n=17)	**Nasal (n=10)**	**Pharyngeal (n=7)**	**Rectal (n=13)**
1	+	Neg	+
2	+	Neg	+
3	+	Neg	+
4	Neg	+	Neg
5	Neg	+	+
6	+	Neg	+
7	Neg	Neg	+
8	+	+	+
9	+	Neg	+
10	Neg	+	Neg
11	+	+	+
12*	Neg	Neg	+
13	+	Neg	+
14	Neg	+	Neg
15	+	+	+
16	Neg	Neg	+
17	+	Neg	Neg

*MRSA colonized

4.2 Acknowledgements

The authors would like to thank Drs. Patricia Dowling, Janet Hill and Bonnie Chaban for their technical and editorial assistance and the Companion Animal Health Fund for funding. The University of Saskatchewan Committee on Animal Care and Supply and the Biomedical Research Ethics Board approved this study. The authors have no conflicts of interests to declare.

Chapter 5

Antimicrobial Susceptibility of Canine and Human *Staphylococcus aureus* Collected in Saskatoon, Canada

J.E. Rubin and M. Chirino-Trejo

Zoonoses and Public Health, In Press 2011

5.1 Summary

Staphylococcus aureus is one of the most common causes of infection in people and is increasingly recognized in dogs. The increasing prevalence of methicillin resistant *S. aureus* (MRSA) is complicating the treatment of these infections. Panton Valentine leukocidin (PVL), a toxin involved in the pathogenesis of necrotic syndromes in people may be partially responsible for the rise of MRSA. Canine and human *S. aureus* isolates from the same geographic area are genetically similar, indicating a common population and probably transmission. The implications of increasing antimicrobial resistance complicated by interspecies transmission necessitates including both dogs and humans in *S. aureus* resistance surveillance studies. A collection of 126 *S. aureus* isolates from people (51 MRSA and 48 MSSA) and dogs (18 colonizing isolates and 9 clinical MRSA) were included. The minimum inhibitory concentrations (MIC) of a panel of 33 antimicrobials used in human and veterinary medicine were determined for all isolates. No resistance to vancomycin, linezolid, daptomycin, quinupristin/dalfopristin or nitrofurantoin was found. A wide range of antibiograms were found including resistance to between 0-12 drugs (0-6 drug classes). Outstanding antibiograms included a canine MRSA resistant to rifampin and a human MRSA resistant to chloramphenicol. Inducible clindamycin resistance (iCR) was found among 78% and 4% of canine and human MRSA and 17% and 25% of canine colonizing and human methicillin susceptible *S. aureus* (MSSA) respectively. Resistance to mupirocin was only found among human isolates including 20% of MRSA and 4% of MSSA. While no canine isolates were PVL positive, 39% of human MRSA and 2% of MSSA carried the gene. The bidirectional transmission of *S. aureus* between people and dogs necessitates the inclusion of isolates from both species in future studies.

5.2 Introduction

Staphylococcus aureus commonly causes infections in people and is increasingly recognized in dogs (Weese and van Duijkeren 2010). Readily adapting to antimicrobial selection pressure, penicillin resistance emerged shortly after its introduction, initially in hospitals followed by dissemination into the community (Woodford 2005). Similarly, the development of MRSA in hospitals was followed by community dissemination, where it now predominates as a cause of skin and soft tissue infections in some areas (Stenstrom, Grafstein et al. 2009). The ecology of *S. aureus* is complex and includes multiple host species. Genetic studies have shown that canine and human MRSA from the same region tend to be related, indicating a shared population (Weese and van Duijkeren 2010). Furthermore, indistinguishable isolates recovered from infections in people and dogs highlights the clinical importance of this shared population (Manian 2003; Faires, Tater et al. 2009). Despite the increasing incidence in people, canine MRSA infections are still rarely identified in Western Canada. This report includes the first clinical canine isolates from our region (Simor, Gilbert et al. 2010). Interspecies transmission of *S. aureus* highlights the importance of including human and canine isolates in future resistance surveillance.

Often associated with community associated MRSA (CA-MRSA), Panton Valentine leukocidin (PVL) plays a role in the pathogenesis of certain necrotic syndromes in people (Labandeira-Rey, Couzon et al. 2007). While the activity of PVL is species specific, and its role in canine infection is unknown, its human health implications necessitate monitoring the dissemination of this toxin (Loffler, Hussain et al. 2010).

While antimicrobial resistance surveillance programmes in Canada exist for human pathogens, these data are reported for large geographic regions, making province specific

analysis difficult (Canada 2008). Foodborne pathogens are actively surveilled in Canada and the United States, while other veterinary resistance reservoirs including companion animal staphylococci are not addressed (Canada 2007; FDA 2007). While canine staphylococci in Saskatoon have historically been remarkably susceptible, the recent isolation of methicillin resistant organisms indicates that this may be changing (Ball, Rubin et al. 2008; Rubin, Ball et al. 2011; Rubin and Gaunt 2011). The purpose of this study was to determine the susceptibility of canine and human *S. aureus* to a panel of 33 antimicrobials and to determine the frequency of PVL positive isolates in this collection.

5.3 Materials and methods

5.3.1 Bacterial collection

A collection of 126 canine and human *S. aureus* from Saskatoon, Canada were tested. The 27 canine samples included the nine MRSA isolates (CCMR) corresponding to all canine MRSA infections identified at our hospital in 2006 and 2008. These MRSA, while cultured from different patients, included isolates from three outbreaks and are therefore not all independent samples. Eighteen colonizing isolates (CCOL) were also tested including one MRSA. These were isolated as previously described from epidemiologically unrelated healthy dogs presenting to the teaching hospital for routine health checks in 2008 (Rubin and Chirino-Trejo 2010). Isolates from human infections were collected from the diagnostic lab at Royal University Hospital in Saskatoon, Canada. Fifty one MRSA (HCMR) and 48 methicillin susceptible *S. aureus* (MSSA) (HCMS) were collected from serial, diagnostic samples from unique patients in late 2008 and early 2009. Isolates were identified using standard biochemical tests, and were stored at -80C in skim milk (Winn, Allen et al. 2006).

5.3.2 Antimicrobial susceptibility testing

Using the Sensititre system (Trek Diagnostics, Cleveland, OH), antimicrobial MICs of 31 drugs were determined (Table 5.1). Antimicrobial MICs were classified as susceptible or resistant according to CLSI guidelines (CLSI 2008; CLSI 2008). Mupirocin (MUP) and fusidic acid (FUS) susceptibility was tested by disk diffusion according to BSAC guidelines (Andrews 2008). Mupirocin MICs were determined by E-test (AB biomérieux, Solna, Sweden) for all resistant isolates to differentiate between high and low level resistance. Isolates resistant to erythromycin and susceptible to clindamycin (E^RC^S) were tested for inducible resistance (iCR) according to CLSI guidelines (CLSI 2008). For quality control, *S. aureus* ATCC 29213 and ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 were used.

5.3.3 Screening for Panton Valentine Leukocidin and *mecA*

All isolates were screened by PCR for *mec*A using previously published primers *S. aureus* ATCC 43300 and *S. aureus* ATCC 29213 were used as positive and negative controls respectively (Table 5.2) (de Neeling, van Leeuwen et al. 1998). All isolates were screened for PVL by PCR using previously published primers; *S. aureus* ATCC 49775 and *S. aureus* ATCC 29213 was used as positive and negative controls respectively (Table 5.2) (Lina, Piemont et al. 1999). All reactions were run in duplicate on individual colonies picked from fresh overnight cultures.

Table 5.1. Antimicrobial classes, drugs and abbreviations of compounds for which minimum inhibitory concentrations were determined.

College	Drug	Abbreviation
β-Lactams	Ampicillin	AMP
	Ceftiofur	CEF
	Oxacillin	OXA
	Penicillin	PEN
Macrolides-Lincosamides-Ketolides	Clindamycin	CLI
	Erythromycin	ERY
	Telithromycin	TEL
	Tilmicosin	TIL
	Tulathromycin	TUL
	Tylosin	TYL
Fluoroquinolones	Ciprofloxacin	CIP
	Danofloxacin	DAN
	Enrofloxacin	ENR
	Gemifloxacin	GEM
	Moxifloxacin	MOX
Tetracyclines	Chlortetracycline	CLO
	Oxytetracycline	OXY
	Tetracycline	TET
Aminoglycosides	Gentamycin	GMS
	Neomycin	NEO
	Spectinomycin	SPT
Phenicols	Chlroamphenicol	CFC
	Florfenicol	FFN
Peptide	Daptomycin	DAP
Oxazolidonone	Linezolid	LZD
Nitroimidazole	Nitrofutantoin	NIT
Streptogramin	Quinupristin/Dalfopristin	QDA
Rifamycin	Rifampin	RIF
Sulfonamide	Trimethoprim/Sulfamethoxazole	SXT
Pleuromutilin	Tiamulin	TIA
Glycopeptide	Vancomycin	VAN

Table 5.2 Primer sequences used to amplify *mec*A and PVL

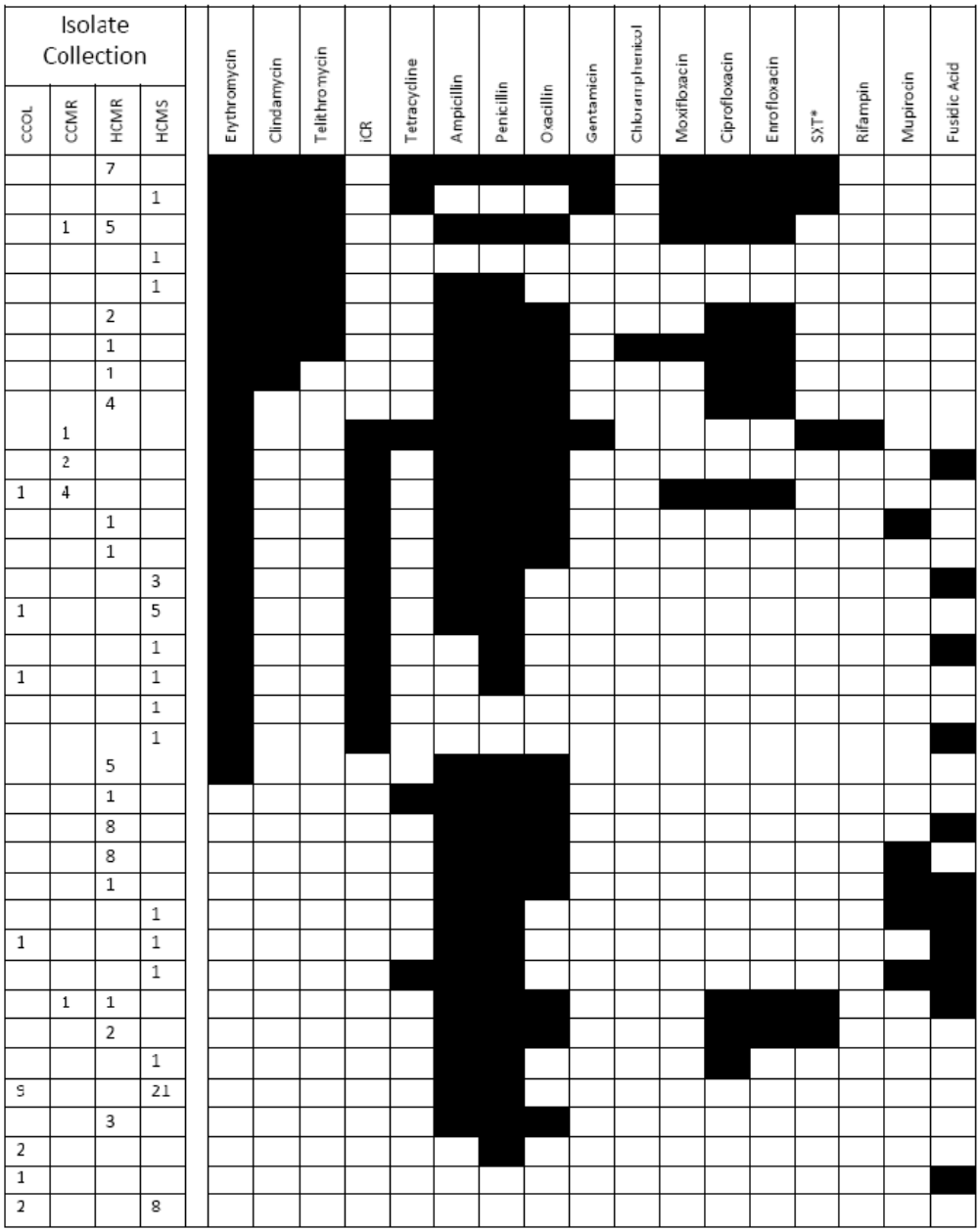
Name	Sequence	Reference
Primers used to amplify *mec*A		(de Neeling, van Leeuwen et al. 1998)
MecA1	5’ GTT GTA GTT GTC GGG TTT GG -3’	
MecAC3	5’ CTT CCA CAT ACC ATC TTC TTT A -3’	
Primers used to amplify PVL		(Lina, Piemont et al. 1999)
luk-PV-1	5’ ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A -3’	
luk-PV-2	5’ GCA TCA AST GTA TTG GAT AGC AAA AGC -3’	

5.4 Results

While resistance to 10 antimicrobial classes including macrolide/lincosamide/ketolides, tetracyclines, β-lactams, aminoglycosides, phenicols, fluoroquinolones, rifamycins, sulfonamides, mupirocin and fusidic acid was found, no isolates resistant to VAN, LZD, QDA, DAP or NIT were identified. CCOL isolates were resistant to 0-8 drugs (0-3 classes) while HCMS were resistant to 0-9 drugs (0-5 classes) (Figure 5.1). Multidrug resistance (resistance to three or more classes) was found in 6% of CCOL and 15% of HCMS. Among CCOL, 11% were pan-susceptible and a further 6% and 11% were only resistant to FUS and PEN respectively. Similarly, 17% of HCMS were pan-susceptible.

Resistance to PEN and AMP were commonly encountered among HCMS (77% and 73%) (Figure 5.2) and CCOL (78% and 67%) (Figure 5.3) respectively. Half of CCOL and 44% of HCMS were only resistant to PEN and AMP. Resistance to ERY and CLI was next most common among HCMS and CCOL, occurring in 31% and 17% of isolates, respectively. The majority of these isolates, 80% and 100% respectively, were iCR. Fluoroquinolone resistance was rare among both HCMS and CCOL, only a single isolate of each was MOX resistant. No CCOL were resistant to GMS, TET or SXT (Figure 5.3), while a single HCMS was resistant to all three of these drugs (Figure 5.2). Neither CFC nor RIF resistance was found among HCMS or CCOL.

Figure 5.1 Antibiograms of canine colonizing *Staphylococcus aureus* (CCOL), canine clinical MRSA (CCMR), human MRSA (HCMR) and human MSSA (HCMS) for drugs with recognized resistance breakpoints.



Drugs with British Society for Antimicrobial Chemotherapy (mupirocin and fusidic acid) or Clinical and Laboratory Standards Institute (all others) resistance breakpoints are included. Resistance is indicated by blacked out cells in that drugs column. Inducible clindamycin resistance (iCR) is included in a column separate from clindamycin. The number of isolates in each isolate collection with a given antibiogram is indicated on the left in each isolate collections column.

* Trimethoprim/sulfamethoxazole

Figure 5.2
Antimicrobial minimum inhibitory concentration (MIC) distribution of human MSSA (n=48)

Drug Class	Drug	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC_{50}	MIC_{90}	% R
β-Lactams	Ampicillin				9	4	1	13	8	5	5	3				1	8	72.9
	Ceftiofur					1	38	9								1	2	0
	Oxacillin					31	15	1	1							≤0.25	0.5	0
	Penicillin			10	1	2	2	5	6	12	3	7				2	≥16	77.1
Macrolides – Lincosamides - Ketolides	Clindamycin					45							3			≤0.25	≤0.25	6.3
	Erythromycin*					15	18				15					0.5	8	31.2
	Telithromycin*					44	1			3						≤0.25	≤0.25	6.2
	Tilmicosin									45					3	≤4	≤4	
	Tulathromycin									21	17	2	2		6	8	≥128	
	Tylosin							14	30		1			3		2	2	
Fluoroquinolones	Ciprofloxacin*							45	1	2						≤1	≤1	4.1
	Danofloxacin				19	22	5		2							0.25	0.5	
	Enrofloxacin*				29	15	3		1	1						≤0.12	0.5	2
	Gemifloxacin	12	28	6			2									0.03	0.06	
	Moxifloxacin					46		1			1					≤0.25	≤0.25	2
Tetracyclines	Chlortetracycline						45	1		1		1				≤0.5	≤0.5	
	Oxytetracycline						42	4				2				≤0.5	1	
	Tetracycline								46			1	1			≤2	≤2	4.2
Aminoglycosides	Gentamicin							47					1			≤1	≤1	2
	Neomycin									43	1	1	1	2		≤4	8	
	Spectinomycin													13	35	≥128	≥128	
Phenicols	Chloramphenicol*										43	5				8	16	0
	Florfenicol								1	46	1					4	4	
Peptide	Daptomycin						48									≤0.5	≤0.5	0
Oxazolidonone	Linezolid							3	44	1						2	2	0
Nitroimidazole	Nitrofurantoin*												48			≤32	≤32	
Streptogramin	QDA†						47	1								≤0.5	≤0.5	0
Rifamycin	Rifampin						48									0.5	0.5	0
Sulfonamide	SXT§						46			2						≤0.5	≤0.5	4.1
Pleuromutilin	Tiamulin						10	36	2							1	1	
Glycopeptide	Vancomycin							45	3							≤1	≤1	0

Cells corresponding to drug concentrations (µg/ml) tested are un-shaded. Isolates inhibited at the lowest drug concentration should be considered to have an MIC of less than or equal to that concentration. Isolates not inhibited at the highest concentration tested should be considered to have an MIC of greater than or equal to the first concentration beyond the tested range. For example, isolates uninhibited by 8µg/ml of ampicillin are presumed to have an MIC of ≥168µg/ml. Cells with double borders correspond to Clinical and Laboratory Standards Institute resistance breakpoints for all drugs except daptomycin and linezolid where susceptible breakpoints are defined. *Resistance breakpoint is the first concentration above the tested range †Quinupristin/dalfopristin §Trimethoprim/sulfamethoxazole

Figure 5.3
Antimicrobial minimum inhibitory concentration (MIC) distribution of canine colonizing *Staphylococcus aureus* (n=18)

Drug Class	Drug	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC_{50}	MIC_{90}	% R
β-Lactams	Ampicillin				4	2		3	2	6		1				1	4	67
	Ceftiofur						1	14	2			1				1	2	6
	Oxacillin					11	6					1				≤0.25	0.5	6
	Penicillin			3	1	1	1	1	1	7	2	1				4	8	78
Macrolides – Lincosamides - Ketolides	Clindamycin					17		1								≤0.25	≤0.25	0
	Erythromycin*					7	8				3					0.5	8	17
	Telithromycin*					17			1							≤0.25	≤0.25	0
	Tilmicosin									17			1			≤4	≤4	
	Tulathromycin									12	5				1	4	8	
	Tylosin							8	9					1		1	2	
Fluoroquinolones	Ciprofloxacin*							17		1						≤1	≤1	6
	Danofloxacin				11	5	1		1							≤0.12	0.5	
	Enrofloxacin*				13	2	1	1		1						≤0.12	1	6
	Gemifloxacin	5	9	3			1									0.03	0.06	
	Moxifloxacin					17					1					≤0.25	≤0.25	6
Tetracyclines	Chlortetracycline						18									≤0.5	≤0.5	
	Oxytetracycline						17	1								≤0.5	≤0.5	
	Tetracycline								18							≤2	≤2	0
Aminoglycosides	Gentamicin							17	1							≤1	≤1	0
	Neomycin									17			1			≤4	≤4	
	Spectinomycin													9	9	64	≥128	
Phenicols	Chloramphenicol*										17	1				≤8	≤8	0
	Florfenicol								1	17						≤4	≤4	
Peptide	Daptomycin						18									≤0.5	≤0.5	0
Oxazolidonone	Linezolid								15	3						2	4	0
Nitroimidazole	Nitrofurantoin*												18			≤32	≤32	0
Streptogramin	QDA†						16	2								≤0.5	1	0
Rifamycin	Rifampin						18									0.5	0.5	0
Sulfonamide	SXT§						18									≤0.5	≤0.5	0
Pleuromutilin	Tiamulin						8	8			2					0.25	8	
Glycopeptide	Vancomycin							18								≤1	≤1	0

Cells corresponding to drug concentrations (µg/ml) tested are un-shaded. Isolates inhibited at the lowest drug concentration should be considered to have an MIC of less than or equal to that concentration. Isolates not inhibited at the highest concentration tested should be considered to have an MIC of greater than or equal to the first concentration beyond the tested range. For example, isolates uninhibited by 8µg/ml of ampicillin are presumed to have an MIC of ≥168µg/ml. Cells with double borders correspond to Clinical and Laboratory Standards Institute resistance breakpoints for all drugs except daptomycin and linezolid where susceptible breakpoints are defined. *Resistance breakpoint is the first concentration above the tested range †Quinupristin/dalfopristin §Trimethoprim/sulfamethoxazole

The CCMR were resistant to 6-9 drugs (3-6 classes) and HCMR were resistant to 3-12 drugs (1-6 classes); 100% and 45% were multidrug resistant, respectively (Figure 5.1). All MRSA (*mec*A positive) were phenotypically resistant to PEN, AMP and OXA while all OXA susceptible isolates were *mec*A negative. Resistance to CLI including iCR was very common (89% of CCMR and 35% of HCMR isolates) (Figure 5.1). Resistance to fluoroquinolones was also common; 56% of CCMR (Figure 5.5) and 26% of HCMR were MOX resistant (Figure 5.4). Resistance to CFC and RIF were found in single HCMR (Figure 5.4) and CCMR (Figure 5.5) isolates, respectively. While TET and SXT resistance was found only in a single CCMR, these phenotypes were common among HCMR, occurring in 16% and 14% of isolates respectively. Resistance to GMS was found in 14% of HCMR and 11% of CCMR.

For drugs without CLSI resistance breakpoints, categorical analyses were not done. Isolates resistant to ERY, CLI and TEL had elevated TYL, TUL and TIL MICs compared to ERY, CLI and TEL susceptible isolates. Likewise, OXY and CLO MIC distributions were similar to TET but those isolates resistant to TET were not inhibited by the highest concentrations of OXY and CLO tested. MICs of NEO varied widely, and inconsistently compared to GMS. Isolates susceptible and resistant to GMS inhibited at the lowest concentration of NEO, or entirely uninhibited by NEO were identified. With the exception of two CCOL and one HCMR, all isolates were inhibited by the lowest three concentrations of TIA tested.

Resistance to MUP was only found among human isolates including 4% of HCMS and 20% of HCMR and all isolates had MICs ≥1024 µg/ml indicating high level resistance (Andrews 2008). Resistance to FUS was found in all collections including 17% of HCMS, 20% of HCMR, 33% of CCMR and 17% of CCOL.

Figure 5.4
Antimicrobial minimum inhibitory concentration (MIC) distribution of human MRSA (n=51)

Drug Class	Drug	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC_{50}	MIC_{90}	% R
β-Lactams	Ampicillin								1	1	4	45				≥16	≥16	100
	Ceftiofur								2	13	20	16				8	≥16	70.6
	Oxacillin										2	49				≥16	≥16	100
	Penicillin								1	1	3	46				≥16	≥16	100
Macrolides – Lincosamides - Ketolides	Clindamycin					34	1						16			≤0.25	≥32	31.4
	Erythromycin*					2	22				27					8	8	52.9
	Telithromycin*					35			1	15						≤0.25	≥4	29.4
	Tilmicosin									35				1	15	≤4	≥128	
	Tulathromycin									6	27				18	8	≥128	
	Tylosin							6	29					15		2	≥64	
Fluoroquinolones	Ciprofloxacin*							28		23						≤1	≥4	45.1
	Danofloxacin				7	21			23							0.25	≥2	
	Enrofloxacin*				10	18				23						0.25	≥4	45.1
	Gemifloxacin	3	25				23									0.03	≥0.5	
	Moxifloxacin					28			10		13					≤0.25	≥8	25.5
Tetracyclines	Chlortetracycline						39		4			8				≤0.5	≥16	
	Oxytetracycline						34	5		4		8				≤0.5	≥16	
	Tetracycline								39	4			8			≤2	≥32	15.7
Aminoglycosides	Gentamicin							44				1	6			≤2	≥32	13.7
	Neomycin									29		1		21		≤4	≥64	
	Spectinomycin													27	24	64	≥128	
Phenicols	Chloramphenicol*								1		30	19	1			8	16	2
	Florfenicol									39	12					4	8	
Peptide	Daptomycin						51									≤0.5	≤0.5	0
Oxazolidonone	Linezolid								47	4						2	2	0
Nitroimidazole	Nitrofurantoin*												51			≤32	≤32	0
Streptogramin	QDA†						43	8								≤0.5	1	0
Rifamycin	Rifampin						51									≤0.5	≤0.5	0
Sulfonamide	SXT§						44				7					≤0.5	≥8	13.7
Pleuromutilin	Tiamulin						16	34			1					1	1	
Glycopeptide	Vancomycin							50	1							≤1	≤1	0

Cells corresponding to drug concentrations (µg/ml) tested are un-shaded. Isolates inhibited at the lowest drug concentration should be considered to have an MIC of less than or equal to that concentration. Isolates not inhibited at the highest concentration tested should be considered to have an MIC of greater than or equal to the first concentration beyond the tested range. For example, isolates uninhibited by 8µg/ml of ampicillin are presumed to have an MIC of ≥168µg/ml. Cells with double borders correspond to Clinical and Laboratory Standards Institute resistance breakpoints for all drugs except daptomycin and linezolid where susceptible breakpoints are defined. *Resistance breakpoint is the first concentration above the tested range †Quinupristin/dalfopristin §Trimethoprim/sulfamethoxazole

Figure 5.5.
Antimicrobial minimum inhibitory concentration (MIC) distribution of clinical canine MRSA (n=9)

Drug Class	Drug	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC_{50}	MIC_{90}	% R
β-Lactams	Ampicillin											9				16	16	100
	Ceftiofur											9				16	16	100
	Oxacillin											9				16	16	100
	Penicillin											9				16	16	100
Macrolides – Lincosamides - Ketolides	Clindamycin					8							1			0.25	32	11.1
	Erythromycin*						1				8					8	8	88.9
	Telithromycin*					8				1						0.25	4	11.1
	Tilmicosin									8					1	4	128	
	Tulathromycin									2	5				2	8	128	
	Tylosin							4	4					1		2	64	
Fluoroquinolones	Ciprofloxacin*							3		6						4	4	66.7
	Danofloxacin				1	2			6							2	2	
	Enrofloxacin*				1	2				6						4	4	66.7
	Gemifloxacin		2	1			6									0.5	0.5	
	Moxifloxacin					3			1		5					8	8	55.6
Tetracyclines	Chlortetracycline						8					1				0.5	16	
	Oxytetracycline						4	4				1				1	16	
	Tetracycline								7	1			1			2	32	11.1
Aminoglycosides	Gentamicin							7	1				1			1	32	11.1
	Neomycin									4			4	1		32	64	
	Spectinomycin													2	7	128	128	
Phenicols	Chloramphenicol*										3	6				16	16	0
	Florfenicol									6	3					4	8	
Peptide	Daptomycin						9									0.5	0.5	0
Oxazolidonone	Linezolid								3	6						4	4	0
Nitroimidazole	Nitrofurantoin*												9			32	32	0
Streptogramin	QDA†						7	2								0.5	1	0
Rifamycin	Rifampin						8			1						0.5	4	11.1
Sulfonamide	SXT§						8				1					0.5	8	11.1
Pleuromutilin	Tiamulin						3	6								1	1	
Glycopeptide	Vancomycin							9								1	1	0

Cells corresponding to drug concentrations (µg/ml) tested are un-shaded. Isolates inhibited at the lowest drug concentration should be considered to have an MIC of less than or equal to that concentration. Isolates not inhibited at the highest concentration tested should be considered to have an MIC of greater than or equal to the first concentration beyond the tested range. For example, isolates uninhibited by 8µg/ml of ampicillin are presumed to have an MIC of ≥168µg/ml. Cells with double borders correspond to Clinical and Laboratory Standards Institute resistance breakpoints for all drugs except daptomycin and linezolid where susceptible breakpoints are defined. *Resistance breakpoint is the first concentration above the tested range †Quinupristin/dalfopristin §Trimethoprim/sulfamethoxazole

While no canine isolates possessed PVL, it was found among 2% of HCMS and 39% of HCMR.

5.5 Discussion

The increasing incidence of MRSA in people is resulting in increased costs, length of hospitalization and mortality (Abramson and Sexton 1999; Laupland, Ross et al. 2008; Simor, Gilbert et al. 2010). Perhaps because canine MRSA infections are most frequently associated with non-life threatening conditions, the increased mortality associated with MRSA infections in human beings were not found in dogs (Faires, Traverse et al. 2010). Interspecies MRSA transmission necessitates integrated surveillance programs to actively monitor the emergence and dissemination of resistance among human and veterinary staphylococci. The close contact between people and dogs is underappreciated as a means of transmission for potential pathogens and requires further study.

By definition, MRSA are resistant to all β-lactam antimicrobials, including the penicillins, cephalosporins and carbapenems (Woodford 2005). Orally bioavailable drugs including doxycycline, clindamycin and chloramphenicol are used to treat resistant staphylococcal infections in dogs, though the efficacy of these agents is also threatened. Failure to express clindamycin resistance *in vitro* presents unique challenges in iCR; without exposure to erythromycin the misclassification of an isolate as clindamycin susceptible is possible. *In vivo* induction of resistance and subsequent treatment failure has been described, highlighting the importance of iCR (Siberry, Tekle et al. 2003). Practitioners should suspect iCR in isolates that, without specialized susceptibility tests, appear to be erythromycin resistant and clindamycin susceptible (CLSI 2008). As testing for iCR is not yet standard in veterinary diagnostic labs, and

susceptibility to both clindamycin and erythromycin are not always reported, it may be prudent to suspect iCR in isolates resistant to erythromycin (Faires, Gard et al. 2009). The high proportion of canine MRSA (78%) which were iCR may reflect a bias collection, these isolates are not independent and were collected from three. In contrast, 17% of canine colonizing isolates were iCR, consistent with previous reports (Faires, Gard et al. 2009). The proportion of human MSSA that were iCR (22.9%) was greater than MRSA (3.9%), similar to previously reported studies (Levin, Suh et al. 2005; Yilmaz, Aydin et al. 2007). The prevalence of iCR described in the present study, particularly among canine isolates and human MSSA, highlights the potential impact of this phenotype in our region.

Since the recognition of MRSA in the 1960's, vancomycin has been the mainstay of anti-MRSA therapy (Song 2008). Recently, MRSA isolates with intermediate susceptibility to vancomycin have emerged, and although rare, vancomycin resistant MRSA have also been reported (Finks, Wells et al. 2009). Canine *S. aureus* isolates with intermediate susceptibility or resistance to vancomycin have not yet been reported. No isolates resistant to vancomycin, linezolid, quinupristin/dalfopristin or daptomycin were found in this study.

Fluoroquinolone resistance was common among both HCMR and CCMR. As fluoroquinolone usage has been identified as a risk factor for MRSA acquisition in people and dogs, it may be prudent to avoid these drugs when other therapeutic options are available (Weber, Gold et al. 2003; Dancer 2008; Faires, Traverse et al. 2010). No isolates simultaneously resistant to tetracycline, chloramphenicol and trimethoprim/sulfamethoxazole, drugs commonly used to treat canine MRSA infections, were found. Gentamicin resistance was uncommon among MSSA; only a single HCMS was resistant while 14% of HCMR and 11% of CCMR were

resistant. The gentamicin resistant canine isolate was also the only rifampin resistant isolate from any source.

Topical antimicrobials are commonly used for treating superficial MRSA infections and nasal decolonization (Enoch, Karas et al. 2009; McConeghy, Mikolich et al. 2009). Previously, 50% of MRSA from Northern Saskatchewan were reported to be mupirocin resistant, higher than elsewhere in Canada (Mulvey, MacDougall et al. 2005; Simor, Stuart et al. 2007). Whether the relatively low prevalence of mupirocin resistance found in this study is due to recent changes in mupirocin usage or geographic variability is unknown. No canine isolates were resistant to mupirocin perhaps because mupirocin is infrequently used in dogs due to its oily preparation reducing the selection pressure for resistance. Fusidic acid resistance was found in both canine and human isolates.

The incidence of CA-MRSA infections has rapidly increased in Canada over the last decade (Simor, Gilbert et al. 2010). Shown to play a role in necrotizing syndromes in people, PVL is associated with CA-MRSA and is also increasingly prevalent (Labandeira-Rey, Couzon et al. 2007). Whether reflecting its true prevalence or the small number of samples is unknown but PVL was not found in any canine isolates. The activity of PVL against canine neutrophils is unknown.

The ecology of *S. aureus* is complex, including bidirectional transmission between people and dogs. The public health risks of canine MRSA and the canine health risks of human MRSA, combined with antimicrobial selection pressure in both species requires further study. The risks associated with using 'top-shelf' drugs like vancomycin for treating canine infections are ill defined, suggesting the need for a thorough risk assessment (Weese 2008). Fortunately, one of the most common sites of canine MRSA infection (the ear), allows the application of

topical medications such as Burrow's solution which, by altering the ear environment is effective against a broad spectrum of pathogens irrespective of antimicrobial resistance (Kashiwamura, Chida et al. 2004; Faires, Traverse et al. 2010).

Susceptibility data for this extensive panel of drugs tested will be invaluable for future studies; few reports detailing *S. aureus* susceptibility to many of these drugs are available. Drugs in their infancy of clinical use, pleuromutilins, or without adequately defined resistance breakpoints such as neomycin were included. By presenting MIC distributions in addition to categorical (susceptible versus resistant) data, this study provides maximally comparable information.

While resistance surveillance is routine in Canada, its scope is limited and may not reflect what is seen in the community or by veterinarians. Collaboration between veterinarians and human health professionals is essential for surveillance of this potential pathogen which has little regard for species barriers.

5.6 Acknowledgements

The authors would like to thank Dr. J. Blondeau for the provision of human isolates and the Veterinary Teaching Hospital for supporting this study. This study was funded by the Companion Animal Health Fund. The University of Saskatchewan Biomedical Research Ethics Board and the University Council on Animal Care approved this study. The authors have no conflicts of interest to declare.

Chapter 6

Prevalence, Sites of Colonization, and Antimicrobial Resistance Among *Staphylococcus pseudintermedius* Isolated from Healthy Dogs in Saskatoon, Canada

J.E. Rubin and M. Chirino-Trejo

Journal of Veterinary Diagnostic Investigation, 2011; Vol 23 (2), 351-354

6.1 Abstract

In dogs, *Staphylococcus pseudintermedius* is a common colonizer and is associated with pyoderma, otitis externa and urinary tract infections. In the current study, nasal, pharyngeal and rectal swabs were taken from 175 healthy dogs and cultured for *S. pseudintermedius.* The organism was found in 153 (87.4%) dogs including individuals exclusively colonized in the nares (n=1), pharynx (n=16) and rectum (n=17). Antimicrobial susceptibility testing revealed that a remarkably susceptible population: 46.4% of isolates, were susceptible to all drugs tested, and resistance to penicillin (39.9%) and tetracycline (23.5%) were most common. No methicillin (oxacillin)-resistant isolates were identified. Although 3.3% of isolates were erythromycin resistant, no inducible clindamycin resistance was found. The data provide a baseline for future resistance surveillance and indicate that multiple body sites including at least the pharynx and rectum, should be included.

6.2 The study

Staphylococcus pseudintermedius, first described as distinct from *Staphylococcus intermedius* in 2005, is a coagulase-positive staphylococcal species frequently associated with pyoderma, otitis externa, urinary tract infections (UTI), and opportunistically infected sites in dogs (Werckenthin, Cardoso et al. 2001; Ball, Rubin et al. 2008; Devriese, Hermans et al. 2009; Weese and van Duijkeren 2010). Colonization with *S. pseudintermedius* is common, and is reported in up to 68% of healthy dogs (Griffeth, Morris et al. 2008). Although the nares, mouth, rectum, groin, and forehead have been sampled in previous studies, the prevalence of single site colonization has not been evaluated (Hanselman, Kruth et al. 2007; Griffeth, Morris et al. 2008). In human beings and dogs, multiple body sites including the nares, pharynx and gastrointestinal

tract, play a role in *Staphylococcus aureus* colonization, although some individuals are single site colonized (Eveillard, de Lassence et al. 2006; Mertz, Frei et al. 2007; Batra, Eziefula et al. 2008; Mody, Kauffman et al. 2008; Rubin and Chirino-Trejo 2010). Knowledge of sites of colonization is essential for conducting surveillance studies; failing to sample relevant sites may result in false negatives and an underestimation of itsprevalence. Conversely, the increased expense and time required for excessive sampling is undesirable.

Antimicrobial resistance, including methicillin resistance (MRSP), is increasing among *S. pseudintermedius* (Weese and van Duijkeren 2010). Although clinical *S. pseudintermedius* isolates from the Saskatoon region have remained largely susceptible, the recent identification of a multidrug-resistant MRSP from a canine UTI suggests that the locally prevalent resistance patterns are changing (Ball, Rubin et al. 2008; Rubin, Ball et al. 2011; Rubin and Gaunt 2011).

The purpose of the current study was to determine the relative prevalence of nasal, pharyngeal and rectal colonization of healthy dogs with *S. pseudintermedius,* and to characterize the antimicrobial susceptibility profiles of these isolates to an extensive panel of drugs.

Between May and November 2008, 175 dogs presenting to the Western College of Veterinary Medicine (Saskatoon, Saskatchewan, Canada) for routine health checks and vaccination were sampled. One dog per household was included in the study. Using individual, commercially prepared culturettes,[a] nasal, pharyngeal and rectal samples were taken. Of the 175 dogs sampled, it was not possible to collect all three specimens from eight fractious animals; therefore, only 167 dogs were completely sampled.

All samples were processed within 5 hr of collection. Swabs were streaked on chromogenic *S. aureus* medium[b] and mannitol salt agar[c] both with and without 4 μg/ml oxacillin[d] and tripticase soy agar with 5% sheep's blood.[c] Swabs were then put into a tube

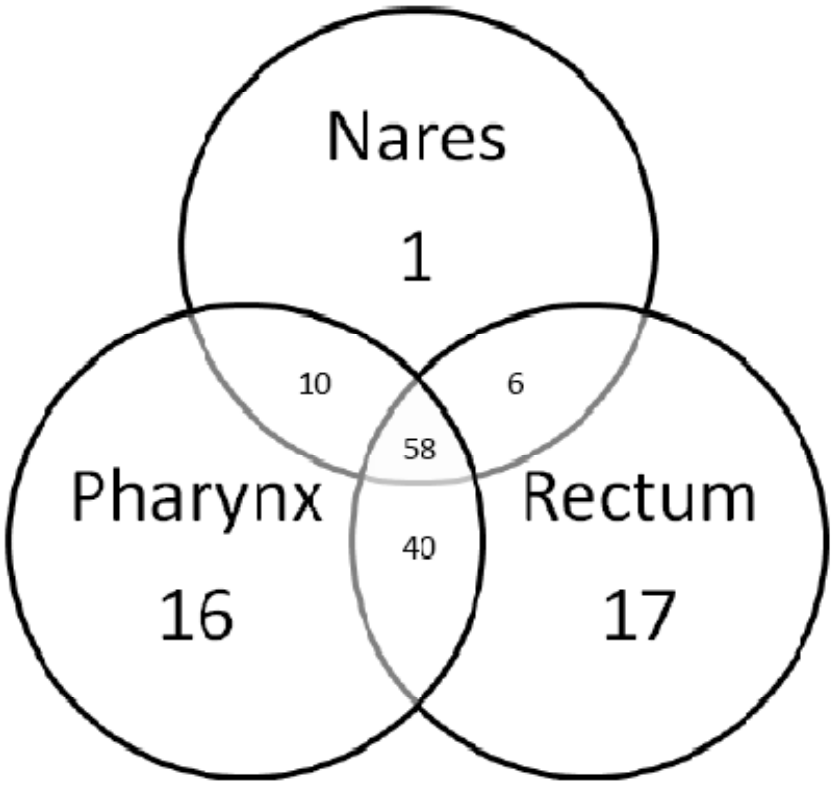
containing 2 ml of enrichment broth with 75 g/l sodium chloride (Weese 2007). After overnight incubation at 35°C, broth cultures were inoculated onto the same five solid media to detect low bacterial numbers. Plates were evaluated for growth at 24, 48 and 96 hr. Identification of *S. pseudintermedius* was based on colony morphology on blood agar; Gram's staining characteristics; the production of catalase, DNase and coagulase; and lack of hyaluronidase or acetoin production (Winn, Allen et al. 2006; Devriese, Hermans et al. 2009).

The susceptibility of 153 *S. pseudintermedius* isolates (one per dog) to 33 antimicrobials was tested using a commercially available system.[e] The minimum inhibitory concentrations (MICs) of the following antimicrobials were determined: β-lactams: ampicillin (AMP), ceftiofur (CEF), penicillin (PEN), and oxacillin (OXA); macrolide/lincosamide/ketolides: clindamycin (CLI), erythromycin (ERY), telithromycin (TEL), tilmicosin (TIL), tulathromycin (TUL), and tylosin (TYL); fluotoquinolones: ciprofloxacin (CIP), danofloxacin (DAN), enrofloxacin (ENR), gemifloxacin (GEM), and moxifloxacin (MOX); tetracyclines: chlortetracycline (CLO), oxytetracycline (OXY), and tetracycline (TET); aminoglycosides: gentamicin (GMS), neomycin (NEO), and spectinomycin (SPT); and other drug classes: chloramphenicol (CHL), daptomycin (DAP), florfenicol (FFC), linezolid (LZD), nitrofurantoin (NIT), quinupristin/dalfopristin (QDA), rifampin (RIF), trimethoprim/sulfamethoxazole (SXT), tiamulin (TIA), and vancomycin (VAN). Clinical and Laboratory Standards Institute (CLSI) testing protocols and interpretive criteria were used for all drugs except oxacillin for which the newly implemented resistance breakpoint (0.5 μg/ml) was used (CLSI 2008; CLSI 2008; Papich 2010). For drugs without CLSI resistance breakpoints, MICs were not categorically analyzed. Mupirocin (MUP) and fusidic acid (FUS) susceptibilities were determined using the disk diffusion method[f] according to BSAC guidelines (Andrews 2008). Isolates resistant to ERY and susceptible to CLI were tested

for inducible clindamycin resistance (iCR) according to CLSI guidelines (CLSI 2008). For quality control *S. aureus* American Type Culture Collection (ATCC) 29213, *S. aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 were used.

Staphylococcus pseudintermedius was identified from 153 of 175 dogs (87.4%) overall and in 148 of 167 (88.6%) of completely sampled dogs, higher than previously reported (Griffeth, Morris et al. 2008). Nasal, pharyngeal and rectal colonization were identified in 75, 124 and 121 dogs respectively (Figure 6.1). A total of 1 (0.7%), 16 (10.5%), and 17 (11.1%) dogs were exclusively nasally, pharyngeally and rectally colonized respectively. Using all three sites as the gold standard, the sensitivity of sampling the nares + rectum was 89.2%, nares + pharynx 88.5% and the pharynx and rectum 99.3%. To maximize the sensitivity of *S. pseudintermedius* detection future studies should include at least the pharynx and rectum.

Figure 6.1.
Numbers of dogs colonized in the nares, pharynx and/or rectum

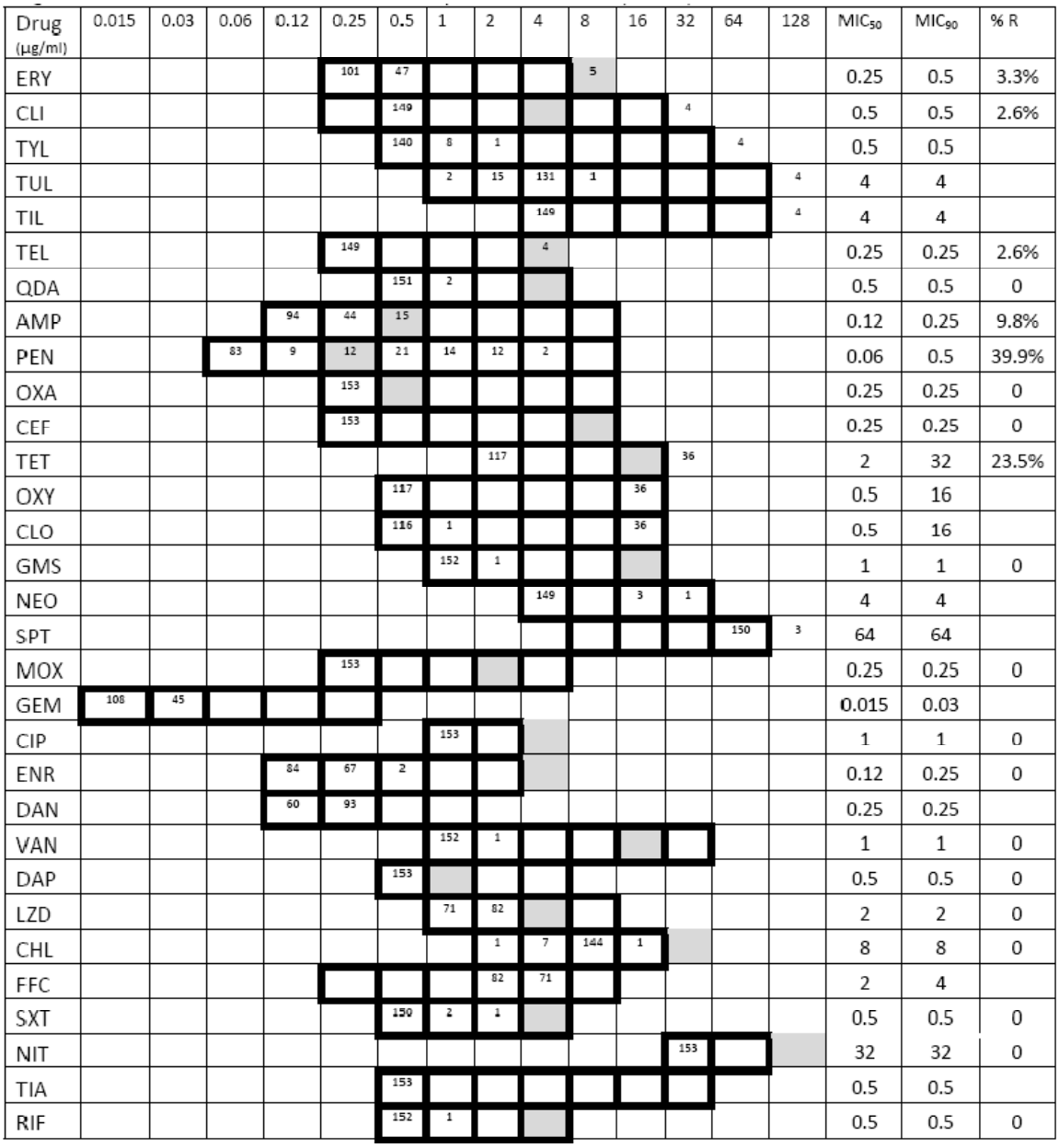


Susceptibility to all drugs was the most common phenotype identified, including 71 isolates (46.4%) (Table 6.1). No resistance to OXA (MRSP), CEF, GMS, MOX, CIP, ENR, CHL, SXT, RIF, NIT, VAN, LZD, QDA, DAP or FUS was found (Figure 6.2). No MRSP were identified, although these isolates were not screened for *mec*A, the gene conferring methicillin resistance. However, susceptibility to oxacillin is reported to be 97% sensitive in detecting methicillin resistance and was considered sufficient for the purposes of this study (Schissler, Hillier et al. 2009). Resistance to PEN and TET was found most commonly, occurring in 39.9% and 23.5% of isolates respectively. However, nearly half of PEN-resistant (30 of 61) and half of TET-resistant (18 of 36) isolates were resistant to only that one drug. Five isolates (3.6%) were macrolide resistant including a single isolate only resistant to ERY. Two of four CLI-resistant isolates were also PEN resistant but remained susceptible to TET. No iCR was found. A single isolate resistant to MUP was identified, which was also resistant to PEN, AMP, and TET, making it the only multidrug-resistant isolate (resistant to three or more antimicrobial classes). High rates of MUP resistance (>50%) have been reported among human MRSA in Saskatchewan, but little is known about the susceptibility of canine staphylococci to this drug (Mulvey, MacDougall et al. 2005). No isolates were simultaneously resistant to β-lactams, tetracyclines, and macrolides. A previous study that included clinical canine *S. pseudintermedius* isolates from Saskatoon had reported lower levels of PEN resistance, but a higher prevalence of TET, ERY, CLI and SXT resistance (Rubin, Ball et al. 2011). The lack of resistance to the fluoroquinolones, GMS, CHL and RIF is consistent with earlier studies from this area(Ball, Rubin et al. 2008; Rubin, Ball et al. 2011).

Table 6.1. Relative frequency of susceptibility profiles

Resistance Phenotype	Number (%) of isolates
Susceptible to all drugs test	71 (46.4%)
PEN	30 (19.6%)
TET	18 (11.8%)
PEN + TET	14 (9.2%)
PEN + AMP	11 (7.2%)
PEN + AMP + TET	3 (2.0%)
PEN + ERY + CLI + TEL	2 (1.3%)
ERY + CLI + TEL	2 (1.3%)
ERY	1 (0.7%)
PEN + AMP + TET + MUP	1 (0.7%)

Figure 6.2 Antimicrobial minimum inhibitory concentration (MIC) distribution of *Staphylococcus pseudintermedius* (n=153)



Drug (µg/ml)	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	MIC_{50}	MIC_{90}	% R
ERY					101	47				5					0.25	0.5	3.3%
CLI						149						4			0.5	0.5	2.6%
TYL						140	8	1					4		0.5	0.5	
TUL							2	15	131	1				4	4	4	
TIL									149					4	4	4	
TEL					149				4						0.25	0.25	2.6%
QDA						151	2								0.5	0.5	0
AMP				94	44	15									0.12	0.25	9.8%
PEN			83	9	12	21	14	12	2						0.06	0.5	39.9%
OXA					153										0.25	0.25	0
CEF					153										0.25	0.25	0
TET								117				36			2	32	23.5%
OXY						117					36				0.5	16	
CLO						116	1				36				0.5	16	
GMS							152	1							1	1	0
NEO									149		3	1			4	4	
SPT													150	3	64	64	
MOX					153										0.25	0.25	0
GEM	108	45													0.015	0.03	
CIP							153								1	1	0
ENR				84	67	2									0.12	0.25	0
DAN				60	93										0.25	0.25	
VAN							152	1							1	1	0
DAP						153									0.5	0.5	0
LZD							71	82							2	2	0
CHL								1	7	144	1				8	8	0
FFC								82	71						2	4	
SXT						150	2	1							0.5	0.5	0
NIT												153			32	32	0
TIA						153									0.5	0.5	
RIF						152	1								0.5	0.5	0

Cells corresponding to concentrations tested are outlined in bold. The number of isolates inhibited at each concentration are noted in each cell; isolates inhibited at the lowest concentration tested should be considered to have an MIC less than or equal to that concentration. Cells corresponding to Clinical and Laboratory Standards Institute resistance breakpoints are shaded; for daptomycin (DAP) and linezolid (LZD), susceptible breakpoints are shaded. Erythromycin (ERY), clindamycin (CLI), tylosin (TYL), tulathromycin (TUL), tilmicosin (TIL), telithromycin (TEL), quinupristin/dalfopristin (QDA), ampicillin (AMP), penicillin (PEN), oxacillin (OXA), ceftiofur (CEF), tetracycline (TET), oxytetracycline (OXY), chlortetracycline (CLO), gentamicin (GMS), neomycin (NEO), spectinomycin (SPT), moxifloxacin (MOX), gemifloxacin (GEM), ciprofloxacin (CIP), enrofloxacin (ENR), danofloxacin (DAN), vancomycin (VAN), DAP, LZD, chloramphenicol (CHL), florfenicol (FFC), trimethoprim/sulfamethoxazole (SXT), nitrofurantoin (NIT), tiamulin (TIA), and rifampin (RIF).

Because the current study is intended to serve as a baseline for *S. pseudintermedius* resistance surveillance, MICs of an extensive panel of drugs were determined for an extensive panel of drugs. Monitoring of MICs allows changes in susceptibility below the resistance breakpoint to be detected compared with categorical analyses, which is relatively insensitive. Nearly uniform susceptibility to RIF, TIA, NIT, LZD, DAP, VAN, CIP, GEM, MOX, GMS, CEF, OXA and QDA was found, with all isolates inhibited by the lowest two concentrations tested, and below the resistance breakpoint, where available. As old drug classes such as the pleuromutilins (retapamulinis now used for treating MRSA in humans) are "re-discovered", historical susceptibility data for related compounds (tiamulin) will be invaluable in detecting the emergence of resistance (Yang and Keam 2008).

The inclusion of *S. pseudintermedius* isolated from healthy dogs may be more indicative of isolates involved in first-time community-associated infections than diagnostic samples, addressing an important gap in the literature. The recognition of highly susceptible *S. pseudintermedius* is important in balancing the publication bias towards resistant organisms. The emphasis on resistance may encourage the empiric use of broad spectrum antimicrobials by both veterinarians and human health professionals. Although empiric therapy is often unavoidable in clinical practice, it should be stressed that culture and susceptibility testing are cornerstones of prudent use.

Continued surveillance of antimicrobial resistance among coagulase positive staphylococci, including *S. pseudintermedius*, is required to monitor the emergence and dissemination of resistance. Relatively little is known about the epidemiology, frequency of zoonotic transmission, and antimicrobial resistance of *S. pseudintermedius* colonizing healthy

dogs. Necessitated by both animal and public health concerns, more research is needed to address these important issues.

6.3 Acknowledgements, Sources and Manufacturers

The authors would like to thank the staff, students and administration of the Western College of Veterinary Medicine teaching hospital for their support in conducting this study, and the Companion Animal Health Fund for funding. The University of Saskatchewan Committee on Animal Care and Supply and the biomedical Research Ethics Board approved this study. The authors have no conflicts of interest to declare.

a. BBL CultureSwab, Becton, Dickinson and Company, Sparks, MD
b. CHROMagar, Paris, France
c. Becton, Dickinson and Company, Sparks, MD
d. Sigma-Aldrich, St. Louis, MO
e. Trek Diagnostic Systems, Cleveland, OH
f. Oxoid Ltd, Basingstock, Hampshire, United Kingdom

Chapter 7

Relatedness of Methicillin Resistant and Susceptible *Staphylococcus aureus* from Saskatoon, Canada and the Netherlands and Human *Staphylococcus aureus* from Saskatoon, Canada

J.E. Rubin, J.A. Wagenaar, M. Chirino-Trejo, E. van Duijkeren and P.H.M. Savelkoul

Manuscript in Preparation

7.1 Introduction

Staphylococcus aureus is an important cause of morbidity and mortality in people, and is one of the most common causes of infection (Moet, Jones et al. 2007). Colonization of healthy people with *S. aureus* is common with approximately 30% of individuals colonized nasally at a given time. In contrast relatively few dogs (10%) are colonized (Kuehnert, Kruszon-Moran et al. 2006; Rubin and Chirino-Trejo 2010).

Antimicrobial resistance has been complicating the treatment of *S. aureus* infections since the recognition of penicillin resistance in the 1940s (Woodford 2005). The incidence of methicillin resistant *Staphylococcus aureus* (MRSA) infections in people has rapidly increased, and in some areas it now predominates in the community over methicillin susceptible *S. aureus* (MSSA) (Stenstrom, Grafstein et al. 2009; Simor, Gilbert et al. 2010; Weese and van Duijkeren 2010). In the last decade, the emergence of canine MRSA has received increasing attention and resistance in canine staphylococci and the implications of these organisms is now an intensively studied area (Leonard and Markey 2008; Weese and van Duijkeren 2010).

The genetic similarity of human and canine *S. aureus*, both MRSA and MSSA, suggests that there is a common bacterial population (Weese and van Duijkeren 2010). Cases of MRSA transmission between people and dogs have been published, documenting the interspecies mobility of this organism (Simoons-Smit, Savelkoul et al. 2000; Manian 2003; van Duijkeren, Wolfhagen et al. 2005). Transmission to immunocompromised people with increased susceptibility to infection is particularly concerning and institutionalized patients in contact with dogs as part of animal assisted therapy program may be at elevated risk (Lefebvre, Reid-Smith et al. 2009). While the human health implications of zoonotic MRSA transmission are concerning, the risks to canine health from human borne MRSA are ill defined and warrant further study.

Despite extensive investigations into the molecular epidemiology of human MRSA, relatively few studies comparing human and canine *S. aureus* have been published (Chambers and Deleo 2009; Grundmann, Aanensen et al. 2010; Weese and van Duijkeren 2010). The purpose of this study was to compare canine and human *S. aureus* isolates using DNA fingerprinting techniques to address the hypothesis that dogs and people share a population of *S. aureus*. Additionally, we aimed to define the common *spa* types found in dogs in both the Netherlands and Saskatoon, Canada, and those prevalent in humans in Saskatoon, Canada.

A collection of 144 *S. aureus* isolates from people and dogs in Saskatoon, Canada collected in 2008 and 2009 (n = 124) as well as canine isolates collected in Utrecht, the Netherlands between 2000 and 2008 (n = 20) were examined. Four different techniques were employed: amplified fragment length polymorphism (AFLP), 16S-23S inter-genic spacer typing (IS-typing), sequencing the hyper variable x-region of the Staphylococcal protein A gene (*spa* typing) and multilocus sequence typing (MLST).

Closely related and indistinguishable canine and human isolates were found, suggesting a common *S. aureus* population and interspecies transmission. Two MRSA ST398, one human and one Dutch canine, and two human MSSA ST398 were identified. Very little is known about the locally prevalent *S. aureus* lineages in Saskatoon, Canada and more research is required so that changes in *S. aureus* epidemiology can be better understood. Furthermore, the complex epidemiology of *S. aureus* necessitates collaboration between veterinarians and human health professionals in future studies.

7.2 Materials and methods

Twenty five canine isolates from the Western College of Veterinary Medicine (WCVM) in Saskatoon, Canada were included. Nine clinical MRSA isolates from three nosocomial outbreaks at the WCVM in 2006 and 2008 and 15 MSSA and a single MRSA cultured in 2008 from clinically healthy, epidemiologically unrelated, colonized dogs (nasal, pharyngeal or rectal colonization) were tested. Fifty one MRSA and 48 MSSA were collected from the diagnostic laboratory at Royal University Hospital in Saskatoon, Canada in 2008. Isolates were collected from serial lab submissions from unique patients and were not selected based on site of infection. Finally, nine MRSA and 11 MSSA isolated from canine infections at a variety of body sites at the Faculty of Veterinary Medicine at Utrecht University from 2000 through 2009 were included.

DNA was isolated from overnight cultures on 5% Sheep's Blood TSB agar (Oxoid, Cambridge, United Kingdom). Bacterial suspensions were made to a density of McFarland 1 in Tris EDTA Buffer pH 8.0 and lysed with 25 µl lysostaphin (20 µg/ml) at 37°C for 30 minutes. Extraction and purification of DNA was done using the DNeasy kit (Qiagen, Germany). This DNA preparation was used as template for AFLP. For IS-typing, the DNA was diluted 1/10,000 prior to PCR to optimize the amplicon signal.

An AFLP technique using specifically designed primers and adaptors was developed from previously published protocol (Savelkoul, Aarts et al. 1999). First, a simultaneous restriction-ligation step including equal parts (5 µl) purified genomic DNA and restriction-ligation reaction mixture (Table 7.1) was carried out for 3 hours at 37°C. Subsequently, ligated restriction fragments were diluted 1/20 and amplified by PCR using a FAM labeled Eco-C primer and an unlabeled Hha-A primer (Table 7.2). Labeled PCR amplicons were then resolved

by capillary gel electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

PCR amplification of the variable length 16S-23S inter-genic spacer was done using published primers and cycle conditions (Table 7.3) (Budding, Vandenbroucke-Grauls et al. 2010). Labeled PCR products were resolved by capillary gel electrophoresis on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

Previously published primers and thermocycler conditions were used for *spa* typing and MLST (Table 7.3) (Enright, Day et al. 2000; Harmsen, Claus et al. 2003; Hallin, Friedrich et al. 2009). Reactions were carried out on single well isolated colonies grown overnight on trypticase soy agar + 5% sheep blood (Becton, Dickinson and Company, Sparks, MD). PCR products were purified using the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Markham, Canada) according to the manufacturer's instructions, and sent to a commercial facility for sequencing (NRC Plant Biotechnology Institute, Saskatoon, Canada).

Table 7.1 Reagents used in the restriction-ligation step of AFLP

Reagent	Concentration	Volume per Sample
T4 ligase buffer	10X	1.0 µl
NaCl	0.5 M	1.0 µl
Bovine serum albumin	1 mg/ml	0.5 µl
Eco-AD adaptor	5 pmol/µl	0.2 µl
Hha-AD adaptor	50 pmol/µl	0.2 µl
T4 ligase	400 U/µl	0.2 µl
EcoR1 restriction enzyme	20 U/µl	0.05 µl
Hha1 restriction enzyme	20 U/µl	0.05 µl
Ultra-pure water	Pure	1.5 µl

5.0 µl of restriction-ligation mixture is combined with 5.0 µl of purified genomic DNA.

Table 7.2 PCR master-mix used in the amplification step of AFLP

Reagent	Concentration	Volume per Sample
SuperTaq Buffer	10X	1.0 µl
dNTP	10 mM	0.2 µl
Eco-C-FAM primer	50 ng/µl	0.4 µl
Hha-A primer	50 ng/µl	1.2 µl
SuperTaq	5 U/µl	0.2 µl
Ultra-pure water	Pure	2.0 µl

5.0 µl of PCR master-mix is combined with 5.0 µl of diluted (1/20) restriction-ligation product

Table 7.3 Primer sequences used for IS-typing and to amplify *spa* and the seven genes amplified in MLST

Name	Sequence	Reference
Primers used for IS-typing		(Budding, Vandenbroucke-Grauls et al. 2010)
FirlSf	5’ CTG GAT CAC CTC CTT TCT AAG -3’	
DUISr1	5’ AGG CAT CCA CCG TGC GCC CT -3’	
Primers used for MLST		(Enright, Day et al. 2000)
*arc*UP	5' TTG ATT CAC CAG CGC GTA TTG TC -3'	
*arc*DN	5' AGG TAT CTG CTT CAA TCA GCG -3'	
*aro*UP	5' ATC GGA AAT CCT ATT TCA CAT TC -3'	
*aro*DN	5' GGT GTT GTA TTA ATA ACG ATA TC -3'	
*glp*UP	5' CTA GGA ACT GCA ATC TTA ATC C -3'	
*glp*DN	5' TGG TAA AAT CGC ATG TCC AAT TC -3'	
*gmk*UP	5' ATC GTT TTA TCG GGA CCA TC -3'	
*gmk*DN	5' TCA TTA ACT ACA ACG TAA TCG TA -3'	
*pta*UP	5' GTT AAA ATC GTA TTA CCT GAA GG -3'	
*pta*DN	5' GAC CCT TTT GTT GAA AAG CTT AA -3'	
*tpi*UP	5' TCG TTC ATT CTG AAC GTC GTG AA -3'	
*tpi*DN	5' TTT GCA CCT TCT AAC AAT TGT AC -3'	
*yqi*UP	5' CAG CAT ACA GGA CAC CTA TTG GC -3'	
*yqi*DN	5' CGT TGA GGA ATC GAT ACT GGA AC -3'	
Primers used for *spa* typing		(Hallin, Friedrich et al. 2009)
*spa*1113f	5’ TAA AGA CGA TCC TTC GGT GAG -3’	
*spa*1514r	5’ CAG CAG TAG TGC CGT TTG CTT -3’	

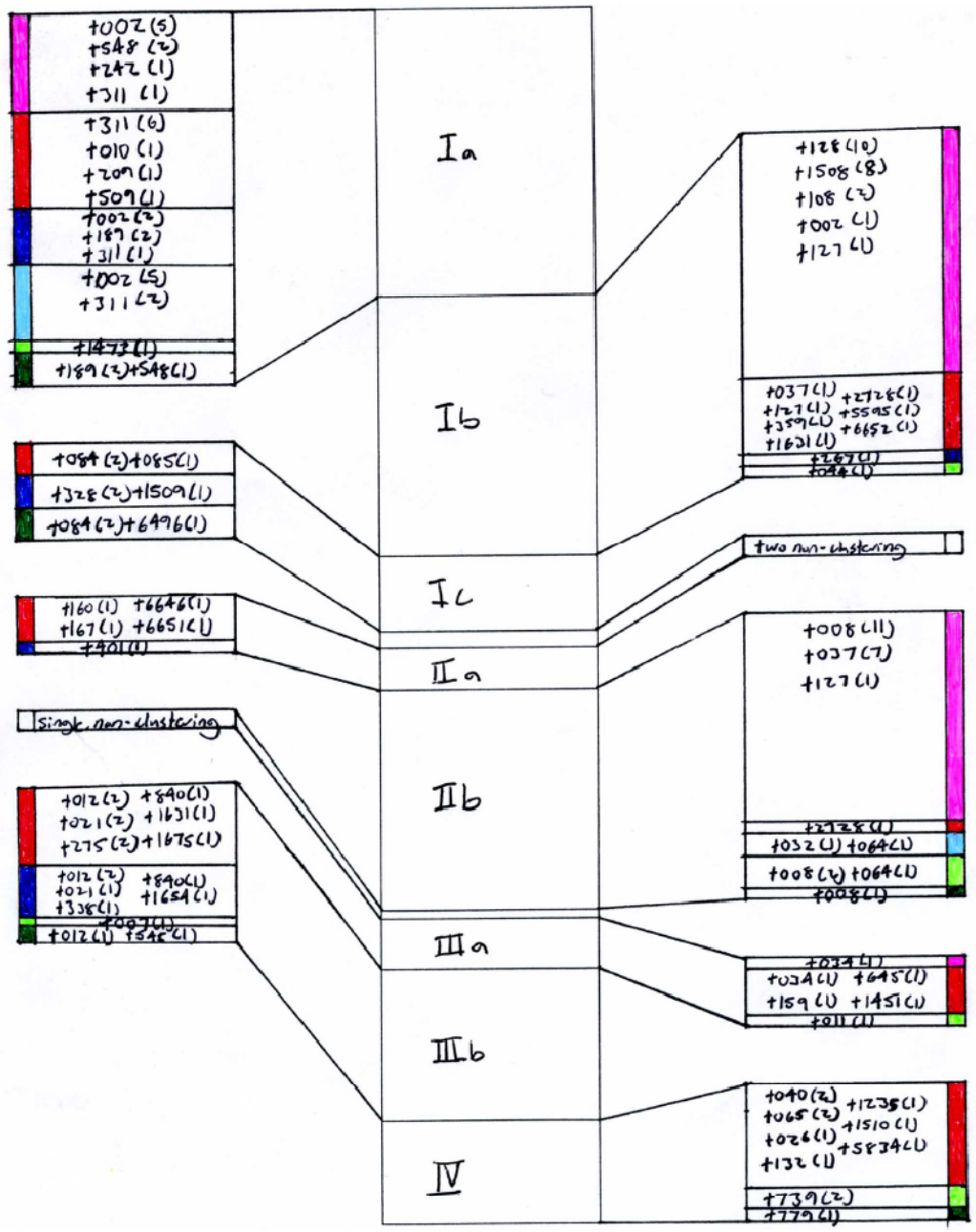
AFLP, IS-typing and *spa* data were analyzed using the BioNumerics platform (Applied Maths, Sint-Martens-Latem, Belgium). For AFLP and IS-typing, digitized gel images from the automated sequencer were analyzed using the Bionumerics software, while computerized sequence chromatograms were uploaded into Bionumerics for spa typing.

AFLP bands were assigned to classes (n = 63) allowing binary profiles to be defined which were then analyzed using the Dice coefficient and the un-weighted pair group method with arithmetic means (UPGMA) as previously described (Melles, Tenover et al. 2008; Melles, Schouls et al. 2009). Clusters were defined as 80% similar and subclusters were defined at the 86% level of similarity. Amplicons from IS-typing PCR were assigned to one of 15 band classes; binary profiles were generated and compared using the Dice coefficient and UPGMA as previously described (Budding, Vandenbroucke-Grauls et al. 2010). Briefly, s*pa* sequences were analyzed using the *spa* plugin for Bionumerics; newly identified types were submitted to the *spa* server for classification (spaserver2.ridom.de). Sequence types (MLST) were defined by concatenating sequences with the MLST *S. aureus* database (www.mlst.net).

7.3 Results

Four AFLP clusters (I-IV) and seven subclusters (Ia,b,c;IIa,b;IIIa,b) were identified. Canine (Dutch and Canadian) and human isolates, both methicillin resistant and susceptible, clustered together including isolates with identical AFLP binary band patterns (Figure 7.1). Human MRSA were concentrated in clusters Ia (9/51), Ib (22/51) and IIB (19/51), while MSSA were distributed throughout all clusters. Clinical canine MRSA from Saskatoon were found in clusters Ia (7/9) and IIb (2/9), while canine MRSA from the Netherlands were found among six of eight clusters. Although all eight subclusters contained both human and canine isolates,

Figure 7.1 AFLP clusters broken down by origin with *spa* types defined



AFLP clusters broken down by origin, each coloured section is sized proportionately to the number of isolates in that group. Pink corresponds to human MRSA, red to human MSSA, dark blue to colonizing *S. aureus* from Saskatoon, light blue to clinical canine MRSA from Saskatoon, light green to canine MRSA from the Netherlands and dark green to canine MSSA from the Netherlands. Three non-clustering isolates, two between clusters Ic and IIa, a human MSSA from Saskatoon (t941) and a canine MSSA from the Netherlands (t6495), and one between clusters IIb and IIIa, a human MSSA from Saskatoon (t216). The *spa* types, and number of isolates with each type, are also included.

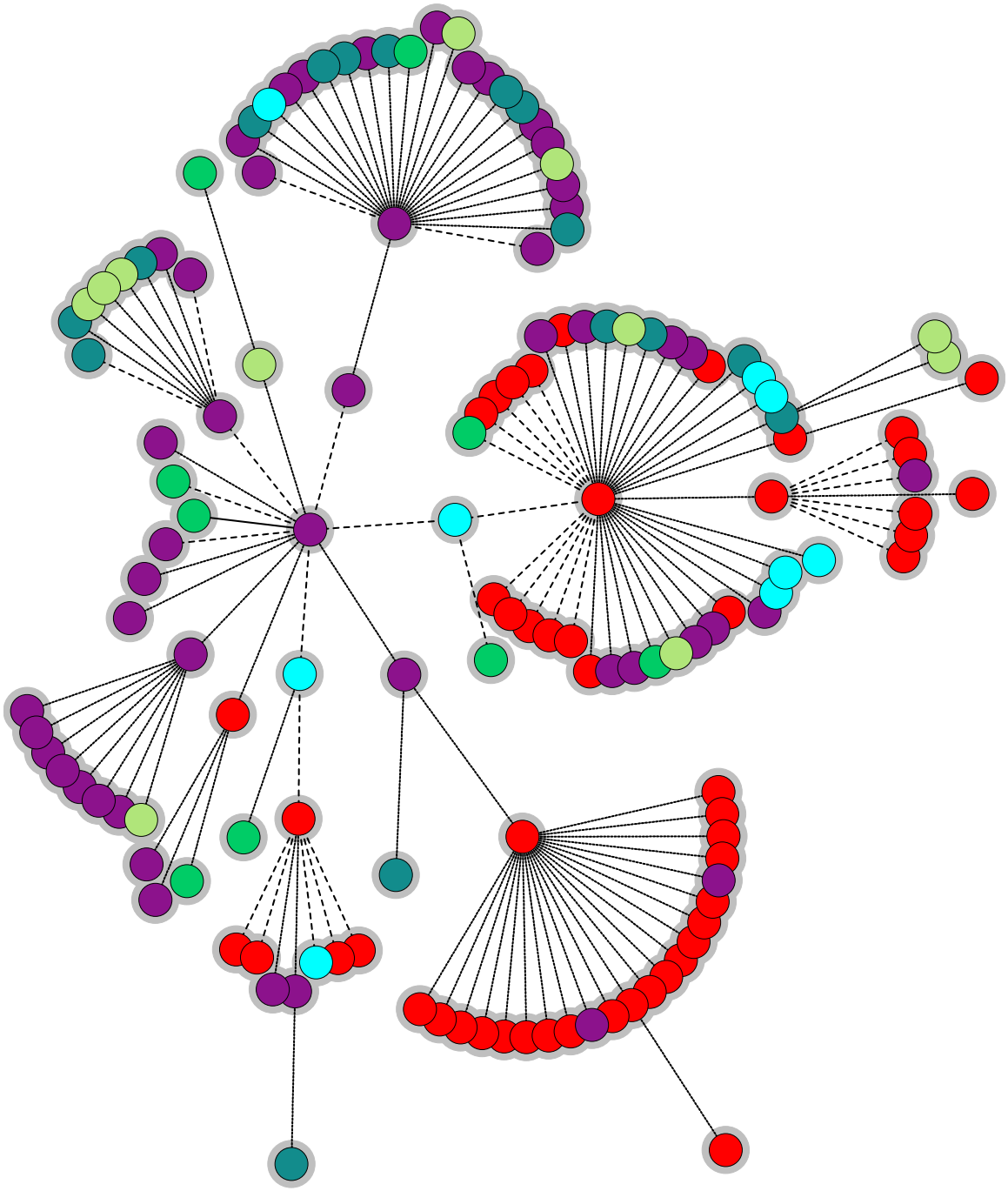
human isolates predominated in clusters Ib and IIb. Cluster Ia was the only group containing isolates from all sources (Figure 7.1).

Forty eight IS-PRO profiles were found containing between one and 21 isolates in each. Canine and human isolates were distributed throughout the minimum spanning tree including many indistinguishable isolates (Figure 7.2). Of the 48 IS-profiles, nine were common to human and canine isolates while 23 were limited to human and 16 were limited to those from dogs.

Fifty seven different *spa* types were identified including five new types: t6495, t6496 among canine MSSA from the Netherlands, and t6646, t6651 and t6652 among human MSSA from Saskatoon (Table 7.4). Human MRSA were limited to 11 *spa* types while HC-MSSA was made up of 32 *spa* types. Seven of nine canine MRSA isolates from Saskatoon were *spa* types common to Human MRSA (t002 n=5 and t311 n=2). Among the 15 *spa* types identified in canine strains from the Netherlands, 10 were unique to the Netherlands, the remainder were found among Canadian isolates.

Good agreement between the techniques was observed. When AFLP clusters were overlaid on IS types (Figure 7.3.) and *spa* types (Figure 7.1.), this agreement was apparent.

Figure 7.2 Minimum spanning tree of IS-types by isolate origin.

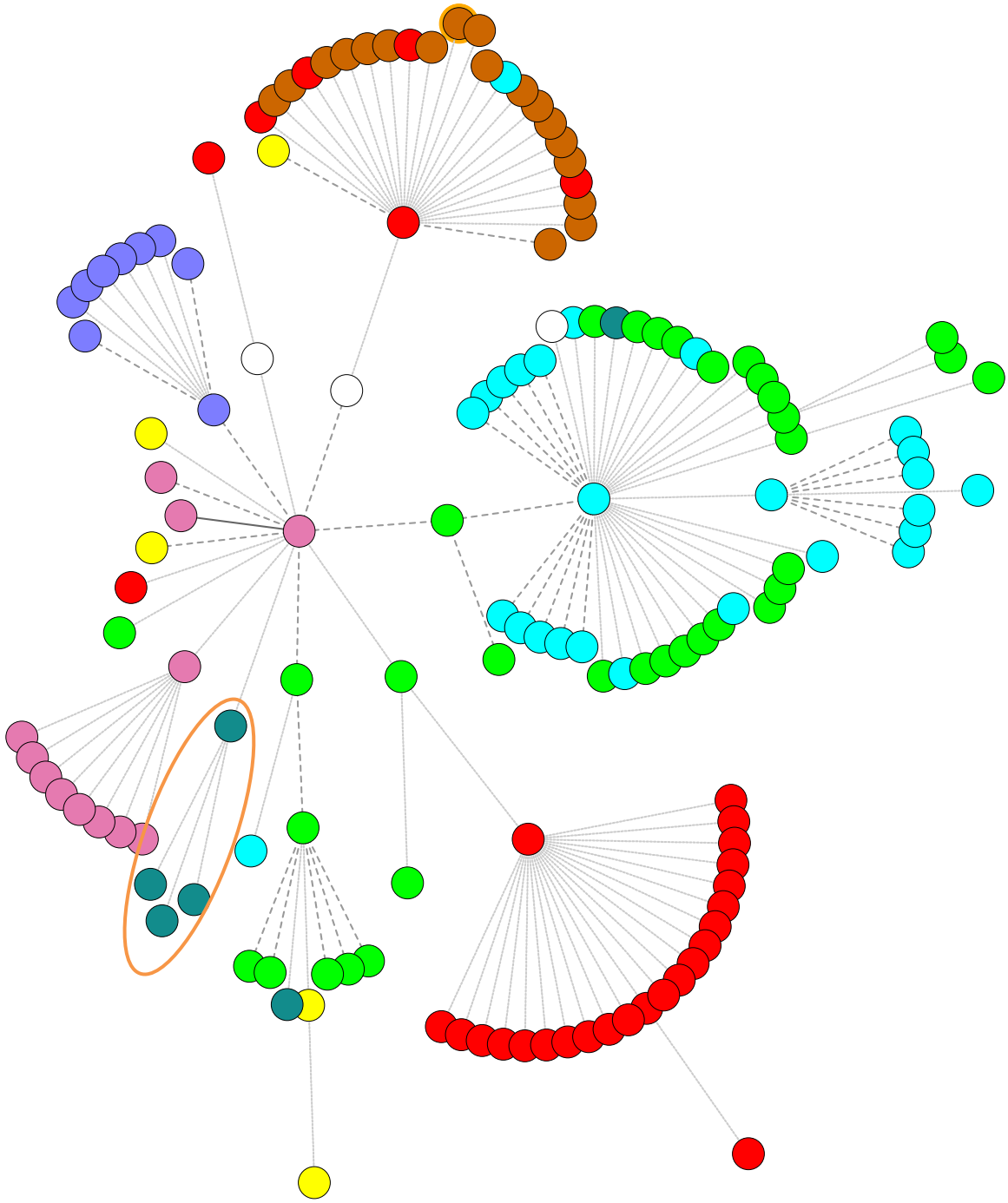


Each node represents a unique isolate. Nodes are coloured by isolate origin: Human MRSA, bright red; human MSSA, purple; Canadian canine colonizing, dark blue; Canadian clinical canine MRSA, bright blue; Dutch canine MRSA, dark green and Dutch canine MSSA, light green.

Table 7.4 Distribution of *spa* types (n) among isolates

Human clinical MRSA	**Human clinical MSSA**	**Canine colonizing isolates**	**Canine clinical MRSA (CAN)**	**Canine MRSA (NL)**	**Canine MSSA (NL)**
t008 (11), t128 (10), t1508 (8), t037 (7), t002 (6), t108 (2), t127 (2), t548 (2), t034 (1), t242 (1), t311 (1)	t311 (6), t012 (2), t040 (2), t065 (2), t084 (2), t275 (2), t2728 (2), t010 (1), t026 (1), t034 (1), t037 (1), t085 (1), t127 (1), t132 (1), t159 (1), t160 (1), t167 (1), t209 (1), t216 (1), t359 (1), t509 (1), t645 (1), t840 (1), t941 (1), t1235 (1), t1451 (1), t1510 (1), t1631 (1), t1675 (1), t5595 (1), t5834 (1), t6646 (1), t6651 (1), t6652 (1)	t002 (2), t012 (2), t189 (2), t012 (1), t228 (1), t267 (1), t311 (1), t328 (1), t338 (1), t401 (1), t840 (1), t1509 (1), t1654 (1)	t002 (5), t311 (2), t032 (1), t064 (1)	t008 (2), t739 (2), t007 (1), t011 (1), t044 (1), t064 (1), t1473 (1)	t084 (2), t189 (2), t008 (1), t012 (1), t548 (1), t582 (1), t779 (1), t6495 (1), t6496 (1)

Figure 7.3. Minimum spanning tree of IS-types by AFLP cluster.



Each node represents a unique isolate. Nodes are coloured by AFLP cluster/sub-cluster: Ia, light green; Ib, red; Ic, purple; IIa, yellow; IIb, light blue; IIIa, dark blue; IIIb, brown and IV, pink. The isolates encircled with an orange oval are the four ST398 identified.

Comparison of IS profiles with an existing database (courtesy of A.E. Budding) revealed close clustering of four isolates with confirmed MRSA ST398 samples. These four isolates all possessed a previously described ST398 specific band class (Budding, Vandenbroucke-Grauls et al. 2010). By AFLP, these isolates were closely related belonging to the IIIa cluster (Figure 7.1). These isolates were all *spa* types commonly associated with ST398; the human MRSA was t034, the human MSSA were t034 and t1451 and the Dutch canine MRSA was t011; all were confirmed with MLST to be ST398. Two isolates with *spa* type t108, frequently associated with ST398 were identified that did not cluster with other ST398 isolates with either AFLP or IS-PRO; following MLST this isolate was identified as ST1 (van Duijkeren, Ikawaty et al. 2008).

7.4 Discussion

In this study three techniques were used, allowing a level of analysis not possible with any one of the techniques individually. The use of IS-typing may prove useful in future investigation for detecting ST398; this technique is much less labor intensive and expensive than sequence based methods, and may be an invaluable screening test. The sequence based typing methods, *spa* and MLST, allow comparison of data with a global database. The ridom *spa* server currently contains over 100,000 entries comprising over 8,400 *spa* types from 80 countries (Harmsen 2010). Through it's MLST mapping function, the *spa* server allows associations between *spa* type and sequence type to be seen (Harmsen 2010). Additionally, each entry in the database includes key epidemiological information including data and country of origin, and susceptibility to methicillin, allowing temporal and geographic comparisons (Harmsen 2010).

The recent emergence of MRSA in dogs has raised awareness of interspecies transmission, and its potential canine and human health implications. In this study, the clustering

of canine and human isolates was not mutually exclusive. While several AFLP clusters (Ib and IIB) were dominated by human isolates, those of canine and human origin were found in all clusters (Figure 7.1). Similarly, eight *spa* types including 55 of 144 isolates were common to human and canine isolates (Table 7.1).

Found among both people and dogs in Saskatoon (total n=24), t002, t311 and t010 are associated with ST5, an internationally prevalent lineage commonly isolated from human infections (Lowder, Guinane et al. 2009; Nulens, Stobberingh et al. 2009). Found among human MRSA (n=2) and Dutch canine MSSA (n=2) in this study, t084 is another internationally prevalent type and is the tenth most frequently reported in the ridom database (Wu, Wang et al. ; Harmsen 2010). Among the Dutch canine isolates, *spa* types t008, t044 and t064 are reported to be common among human MRSA, while t012 and t084 are common among human MSSA from the Netherlands (Nulens, Stobberingh et al. 2009; Grundmann, Aanensen et al. 2010). *Spa* type t008 was most frequently found in Saskatoon, accounting for 11 of 51 (22%) of human MRSA tested. The next two most frequently identified *spa* types in Saskatoon, t128 (n=10) and t1508 (n=8), were rarely reported in the ridom database, 0.07% and <0.01% (eight entries) respectively (Harmsen 2010). While the prevalence of *spa* types across Canada remains ill defined, there appears to be regional specificity in the types circulating. Studies of MRSA epidemiology in Canada are hampered by a lack of published province-specific data (Canada 2008). In Western Canada, there are important differences in locally prevalent strains that are not described in the current national surveillance programs (Wylie and Nowicki 2005; Gilbert, MacDonald et al. 2006; Canada 2008).

Similarities were also found among Canadian and Dutch isolates. Dutch canine isolates were found in seven of eight AFLP clusters, and five of 15 spa types including nine of 20

isolates, were common to the Canadian collection. These findings corroborate previous studies documenting related *S. aureus* lineages in the Netherlands and North America (Melles, Tenover et al. 2008).

A common population of *S. aureus* in people and dogs indicates that bidirectional transmission likely occurs. Highly similar and indistinguishable isolates from people and dogs have been reported, including cases of putative transmission (Simoons-Smit, Savelkoul et al. 2000; Manian 2003; Baptiste, Williams et al. 2005; Loeffler, Boag et al. 2005). A 2003 report described multiple transmission events between a dog and its diabetic owners, highlighting the complexity of *S. aureus* ecology and the potential epidemiological role of companion animals (Manian 2003). Recent investigations into canine colonization with *S. aureus* demonstrated indistinguishable strains in human/dog pairs (Boost, O'Donoghue M et al. 2007; Faires, Tater et al. 2009). While the direction of transmission is mainly human to dog, the public health implications of a canine reservoir of MRSA necessitate further study and hygienic vigilance by those in contact with dogs (Boost, O'Donoghue M et al. 2007; Weese and van Duijkeren 2010).

Since it was first described in 2005 in the Netherlands, MRSA ST398 has become increasingly prevalent, and is now responsible for ~30% of human MRSA infections in the Netherlands (van Rijen, Van Keulen et al. 2008; Bosch, de Neeling et al. 2010). Contact with livestock is the main risk factor for acquiring MRSA ST398 and current epidemiological evidence indicates that human-human transmission, even in the hospital setting, is limited (Voss, Loeffen et al. 2005; van Rijen, Van Keulen et al. 2008; Cuny, Nathaus et al. 2009; Bootsma, Wassenberg et al. 2010). In Canada, MRSA ST398 has been found in swine in Ontario and a horse from Alberta (Khanna, Friendship et al. 2008; Tokateloff, Manning et al. 2009). Since 2007, six human cases have also been reported in Canada, five from Saskatchewan and one from

Ontario but unfortunately it is not known if these individuals had contact with livestock (Golding, Bryden et al. 2010).

In this study two human MSSA ST398 (*spa* types t034 and t1451) were found. One of these was the same spa type, t034, as the MRSA previously reported human cases from Saskatchewan and the horse from the neighboring province Alberta (Tokateloff, Manning et al. 2009; Golding, Bryden et al. 2010). There is very little information on MRSA ST398 in Canada, and more studies are urgently needed (Tokateloff, Manning et al. 2009).

A single MRSA ST398, t011, from the Dutch canine collection was identified and while it was possible to determine that the dog resides in Belgium, attempts to contact the owners to ask about livestock contact were unsuccessful. Dogs in Germany and Canada with MRSA ST398 were recently reported but whether these are chance transmission events or represent the emergence of this lineage in dogs is unknown (Nienhoff, Kadlec et al. 2009; Floras, Lawn et al. 2010). Should dogs prove to be a competent host species, the Dutch search and destroy policy could be further challenged as the at-risk population is expanded beyond those with livestock contact.

The magnitude of the role of the canine/human link in the transmission of MRSA is ill defined, but may be a critical control point for interventions. The potential negative health implications for dogs, the occupational hazards of veterinary work and public health concerns necessitate vigilance in monitoring the status of MRSA in dogs (Hanselman, Kruth et al. 2006; Weese and van Duijkeren 2010). As *S. aureus* has little regard for species barriers, collaboration between veterinarians and human health care professionals is essential to define the ecology of this potential pathogen.

7.5 Declarations and Acknowledgments

This study was approved by the University of Saskatchewan Committee on Animal Care and Supply and the Biomedical Research Ethics Board. The authors have no conflicts of interest to declare. The authors would like to thank Dr. J. Blondeau in the Division of Clinical Microbiology at Royal University Hospital in Saskatoon, Canada for the provision of human isolates. At VU medische centrum in Amsterdam, The Netherlands the authors would also like to thank Martine Rijnsburger, Madelone van der Bijl and Bianca Blok-Schuur for their technical assistance and Dr. Budding for access to his IS-type database.

Chapter 8 - General Discussion and Conclusions

8.1 General discussion

The overall aim of these studies was to conduct an investigation into the ecology of coagulase positive staphylooccci in dogs. The description of all aspects of such an endeavour, including an investigation of ill-defined factors (multiple sites of colonization and the use of canine plasma in the tube coagulase test), provides a template for future research. The unparalleled importance of *S. aureus* and *S. pseudintermedius* for canine and human health, combined with the emerging challenges associated with antimicrobial resistance, necessitate vigilence in monitoring these potential pathogens. Our ability to monitor the emergence of resistance requires examination of all relevant reservoirs and sources including clinical and colonizing isolates from interacting host species.

In the clinical microbiology laboratory, rapid and accurate methods of identification are desirable to provide the highest level of patient care as quickly as possible. While the use of selective and differential media aids in the presumptive identification of *S. aureus*, colony morphology even on these media is insufficient for species specific identification (Rubin 2010). The tube coagulase test is perhaps the most important biochemical test for describing staphylococcal species, and is most often done using rabbit plasma (Winn, Allen et al. 2006). In veterinary clinical microbiology, previous studies have shown that rabbit plasma may not be optimal for identifying *S. aureus* isolated from all host species (Adesiyun and Shehu 1985). In the present study, evidence supporting our hypothesis that *S. aureus* of canine origin will more rapidly coagulate canine plasma was presented. Interestingly, the time to clot formation (positive test) was faster for canine plasma than rabbit plasma for human and bovine isolates as well, suggesting that canine plasma may be instrinsically superior to rabbit plasma. However,

the limited availability of canine plasma through animal blood banks makes its use impractical for diagnostic use. Fortunately, all isolates were coagulase positive using rabbit plasma indicating that , despite a significantly longer time until clot formation, this medium is good enough. These findings indicate that the relationship between isolate origin and ability to coagulate various plasmas requires further investigation including large numbers of prospectively collected isolates to remove the bais of previous biochemical identification.

Despite increasing interest in the ecology of coagulase positive staphylococci in dogs, particularly methicillin resistant organisms, studies into canine colonization are confounded by inadequate knowledge of the sites of colonization. Recently, multiple sites of *S. aureus* colonization in human beings, including people who are single site colonized were recognized (Batra, Eziefula et al. 2008). Failing to sample relevant sites results in false negatives and underestimates prevalence, potentially leading to unfounded decisions when carriers are not identified in the hospital setting. In the current study the nares, pharynx and rectum were sampled, and all were found to play a role in both *S. aureus* and *S. pseudintermedius* colonization. Single site nasal, pharyngeal and rectal colonization with *S. aureus* and *S. pseudintermedius* was also identified. For *S. aureus*, the small number of colonized dogs identified (n=17) makes evaluation of the relative importance of each site difficult. Conversely, the sensitivity of pharyngeal + rectal samples for *S. pseudintermedius* was 99.3% compared to sampling all three sites, indicating that future studies of *S. pseudinteremdius* in dogs, may find adequate diagnostic sensitivity with only pharyngeal and rectal samples.

Recent studies in Canada have shown that the incidence of MRSA has increased rapidly in the last decade (Kim, Ferrato et al. 2010; Simor, Gilbert et al. 2010). In all, nine canine MRSA cases have been identified at the Western College of Veterinary Medicine since 2006

when it was first recognized. Methicillin resistance, resulting in pan-β-lactam resistance, renders many of the commonly used, safest and most effective antimicrobials useless. Vancomycin has long been an important component of anti-MRSA therapy for resistant infections in human beings (Woodford and Livermore 2009). This drug along with other 'top-shelf' drugs are infrequently used in dogs and should be reserved for serious infections in human beings (Weese 2008; Woodford and Livermore 2009).

In this present study a collection of 126 *S. aureus* isolates including clinical canine MRSA, canine colonizing *S. aureus* and human clinical MRSA and MSSA. No resistance to vancomycin, linezolid, daptomycin or quinupristin/dalfopristin was found in any *S. aureus*. Two canine and eight human isolates were susceptible to all drugs tested including penicillin. The single colonizing MRSA was also fluoroquinolone resistant and inducibly clindamycin resistant. As canine MRSA is likely acquired from human beings, this it is not surprising given that the macrolide + fluoroquinolone resistance phenotype is common among the human MRSA tested. A number of multidrug resistant *S. aureus* were found, including a canine MRSA resistant to macrolides, β-lactams, tetracyclines, aminoglycosides, sulfonamides and rifampin. Among Canadian MRSA isolates, gentamicin resistance has been reported among 14.5% of HA-MRSA and 1.3% of CA-MRSA while trimethoprim/sulfamethoxazole resistance has been found in 13.5% of HA-MRSA and no CA-MRSA (Zhanel, Adam et al. 2011). The rifampin resistant canine MRSA was the only rifampin resistant isolate identified in this study. While rifampin resistance has been infrequently reported, a recent study from Barcelona, Spain suggests that rifampin resistant MRSA ST228 is emerging in human beings (Mick, Dominguez et al. 2010). Rifampin resistant ST228 was hypothesized to have emerged from MRSA ST247 (Mick, Dominguez et al. 2010). The rifampin resistant isolate described here was *spa* type t064 which

differs by only a single repeat from t008 and t051, *spa* types associated with ST247 (Mick, Dominguez et al. 2010; SpaServer 2011). Whether this isolate represents the emergence of a previously recognized rifampin resistant lineage, or a chance event is unknown. Seven human isolates resistant to macrolides, tetracyclines, β-lactams, aminoglycosides, fluoroquinolones and sulfonamides were also identified. Fortunately, no isolates resistant to all commonly used non-β-lactam drugs: clindamycin, tetracycline, trimethoprim/sulfamethoxazole and chloramphenicol in companion animal medicine were found.

Mupirocin resistance was previously reported to be very common in human MRSA from northern Saskatchewan (50%) (Mulvey, MacDougall et al. 2005) but in this collection only 20% of MRSA and 4% of MSSA isolated from human infections were mupirocin resistant. Whether this difference reflects changes in mupirocin usage or geographic variation between northern areas and the Saskatoon Health Region is unknown. Conversely, no mupirocin resistance was found in any canine isolates possibly reflecting the small canine *S. aureus* collection, or lack of mupirocin use in dogs.

The role of PVL in the pathogenesis of human *S. aureus* infections remains controversial. Although recent evidence suggests that the importance of PVL in pathogenesis has been overstated, its association with CA-MRSA lineages makes screening for PVL a useful epidemiological tool (Boyle-Vavra and Daum 2007; Otto 2011). Consistent with a high and increasing prevalence of CA-MRSA, 39% of human MRSA isolates were PVL positive (Kim, Ferrato et al. 2010; Simor, Gilbert et al. 2010). The activity of PVL on neutrophils varies by species and the susceptibility of canine neutrophils is unknown (Loffler, Hussain et al. 2010). The lack of PVL among this isolate collection suggests that PVL may not play a role in canine disease.

Compared to *S. aureus,* relatively little is known about the antimicrobial susceptibility of *S. pseudintermedius,* particularly isolates collected from healthy colonized dogs. At the Western College of Veterinary Medicine, clinical *S. pseudintermedius* isolates have historically remained overwhelmingly susceptible (Ball, Rubin et al. 2008; Rubin, Ball et al. 2011). One study recently reported only 7% penicillin resistance among clinical *S. pseudintermedius* isolates from 1986 through 2000 (Rubin, Ball et al. 2011). The rapid emergence of MRSP in dogs has, like MRSA in people, challenges the treatment of once simple infections (Ruscher, Lubke-Becker et al. 2010). At the WCVM the discovery of a multidrug resistant MRSP isolate (resistant to macrolides, aminoglycosides, fluoroquinolones, rifampin, trimethoprim/sulfamethoxazole and chloramphenicol) suggests that resistance has recently emerged in this region as well (Rubin and Gaunt 2011). Interestingly, the *S. pseudintermedius* isolated from healthy dogs in the present study were remarkably susceptible; the most common phenotype found in 71 (46.4%) of isolates tested was pan-susceptibility. Although one dog was found to carry MRSA, no MRSP carriers were identified in this study. Only one multidrug resistant *S. pseudintermedius* was identified, resistant to penicillin, ampicillin, tetracycline and mupirocin. Sampling for the present study was done between May and November 2008, before the aforementioned MRSP was identified suggesting that the emergence of resistance in *S. pseudinteremdius* in this region may be a very recent phenomenon. The author is aware of MRSP isolated from dogs in British Columbia, Alberta and Saskatchewan suggesting that this organism is widespread, although perhaps not yet common, in western Canada. Vigilance is needed in monitoring the emergence of antimicrobial resistance in this species particularly because *S. pseudintermedius* infections are often treated empirically in practice.

The recognition of MRSA in companion animals led many to speculate on the origin of these organisms. Currently, the similarity of canine and human *S. aureus* isolated in the same region suggests that transmission is occurring, primarily from people into dogs (Weese and van Duijkeren 2010). In this study, indistinguishable canine and human isolates from Saskatoon were identified using a number of molecular techniques. Canine isolates from the Netherlands, indistinguishable from Canadian isolates were also identified. These data suggest that not only is human-dog transmission occurring, but that internationally prevalent lineages are present in Saskatoon, and the Netherlands. The hypothesis of interspecies transmission is supported by this data, although it must be emphasized that this study was not designed to address the direction of transmission.

A number of human isolates belonging to the ST398 lineage (two MSSA and one MRSA) were identified. This sequence type is strongly associated with pigs and although no data about livestock contact was available, this finding suggests that interspecies transmission of *S. aureus* in Saskatchewan is not limited to humans and dogs (Voss, Loeffen et al. 2005; Bootsma, Wassenberg et al. 2010). While MRSA ST398 has been reported from humans, horses, pigs and dogs in Canada, relatively little is known about its epidemiology and more research is sorely needed (Khanna, Friendship et al. 2008; Tokateloff, Manning et al. 2009; Floras, Lawn et al. 2010; Golding, Bryden et al. 2010). One canine MRSA ST398 from the Netherlands was also identified but again, no data regarding livestock contact was available. At this point, it is unknown if finding ST398 staphylococci in species other than pigs reflects a chance event, or a trend of broadening host range.

8.2 General Conclusions

Canine plasma yields positive results significantly faster than rabbit plasma when testing *S. aureus* using the tube coagulase test.

In dogs, the nares, pharynx and rectum are all sites of colonization for *S. aureus* and *S. pseudintermedius*. Single site colonization of all three sites also occurs for both *S. aureus* and *S. pseudintermedius.*

The *S. pseudintermedius* tested in this study were remarkably susceptible and pan-susceptibility was the most common phenotype identified. These results suggest that empiric ampicillin (amoxicillin), clindamycin or tetracycline therapy is likely to be effective; however, the emergence of multi-drug resistance necessitates culture and susceptiblity testing. Follow up studies including isolates collected from colonized dogs, as well as clinical laboratory submissions should be done to monitor antimicrobial resistance in the Saskatoon region.

The antimicrobial susceptibility profiles of *S. aureus* were highly variable. Although no isolates resistant to all of the commonly used drugs were identified, the unpredictability of resistance in any given isolate makes the selection of rational empiric therapy impossible. To ensure prudent antimicrobial therapy, the necessity of culture and susceptibility testing must be stressed to both veterinarians and physicians. Follow up studies including canine and human *S. aureus* isolates should be done to monitor antimicrobial resistance in the Saskatoon region.

Using DNA fingerprinting techniques (AFLP, *spa* typing and IS-typing) it was clear that canine and human *S. aureus* do not belong to mutually exclusive populations. The hypothesis of a shared population is supported by these data.

Staphylococcus aureus has little regard for species barriers. Collaboration between veterinarians and human health professionals is essential to further our understanding of the ecology of this potential pathogen.

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