METHICILLIN-RESISTANT *STAPHYLOCOCCUS PSEUDINTERMEDIUS* IN ATLANTIC CANADA: EPIDEMIOLOGY AND CULTURE METHODS

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Abstract

The main aim of this thesis was to obtain information on the distribution and prevalence of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) in Atlantic Canada at both a regional Veterinary Teaching Hospital (VTH) and a regional diagnostic laboratory, as well as exploring culture methods for the detection of this pathogen.

The strain type distribution of MRSP using the direct repeat unit (*dru*) typing approach in isolates collected at a regional diagnostic laboratory in Atlantic Canada is described in Chapter 2. Ninety-four isolates were successfully typed and demonstrated to belong to 18 different *dru* types. The majority of isolates belonged to dt11a (30.9%), dt10h (24.5%), dt9a (18.1%), and dt11af (10.6%). The remaining 15.9% isolates were distributed between 13 different *dru* types, nine of which have not been previously identified: dt5k, dt6t, dt8ag, dt9ba, dt9bd, dt10bz, dt10cc, dt10cj, and dt11ca. The predominant *dru* types identified in this study were similar to those found in Ontario, Canada; however, cluster 9a appears to be less common in Atlantic Canada. A significant difference in the distribution of clusters between provinces was detected (p = 0.01). Notably, differences in the *dru* types were observed between Newfoundland and the Maritime provinces (New Brunswick, Nova Scotia, and Prince Edward Island). Cluster 10h was not present in the isolates from Newfoundland, while it was the predominant cluster in samples from PEI. Cluster 11a was the predominant cluster in both New Brunswick and Nova Scotia. Resistance to ≥2 antimicrobials (in addition to β-lactams) was observed in 71.4% of the isolates, with isolates belonging to cluster 9a being significantly more resistant to all antimicrobials tested except fusidic acid,

chloramphenicol, and doxycycline. This study confirms that *dru* type distributions can vary significantly across the same country, and previously unreported *dru* types were identified in Atlantic Canada.

Chapter 3 describes the presence of MRSP in a regional VTH environment after cleaning and disinfection and its potential to cause hospital-acquired infections. MRSP was isolated from 16/137 (12% [95% CI 6.8%–18.3%]) of environmental samples after the first cleaning and disinfection cycle, and 1/14 (7% [95% CI 0.2%–36.0%]) of environmental samples after the second cleaning and disinfection cycle. Culture results and data retrieved from medical records implicated a hospital-acquired MRSP infection involving two canine patients. Pulsed-field gel electrophoresis (PFGE) revealed that the MRSP isolates from these patients were clonal, further supporting a hospital-acquired infection. Since the same hospital personnel handled the MRSP positive patients in this case, transmission between patients was most likely via hospital personnel. PFGE of strains cultured from the environment and patient revealed that environmental strains were clonal to those from the patient associated with that area; however, non-clonal strains were also recovered from the same environmental area. The presence of MRSP in the hospital environment after cleaning and disinfection and a suspected hospital-acquired infection highlights the importance of a robust infection control program in these facilities.

In Chapter 4, the prevalence of MRSP in clinical samples collected from dogs deemed high-risk for MRSP was estimated. This chapter also compared four culture

methods for MRSP: traditional culture, mannitol salt agar with 2µg/mL oxacillin (MSAox), mannitol salt enrichment broth (EB) and MSAox, and EB and traditional culture. A total of 741 samples were analyzed from 556 individual dogs between February 2013 and April 2014. The prevalence of MRSP in samples detected by any method was estimated at 13.4% (95% CI 11.1%–16.0%). When prevalences were compared according to culture methods, EB and MSAox had the highest prevalence (11.2% [9.1%–13.7%]), followed by EB and traditional (10.8% [8.8%–13.2%]), then traditional (10.1% [8.1%–12.5%]), with MSAox having the lowest prevalence (8.9% [7.1%–11.2%]). MRSP prevalence using the traditional culture did not differ significantly from any of the three selective culture methods. Culture with MSAox detected significantly fewer MRSP than either of the enrichment broth methods. The addition of enrichment broth to current methodology is recommended, particularly in patients with a previous history of MRSP positive specimens. These results highlight how differences in culture methodology can influence prevalence estimates; therefore, caution should be exercised when comparing studies investigating prevalence. The MSAox had a low sensitivity for the detection of MRSP at 24 and 48 hours, and is not recommended as a rapid culture-based screening tool. The estimated MRSP prevalence in this study is similar to findings of previous studies in Canada and other parts of the world.

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List of Abbreviations

AHP	Accelerated hydrogen peroxide
AMR	Antimicrobial resistance
AVC	Atlantic Veterinary College
BA	Blood agar (Columbia agar with 5% sheep blood)
bp	Base pair
BPW	Buffered peptone water
CI	Confidence interval
CLSI	Clinical Laboratory Standards Institute
CoNS	Coagulase-negative staphylococci
CoPS	Coagulase-positive staphylococci
DNA	Deoxyribonucleic acid
dru	Direct repeat unit
EB	Enrichment broth
ICU	Intensive care unit
MDR	Multi-drug resistance
MLST	Multi-locus sequence type
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MSAox	Mannitol salt agar with 2 µg/mL oxacillin
MSSP	Methicillin-susceptible *Staphylococcus pseudintermedius*
MST	Minimum spanning tree
NB	New Brunswick
NS	Nova Scotia
PBP2'	Penicillin-binding protein 2'
PCR	Polymerase chain reaction
PEA	Phenylethyl alcohol agar with 5% sheep blood
PEI	Prince Edward Island
PFGE	Pulsed-field gel electrophoresis
SIG	*Staphylococcus intermedius* group
spa	Staphylococcal protein A
SSI	Surgical site infection
ST	Sequence type
VTH	Veterinary Teaching Hospital

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1.0 INTRODUCTION

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has emerged as a major opportunistic pathogen in dogs, and the leading cause of skin disease, otitis, surgical site infections, and wound infections (Perreten et al., 2010; Weese and van Duijkeren, 2010). Methicillin-resistance is mediated by the *mecA* gene, which codes for the expression of a modified penicillin-binding protein, PBP2', and resistance to most β-lactam antimicrobials (Weese and Van Duijkeren, 2010). In addition to the β-lactams, resistance to other antimicrobials has been reported in MRSP isolates, leaving few therapeutic options available (Perreten et al., 2010; van Duijkeren et al., 2011a; Weese and van Duijkeren, 2010). MRSP has been implicated in hospital-acquired infections within veterinary hospitals, and the potential of hospital-wide outbreaks should not be underestimated (Boerlin et al., 2001; Perreten et al., 2010). The accurate and rapid detection and identification of MRSP is essential for delivering effective antimicrobial therapy and the appropriate implementation of infection control measures (Kali et al., 2014).

1.1 Genus *Staphylococcus*

Members of the genus *Staphylococcus* are facultative aerobic gram-positive cocci that appear in characteristic grape-like clusters when stained (Hermans et al., 2010; Songer and Post, 2004). *Staphylococcous* is a diverse genus that evolved along with mammalian host species. Staphylococci are therefore primary constituents of their host commensal microflora and are commonly isolated from the skin and mucosae

(Biberstein et al., 1984; Hermans et al., 2010; Songer and Post, 2004). *Staphylococcus pseudintermedius* is the *Staphylococcus* species most commonly associated with dogs.

The genus *Staphylococcus* has undergone a number of taxonomic reclassifications and species renaming throughout history as the tools to characterize them have expanded (Becker et al., 2014). Early attempts were made to classify staphylococcal-like organisms based on pathogenicity, where organisms considered to be non-pathogenic were placed into a different genus. In the 1940s, a major factor differentiating staphylococcal-like bacteria was discovered: coagulase production (Becker et al., 2014; Fairbrother, 1940). At that time, coagulase-negative staphylococci (CoNS) were considered non-pathogenic, while one strain of coagulase-positive staphylococci (CoPS), *S. aureus*, was considered pathogenic. Testing for coagulase production is still used today in human and veterinary medicine, although the pathogenic potential of the CoNS is now recognized (Becker et al., 2014).

Both coagulase-positive and coagulase-negative staphylococcal species, either methicillin-susceptible or –resistant, have been isolated from animals (Becker et al., 2014; Hermans et al., 2010; Songer and Post, 2004). *S. aureus* is most commonly associated with asymptomatic carriage or disease in humans, but essentially all warm-blooded animal species can be carriers of or be infected with this organism (Peton and Le Loir, 2014). *S. aureus* is a significant cause of mastitis in ruminants, as well as skin and soft tissue infections, urinary tract infections, arthritis and septicemia in horses, cats, pigs, and poultry (Peton and Le Loir, 2014). Besides *S. aureus*, there are a number of

coagulase-positive staphylococci in veterinary medicine that are of significant concern: *S. intermedius* (pigeons), *S. pseudintermedius* (dogs), *S. delphini* (dolphins and mustelids), *S. schleiferi* subsp. *coagulans* (dogs), *S. lutrae* (otters), and the coagulase variable *S. hyicus* (pigs) and *S. agnetis* (cows) (Becker et al., 2014; Devriese et al., 2005; Foster et al., 1997; Guardabassi et al., 2012; Hermans et al., 2010; Igimi et al., 1990; Songer and Post, 2004; Taponen et al., 2012; Varaldo et al., 1988). The coagulase-negative staphylococci (CoNS) make up a far more diverse group, and contain almost 4 times the number of species than the CoPS. *S. epidermidis* is the primary CoNS in humans, along with *S. hominis, S. haemolyticus, S. saprophyticus, S. capitis,* and *S. simulans* to name a few (Becker et al., 2014). Many of these CoNS are shared between humans and animals, but there are also some CoNS that are frequently isolated from animals, but rarely from humans: for example, *S. felis* in cats, *S. gallinarum* in poultry, and *S. caprae* in goats (Devriese et al., 1985; Songer and Post, 2004). The CoNS are a leading cause of subclinical bovine mastitis in dairy operations, and a number of species have been isolated from milk samples including *S. chromogenes, S. xylosus, S. sciuri, S. cohnii, S. warneri,* as well as some of the previously mentioned human CoNS (Vanderhaeghen et al., 2015). Although data from previous studies suggest that the staphylococcal-host association/relationship is absolute, recent reports indicate that certain strains are capable of colonizing a range of animal species (Hermans et al., 2010).

1.2 *Staphylococcus pseudintermedius*

Staphylococcus pseudintermedius is the most common coagulase-positive staphylococcal commensal in dogs, and has been reported to be the most frequent pathogen isolated from canine clinical samples (Weese and Van Duijkeren, 2010). Previously, this organism was classified as *S. intermedius*, and was first described in 1976 as a coagulase-positive species isolated from pigeons, dogs, mink and horses (Hájek, 1976). Molecular work on *S. intermedius* has established that the species is comprised of a group of three distinct taxa known as the *S. intermedius* group (SIG): *Staphylococcus intermedius, S. pseudintermedius,* and *S. delphini* (Bannoehr et al., 2007; Devriese et al., 2009, 2005; Sasaki et al., 2007a). Phenotypically, the organisms in the SIG are difficult to differentiate with traditional biochemical testing providing unreliable results (Bannoehr et al., 2007; Devriese et al., 2009; Sasaki et al., 2007a). However, molecular methods have been developed to successfully differentiate between all coagulase-positive species, including those in the SIG (Sasaki et al., 2010). Results of the molecular typing have suggested that all coagulase-positive staphylococci isolated from canine specimens be identified as *S. pseudintermedius*, unless genetic typing confirms otherwise (Bannoehr et al., 2007; Devriese et al., 2009; Sasaki et al., 2007a).

1.2.1 Emergence of methicillin-resistance

Historically, *S. aureus* was routinely susceptible to all β-lactam antibiotics, and the organism has been described as being naturally susceptible to all antibiotics (Chambers, 1997, Chambers and DeLeo, 2009). The antibiotic era, and the emergence of antimicrobial resistance, was first observed in *S. aureus*. The discovery of penicillin

by Alexander Fleming in 1928 has been considered one of the greatest advances in medicine, providing the ability to treat previously life-threatening infections (Chambers and DeLeo, 2009). By 1942, only a few years after its introduction in to clinical practice, penicillin-resistant *S. aureus* strains in hospitals were reported in the literature (Chambers and DeLeo, 2009; Deresinski, 2005). These penicillin-resistant strains produced a penicillinase that hydrolyzed the β-lactam ring, the active site, of penicillin. By the end of the next decade, penicillin-resistant *S. aureus* had emerged as a serious problem in the community and had become pandemic (Chambers and DeLeo, 2009; Deresinski, 2005).

In response to the growing threat of penicillin-resistant *S. aureus,* methicillin, a semi-synthetic penicillin, was introduced in 1959. A couple of years after methicillin's introduction to market, methicillin-resistant *S. aureus* (MRSA) strains were reported from European hospitals and later from the community and hospitals worldwide (Chambers and DeLeo, 2009; Deresinski, 2005; Enright et al., 2002). The subsequent emergence of MRSA in a number of animal species was not surprising, and can be attributed to the close relationship between humans and animals (Weese and Van Duijkeren, 2010; Weese, 2010). One of the earliest reports of MRSA in an animal was in 1972 from a milk sample in a cow with mastitis (Devriese et al., 1972). Since then, MRSA has been reported in dogs, cats, horses, pigs, and poultry, however, the majority of publications appeared in the early 2000s, approximately 40 years after the emergence of MRSA in humans (Weese and Van Duijkeren, 2010; Weese, 2010). Similarly to *S. aureus*, *S. pseudintermedius* was generally susceptible to the β-lactam antimicrobials

(Van Duijkeren et al., 2011a; Weese and van Duijkeren, 2010). Probable MRSP, based on oxacillin MICs, was first detected in the early 1980s in a retrospective study involving healthy dogs and dogs with pyoderma. However, methicillin-resistance was not confirmed by the detection of *mecA* (Moodley et al., 2014; Pellerin et al., 1998). MRSP confirmed to have the *mecA* gene was first reported in a case of canine pyoderma in the US in 1999 and then in Europe in 2005 (Gortel et al., 1999; Loeffler et al., 2007; Moodley et al., 2014). Since then, MRSP has been increasingly reported from canine specimens, and has emerged as a significant issue in veterinary medicine (Bannoehr and Guardabassi, 2012; Moodley et al., 2014; van Duijkeren et al., 2011a; Weese and van Duijkeren, 2010).

Unlike the penicillin-resistant strains of *S. aureus* that contained a penicillinase, it was noted that MRSA strains had a different resistance mechanism as resistance to other classes of antimicrobials was reported and drug inactivation was not observed (Chambers and DeLeo, 2009). The *mecA* gene, which is located on a mobile genetic element, the staphylococcal cassette chromosome (SCC), mediates methicillin-resistance. This portion of the SCC is not present in methicillin-susceptible isolates (Chambers, 1997). This mechanism of resistance is similar for MRSA and MRSP (Weese and Van Duijkeren, 2010). The *mecA* gene encodes a modified penicillin-binding protein (PBP2'). The β–lactam antimicrobials act by disrupting cell wall synthesis of staphylococci through binding to the penicillin-binding proteins at the β-lactam ring. PBP2' has a lowered affinity for the β-lactam antimicrobials, so they do not bind, and cell wall production is not inhibited (Chambers, 1997; Frank and Loeffler,

2012; van Duijkeren et al., 2011a). The presence of *mecA* and the expressed PBP2' consequently confer resistance to virtually all β–lactam antimicrobials, including penicillins, almost all cephalosporins, and carbapenems (Chambers, 1997).

1.2.2 Additional resistance patterns

Multi-drug resistance (MDR) to classes other than the β-lactams has been historically reported with health care-associated MRSA (HA-MRSA) infections. In contrast, MDR is less common in community-acquired MRSA (CA-MRSA) infections, which commonly occur in otherwise healthy individuals (Chambers and DeLeo, 2009). As in HA-MRSA, MRSP has acquired additional drug resistance mechanisms, leaving very few veterinary approved antimicrobials available for clinical use (Loeffler et al., 2007; Ruscher et al., 2009). These resistance patterns in MRSP have not been documented in methicillin-susceptible *S. pseudintermedius* (MSSP) isolates (Perreten et al., 2010). Resistance in MRSP isolates from dogs and cats to the aminoglycosides, tetracyclines, fluoroquinolones, macrolides, lincosamides, trimethroprim, and sulfonamides has been documented and well reviewed (De Lucia et al., 2011; Loeffler et al., 2007; Moodley et al., 2014; Perreten et al., 2010; Ruscher et al., 2009; van Duijkeren et al., 2011a; Weese and van Duijkeren, 2010). The temporal trends of antimicrobial resistance in methicillin-susceptible and methicillin-resistant *S. pseudintermedius* isolated from dogs in 27 countries, and published in 57 studies between 1984 and 2013 in the English language, were recently systematically reviewed. Seven major antimicrobial classes for treatment of veterinary staphylococcal infections were included in the review, and trends in penicillin/ampicillin resistance were also

analyzed for MSSP. No significant temporal changes in resistance to any of the 7 antimicrobial classes were observed for methicillin-susceptible and methicillin-resistant strains. A significant increase in resistance in methicillin-susceptible strains was observed to penicillin/ampicillin (Moodley et al., 2014).

1.3 Pathogenesis of *S. pseudintermedius*

1.3.1 Host factors

As a commensal organism, *S. pseudintermedius* does not cause disease unless host defenses are compromised, such as with skin allergies, skin abrasions, or surgical procedures (Bannoehr and Guardabassi, 2012; Hermans et al., 2010; Songer and Post, 2004). Thus, host factors play a critical role in the pathogenesis of *S. pseudintermedius* infections. *S. pseudintermedius* is the primary cause of canine dermatologic infections, including pyoderma, folliculitis, atopic dermatitis, otitis externa, as well as post-operative surgical site infections (SSI) (Bannoehr and Guardabassi, 2012; Hermans et al., 2010; Perreten et al., 2010; Quinn et al., 1994; Songer and Post, 2004; Weese and van Duijkeren, 2010). Like *S. aureus* in humans, *S. pseudintermedius* has also been isolated from other clinical specimens and has been reported to cause endometritis, cystitis, mastitis, and osteomyelitis in dogs (Bannoehr and Guardabassi, 2012; Quinn et al., 1994; Songer and Post, 2004). The role of colonization in *S. pseudintermedius* infections is unclear, but it is known that *S. aureus* nasal colonization in humans plays an important role in pathogenesis.

1.3.2 Virulence factors

Information on the virulence factors of *S. pseudintermedius* is limited, compared to what is known about virulence factors associated with *S. aureus* (Fitzgerald, 2009). Some *S. pseudintermedius* virulence factors are similar to those of *S. aureus* (Bannoehr and Guardabassi, 2012; Ben Zakour et al., 2011; Fitzgerald, 2009; van Duijkeren et al., 2011a). *S. pseudintermedius* has numerous virulence factors, but only the following will be discussed: hemolysins, leukotoxins, exfoliative toxins, enterotoxins, and biofilm production

In the literature reviewed for this thesis, no differences in the presence of virulence factors between methicillin-resistant and methicillin-susceptible strains were described (Couto et al., 2015; Singh et al., 2013) and this is comparable to studies investigating these associations with *S. aureus* (Gordon and Lowy, 2008; Melzer et al., 2003). One study found no significant difference in the mortality rates between canine patients with MRSP and canine patients with MSSP, further substantiating that MRSP strains are no more virulent than their MSSP counterparts (Weese et al., 2012). Although the information on *S. pseudintermedius* virulence is limited, it can be extrapolated that the success of methicillin-resistant strains is likely not related to virulence.

1.3.2.1 Hemolysins

The hemolysin toxins produced by *S. pseudintermedius* cause hemolysis of rabbit erythrocytes and hot-cold hemolysis of sheep erythrocytes. They possess a

similar enzymatic activity to the hemolysins produced by *S. aureus* but their amino acid composition are different (Bannoehr and Guardabassi, 2012; Dziewanowska et al., 1996; Fitzgerald, 2009; Futagawa-Saito et al., 2006; van Duijkeren et al., 2011a).

1.3.2.2 Leukotoxins

Luk-I, a cytotoxin produced by *S. pseudintermedius*, has similar activity to *S. aureus'* Panton-Valentine leukocidan (PVL), and has been shown to be leukotoxic to polymorphonuclear cells (Bannoehr and Guardabassi, 2012; Fitzgerald, 2009; Futagawa-Saito et al., 2004a; Prevost et al., 1995; van Duijkeren et al., 2011a). One study showed that there was no significant difference in the leukotoxicity of *S. pseudintermedius* isolated from healthy or diseased dogs (Futagawa-Saito et al., 2004a), while another showed no significant difference in Luk-I presence between methicillin-resistant and methicillin-susceptible strains (Couto et al., 2015). PVL is more likely to be carried by CA-MRSA versus HA-MRSA strains, but its role in the increased virulence of CA-MRSA is unclear (Chambers and DeLeo, 2009). The presence of Luk-I in both methicillin-resistant and –susceptible strains isolated from healthy dogs highlights the important role this toxin may have in a commensal *S. pseudintermedius* becoming an opportunistic pathogen (Bannoehr and Guardabassi, 2012; Couto et al., 2015; Gómez-Sanz et al., 2011).

1.3.2.3 Exfoliative toxins

The *S. pseudintermedius* exfoliative toxin (SIET) has been identified as a virulence factor in canine skin disease (Bannoehr and Guardabassi, 2012; Fitzgerald,

2009; Terauchi et al., 2003; van Duijkeren et al., 2011a). *In vitro* studies with a purified toxin demonstrated a rounding, or cytotoxic effect, on human keratinocytes, similar to the exfoliative toxins (ET) of *S. aureus* (ETA, ETB, and ETD) and *S. hyicus* (*S. hyicus* [SH] ETA and SHETB), but also showed that SIET, ETA/B, and SHETA/B were serologically different proteins (Bannoehr and Guardabassi, 2012; Fitzgerald, 2009; Terauchi et al., 2003; van Duijkeren et al., 2011a). Injection of purified SIET in dogs produced symptoms of erythema, exfoliation, and crusting, similar to the symptoms of canine *S. pseudintermedius* pyoderma, human staphylococcal scalded skin syndrome (SSSS) caused by *S. aureus*, and porcine exudative epidermitis caused by *S. hyicus* (Bannoehr and Guardabassi, 2012; Fitzgerald, 2009; Terauchi et al., 2003; van Duijkeren et al., 2011a). One study determined that over half of the *S. pseudintermedius* strains isolated from skin and wound infections were positive for the SIET encoding genes (Lautz et al., 2006), while more recent studies detected SIET encoding genes in all isolates tested, regardless if isolated from dogs with clinical infection or subclinical carriage or if the isolate was methicillin-resistant (Bannoehr and Guardabassi, 2012; Bardiau et al., 2013; Couto et al., 2015; Gómez-Sanz et al., 2011; Yoon et al., 2010).

Other *S. pseudintermedius* exfoliative toxins have also been identified, and provide conflicting evidence for the role of SIET in canine pyoderma (Bannoehr and Guardabassi, 2012; Fitzgerald, 2009; Futagawa-Saito et al., 2009; Iyori et al., 2011, 2010). Exfoliative toxin of pseudintermedius (EXI) was reported as a novel exfoliative toxin of *S. pseudintermedius* that is closely related to ETB in *S. aureus*, however the authors did not reference the previously discovered SIET (Futagawa-Saito et al., 2009).

It is possible that SIET was not considered because of its lack of homology to any of the previously reported exfoliative toxins (Terauchi et al., 2003). EXI was found in 23% *S. pseudintermedius* strains cultured from dogs with pyoderma (Futagawa-Saito et al., 2009). Once a second novel EXI was discovered the first EXI was renamed ExpA, and the newly reported exfoliative toxin was named ExpB, to be consistent with the naming of exfoliative toxins in other staphylococci (Iyori et al., 2010). ExpB was reported to have significant homology to ExpA, SHETB, and ETD, and injection of the purified toxin into healthy canine skin induced exfoliation (Bannoehr and Guardabassi, 2012; Iyori et al., 2010). Similarly, as with ExpA, ExpB was present in 23% of isolates collected from dogs with pyoderma, while it was only present in 6% of healthy dogs. One study where both ExpA (reported as EXI) and SIET were injected into the skin of healthy dogs, demonstrated that ExpA, not SIET, was responsible for the intradermal splitting at the superficial epidermis seen in cases of canine skin disease (Bannoehr and Guardabassi, 2012; Iyori et al., 2011). The inability of SIET to cause this intradermal splitting suggests that it is not a true exfoliative toxin. It was concluded that ExpA and ExpB play an important role in the pathogenesis of staphylococcal skin disease in the dog, while the role of SIET in *S. pseudintermedius* pathogenesis is still unclear (Iyori et al., 2011).

1.3.2.4 Enterotoxins

Staphylococcal enterotoxin (SE) production is most commonly associated with foodborne illness, but has also been associated with cases of human pyoderma and dermatitis (Hendricks et al., 2002; Kadariya et al., 2014; Tanabe et al., 2013). Isolates of

S. pseudintermedius have demonstrated the ability to produce enterotoxins and to activate T cell proliferation (Bannoehr and Guardabassi, 2012; Fitzgerald, 2009; Hendricks et al., 2002). An SE produced by isolates of *S. pseudintermedius* from canine pyoderma cases, the canine type C (SEC_{canine}), is similar to the SEC produced by *S. aureus* (Bannoehr and Guardabassi, 2012; Edwards et al., 1997; Fitzgerald, 2009). As with other SEs, SEC_{canine} is capable of inducing an emetic response and stimulating T cell proliferation (Edwards et al., 1997). The SEs (SEA, SEB, SEC, and SED) and toxic shock syndrome toxin 1 (TSST-1) of *S. aureus* have been referred to as 'superantigens' because of this ability to stimulate T cells and immunomodulatory properties (Hendricks et al., 2002). One study reported that 24% of *S. pseudintermedius* isolates from cases of canine pyoderma were positive for SEC_{canine}, another reported that 12% of strains tested were SEC_{canine} positive, while in a third study this enterotoxin was detected in only 0.5% of isolates tested (Becker et al., 2001; Yoon et al., 2010; Youn et al., 2011b). An association of SEC_{canine} production by isolates from dogs with and without pyoderma was not detected (Tanabe et al., 2013). Similarly, SEC_{canine} production was not associated with strains that were either methicillin-susceptible or methicillin-resistant, although the gene was only detected in two isolates (Couto et al., 2015). A novel enterotoxin of *S. pseudintermedius* se-int, has been described and was detected in all isolates tested, regardless of whether the sample was from an infected or colonized animal or if the strain was methicillin-resistant or sensitive (Bannoehr and Guardabassi, 2012; Fitzgerald, 2009; Futagawa-Saito et al., 2004b). Although the potential role of enterotoxins in human foodborne illness caused by *S. pseudintermedius* has been

established, the role of enterotoxins in canine pyoderma remains unclear (Becker et al., 2001).

1.3.2.5 Biofilm Production

Recently, biofilms have been recognized as an important virulence factor for staphylococci, including *S. pseudintermedius* (Bardiau et al., 2013; Casagrande Proietti et al., 2015; Couto et al., 2015; Futagawa-Saito et al., 2006; Osland et al., 2012; Pompilio et al., 2015; Singh et al., 2013). Biofilms are a community of microbial cells growing within a self-produced extracellular polymeric substance matrix (EPS) that has become irreversibly associated with a surface (Donlan, 2002). The production of the polysaccharide intracellular adhesion molecule (PIA) in the EPS is critical for biofilm formation and is, at least in part, encoded by the intracellular adhesion (*ica*) operon in staphylococci (Casagrande Proiettti et al., 2015; Singh et al., 2013). The biofilm matrix plays a key role in the success of the bacterial community by enhancing cell-to-cell communication and providing protection from host immune cells and antimicrobials. The majority of *S. pseudintermedius* isolates have been reported to be strong or moderate biofilm producers, although the mechanism for biofilm formation is not yet understood (Casagrande Proietti et al., 2015; Singh et al., 2013). There is conflicting data on the role *ica* operon plays in the formation of biofilms in *S. pseudintermedius*, where it has been reported that one, both, or neither of the *ica*A and *ica*D genes must be present (Casagrande Proietti et al., 2015; Singh et al., 2013). No significant associations between biofilm production and disease or colonization in dogs have been reported (Couto et al., 2015; Singh et al., 2013). The association between methicillin resistance

and biofilm production was investigated in two independent studies with contradictory results. Singh et al. (2013) reported no association between methicillin resistance and biofilm production, while Couto et al. (2015) reported increased biofilm production in methicillin-susceptible strains (Couto et al., 2015; Singh et al., 2013). The first study was a convenience sample set of 140 samples, and did not include many methicillin-susceptible strains (14%), while the second study was a matched case-control design with a smaller sample size (42 isolates), where consecutive methicillin-resistant and methicillin-susceptible strains were matched. Both studies used the microtitre method for quantifying biofilm production, but there were differences such as growth medium and primary stain used (Couto et al., 2015; Singh et al., 2013). The relatively small sample sizes of both studies, along with the differences in methodology and isolate source, likely explain the disagreement in results. The strong biofilm production of *S. pseudintermedius* has been implicated in the rapid emergence of MRSP in veterinary hospitals worldwide (Singh et al., 2013).

1.4 Laboratory detection of MRSP

In veterinary and human clinical diagnostic laboratories, coagulase-positive staphylococci are, in general, cultured and identified using similar methods. Specimens are plated onto a nutrient agar supplemented with 5% sheep blood (Bannerman and Peacock, 2007; Quinn et al., 1994). After incubation, plates are evaluated for typical colonial morphology, i.e., white-cream coloured colonies surrounded by a small zone of complete hemolysis and a larger zone of incomplete hemolysis (Bannerman and Peacock, 2007; Quinn et al., 1994; Songer and Post, 2004). One typical colony is

selected and tested for coagulase production. In veterinary laboratories, a battery of biochemical tests is more commonly run to discriminate between *S. aureus* and the *S. intermedius* group organisms including fermentation of different carbohydrates (mannitol, maltose, and trehalose), utilization of arginine dihydrolase, and DNase activity (Devriese et al., 2009, 2005; Quinn et al., 1994; Songer and Post, 2004).

Methicillin-resistance testing is often performed together with routine antimicrobial susceptibility testing, and is done by disk diffusion or microbroth dilution. Most clinical microbiology laboratories in North America perform antimicrobial susceptibility testing following guidelines set by the Clinical Laboratory Standards Institute (CLSI), which will be referred to as "the guidelines" in the following paragraphs. Oxacillin was traditionally used as the recommended surrogate for methicillin-resistance testing because of its greater stability *in vitro* (Bemis et al., 2009). Testing with cefoxitin was shown to be superior to oxacillin to detect methicillin-resistance in *S. aureus* and CoNS (Swenson et al., 2005), and is the method in the published laboratory guidelines (CLSI, 2015a, CLSI, 2015b). At first, no specific breakpoint data for oxacillin or cefoxitin were established for CoPS of veterinary origin, including *S. pseudintermedius,* and the use of human guidelines for *S. aureus* was recommended (Bemis et al., 2006).

For veterinary staphylococci, including *S. pseudintermedius*, it was shown that using zone diameters and breakpoints for CoNS were more accurate indicators of methicillin resistance than those recommended for *S. aureus* (Bemis et al., 2009). These

investigators also demonstrated that cefoxitin susceptibility testing was unacceptable for detecting methicillin-resistance in canine staphylococci. When compared to the result of penicillin-binding protein 2' (PBP2') production as the gold standard, cefoxitin had a 25% sensitivity and 100% specificity for detecting methicillin resistance in canine CoPS, using human break point data. Oxacillin disk diffusion was interpreted using human CoNS breakpoints, and had 97% sensitivity and 98% specificity when compared to the PBP2' result using canine CoPS isolates (Bemis et al., 2006). In one study, the authors proposed an epidemiological breakpoint for cefoxitin zone diameter (≤30 mm = resistant), but acknowledged the need for further study before this breakpoint was implemented in a clinical setting (Bemis et al., 2012). The guidelines have now been amended to reflect the proposed changes: for non-*S. aureus* coagulase-positive veterinary staphylococci, oxacillin disk diffusion or microbroth dilution is the recommended method of testing and test results are interpreted using breakpoint data for human CoNS (≤17 mm and ≥0.5 μg/mL = resistant) (CLSI, 2015a). Once resistance to oxacillin (or cefoxitin for *S. aureus*) is detected using phenotypic methods, it is recommended that resistance be confirmed by detection of either *mecA* by PCR or PBP2' antigens by latex agglutination (Bannerman and Peacock, 2007; Brown et al., 2005; CLSI, 2015a; Kali et al., 2014).

Selective culture media have been widely used for the detection of MRSA in specimens obtained from humans (Chan and Seales, 2013; Denys et al., 2013; Fwity et al., 2011; Kali et al., 2014; Morris et al., 2012). A number of MRSA selective agar plates are commercially available, but no products are specifically manufactured for the

detection of MRSP. One study compared 6 commercially available MRSA selective media for the detection of MRSP using previously isolated and stored strains. The results of this study suggested that the oxacillin resistance screening agar base (ORSAB; Oxoid Company, Nepean, Canada) or Brilliance MRSA agar (Oxoid Limited, Basingstoke, UK) be used for routine culture of canine samples (Horstmann et al., 2012). However, this study used isolates of MRSP, a situation that may not be representative of clinical sample testing (Horstmann et al., 2012). Three of the media investigated in this study are not available for sale in Canada, including Brilliance MRSA agar. The ORSAB medium is similar to a popular medium promoted in the literature for the selective detection of MRSP, i.e., mannitol salt agar with 2µg/mL oxacillin. To date, no published studies exist on evaluation of diagnostic test characteristics for selective culture media for the detection of MRSP in clinical specimens.

Both humans and animals can be infected, colonized, or contaminated with methicillin-resistant staphylococci. Infection occurs when an organism has invaded a body site, multiplies, and causes clinical manifestations of disease. On the contrary, colonization occurs when an organism is present, grows, and proliferates in a host without causing clinical signs or an immune response. Carrier status is used to refer to a colonized individual, either persistently colonized or transiently colonized. Contamination of an organism on an animal's coat, skin, or nose can occur, but these organisms can easily be washed off (Van Duijkeren et al., 2011a). These definitions are not concrete, and it can be quite difficult to discriminate between them *in vivo*. The

detection of MRSA and MRSP from clinically normal individuals (either colonized or contaminated) has been studied in both humans and dogs. In humans, nasal and perianal swabs are considered the optimal specimens for detection of *S. aureus* carriage (Warnke et al., 2014; Wertheim et al., 2005). Although there is no harmonized protocol for detection of staphylococci in dogs, at least five studies have concluded that nasal swabs have a lower sensitivity for detection *S. pseudintermedius*, and a mouth swab and the perianal swab are recommended for maximal recovery of *S. pseudintermedius* (Bannoehr and Guardabassi, 2012; Fazakerley et al., 2009; Iverson et al., 2015; Paul et al., 2012; Rubin and Chirino-Trejo, 2011; van Duijkeren et al., 2011a; Windahl et al., 2012). Enrichment broths have been used in studies investigating subclinical carriage because they have been shown to increase the sensitivity of screening by reducing overgrowth of other bacterial and fungal species (Brown et al., 2005; Windahl et al., 2012). A typical staphylococcal enrichment broth includes a high concentration of sodium chloride with or without added β-lactam antimicrobials (Iverson et al., 2015). Differences in the culture-based methodology for the detection of methicillin-susceptible and methicillin-resistant *S. pseudintermedius* make it difficult to compare the frequency of staphylococcal colonization between studies. Further work is required to evaluate the diagnostic test characteristics of staphylococcal enrichment broths and selective culture media for the detection of MRSP carriers and infected dogs.

1.5 Molecular Typing

Molecular typing of samples obtained from colonized and diseased dogs worldwide has revealed great diversity in *S. pseudintermedius* strain types (Black et al.,

2009; Fazakerley et al., 2010; Hartmann et al., 2005; Kadlec et al., 2015; Paul et al., 2012; Perreten et al., 2010; Pinchbeck et al., 2006; Sasaki et al., 2005; Solyman et al., 2013; Windahl et al., 2012). To date, no standardized typing protocol has been developed for *S. pseudintermedius,* and there is no consensus on the approach for epidemiologic investigations (Kadlec et al., 2015). Historically, comparative typing methods, such as ribotyping, pulsed-field gel electrophoresis (PFGE), and analysis of intergenic ribosomal DNA spacer polymorphisms, have been successfully used to investigate diversity of *S. pseudintermedius* (Bes et al., 2002; Hesselbarth and Schwarz, 1995; Moodley et al., 2009; Shimizu et al., 1996). PFGE holds the highest discriminatory power besides whole genome sequencing, and remains the standard method used to investigate outbreaks in epidemiologic studies, however, there is no standard PFGE protocol for MRSP (Bannoehr and Guardabassi, 2012; Moodley et al., 2009; Perreten et al., 2010). Further, PFGE methods are laborious, subjective, and have limited reproducibility, making them poor candidates for any large-scale or multi-institutional investigations.

Single- and multi-locus sequence analyses have emerged as promising definitive typing tools for *S. pseudintermedius.* Multilocus sequence typing (MLST) has been used extensively to study the population genetic structure of *S. aureus*, and two multilocus sequence typing (MLST) schemes, one with 5 loci (MLST-5) and the other with 7 loci (MLST-7), have been developed for *S. pseudintermedius* (Bannoehr et al., 2007; Solyman et al., 2013). Two predominant sequence types (ST) have been identified: ST71, a European clone, and ST68, a North American clone (Perreten et al.,

2010; Solyman et al., 2013). Although MLST has high inter-laboratory reproducibility and provides good discriminatory power, it is costly and time consuming. In the case of MRSP it can require 5 or 7 individual PCR reactions per isolate (Kadlec et al., 2015). MLST databases are available online, and provide a definitive for comparing isolates from different geographic locations.

Sequence analysis of the X region of the staphylococcal protein A gene (*spa* typing) has been used extensively for *S. aureus* investigations, replacing PFGE in many reference laboratories (Moodley et al., 2009). One research group found a protein A homolog in the *S. pseudintermedius* genome, and developed a *spa* typing protocol. When compared to MLST-5, *spa* typing was able to further discriminate MRSP isolates of the same ST to different *spa* types, and therefore was shown to have a higher discriminatory power than MLST for MRSP (Moodley et al., 2009). However, the *spa* typing approach is unable to provide results for all isolates of *S. pseudintermedius* (i.e. it is does not have 100% typeability)(Kadlec et al., 2015).

Recently, sequence analysis of the *mec*-associated direct repeat units (*dru* typing) was reported to be useful for discriminating highly clonal MRSA, and was further evaluated for typing MRSP isolated from dogs and cats (Goering et al., 2008; Kadlec et al., 2015). Kadlec *et al*. (2015) reported that *dru* typing is a convenient, objective, and discriminatory typing method for MRSP, with 100% typeability and good discriminatory power (Kadlec et al., 2015). In this study, *dru* typing was compared to MLST-5 and *spa* typing. Good agreement between MLST and *dru*-typing of MRSP

was noted, with significant associations between ST71 and *dru* cluster 9a, and ST68 and *dru* cluster 11a (Kadlec et al., 2015). The two predominant clones identified in Kadlec's study were the same as those identified in a previous multi-institutional study, with clear differences in regional distribution between European and American MRSP isolates. Canadian MRSP isolates were a hybrid of European and American dominant-strains (Kadlec et al., 2015; Perreten et al., 2010). It was concluded that ST71 (*dru* type 9a) should be named the "international" MRSP clone, rather than the "European" clone because of its apparent widespread dissemination (Kadlec et al., 2015).

In summary, definitive typing methods, or those methods that are based on sequence analysis, are the best choices for multi-institutional or large-scale research. Because of its good discriminatory power and ability to type all isolates, *dru* typing has become the preferred method for typing of MRSP. PFGE remains the gold standard when dealing with small-scale outbreaks involving one institution and testing laboratory, for example when dealing with the spread of a pathogen in a hospital setting.

1.6 Epidemiology

1.6.1 Dogs

Colonization of the canine skin and mucosa with both methicillin-susceptible and methicillin-resistant strains of *S. pseudintermedius* has been well documented. Most healthy dogs are colonized with MSSP, with rates ranging from 37%-92% (Cox et al., 1988; Fazakerley et al., 2009; Garbacz et al., 2013; Griffeth et al., 2008; Hanselman et al., 2009; Priyantha et al., 2016; Rubin and Chirino-Trejo, 2011).

Reported prevalence of MRSP colonization of healthy dogs in the community, or at hospital admission, have ranged from 0-4.5%, with one study in Japan reporting MRSP carriage in 30% of dogs at a veterinary teaching hospital (Garbacz et al., 2013; Griffeth et al., 2008; Hanselman et al., 2009, 2008; Murphy et al., 2009; Nazarali et al., 2015; Priyantha et al., 2016; Rubin and Chirino-Trejo, 2011; Sasaki et al., 2007b; van Duijkeren et al., 2011a; Vengust et al., 2006; Weese and van Duijkeren, 2010). A primary limitation discussed by the authors of most colonization studies is the one time sampling approach making it difficult to discriminate between colonization and contamination (Bannoehr and Guardabassi, 2012). The variability in reported colonization rates can be attributed to a number of factors, including the number of samples (between 1 and 5) collected, the anatomical sites (nasal, perianal, mouth, skin) sampled, differences in study populations (e.g. geographic location, inclusion criteria), and culture methodology (e.g. enrichment broth or selective culture) (Bannoehr and Guardabassi, 2012).

S. pseudintermedius is the most common cause of canine skin disease, and is the most frequently isolated pathogen from diagnostic samples collected from dogs at diagnostic laboratories (Bannoehr and Guardabassi, 2012; Weese and van Duijkeren, 2010). In the past five years a number of studies have reported the prevalence of MRSP in canine diagnostic samples from a variety of populations worldwide with ranges from 0-41% (Bardiau et al., 2013; Beck et al., 2012; Detwiler et al., 2013; Garbacz et al., 2013; Hariharan et al., 2014; LoPinto et al., 2014; Maluping et al., 2014; Siak et al., 2014; Wang et al., 2012; Windahl et al., 2012). These studies, however, often target

high-risk populations, such as those visiting referral hospitals, or patients with a previous history of disease caused by MRSP. One study looking at pyoderma in dogs found no difference in the frequency of isolation of methicillin-resistant staphylococci at primary care versus tertiary referral facilities (Eckholm et al., 2013). Results of these studies are often incomparable because of differences spatially, as well as differences in specimen collection sites and detection methodology (Bannoehr and Guardabassi, 2012).

Two longitudinal studies have reported information on long-term carriage of both MSSP and MRSP. Paul *et al.* (2012), investigating *S. pseudintermedius* colonization in Denmark, collected and followed samples from 16 dogs over a six-month period. Six (37.5%) of the dogs followed were classified as persistent carriers, that is, having *S. pseudintermedius* isolated at all sampling times over the study period. Of the remaining 10 dogs, five were classified as transient carriers (positive in at least three consecutive samplings), four as sporadic carriers (positive in only one or two samplings), and one as a non-carrier (negative in all samplings), respectively (Paul et al., 2012). Windahl *et al.* (2012) investigated MRSP carriage in 31 dogs with a previous history of MRSP infection, over a period of eight months or longer. They reported that dogs remain colonized for several months post-infection, with 61% of dogs culturing positive for a least 8 months. Of the dogs that were positive for 8 months, 5 were followed for a total of 14 months and continued to culture positive for MRSP (Windahl et al., 2012). The authors of this study emphasized that re-infection (from other positive dogs, the environment, or humans) rather than prolonged colonization could not be ruled

out. Similar findings were reported in a study that followed 16 dogs infected with MRSP for 6 months (Laarhoven et al., 2011).

Risk factors for the isolation of MRSP from infected dogs have been investigated. No risk factors associated with patient signalment have been reported, except in one study that found that castrated males were more likely to be MRSP positive when compared to intact males and spayed or intact females (Weese et al., 2012). The authors were unable to explain this phenomenon and this finding has not been duplicated in other studies. Hospitalization within 6 months to a year, and frequent visitation to a veterinary clinic, have been strongly associated with the isolation of MRSP from dogs, highlighting the potential for MRSP to be implicated in hospital-acquired infections (Beck et al., 2012; Eckholm et al., 2013; Grönthal et al., 2014; Lehner et al., 2014; Nienhoff et al., 2011; Weese et al., 2012). One study investigating a large outbreak of MRSP in dogs reported that the risk of becoming infected or colonized with MRSP was increased if the patient was treated in the intensive care units (ICU) or surgery wards (Grönthal et al., 2014). Antimicrobial treatment within 30 days to 6 months has also been strongly associated with the isolation of MRSP from dogs, regardless of duration of treatment or antimicrobial used (Beck et al., 2012; Eckholm et al., 2013; Lehner et al., 2014; Nienhoff et al., 2011; Weese et al., 2012). One study did not find any association between history of antimicrobial therapy and MRSP isolation, however it documented an association between systemic glucocorticoid administration and MRSP isolation (Lehner et al., 2014). This latter finding has been reported elsewhere (Nienhoff et al., 2011).

1.6.2 Hospital Environment

Since their emergence, MRSA and MRSP have become major clinical and epidemiological problems in human and veterinary hospitals alike. Several studies have reported MRSP contamination of veterinary hospital environments, even after cleaning and disinfection, with estimates of environmental contamination ranging from 6-16% (Ishihara et al., 2010; van Duijkeren et al., 2011b; Youn et al., 2011a, 2011b). A number of areas within veterinary clinics or hospitals were sampled, including, but not limited to, waiting areas, examination rooms, ICU, wards, and surgical suites, as well as small and large pieces of equipment (Ishihara et al., 2010; van Duijkeren et al., 2011b; Youn et al., 2011b). It is not stated whether areas and equipment were cleaned and disinfected prior to sampling. One study resampled MRSP positive areas one month after the initial positive result after a cycle of cleaning and disinfection, however, it is not specified if cleaning and disinfection was performed for the first sample (Van Duijkeren et al., 2011b). After the first round of sampling, 16% of environmental samples (n = 200) in 54% of the clinics (n = 13) were MRSP positive. At the time of resampling 14% of samples (n= 101) in 50% (n = 6) clinics were MRSP positive (Van Duijkeren et al., 2011b). In this study, clinics were required to clean and disinfect according to site protocols, with no intervention from the study personnel. To investigate the persistence of MRSP in hospital environments, variations in cleaning and disinfection should be eliminated. Only one of the aforementioned studies described actual areas that were MRSP positive: the ICU, diagnostic imaging suites, and cages (Ishihara et al., 2010). MRSP strain typing was performed in one of these studies and

revealed that isolates from patients and the hospital environment had indistinguishable PFGE band patterns (Youn et al., 2011a, 2011b). A large outbreak involving a MRSP strain (sequence type 71) at a Finnish veterinary teaching hospital was reported with over 60 cases of infection and colonization occurring in canine patients (Grönthal et al., 2014). Environmental surface samples were collected post-cleaning and disinfection from 65 high-touch areas and only 1 sample was MRSP-positive. The MRSP isolated from the hospital environment and the outbreak strain had different antimicrobial susceptibility patterns (Grönthal et al., 2014). No common source of exposure was identified, but the authors inferred that patient-to-patient transmission was the likely route. Results from the above studies confirm that veterinary clinics and hospitals are often contaminated with MRSP. The wide distribution of MRSP in veterinary settings provides considerable exposure to the canine patients examined in these areas. Further work is necessary to understand the dynamics of MRSP transmission in hospital environments, and whether or not environmental contamination plays a role in its transmission.

1.6.3 Humans

Until the last decade, reports of *S. pseudintermedius* colonization in humans were limited. More recently, there have been a number of studies investigating the zoonotic transmission of *S. pseudintermedius* from dogs to veterinary personnel or their owners. Colonization of humans by methicillin-susceptible and methicillin resistant strains of *S. pseudintermedius* is considered rare or uncommon, with prevalence estimates in humans with animal contact ranging from 0.4% - 8% (Boost et al., 2011;

Frank et al., 2009; Hanselman et al., 2009; Harvey et al., 1994; Ishihara et al., 2010; Mahoudeau et al., 1997; Morris et al., 2010; Paul et al., 2011; Sasaki et al., 2007b; Talan et al., 1989c; van Duijkeren et al., 2011b). The colonization or contamination of veterinarians with MRSP is within the reported estimates for MRSA (3.9%-32%) (Weese and Van Duijkeren, 2010). One study reported that humans were significantly more frequently colonized with *S. aureus* compared to *S. pseudintermedius* (Hanselman et al., 2009). Molecular typing of isolates from humans and dogs shows genetic relatedness between strains suggesting transmission from dog to human (Guardabassi et al., 2004; Hanselman et al., 2009; Morris et al., 2010; Sasaki et al., 2007b; Soedarmanto et al., 2011). Most of the reports were cross-sectional studies that describe a one-time sampling approach, therefore, it is impossible to distinguish between colonization (either persistent or intermittent) and contamination. Resampling approaches for detecting human colonization or contamination with MRSP in households with infected dogs have been reported (Frank et al., 2009; Guardabassi et al., 2004; Laarhoven et al., 2011). In one of these studies, resistance to methicillin (oxacillin) was not assessed, but the multi-drug resistance reported in the *S. pseudintermedius* isolates is characteristic of MRSP (Guardabassi et al., 2004). In all of the studies investigating MRSP colonization in humans in households with infected dogs, no human tested positive more than once, however, the number of test positive owners was small ($n \leq 2$) (Frank et al., 2009; Guardabassi et al., 2004).

One longitudinal study investigated the carriage of MRSP in 16 previously infected dogs, as well as the household environment, contact animals and humans

(Laarhoven et al., 2011). Sample collection started within 7 months of initial diagnosis of a household dog with an MRSP infection. Nasal samples were collected from 25 persons (owners/household members) in 16 households for 6 months. Three humans from different households were positive at the initial sampling, while no humans were positive over the next four samplings. Two humans from the same house were MRSP positive at the last sampling. No human tested positive more than once, including 2 that were positive at the start of the study and remained negative for the duration. In all cases where humans were MRSP positive, MRSP was also isolated from the index dog, in contact animals, and the household environment. Throughout the study, 10 dogs had active clinical disease, suggesting that even when the bacterial load is high, the risk of zoonotic transmission is low. The strains recovered from humans were always identical to strains isolated from the index canine case. Various MRSP sequence types were isolated from humans in this study, including ST71. This study reaffirmed that transmission of MRSP from dogs to humans is rare (5/140 human samples MRSP positive). Since MRSP was never isolated more than once from the same person, it was concluded that people are contaminated rather than colonized with MRSP (Laarhoven et al., 2011). A longitudinal study with more frequent sampling would provide additional insight into to the duration of contamination or transient colonization of humans in households with MRSP positive dogs.

Although reports of *S. pseudintermedius* infection in humans exist *S. pseudintermedius* is primarily considered a canine pathogen. Infected dog bite wounds are a common cause of *S. pseudintermedius* infections in humans (Bannoehr and

Guardabassi, 2012; Lee, 1994; Talan et al., 1989a, 1989b; van Duijkeren et al., 2011a; Weese and van Duijkeren, 2010). Less commonly, *S. pseudintermedius* has been associated with bacteremia, pneumonia, skin and ear infections, endocarditis, infected surgical site, and varicose leg ulcers (Atalay et al., 2005; Gerstadt et al., 1999; Hatch et al., 2012; Kempker et al., 2009; Kikuchi et al., 2004; Lee, 1994; Pottumarthy et al., 2004; Riegel et al., 2011; Stegmann et al., 2010; Tanner et al., 2000; Van Hoovels et al., 2006; Vandenesch et al., 1995). Exposure to dogs at home was documented in a few cases of infection; strains isolated from household dogs and the patient were indistinguishable by PFGE (Kempker et al., 2009; Kikuchi et al., 2004; Tanner et al., 2000). In the other cases of *S. pseudintermedius* infections in humans, animal contact was either not documented or followed up by sampling household pets or animal exposure was not documented or available at the time of the report. Most human clinical laboratories do not routinely take the additional steps to detect *S. pseudintermedius*, so it is possible that these isolates, once being identified as coagulase positive staphylococci, were mistakenly identified as *S. aureus* (Pottumarthy et al., 2004; Talan et al., 1989b; Van Hoovels et al., 2006). Thus it is conceivable that the true incidence of human *S. pseudintermedius* infections is underreported (Weese and Van Duijkeren, 2010).

1.7 Aims of this study

Currently, there is no published information on MRSP in Atlantic Canada. Since the fall of 2009, the Diagnostic Services Bacteriology Laboratory at the Atlantic Veterinary College started routinely screening all coagulase-positive staphylococci for methicillin-resistance. Similar to other regions of Canada and the world, the recovery of

MRSP increased dramatically between 2010 and 2011; less than 10 infections were documented in 2010 while 46 cases were confirmed in 2011 (unpublished data). To further understand the dynamics of this pathogen, and its implications to human and animal health, the distribution of MRSP in Atlantic Canada was investigated. This thesis focused on studying the frequency and distribution of MRSP at a Veterinary Teaching Hospital (VTH) and regional diagnostic laboratory in Atlantic Canada, as well as on exploring culture methods for rapid detection of this pathogen. This thesis had five aims: first, to explore the strain type diversity of MRSP isolates from dogs using the *dru* typing method and to describe the antimicrobial resistance (AMR) pattern of these MRSP isolates from submissions to a regional diagnostic laboratory in Atlantic Canada (Chapter 2); second, to investigate the persistence of MRSP in the VTH environment after cleaning and disinfection of areas that were in contact with MRSP positive patients and to explore clonal relationships between MRSP recovered from the environment and MRSP positive patients associated with that area (Chapter 3); third, to investigate potential hospital-acquired MRSP infections in the VTH (Chapter 3); fourth, to determine if using selective culture methods would detect more MRSP when compared to the traditional culture methods, and to evaluate the utility of a selective culture medium for rapid culture-based detection and/or presumptive identification of MRSP in clinical specimens (Chapter 4); and finally to estimate the prevalence of MRSP in clinical samples obtained from canine patients in Atlantic Canada deemed at high risk of being infected or colonized with MRSP (Chapter 4).

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2.0 DIRECT REPEAT UNIT (*DRU*) TYPING AND ANTIMICROBIAL RESISTANT PROFILES OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS PSEUDINTERMEDIUS* ISOLATED FROM DOGS IN ATLANTIC CANADA.

Abstract

MRSP has emerged as a major pathogen in dogs, being primarily isolated from skin, ears, surgical site infections, and wounds. Currently, there are few reports investigating the molecular characterization of MRSP in dogs in Canada, with none from Atlantic Canada. Sequence analysis of the *mec*-associated direct repeat units (*dru* typing) has been recommended as an ideal method for strain typing MRSP. The objectives of this study were to strain type MRSP isolates cultured at the Atlantic Veterinary College (AVC) Diagnostic Bacteriology Laboratory using *dru* typing and to describe their antimicrobial resistance profiles. Coagulase positive staphylococcal isolates recovered from dogs between January 2010 and December 2012 were tested. Isolates were typed by analyzing sequence data from the direct repeat units and a cluster analysis was performed to compare *dru* type relatedness. Ninety-eight isolates were retained for this study, and 94 were successfully identified as belonging to 18 different *dru* types. The majority of isolates belonged to type dt11a (30.9%), dt10h (24.5%), dt9a (18.1%), and dt11af (10.6%). The remaining 15.9% isolates were distributed between 13 different *dru* types, nine of which have not been previously identified: dt5k, dt6t, dt8ag, dt9ba, dt9bd, dt10bz, dt10cc, dt10cj, and dt11ca. Each of the previously unidentified *dru* types was represented by one isolate. The predominant *dru* types identified in this study were similar to those found in Ontario, Canada, however cluster 9a appears to be less

common in Atlantic Canada. A significant difference in the distribution of clusters between provinces was detected ($p = 0.01$), while no difference was detected between specimen source ($p = 0.91$) or whether the specimen originated from a patient at AVC Veterinary Teaching Hospital versus an outside submission ($p = 0.18$). Resistance to ≥2 antimicrobials (in addition to β-lactams) was observed in 71.4% of the isolates, with isolates belonging to cluster 9a being significantly more resistant to all antimicrobials tested except fusidic acid, chloramphenicol, and doxycycline. However, the MRSP isolates from this study were notably less resistant than those reported in the literature. This study confirms that *dru* type distributions can vary significantly across the same country, and previously unreported *dru* types were identified in Atlantic Canada. A larger, more comprehensive study of the MRSP *dru* types could help further elucidate the distribution of this pathogen in Canada.

2.1 Introduction

Over the last decade, MRSP has emerged as a major opportunistic pathogen in dogs, and as the leading cause of skin disease, otitis, surgical site infections, and wound infections (Perreten et al., 2010; Weese and van Duijkeren, 2010). Methicillin-resistance is mediated by the *mecA* gene, which codes for the expression of a modified penicillin-binding protein, PBP2', and resistance to most β-lactam antimicrobials (Weese and Van Duijkeren, 2010). Increasing antimicrobial resistance to antimicrobials other than β-lactams, has been reported in MRSP isolates, leaving few therapeutic options available in many instances (Perreten et al., 2010; van Duijkeren et al., 2011; Weese and van Duijkeren, 2010). MRSP has been implicated in hospital-acquired infections within veterinary hospitals, and the potential of hospital-wide outbreaks should not be under estimated (Boerlin et al., 2001; Perreten et al., 2010). Not only has MRSP been reported in the canine population, but there have been several reports of humans infected or colonized with MRSP (Boost et al., 2011; Gerstadt et al., 1999; Morris et al., 2010; Paul et al., 2011; Sasaki et al., 2007; Starlander et al., 2014; Stegmann et al., 2010). Understanding the diversity and dissemination of MRSP is essential. Currently, there is no study characterizing MRSP isolates in Atlantic Canada.

A number of molecular tools have been reported to genetically discriminate between staphylococci. While most of these tools were first developed and standardized for methicillin-resistant *Staphylococcus aureus* (MRSA), few have been further refined for discriminating MRSP (Bannoehr and Guardabassi, 2012; Black et al., 2009; Moodley et al., 2009; Murchan et al., 2003; Perreten et al., 2010; Solyman et al., 2013).

While pulsed-field gel electrophoresis (PFGE) remains the gold standard for MRSA outbreak investigations, it has been shown to be less useful for MRSP investigations (Bannoehr and Guardabassi, 2012; Goering et al., 2008; Moodley et al., 2009; Strandén et al., 2003). Historically, multi-locus sequence typing (MLST) has been the primary tool for investigating the population genetic structure of MRSP, and two major sequence types (ST) have been identified (Bannoehr et al., 2007; Perreten et al., 2010; Solyman et al., 2013). PFGE and MLST are laborious, costly, and require the use of reference strains, thus making them impractical tools for implementation in smaller diagnostic laboratories.

The use of a single-locus marker for isolate discrimination has also been explored for MRSA, and provides a less expensive and less laborious approach to strain typing (Koreen et al., 2004; Shopsin et al., 1999). Sequence analysis of the staphylococcal protein A region (*spa* typing) has been shown to have greater discriminatory power than MLST and PFGE for MRSA, but it is less successful for strain typing all MRSP isolates (Kadlec et al., 2015; Koreen et al., 2004; Moodley et al., 2009; Shopsin et al., 1999). The *mec*-associated direct repeat unit (*dru*) typing was first demonstrated to be useful in discriminating highly-clonal MRSA isolates in Scotland (Goering et al., 2008), and has more recently been successfully applied to MRSP isolates from Canada, the United States of America, Europe, and Australia (Kadlec et al., 2015; Siak et al., 2014; Weese et al., 2012). In a previous study, significant associations between *dru* cluster and MLST were established, where cluster 9a was associated with ST71, the "International clone" and cluster 11a was associated with

ST68, the "North American clone" (Kadlec et al., 2015; Perreten et al., 2010). This single-locus sequence-based method is rapid, standardized, and cost effective, making it an ideal candidate for use in small-scale laboratories (Kadlec et al., 2015; Siak et al., 2014).

In a previous study significant differences have been reported in the distribution of the two main *dru* clusters, 9a and 11a, between Canada, the USA, and Europe (Kadlec et al., 2015). The report also inferred that variation within a country may exist, as some *dru* types were detected in some US states but not the others included in the study. To date this has been the only study investigating *dru* types in Canada, therefore, genetic information on MRSP isolates from Canadian regions other than Ontario is needed. Thus, the primary objective of this current study was to explore the strain type diversity of MRSP isolates from dogs using the *dru* typing method from submissions to a regional diagnostic laboratory in Atlantic Canada. A secondary objective was to describe the antimicrobial resistance (AMR) pattern of these MRSP isolates.

2.2 Materials and Methods

2.2.1 Isolate screening and collection

Isolates were recovered from canine specimens submitted to the AVC Diagnostic Services Bacteriology laboratory for routine culture and susceptibility testing. Staphylococci were identified by colony morphology, including hemolysis, a positive tube coagulase test and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Isolates were frozen and further identified using

molecular approaches. Antimicrobial susceptibilities to the following drugs were determined using the Kirby Bauer disk diffusion method, following Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008): ampicillin (10 μg), amikacin (30 μg), amoxicillin-clavulanic acid (30 μg), cephalexin (30μg), cefovecin (30 μg), chloramphenicol (30 μg), clindamycin (30 μg), doxycycline (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), fusidic acid (10 μg), gentamicin (10 μg), penicillin (10 μg), and trimethoprim-sulfamethoxazole (TMS) (25 μg). Methicillin resistance was detected by oxacillin (1 μg) disk diffusion, and confirmed by *mecA* expression using the PBP2' latex agglutination test (PBP2' Latex Agglutination Test; Oxoid Company, Nepean, Canada). All isolates identified presumptively as MRSP were placed in a medium containing 15% glycerol and frozen at -80ºC.

Patient province of residence, submitting clinic, anatomical source/site of specimen, and antimicrobial susceptibility data were extracted from the laboratory data management system, and descriptive statistics were computed. One isolate from each patient was included for analysis. If multiple isolates were recovered, the first isolate was included for analysis, unless a different *dru* type was identified in subsequent isolates.

2.2.2 DNA extraction

Frozen isolates were subcultured to blood agar plates and incubated aerobically at 35ºC for 18-20 hours. Two subcultures in series were made to blood agar plates to ensure purity. Genomic material was extracted using the InstaGene™ Matrix (Bio-Rad

Laboratories, Montréal, Canada). Manufacturer's guidelines were followed except a large loopful of bacterial colonies were suspended in 1.0 mL of PCR water, and the incubation time at 56ºC was increased from 30 min to 1 hour. Isolated DNA was frozen at -20ºC until use.

2.2.3 Molecular identification and typing

A multiplex PCR reaction was used to identify coagulase-positive staphylococci to the species level based on partial amplification of the *nuc* gene locus (Sasaki et al., 2010). A 25 µL reaction was prepared containing 2 µL of template DNA, 0.5 U of KAPA2G Fast Hot Start DNA polymerase, buffer mixture containing 1.5 mM $MgCl_2$ and 200 µM of each dNTP (KAPA Biosystems, Boston, USA), additional 0.5 mM $MgCl_2$, and 0.2 µM of each primer set for *S. intermedius, S. pseudintermedius,* and *S. schleiferi* (Table 2.1). Reaction mixtures were thermally cycled for an initial denaturation step of 95ºC for 2 min followed by 30 cycles at 95ºC for 30 s, 56ºC for 35s, and 72ºC for 1 min, and one cycle at 72ºC for 2 min (Sasaki et al., 2010). Control strains were provided by Dr. J. S. Weese's laboratory at the Ontario Veterinary College at the University of Guelph, Guelph, Canada.

S. pseudintermedius isolates were characterized using the direct repeat unit (*dru*) typing method, as previously described (Goering et al., 2008). A 40 µL reaction was prepared containing 4 µL of template DNA, 0.8 U of KAPA2G Fast Hot Start DNA polymerase, buffer mixture containing 1.5 mM $MgCl_2$ and 200 µM of each dNTP (KAPA Biosystems, Boston, USA), additional 0.3 mM $MgCl_2$, and 0.8 µM of the

forward primer *dru*GF (5′-GTTAGCATATTACCTCTCCTTGC-3′) and the reverse primer *dru*GR (5′-GCCGATTGTGCTTGATGAG-3′). Reaction mixtures were thermally cycled for an initial denaturation step of 94ºC for 2 min followed by 30 cycles at 94ºC for 1 min, 52ºC for 1 min, and 72ºC for 1 min (Goering et al., 2008). PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen Inc., Mississauga, Canada) prior to sequencing. *Dru* repeats and types were assigned using www.dru-typing.org following the previously described nomenclature (Goering et al., 2008). Cluster analysis was performed to compare the relatedness between *dru* types, and a minimum spanning tree (MST) was generated. Distance intervals (or similarity values) were created using a bin distance of 1.0%, where *dru* types separated by an MST distance of ≤2 repeats (>98.5% similarity) were considered closely related and assigned to the same cluster. Root nodes were assigned to the *dru* type with the greatest number of isolates. Cluster analysis was completed using the TRST plug-in tool of BioNumerics v6.6 (Applied Maths, Austin, USA), as described previously for MRSP *dru* typing (Bartels et al., 2013; Kadlec et al., 2015; Siak et al., 2014; Weese et al., 2012).

2.2.4 Statistical analysis

Multi-drug resistant (MDR) MRSP were defined as being resistant to ≥2 antimicrobials classes in addition to β-lactams. *Dru* clusters were used for comparisons to decrease the number of tested groups. Unconditional associations between *dru* cluster and patient data and *dru* cluster and resistance to the non-β-lactam antimicrobials were assessed using chi-square or Fisher's exact tests, where appropriate, with significance

set at $p \leq 0.05$. Subtables were explored for any significant association to determine where the differences were. All statistical computations were performed in Stata/IC 13.1 for Mac (StataCorp, College Station, Texas).

2.3 Results

2.3.1 Isolate collection

The diagnostic laboratory collected 129 isolates from 90 dogs between January 2010 and December 2012. Of those, 98 isolates were retained for analysis. Twenty-three patients had multiple submissions. Isolates were collected from a variety of specimens ($n = 98$): skin (52, 53.1%), ears (18, 18.4%), wounds (8, 8.2%), surgical sites (6, 6.1%), urine (5, 5.1%), abscesses (2, 2.0%), and other (7, 7.1%). Most of the patients were seen by private veterinary clinics in the region (71.4%) compared to being seen by the AVC Veterinary Teaching Hospital (28.6%).

2.3.2 Antimicrobial resistance patterns

Only 8.2% ($n = 8$) of isolates were susceptible to all tested drugs with the exception of β–lactams (Table 2.2). 20.4% ($n = 20$) of isolates were resistant to at least 1 non-β-lactam antimicrobial, while 71.4% ($n = 70$) were resistant to ≥2 non-β-lactam antimicrobials. The most common resistance was to TMS (74.5%), followed by erythromycin (68.4%), clindamycin (55.1%), enrofloxacin (46.9%), gentamicin (34.7%), chloramphenicol (23.5%), doxycycline (15.3%), and fusidic acid (3.1%). In isolates that displayed multi-drug resistance ($n = 70$), 68.6% of isolates were resistant to

TMS, erythromycin, clindamycin, while 55.7% of those also resistant to enrofloxacin. Amikacin resistance was not detected in any of the isolates.

2.3.3 Direct repeat unit (*dru*) typing

Dru types were determined for 94/98 isolates, because 4 isolates were not available for typing. From 94 isolates, 18 *dru* types were recovered with three predominant *dru* types contributing more than 70% of the distribution: dt11a (30.9%), dt10h (24.5%), and dt9a (18.1%). The frequency of each *dru* type can be found in Table 2.3. Nine of the *dru* types were previously unidentified at the time of analysis and are represented by one isolate each: dt5k, dt6t, dt8ag, dt9bd, dt10cc, dt10cj, dt11ca, and dt11cm.

A minimum spanning tree (MST) was constructed to identify relatedness between *dru* types, and to determine the predominate clusters (Figure 2.1). The cluster analysis identified three main *dru* clusters that included 90/94 typed isolates (Figure 2.1). Cluster 11a contained the most diversity, comprising 9 *dru* types and 47.9% of the typed isolates. Cluster 10h contained 3 *dru* types and 28.7% of the isolates, while cluster 9a contained 2 *dru* types and 19.2% of the isolates. Table 2.3 contains the *dru* type and cluster association.

2.3.4 Associations between *dru* clusters, patient demographics and AMR

Significant differences were determined between the distributions of *dru* clusters in the 4 Atlantic Canadian provinces ($p = 0.01$) (Table 2.4). In Newfoundland and

Labrador, cluster 9a was over-represented with 5/7 strains belonging to dt9a. Cluster 10h was present in NS, NB, but not NL, and was the predominant cluster from PEI isolates (7/12). Both mainland provinces, NB and NS, shared the same predominant cluster, 11a, which comprised over 50% of the isolates from each province.

Dru cluster was not significantly different between the VTH and private practice ($p = 0.18$) or specimen types ($p = 0.91$). Significant associations between AMR and *dru* clusters were detected for all antimicrobials except fusidic acid ($p = 0.31$)(Table 2.2). For clindamycin, erythromycin, enrofloxacin, and TMS, cluster 9a had a significantly higher proportion of resistant isolates ($p \leq 0.01$). Cluster 10h had significantly higher proportion of isolates resistant to doxycycline ($p = 0.04$), while cluster 11a had a significantly higher proportion of chloramphenicol resistance ($p < 0.001$). The proportion of MDR isolates was significantly different between *dru* clusters ($p = 0.03$), with cluster 9a having the highest proportion of MDR (94.4%), followed by 10h (55.6%) and 11a (68.8%).

2.4 Discussion

The majority of isolates (73.5%) collected as part of this convenience sampling at the AVC Diagnostic Services Bacteriology Laboratory were represented by *dru* types dt11a, dt10h, and dt9a, which is similar to the findings in a previous multi-national study (Kadlec et al., 2015). However, in the current study, clusters 11a and 10h comprised more than 75% of the isolates collected, whereas in the Ontario study, cluster 11a (including dt10h) and 9a were evenly distributed (Kadlec et al., 2015). The

distribution of MRSP *dru* clusters in Atlantic Canada was more similar to what has been reported in California, Illinois, North Carolina, Tennessee, and Texas (Kadlec et al., 2015). Differences between geographic regions within the US were also noted in the previous study (Kadlec et al., 2015).

Significant differences in the diversity of strains were detected between provinces of Atlantic Canada (Table 2.4). It should be noted that this study was not designed for prevalence estimation because of the convenience sampling approach. Although it may appear that the incidence of MRSP is higher in certain provinces, these numbers are reflective of the sample submission demographics from these regions (*i.e.* the laboratory receives approximately 45% of their canine samples from NS, but only 5% are from NL). These provincial differences in the distribution of *dru* types could be attributed to geographic separation, since differences between Newfoundland and the Maritime provinces (NB, NS, and PEI) exist, as well as population density, since provinces with larger populations have greater *dru* type diversity. The small sample size of isolates from Newfoundland and Labrador may bias its estimate. It is possible that the true diversity of the isolates from some provinces is underestimated, since each province except PEI has its own animal health microbiology laboratory, decreasing submissions from these provinces to the AVC diagnostic laboratory.

This study detected nine *dru* types that had not been previously identified (Table 2.3), and required entry into the *dru* database. These new *dru* types are results of a random single-nucleotide polymorphism in one of the 40bp *dru* repeat sequences,

subsequently creating a new *dru* repeat and thus new *dru* type. Novel *dru* types have also been identified in a previous study (Kadlec et al., 2015). Although there were no significant differences in the distribution of the new *dru* types, the majority of the novel *dru* types were isolated from Nova Scotia (5/9), and from skin samples (5/9).

More than 70% of the isolates recovered in this study were resistant to ≥2 non-β-lactam antimicrobial classes, highlighting the clinical concerns regarding management of MRSP infections. Multi-drug resistance has been frequently reported in MRSP isolated from canine specimens (Moodley et al., 2014; Perreten et al., 2010). The isolates recovered in this study were from submissions to a regional diagnostic bacteriology laboratory that also is the diagnostic laboratory for the AVC-VTH, thus a selection bias is possible, based on submission of specimens from complicated referral cases from the AVC-VTH. However, most samples in this study were from primary care practices. Submissions to a diagnostic laboratory for bacterial culture and susceptibility testing can be from cases proving difficult to treat, with the primary reason being non-response to antimicrobial therapy. Information about prior antimicrobial exposure was not always available. There is a likelihood that diagnostic laboratory submissions are biased to be more resistant.

Interestingly, cluster 9a isolates had the highest proportion of MDR at 94.4%, and also had a significantly higher proportion of resistant isolates for each antimicrobial except fusidic acid, chloramphenicol, and doxycycline. Strong associations between cluster 9a and ST71 have been shown (Kadlec et al., 2015), and reports have also shown

ST71 to have increased antimicrobial resistance compared to other STs isolated (Osland et al., 2012; Perreten et al., 2010). MLST was not performed on the isolates in this study, but the cluster 9a isolates in this study likely belong to ST71. Although this study was not designed for prevalence estimation, the ST71 clone associated *dru* cluster 9a seems to have not disseminated into Atlantic Canada (18.1%) to the degree that it has in Europe (90%), the US (66%) and Ontario, Canada (47%) (Kadlec et al., 2015).

Resistance of the MRSP isolates to the non-β-lactam antimicrobials in this study was lower than in previous reports of multi-drug resistance in MRSP, which could be explained by the low prevalence of cluster 9a isolates. Caution, however, should be taken when comparing studies because of differences in testing methodology. Specifically, Weese *et al.* (2012) reported that 62% (n = 107) of study isolates were resistant to doxycycline, while in our study doxycycline resistance of isolates was 15% (n = 94). One study in Australia and another in the United Kingdom (UK) found similarly high tetracycline resistance at over 50% and 35%, respectively, compared to 14.9% doxycycline resistance in the current study (Maluping et al., 2014; Siak et al., 2014). Similar trends can be observed where the proportion of resistant isolates in Atlantic Canada is much lower than those reported in Australia and the UK. A recent study completed at a Texas (USA) Veterinary Medical Teaching hospital reported amikacin resistance in 36% of their MRSP isolates from dogs, whereas in our study amikacin resistance was not detected (Gold et al., 2014). A systematic literature review of antimicrobial resistance in MRSP isolates reported individual antimicrobial resistance ranging between 0 and 100%, with most studies reporting AMR estimates >50% for

most antimicrobials, except for chloramphenicol and amikacin (Moodley et al., 2014). Thus, it can be inferred that the MRSP isolates in Atlantic Canada are typically less resistant, specifically to doxycycline and amikacin, than their counterparts in some other regions, even though multi-drug resistance is still common. A possible explanation for this low-level resistance could be the low population density of the region as a whole, when compared to larger, more densely populated regions, which could mean less exposure to AMR organisms and less total antimicrobial use.

The overall picture of MRSP in Atlantic Canada is similar to reports elsewhere in Canada and the world. The distribution of the *dru* types reported in this study are similar to other reports from North America, however, the distribution is more similar to what was observed in the US versus Ontario, Canada. The International MRSP clone, ST71, which is disseminated throughout Europe, and made up almost half the isolates in Ontario, Canada, was less common in Atlantic Canada. This confirms that *dru* type distributions can vary significantly across the same country, and a larger, more comprehensive study of the *dru* types in Canada could help further clarify the dissemination of this pathogen. Multi-drug resistance in these isolates is common, especially within cluster 9a isolates, but resistance to the non-β-lactam antimicrobials is still considerably lower than has been previously reported.

Table 2.1. Oligonucleotide primers for species identification of coagulase-positive staphylococci using a multiplex PCR.

Species	Primer	Sequence (5'-3')	Product size (bp)
S. intermedius	in-F	CATGTCATATTATTGCGAATGA	430
	in-R3	AGGACCATCACCATTGACATATTGAAACC	
S. pseudintermedius	pse-F2	TRGGCAGTAGGATTCGTTAA	926
	pse-R5	CTTTTGTGCTYCMTTTTGG	
S. schleiferi	sch-F	AATGGCTACAATGATAATCACTAA	526
	sch-R	CATATCTGTCTTTCGGCGCG	

Table 2.2. Number (and percent) of antimicrobial resistant MRSP isolates overall and by *dru* cluster, following CLSI guidelines. Multidrug resistance (MDR) in each cluster is also reported.

Antimicrobial	Overall (n = 94)	9a (n = 18)	10h (n = 27)	11a (n = 45)	No cluster (n = 4)
Amikacin	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0%)
Chloramphenicol	23 (24.5%)	3 (16.7%)	0 (0%)	19 (42.2%)	1 (25%)
Clindamycin	54 (57.5%)	16 (88.9%)	7 (25.9%)	29 (64.4%)	2 (50%)
Doxycycline	14 (14.9%)	1 (5.6%)	7 (25.9%)	4 (8.9%)	2 (50%)
Erythromycin	63 (67.0%)	17 (94.4%)	11 (40.7%)	32 (71.1%)	3 (75%)
Enrofloxacin	46 (48.9%)	16 (88.9%)	0 (0%)	29 (64.4%)	1 (25%)
Fusidic Acid	2 (2.1%)	1 (5.6%)	1 (3.7%)	0 (0%)	0 (0%)
Gentamicin	34 (36.2%)	10 (55.6%)	2 (7.4%)	20 (44.4%)	2 (50%)
TMS	70 (74.5%)	18 (100%)	23 (85.2%)	27 (60.0%)	2 (50%)
MDR	66 (70.2%)	17 (94.4%)	15 (55.6%)	31 (68.9%)	3 (75%)

Table 2.3. Frequency of methicillin-resistant *S. pseudintermedius dru* types from 94 isolates collected at a regional diagnostic laboratory between 2010 and 2012. Novel *dru* types are marked with an asterisk (*).

dru type	Frequency (n = 94)	Cluster
dt11a	29 (30.9%)	11a
dt10h	23 (24.5%)	10h
dt9a	17 (18.1%)	9a
dt11af	10 (10.6%)	11a
dt10as	3 (3.2%)	10h
dt10cc*	1 (1.1%)	10h
dt10cj*	1 (1.1%)	11a
dt11bn	1 (1.1%)	11a
dt11ca*	1 (1.1%)	11a
dt11v	1 (1.1%)	11a
dt11y	1 (1.1%)	11a
dt5k*	1 (1.1%)	–
dt6t*	1 (1.1%)	–
dt8ag*	1 (1.1%)	–
dt9ba*	1 (1.1%)	9a
dt9bd*	1 (1.1%)	–
dt11cm*	1 (1.1%)	11a

Table 2.4. Number (and percent) of MRSP isolates in each province overall and by *dru* cluster.

	NB	NL	NS	PEI
Overall (n = 94)	23 (24.5%)	7 (7.4%)	52 (55.3%)	12 (12.8%)
9a (n = 18)	6 (33.3%)	5 (27.8%)	6 (33.3%)	1 (5.6%)
10h (n = 27)	5 (18.5%)	0 (0.0%)	15 (55.6%)	7 (25.9%)
11a (n = 45)	12 (26.7%)	2 (4.4%)	28 (62.2%)	3 (6.7%)
No cluster (n = 4)	0 (0.0%)	0 (0.0%)	3 (75.0%)	1 (25.0%)

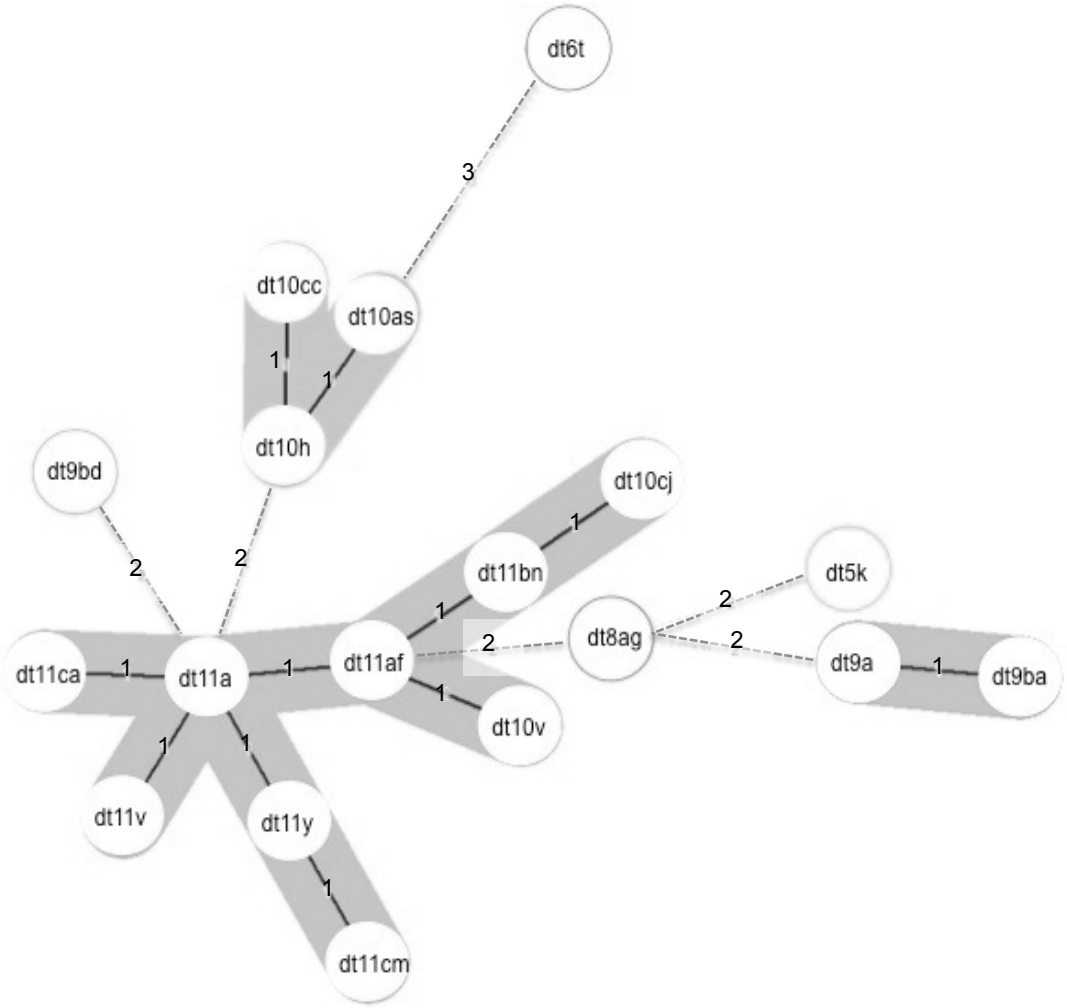

Figure 2.1. Minimum spanning tree (MST) of methicillin-resistant *S. pseudintermedius* in Atlantic Canada. Numerical values on the branches indicate the similarity (MST distance) between different *dru* types. *Dru* types separated by an MST distance of ≤2 (≥98.5% similar) were considered closely related and assigned to the same cluster.

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3.0 METHICILLIN-RESISTANT *STAPHYLOCOCCUS PSEUDINTERMEDIUS* AT A VETERINARY TEACHING HOSPITAL IN ATLANTIC CANADA.

Abstract

Methicillin-resistant coagulase-positive staphylococci are important causes of hospital-associated infections in human and veterinary hospitals. Recently, MRSP has emerged in dogs; its potential to cause hospital-associated infections should not be underestimated. Few studies have investigated MRSP contamination in veterinary hospital environments other than for a single report of a large MRSP outbreak in a Veterinary Teaching Hospital (VTH). An infection prevention and control program at a VTH in Atlantic Canada has focused its efforts on reducing the transmission and controlling the persistence of MRSP in patients and in the hospital environment. The objectives of this study were (i) to investigate the persistence of MRSP in the VTH environment after cleaning and disinfection of areas that were in contact with MRSP positive patients, (ii) to explore clonal relationships between MRSP recovered from the environment and MRSP positive patients associated with that area, and (iii) to investigate potential hospital-acquired MRSP infections in the VTH. Patient isolates were collected as clinical specimens and submitted to the diagnostic laboratory for routine testing. Hospital environmental isolates were collected using a Swiffer® cloth after cleaning and disinfection, and recovered using an enrichment broth technique. Most isolates were *dru* typed. Pulsed-field gel electrophoresis (PFGE) using *Sma*I digestion was performed to determine clonality among isolates with the same *dru* type. MRSP was isolated from 27 patients during the study period, while MRSP was isolated

from 16/137 (12% [95% CI 6.8%–18.3%]) of environmental samples after the first cleaning and disinfection cycle, and 1/14 (7% [95% CI 0.2%–36.0%]) of environmental samples after the second cleaning and disinfection cycle. Culture results and data retrieved from medical records implicated a hospital-acquired MRSP infection involving two canine patients. PFGE revealed that the MRSP isolates from these patients were clonal, further supporting a hospital-acquired infection. PFGE of strains cultured from the environment and patient revealed that environmental strains were clonal to those from the patient associated with that area; however, non-clonal strains were also recovered from the same environmental area. This work describes the role of MRSP in a veterinary hospital setting.

3.1 Introduction

Methicillin-resistant coagulase positive staphylococci are important causes of hospital-associated infections in human and veterinary hospitals. Healthcare-associated infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are a large burden in human medicine (Dantes et al., 2013; Nelson et al., 2015; Tadros et al., 2013; Zimlichman et al., 2013). Recently, MRSP, associated with infection or colonization, has emerged in dogs (Perreten et al., 2010; Weese and van Duijkeren, 2010). The risk of MRSP transmission between infected or colonized dogs and other animals or the household environment has been reported as high, but the risk of transmission between infected or colonized animals and humans is low (Laarhoven et al., 2011; van Duijkeren et al., 2011). This ease of transmission of MRSP between household pets highlights the potential of MRSP to cause veterinary hospital-associated infections (Perreten et al., 2010; Youn et al., 2011b).

There are a few published studies of MRSP contamination in veterinary hospital environments (Ishihara et al., 2010; Youn et al., 2011a, 2011b). In one Japanese study, 75 and 81 environmental swabs were collected on two sampling days; MRSP was identified in 13.3% of environmental samples on the first sampling day, but none on the second day (Ishihara et al., 2010). No associations between positive environmental samples and MRSP-positive animals were made in this study. In South Korea, samples were collected from hospitalized patients, veterinary personnel, and hospital environments in 8 veterinary hospitals. MRSP was identified in 5.7% (n = 157) of all hospital environment samples, with high variability noted in the isolation of MRSP

between hospitals in different regions (Youn et al., 2011b). In a second South Korean study, the genetic relatedness of the isolates obtained from the patients, the staff, and the environment was assessed using *spa* typing, MLST, and *Sma*I digestion in a pulsed-field gel electrophoresis (PFGE) analysis (Youn et al., 2011a). The PFGE banding patterns between *S. pseudintermedius* isolates were indistinguishable, suggesting potential contamination from infected animals (Youn et al., 2011a). In this study, it was not clear whether the isolates tested were methicillin-resistant or susceptible, but it does emphasize the potential of *S. pseudintermedius* contamination of the hospital environment from infected or colonized patients. In neither of these studies was it specified whether environmental surfaces had been cleaned and/or disinfected prior to sample collection.

Recently, a hospital outbreak of MRSP sequence type (ST) 71 spanning from November 2010 to January 2012 was described at the Veterinary Teaching Hospital of the University of Helsinki (Grönthal et al., 2014). During the outbreak period, a total of 63 cases with the outbreak strain were identified in infected (43%) and colonized (57%) patients. Infections in patients were related to hospital practices including surgical site infections and complications associated with prolonged hospital stays. The case strains were identified to be clonal using MLST, SCC*mec* typing and *Sma*I digested PFGE analysis. Environmental surface samples were collected post-cleaning and disinfection from 65 high-touch areas, and only 1 sample was MRSP-positive with an antibiogram different from the outbreak strain. Although a common source for MRSP was not

identified, it was suggested that patient-to-patient transmission was the likely route of spread (Grönthal et al., 2014).

MRSP is the most commonly isolated methicillin-resistant coagulase positive *Staphylococcus* spp. at the Atlantic Veterinary College Veterinary Teaching Hospital (AVC-VTH), the primary veterinary referral hospital for Atlantic Canada. The objectives for this study were threefold: (i) to investigate the persistence of MRSP in the VTH environment after cleaning and disinfection of areas that were in contact with MRSP positive patients, (ii) to explore clonal relationships between MRSP recovered from the environment and MRSP positive patients associated with that area, and (iii) to investigate potential hospital-acquired MRSP infections in the VTH.

3.2 Materials and Methods

3.2.1 Patient selection

Patients admitted to the Veterinary Teaching Hospital of the Atlantic Veterinary College (AVC-VTH) at the University of Prince Edward Island, the regional tertiary care and referral veterinary hospital for Atlantic Canada were eligible for enrolment. The Small Animal Hospital admits approximately 4,200 dogs per year. All dogs with MRSP cultured from clinical samples between January 2010 and December 2012 were enrolled in this study.

3.2.2 Sample Collection and Isolate Identification

Patient specimens were sent to the AVC Diagnostic Services Bacteriology Laboratory for routine culture and susceptibility testing. A positive MRSP patient in the AVC-VTH initiated an infection control response. All areas in contact with the patient were closed or restricted, thoroughly cleaned with soap and water, and disinfected with two applications of either potassium monopersulfate (Virkon®) or accelerated hydrogen peroxide (AHP, Peroxigard™) as per the manufacturer's guidelines for contact time. Areas were cleaned and disinfected within 24 hours of the area being closed, and the area remained closed until culture results were finalized. Once cleaned areas were dry, environmental specimens were collected using a sterile Swiffer® cloth. If an area tested positive for MRSP, an additional cleaning and disinfection cycle was performed, and an additional environmental sample was collected for culture. This was repeated until each area no longer tested positive for MRSP.

Samples from floors or runs were collected using the Swiffer® Sweeper (The Procter & Gamble Company, Toronto, Canada), which was cleaned and disinfected after each use. Cages, examination/surgical/treatment tables, and other hand-touch surfaces were sampled by hand wearing a clean exam glove that was changed after a specific area was sampled. Areas were selected for sampling based on their likelihood for exposure from the MRSP positive patient. Each cloth was placed into a sterile sampling bag and processed by the laboratory within 30 minutes of collection. In the laboratory, 100 mL of buffered peptone water (BPW) was added to the cloth in the bag. The bag was manually mixed for one minute ensuring the entire cloth was wet, and then placed

into a Seward® 400 Circulator for 30 seconds at 125 rpm. Two mL of the BPW from the sample was added to 2 mL of double-strength staphylococcal enrichment broth (containing 1% tryptone, 7.5% sodium chloride, 1% mannitol, and 2.5% yeast per 500 mL of water), resulting in a single-strength solution. The enrichment broth was incubated aerobically at 35ºC for 18-24 h. Using a sterile swab, an inoculum of the enrichment broth was plated to phenylethyl alcohol agar supplemented with 5% defibrinated sheep blood, and the plate was incubated aerobically at 35ºC for 18-24 h.

Staphylococcal isolates were identified using conventional and molecular methods, and *dru* typed as described in Chapter 2 of this thesis. Suspect hospital-acquired infections were investigated if the patients had no signs of MRSP infection at the time of admission, were hospitalized for at least 48 h and were epidemiologically related. Epidemiologically related patients were defined by one or more of the following criteria: patients were in the VTH at the same time, had exposure to the same contaminated areas, and/or had contact with the same hospital personnel.

3.2.3 Pulsed-field gel electrophoresis

A modified version of the Canadian standardized protocol developed for sub-typing methicillin-resistant *S. aureus* was used in this study (Mulvey et al., 2001). One colony was collected from a fresh culture plate into 3.0 mL brain heart infusion broth, and incubated aerobically at 35ºC for 18-24 h. A 1.5 mL aliquot of the incubated broth was centrifuged at 12100 x g, and the pellet was resuspended and washed with PIV buffer (0.1 M Tris HCl and 1 M NaCl in dH_2O). The sample was centrifuged for 2 min

at 12100 x g, and the remaining supernatant was discarded. The pellet was resuspended in 750 µL of PIV buffer, and 300 µL was transferred to a clean microcentrifuge tube. A 2% low-melting temperature agarose solution was prepared, and 300 µL was added to each sample. Plugs were prepared by adding 100 µL of the agarose suspension into the Bio-Rad CHEF disposable plug mold (Bio-Rad Laboratories, Montréal, Canada).

Clean tubes were prepared with 2 mL EC lysis buffer (6 mM Tris HCl, 1 M NaCl, 0.1 M EDTA, 0.5 mM deoxycholate, 17 mM Sarkosyl in distilled H_2O), 10 µL lysostaphin (1 mg/mL) and 50 µL lysozyme (10 mg/mL). Plug molds were added to their own tube, and incubated in a water bath for 4 h at 37ºC shaking at 50 rpm. The lysis buffer and enzyme solutions were removed, the plugs washed with 0.5 mL PK buffer (0.48 M EDTA, 1.7 mM Sarkosyl), 2 mL PK buffer and 40 µL proteinase K were added, and the plugs were incubated overnight at 50ºC. The following day, the PK buffer was removed and the plugs were incubated in 2mL TE buffer (0.8 M Tris HCl and 8 M EDTA) at 50ºC for 3.5 h. The TE buffer was replaced with fresh TE buffer, and incubated for an additional hour, and repeated an additional time. The TE buffer was removed and plugs were washed in 200 µL 1X NE Buffer (New England BioLabs) for 30 minutes at room temperature. For restriction enzyme digestion, wash buffer was removed and a 200 µL solution containing 10% 10X NE Buffer and 1000 units of *Sma*I restriction enzyme were added to each tube and tubes were incubated overnight at 25ºC.

The following day, the enzyme solution was removed, and the tubes were placed and held at 65ºC to melt agarose plugs. A 1% PFGE agarose gel was prepared and 30

µL of each melted plug was added to the poured gel. After all the samples had solidified, the gel was placed into the buffer chamber. Electrophoresis was performed using the CHEF DR-II apparatus (Bio-Rad Laboratories, Montréal, Canada) using switch times of 2 s to 5 s for 24 h at 5.6 V/cm and 14ºC in 0.5X TBE. The gel was stained for 45 minutes in ethidium bromide, and was destained for at least 30 minutes in fresh dH_2O. Samples were visualized using UV light and images generated using the VersaDoc™ imaging system (Bio-Rad Laboratories, Montréal, Canada). PFGE analysis was performed manually comparing banding patterns.

3.3 Results

MRSP was isolated from 27 patients during the study period: 59.3% from surgical site infections, 33.3% from skin or ear disease, and 7.4% from urinary tract infections. Environmental surveillance was performed after the conclusion of each visit or hospitalization by the 27 patients. In total, 151 hospital environment samples were collected, where each sample represents a single Swiffer® cloth used on either a floor or surface. After the first cleaning and disinfection cycle, 16/137 (11.7% [95% CI 6.8%–18.3%]) of samples were positive for MRSP representing areas in contact with 11 MRSP culture positive patients. After the second cleaning and disinfection cycle, MRSP was isolated from 1/14 (7.1% [95% CI 0.2%–36.0%]) of samples. All environmental samples were negative after the third cleaning and disinfection cycle. Two areas were lost to follow up, and were not resampled after a second cleaning and disinfection cycle. Six of the 16 environmental isolates collected were available for *dru*

typing, and the same *dru* types were found for all five environmental isolates that had a corresponding typed animal isolate (Table 3.1).

A hospital acquired infection was suspected to have occurred through spread from one patient (patient A) seen by the surgical service to another patient (patient B) seen by the same service and housed in an adjacent run. Patient A was from Nova Scotia, while Patient B was from Newfoundland and Labrador. Patient A and B had culture samples submitted on June 22, 2011 from infected surgical sites. The sample from Patient A was MRSP positive, while the sample from Patient B was MRSP negative. On July 2, 2011, a second sample was submitted from Patient B because the surgical site infection was not responding to treatment with antimicrobials. This second sample was MRSP positive. Strain typing revealed the isolates from both patients were *dru* type (*dt*)9a. Environmental samples collected from facilities after cleaning associated with these patients were negative for MRSP.

PFGE analysis was performed on environmental samples (n = 5) and on isolates from patients (n = 3) associated with these environments, as well as the two patient isolates involved in the potential hospital acquired infection (n = 2). Not all isolates were available for testing. Indistinguishable banding patterns were observed between 3 environmental strains and their associated patient strains (Figure 3.1, lanes 1,3, lanes 6, 7, lanes 8,10), while 2 environmental strains were different from the associated patient strains (Figure 3.1, lanes 1,2 and lanes 8,9). Indistinguishable banding patterns were

also observed between the two patient isolates involved in the potential hospital-acquired infection indicating that these strains were clonal (Figure 3.1, lanes 4,5).

3.4 Discussion

The presence of MRSP strains isolated from the hospital environment after cleaning and disinfection that are clonal to MRSP strains isolated from patients associated with that area highlights the potential role the environment plays in MRSP transmission. MRSP was detected in 12% (95% CI 6.8%–18.3%) of environmental samples after the first round of cleaning and disinfection, with one sample remaining positive after two rounds of cleaning and disinfection. PFGE analysis of the MRSP isolates in the present study demonstrated that the isolates recovered from the hospital environment were clonal with the isolates recovered from patients associated with that area, as confirmed by indistinguishable banding patterns. This implied that patients were contaminating the hospital environment, and highlights the importance of mitigation strategies to prevent persistence and spread of MRSP in hospital environments. However, in this study some MRSP isolates from the hospital environment were distinct from those recovered from patients associated with that area. The recovery of non-clonal environmental MRSP strains indicates that there were undetected sources of contamination in the hospital. This contamination could have been attributable to MRSP colonized patients or patients with MRSP infections that were not culture confirmed entering the AVC-VTH.

A limitation of this study was the lack of information on patients that might have been colonized with MRSP admitted to the AVC-VTH. The purpose of this study, along with the policies and procedures in place at this institution, focused on controlling the spread of MRSP from clinically infected patients. The prevalence of colonization by MRSP in dogs has been well documented, with estimates ranging 0-7% (Davis et al., 2014; Gómez-Sanz et al., 2011; Hanselman et al., 2008; Morris et al., 2010; Mouney et al., 2013; Rubin and Chirino-Trejo, 2011; Vengust et al., 2006) in most studies, and as high as 45% in one Thai study (Chanchaithong et al., 2014). Colonized animals may contribute to the bacterial burden of MRSP in hospital environments; therefore, the prevalence of patients colonized with MRSP at the AVC-VTH needs to be further explored. In an outbreak of MRSP infections at the University of Helsinki VTH, the majority of cases were patients colonized with the outbreak strain, and in some cases colonization preceded infection which could suggest that MRSP colonization is a risk-factor for developing an MRSP infection (Grönthal et al., 2014). A recent practice at the AVC-VTH requires collection of oral and perianal swabs from patients potentially exposed to MRSP during hospitalization through contact with infected patients, environments or hospital personnel who have handled known MRSP culture positive patients.

There was evidence to support spread of infection from patient A to patient B during hospitalization. The isolate from both patients was *dt*9a. In addition the isolates had indistinguishable banding patterns on PFGE. The patients were epidemiologically related in the hospital, both being surgical patients during the same time period and

housed side-by-side in the wards. Outside the hospital environment the patients were unrelated since they originated from different referring veterinarians and provinces. MRSP colonization was not tested for at the time of admission, therefore the MRSP status of these patients prior to hospital admission was unknown. Colonization of Patient B with the same MRSP strain as Patient A prior to hospital admission would have been unlikely for reasons already stated, further suggesting that Patient B acquired the MRSP strain from Patient A, directly or indirectly during hospitalization.

Because samples were not collected from the hospital environment before cleaning and disinfection, transmission from the hospital environment to Patient B could not be explored. In the report of a larger outbreak at a Finnish Veterinary Teaching Hospital, no common environmental source of MRSP could be established, implying patient-to-patient transmission via hospital personnel (Grönthal et al., 2014). Two studies conducted at the Ontario Veterinary College investigated the contamination of hospital personnel clothing (Singh et al., 2013) and cellular phones (Julian et al., 2012). MRSP was isolated from 14% of clothing samples (n = 114), and 1.6% of cellular phones tested (n = 123) (Julian et al., 2012; Singh et al., 2013). The authors of those studies concluded that clothing and cellular phones of hospital personnel can serve as fomites for hospital-acquired infections. It is clear that personnel have the potential to play a role in the transmission of MRSP in a hospital setting, and it is possible that hospital personnel were involved in the transmission of MRSP in our study.

There have been no reports of resistance of MRSP to either Virkon® or accelerated hydrogen peroxide (AHP) products, such as Peroxigard™. One previous study determined the minimum inhibitory concentrations (MICs) of topical biocides, including AHP, for methicillin-susceptible *S. pseudintermedius* (MSSP) and MRSP clinical isolates from dogs. The study reported no difference in the MICs of MRSP or MSSP, and noted that the MICs for AHP were more than 1000-fold lower than concentrations used in practice (Valentine et al., 2012). This suggests that the environmental persistence of MRSP isolates in this study was not because of resistance to the disinfectants, but because of imperfect cleaning and disinfection practices in contaminated areas or because of recontamination after successful disinfection. Although individuals performed cleaning and disinfection and environmental sample collection were not the same person, there was communication to confirm that areas environmentally cultured were the areas that were previously cleaned and disinfected. Although staff was instructed on how to carry out the cleaning and disinfection, they were not monitored to verify that they had appropriately performed the task.

The results of this study have been used to update current policies and to develop new policies for dealing with possible or known MRSP colonized or infected patients at the AVC-VTH. Identifying patients at high-risk for MRSP infection has become the foundation of the infection prevention and control program. Medical records of patients with a previous history of MRSP are now flagged to ensure early recognition and appropriate handling of these patients in the hospital. Clinical personnel in the AVC-VTH have been trained to recognize factors in patients that are likely to be associated

with MRSP infection, such as lack of response to antimicrobial therapy for any infection, patients presenting with chronic skin disease or patients that develop surgical site infections. During follow up environmental sampling in this study, it was noted that a single patient contacted numerous areas in the hospital, more than was necessary for appropriate case management. This resulted in a large number of areas being quarantined until cleaning and disinfection and re-sampling could be concluded affecting hospital operation. To decrease the number of areas closed for cleaning or disinfection after a MRSP positive patient is identified, the movement of patients at high-risk for MRSP or positive for MRSP in the AVC-VTH is now restricted and barrier precautions on surfaces and personnel are used. A rapid point-of-care test, or more sensitive culture-based method for detecting MRSP in clinical specimens could be explored as a mechanism to initiate a quicker infection control response by earlier presumptive identification of MRSP.

This work describes the importance of MRSP in a veterinary hospital setting and supports the implementation of an infection prevention and control program in veterinary clinics. The persistence of MRSP in the hospital environment post-cleaning and disinfection is concerning, implying that veterinary clinics should review infection control practices and periodically monitor staff responsible for implementation. Further work to investigate MRSP colonization in patients and hospital personnel is necessary to clarify epidemiology of this organism in a hospital environment. An enhanced surveillance program that monitors patient and personnel colonization with MRSP,

along with current surveillance practices, could be used to determine quantitative risk of hospital-acquired MRSP infections in canine patients.

Table 3.1. MRSP isolates collected from the hospital environment and their associated patient strains.

Isolate ID	Environment *dru* type	Area	Source	Associated Patient	Patient *dru* type
2010-004	–	Clinic lab	Surface	C22358-2010	9a
2010-007	–	Clinic lab	Floor	C29756-2010	10h
2010-008*	–	Clinic lab	Floor	C29756-2010	10h
2011-005a	–	Isolation	Surfaces	C12045-2011	9a
2011-005b	–	Quarantine	Cage	C12045-2011	9a
2011-012a	10h	Surgery	Floor	C18120-2011	10h
2011-012b	–	Treatment	Surfaces	C18120-2011	10h
2011-021	10as	Exam	Floor	Dermatology	10as
2012-003	–	ICU	Cage	C34095-2011	11a
2012-005	9a	ICU	Sink	C481-2012	9a
2012-015	9a	Treatment	Floor	C5487-2012	9a
2012-016	11b	Exam	Floor	2012-016-PC	–
2012-021	–	Wards	Run	C5487-2012	9a
2012-024	10h	Surgery	Surfaces	C11733-2012	10h
2012-041a	–	Wards	Cage	C30531-2012	11v
2012-041b	–	Bandage change	Floors	C30531-2012	11v
2012-041c	–	Bandage change	Surfaces	C30531-2012	11v

*Isolated after second cleaning and disinfection cycle.

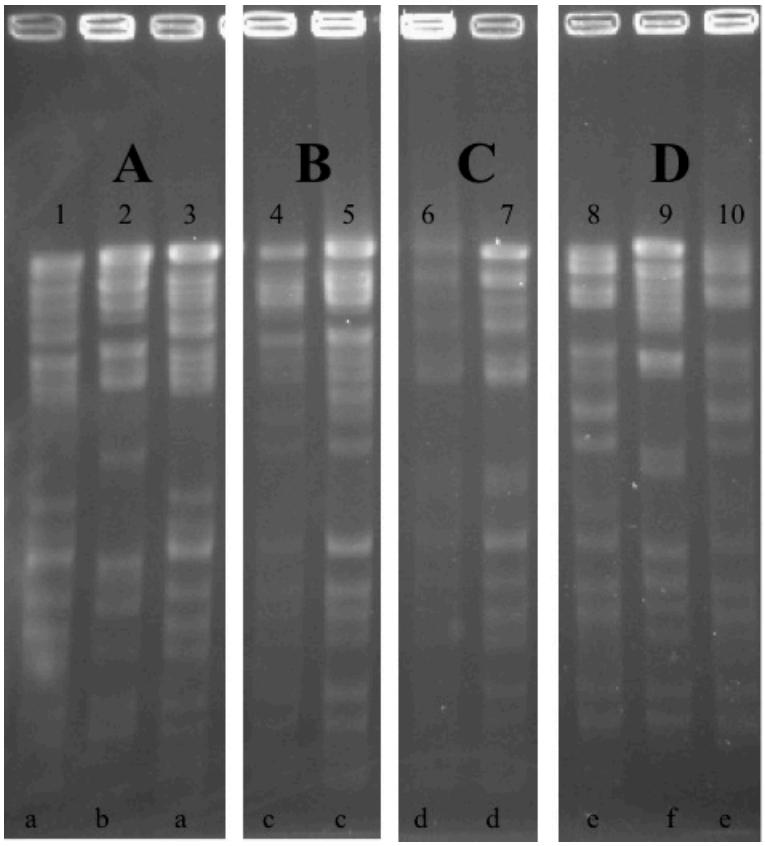

Figure 3.1. Results from pulsed-field gel electrophoresis using *Sma*I DNA restriction on a gel stained in ethidium bromide, where similar PFGE patterns are identified by lower-case Latin characters. (A) Lane 1: patient C18120-2011, Lane 2: treatment room surfaces, Lane 3: surgery floors. (B) Lane 4: patient A, Lane 5: patient B. (C) Lane 6: patient C11733-2012, Lane 7: surgery surfaces. (D) Lane 8: patient C5487-2012, Lane 9: ward run, Lane 10: treatment floor.

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4.0 COMPARING CULTURE METHODOLOGY FOR THE DETECTION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS PSEUDINTERMEDIUS* IN CLINICAL SPECIMENS COLLECTED FROM DOGS

Abstract

MRSP has emerged as a major pathogen in dogs and has been implicated as a hospital-acquired pathogen in veterinary hospitals. The accurate and rapid detection and identification of MRSP is essential for delivering effective antimicrobial therapy and for the appropriate implementation of infection control measures. The objectives of this study were (1) to determine if selective culture methods will detect more MRSP when compared to the traditional culture methods, (2) to evaluate use of a selective culture medium as a rapid culture-based method for the detection and/or presumptive identification of MRSP in clinical specimens, and (3) to estimate the prevalence of MRSP in clinical samples from dogs in Atlantic Canada with a high risk for MRSP infection. Study samples were submitted to the AVC Diagnostic Bacteriology Laboratory for routine culture and susceptibility testing. Dogs considered being at high risk for MRSP infection (specimens from skin, ears, surgical sites, and wounds), or dogs with a history of previous infection with MRSP and/or unsuccessful antimicrobial treatment, were included in the study. Each study sample was tested using four culture methods: traditional culture, mannitol salt agar with 2µg/mL oxacillin (MSAox), mannitol salt enrichment broth (EB) and MSAox, and EB and traditional culture. A total of 741 samples were analyzed from 556 individual dogs between February 2013 and April 2014. The prevalence of MRSP in samples detected by any method was

estimated at 13.4% (95% CI 11.1%–16.0%). When prevalences of MRSP were determined according to each culture method, EB and MSAox had the highest prevalence (11.2% [9.1%–13.7%]), followed by EB and traditional (10.8% [8.8%–13.2%]), then traditional (10.1% [8.1%–12.5%]), with MSAox having the lowest prevalence (8.9% [7.1%–11.2%]). The prevalence using the traditional culture method did not differ significantly from any of the three selective culture methods. Culture with MSAox detected significantly fewer MRSP than either of the enrichment broth methods. The addition of enrichment broth to current methodology is recommended, particularly in patients with a previous history of MRSP positive specimens. The MSAox had a low sensitivity for the detection of MRSP at 24 and 48 hours, and is not recommended as a rapid culture-based screening tool.

4.1 Introduction

Clinical microbiology laboratories play a vital role in the management of clinically diseased animals, through providing valid and reliable results in a timely manner. The accurate and rapid detection and identification of methicillin-resistant MRSP is essential for delivering effective antimicrobial therapy and the appropriate implementation of infection control measures (Kali et al., 2014). *S. pseudintermedius* is the organism most frequently isolated from clinical specimens collected from dogs. Recently MRSP has emerged as a major pathogen in dogs primarily associated with skin, surgical site, and wound infections (Bannoehr and Guardabassi, 2012; Perreten et al., 2010; Weese and van Duijkeren, 2010). The potential for MRSP to be involved in veterinary hospital-acquired infections has been reported, and its epidemiology in hospital environments continues to be investigated (Grönthal et al., 2014; Perreten et al., 2010; Youn et al., 2011).

The prevalence of MRSP in subclinically affected (colonized/carrier patients) and in clinical specimens has been well documented. In clinically normal animals, prevalence estimates of MRSP range from 0-5% except for one study reporting a 45% prevalence (Chanchaithong et al., 2014; Gharsa et al., 2013; Gingrich et al., 2011; Mouney et al., 2013; Rubin and Chirino-Trejo, 2011; Wedley et al., 2014). Reports of MRSP prevalence in dogs with clinical infections have had a wider spread ranging from 0-41% (Bardiau et al., 2013; Beck et al., 2012; Detwiler et al., 2013; Garbacz et al., 2013; Hariharan et al., 2014; LoPinto et al., 2014; Maluping et al., 2014; Siak et al., 2014; Wang et al., 2012; Windahl et al., 2012). It should be noted, that direct

comparisons of prevalence rates between studies are difficult because of differences in culture methodology. Specifically, some studies used a selective staphylococcal enrichment broth or solid medium with or without oxacillin, the recommended surrogate for methicillin resistance testing in *S. pseudintermedius* (CLSI, 2015). Enrichment broths have been used in studies investigating subclinical carriage because they have been shown to increase the sensitivity of screening (Brown et al., 2005). Differences between selective and non-selective culture methods for the recovery of MRSP from clinical specimens in dogs have not been reported.

Selective culture methodologies have the potential to be beneficial as rapid screening tools for the identification of MRSP in clinical specimens. Selective culture media would allow presumptive identification of MRSP as early as 24 hours after receipt of a specimen, compared to at least 48 or 72 h using traditional culture methods. Molecular assays have been designed for rapid detection for *mec*A, which is considered the gold standard for methicillin-resistance testing (Brown et al., 2005; Kali et al., 2014). However, these assays do not differentiate between staphylococcal species (e.g. MRSP versus methicillin-resistant *S. aureus* [MRSA]) and are not practical in smaller diagnostic laboratories. A culture-based assay for the rapid detection of MRSP in clinical specimens would be easy to implement and use in most veterinary diagnostic laboratories.

Diagnostic test characteristics of selective culture methods for MRSA testing in humans have been investigated (Denys et al., 2013), but have not been explored in

veterinary medicine. Numerous commercial media are available for detecting MRSA in humans, but none are commercially available for MRSP detection in dogs. One study compared 6 commercially available MRSA selective media for the detection and isolation of MRSP. The authors of this study concluded that the oxacillin resistance screening agar base (ORSAB; Oxoid Company, Nepean, Canada) or Brilliance MRSA agar (Oxoid Limited, Basingstoke, UK) could be used routinely for culture of diagnostic samples obtained from canine patients (Horstmann et al., 2012). However, this study used stored isolates of MRSP, a situation that may not be representative of clinical sample testing. The ORSAB medium is a modified mannitol salt agar with 2 µg/ml of oxacillin and 50,000 IU/L of polymyxin B ("Oxoid Canada - Product Detail," n.d.). Mannitol salt agar with 2µg/mL oxacillin (MSAox) was chosen as the selective medium in this study because of the performance of the similar ORSAB in the previous study (Horstmann et al., 2012), its use in previous studies investigating MRSP in dogs, and the ease and the low cost of preparing the media. The objectives of this study were (1) to compare different culture methods for detection of MRSP, (2) to evaluate the utility of a selective culture medium for rapid culture-based detection and/or presumptive identification of MRSP in clinical specimens, and (3) to estimate the prevalence of MRSP in clinical samples obtained from canine patients in Atlantic Canada deemed at higher risk for MRSP infections.

4.2 Materials and Methods

4.2.1 Study population

Samples analyzed in this study were submitted to the AVC Diagnostic Services Bacteriology Laboratory for routine culture and susceptibility testing. Specimens from dogs at higher risk of having MRSP infection were included in the study. Specimens defined as being high risk for MRSP infections included samples collected from abscesses, surgical site infections (SSI), wounds or the ears, nose, eyes, and skin of animals. Any canine patient with a previous history of MRSP was also included in this study, regardless of specimen site.

4.2.2 Bacteriologic Culture

A single swab (sample) was analyzed using four culture methods, in the following order: (i) traditional culture, (ii) MSAox, (iii) mannitol salt enrichment broth (EB) with MSAox, and (iv) EB with traditional culture. The EB contained 1% tryptone, 7.5% NaCl, 1% mannitol, and 2.5% yeast per liter of water. Traditional cultures were performed by the AVC Diagnostic Services Bacteriology Laboratory and involved plating the sample to Columbia agar with 5% sheep blood (BA), MacConkey agar (MAC), and phenylethyl alcohol agar with 5% sheep blood (PEA). For samples collected from abscesses, SSIs, and wounds a thioglyocolate enrichment broth and smear for Gram staining was also included. The latter three culture methods were conducted in a research laboratory on the same day. Samples were plated to the MSAox, and placed into the EB. After 24 h incubation at 35ºC, 10 µL of EB was plated to both the MSAox and PEA. All agar plates were incubated for 48 hours and growth

was checked at 24 hours. Any growth on MSAox was purified on PEA. Isolates with hemolysis characteristic for MRSP were further identified with tube coagulase and mannitol fermentation. All tube coagulase positive staphylococci were confirmed to species level by a multiplex PCR reaction, as described in Chapter 2. Methicillin-resistance was detected by antimicrobial susceptibility to oxacillin (1ug) by disk diffusion (CLSI, 2015) and confirmed by detecting the penicillin-binding protein 2' (PBP2') by latex agglutination (PBP2' Latex Agglutination Test Kit; Oxoid Company, Nepean, Canada).

4.2.3 Statistical analyses

The data structure was hierarchical, where individual samples were clustered by patients. The apparent prevalence of MRSP was estimated at sample- and patient-levels for each of the testing methods. At the sample-level, MRSP detected by any one of the four culture methods was classified as an MRSP positive sample. This sample-level result was used as the overall MRSP result (i.e. the reference standard) in further analyses. At the patient level, a patient was considered MRSP positive if any one of the samples submitted over the study period was positive for MRSP using any of the four culture methods. Descriptive statistics were applied to describe sample-level factors, such as specimen source, and patient-level factors, including age, breed, sex, province of residence, and previous antimicrobial therapy. Patient information on the laboratory requisition form was recorded. Not all fields on the requisition form were completed by all veterinarians submitting samples. Breeds were categorized using the Canadian Kennel Club Guidelines ("CKC Breed Standards," n.d.).

Logistic generalized estimating equations (GEE) were used to estimate the sensitivity and specificity of each of the culture methods (model 1). The overall MRSP result was used as the reference standard for estimating diagnostic test characteristics. Model 1 was restricted to true positive or true negative samples for sensitivity and specificity, respectively, based on the reference standard. Similarly, logistic GEE were used to determine discordance between the culture methods (model 2). Model 2 included only samples with disagreement between two test methods. In this model, the intercept represented the log-odds of the proportion of one disagreement scenario among all samples with test results that disagreed. Based on this model, it was tested whether the probability of one disagreement scenario was equal to 50%, or equivalently that the two tests had equal apparent prevalence. Significant discordance was detected if $p \leq 0.05$, corresponding to systematic overrepresentation of one disagreement scenario over the other. Both GEE models included clustering on patients. The effect of test order was investigated by evaluating the sequence of positive results obtained. For all culture methods, the sensitivity was estimated including MRSP confirmation steps, while specificity was not estimated because confirmed samples cannot have a false positive result. To evaluate selective culture methods as a rapid screening tool, the sensitivity and specificity was estimated for MSAox and EB with MSAox at 24 h and 48 h based on growth or no growth without confirmatory steps. The true prevalence of MRSP in all diagnostic submissions from dogs was estimated by adjusting the apparent prevalence obtained by the traditional method using the sensitivity of the traditional method relative to the four test methods (equation 5.7 in Dohoo et al. 2009.) Confidence intervals for the

estimate of true prevalence were calculated using equation 4 and 5 as described by Lang and Reiczigel (2014). All statistical computations were performed in Stata/IC 13.1 for Mac (StataCorp, College Station, Texas).

4.3 Results

4.3.1 Study population

A total of 741 samples from 556 individual dogs were collected between February 2013 and April 2014. The majority of samples were from ears (48.9%), followed by skin (21.7%), surgical sites (10.4%), wounds (8.4%), abscesses (7.4%), and other body sites (3.2%). Other body sites included anal glands (2), eyes (4), nose (12), peritoneal cavity (1), urine (1), uterus (1), and vagina (1). The specimens from other body sites were included and considered high risk for being MRSP positive because of a history of previous MRSP infection or antimicrobial treatment failure.

The median age of dogs in the study was 6.7 years with a range of 0.13 to 15.8 years. Age for 40 dogs was not recorded on the submission form. The study samples included 82 breeds that were then subdivided into eight breed categories. Breed was not recorded for 30 dogs. The study samples included 300 male patients and 242 female patients; sex was not recorded for 14 patients. The majority of patients visited veterinary clinics in Nova Scotia (256), followed by New Brunswick (136), Prince Edward Island (105), Newfoundland and Labrador (57), and Ontario (2). The distribution of MRSP across ages, breeds, sex, and provinces is presented in Table 4.1.

4.3.2 Isolation of MRSP

MRSP was detected in 13.4% (95% CI 11.1%–16.0%) samples, while 12.3% (95% CI 9.6%–15.2%) patients were MRSP positive. SSI submissions had the highest prevalence of MRSP (33.8%), followed by skin (22.4%), wounds (14.5%), ears (7.2%), other (4.2%), and abscesses (1.8%). Differences in prevalence estimates between testing methods were observed, and the prevalence estimates for each testing method at the sample-level are presented in Table 4.2. Evaluation of discordance between testing methods revealed significant differences in the recovery of MRSP between MSAox and EB with MSAox (p = 0.003) and MSAox and EB with the traditional method (p = 0.015) (Table 4.3). MRSP was not detected in 24 samples (41.7% ears, 37.5% skin, 8.3% surgical, 8.3% wounds, and 4.2% nasal flush) using the traditional culture method: 16 samples had methicillin-susceptible *S. pseudintermedius*, 5 samples had non-staphylococci, while 3 samples had no microbial growth. MRSP was not detected in 16 samples (37.5% ears, 18.8% skin, 31.3% surgical, 6.2% wound, and 6.2% other) using the EB and MSAox, and in 6 of those samples MRSP growth was scant to light on traditional culture.

During the study period, urine specimens accounted for 49% and fecal specimens accounted for <1% of canine sample submissions to this laboratory. Most urine specimens (64.8%) yielded no microbial growth, while urine specimens that were culture positive grew mainly *E. coli* (43.6%). *S. pseudintermedius* was isolated from 20.06% of culture-positive urine specimens using the traditional culture methods, while

MRSP was isolated from 1.4% of those specimens based on traditional culture methods. MRSP was not isolated from fecal specimens during the study period.

4.3.3 Diagnostic Test Characteristics

The diagnostic sensitivities of each test method, including the confirmatory steps, are presented in Table 4.2. The test methods that included enrichment broth had the highest sensitivities, while the test method with only the selective medium, MSAox, had the lowest sensitivity. The diagnostic sensitivity and specificity of MSAox and EB with MSAox as rapid screening tools at 24 h and 48 h are presented in Table 4.4. The EB with MSAox at both time points had the highest sensitivity and lowest specificity, while MSAox at both time points had the lowest sensitivity and highest specificity. For MSAox and EB with MSAox, an additional 24 hours of incubation increased the sensitivity but decreased the specificity.

4.3.4 Apparent Prevalence of MRSP

During the study period, a total of 2037 canine samples were submitted to the AVC Diagnostic Laboratory for testing. These samples included samples from canine patients not considered to be at high risk for MRSP. MRSP was isolated from 1.0% (n = 1296) of these samples. The traditional method detected 75 (n = 741) MRSP positive study samples and 13 (n = 1296) MRSP positive samples not included in the study. The estimated true prevalence of MRSP in all diagnostic submissions from dogs is 5.8% (95%CI 4.6%–7.0%).

4.4 Discussion

MRSP was detected in a total of 13.4% (n = 741) of high-risk clinical samples from dogs submitted to the AVC Diagnostic Services Bacteriology Laboratory during the time period of this study. In the study, samples were submitted from 556 individual dogs; 12.3% of the dogs had at least one MRSP positive sample. The estimated true prevalence of all diagnostic submissions from dogs at this laboratory was 5.8% (n = 2037). This is the first study investigating the prevalence of MRSP in Atlantic Canada. The prevalence estimates in this study are similar to previous reports of prevalence in samples from clinically ill dogs (Bardiau et al., 2013; Beck et al., 2012; Detwiler et al., 2013; Garbacz et al., 2013; Hariharan et al., 2014; LoPinto et al., 2014; Maluping et al., 2014; Siak et al., 2014; Wang et al., 2012; Windahl et al., 2012). The cumulative prevalence in this study may have biased this comparison, as most studies in the literature are point-prevalence studies. The estimates of MRSP prevalence reported here likely overestimate the prevalence of MRSP in canine submissions at this laboratory as certain sample types considered low risk were excluded, however those sample types had a low prevalence of MRSP.

MRSP has been reported to be the leading cause of surgical site infections and skin disease. This trend was supported by the current study, which was in agreement with previously reported estimates of 33.8% and 22.4%, respectively (Perreten et al., 2010). In a recent publication by the Ontario Veterinary College Health Sciences Centre, MRSP was isolated the most frequently from SSIs (47.4%, n = 26) (Turk et al., 2015). A high prevalence of methicillin-susceptible *S. pseudintermedius* in SSIs was

reported in a Swedish study, with only 4 of those isolates being methicillin-resistant (Windahl et al., 2015). Many studies have investigated the prevalence of MRSP in dogs with skin disease, with prevalence estimates ranging from 0% – 40.5% (Bardiau et al., 2013; Beck et al., 2012; Hariharan et al., 2014; Siak et al., 2014; Wang et al., 2012). The study reporting the highest prevalence of 40.5% involved dogs being evaluated by a dermatology service, which could explain this high prevalence (Beck et al., 2012).

Differences in the prevalence estimates between testing methods was observed. Although the discordance between the traditional method and any other culture method was not statistically significant, both testing methods that included an enrichment broth (EB with MSAox and EB with traditional culture) detected more MRSP than traditional culture alone. The MSAox selective medium alone detected significantly less MRSP when compared to either method containing the enrichment broth. This shows that the addition of enrichment broth plays a key role in enhancing the detection of MRSP in clinical specimens from dogs. The prevalence of MRSP in studies that only used a selective agar plate without enrichment broth could therefore have underestimated prevalence, and caution should be exercised when making comparisons to such studies (Bardiau et al., 2013; Chanchaithong et al., 2014; Davis et al., 2014; Mouney et al., 2013; Wang et al., 2012; Windahl et al., 2015).

When compared to each sample's overall MRSP result (if positive by any 4 test methods), EB with MSAox had the highest sensitivity. MSAox did not perform as well, detecting MRSP in only 8.9% of samples, achieving the lowest sensitivity of the tested

methods (66.4%). Studies investigating MRSA culture methods using human isolates found that MSA without oxacillin had the lowest sensitivity (Kali et al., 2014; Stoakes et al., 2006). It should be noted that the current study design likely underestimated the sensitivity of the selective culture methods. The samples used in this study were conveniently collected from submissions to the diagnostic laboratory; no additional samples were collected exclusively for use in this study. In order to not interfere with the routine diagnostic process, samples were first set up in the diagnostic laboratory, which include plating in up to four different culture media as well as preparation of a smear for Gram staining. Upon inspection of the sequences of positive results obtained using the specified test order and the sensitivities of each of the four tests, no substantial effect of testing order was noted. It is possible that all viable bacterial organisms present in the specimen were plated to traditional culture media, with none remaining for the selective culture methods. This could explain the cases where the selective culture methods did not detect MRSP in 16 samples that were positive by traditional culture methods.

When MSAox and EB with MSAox were evaluated as rapid screening tests, MSAox was out-performed by methods that included an enrichment broth step. The MSAox selective medium would not be ideal for use as a rapid screening test, as a large proportion of positive samples would not be detected at either 24 h or 48 h of incubation. Addition of enrichment broth does increase the sensitivity of MSAox, however, the extra day of incubation required slows the reporting time of the MRSP

result. When the sensitivity of MSAox as a screening test was estimated for critical specimen types, such as SSIs, the sensitivity did not change (data not shown.)

In this study, there was an intrinsic selection bias that over-estimated the reported prevalence of MRSP. Samples in this study were conveniently collected from routine submissions to the main veterinary diagnostic bacteriology laboratory for the Atlantic region. Samples are usually submitted to this laboratory because previous empirical antimicrobial therapy failed (*e.g.* non-response to antimicrobials), therefore, it can be assumed that such samples would have a higher likelihood of being positive for antimicrobial resistant organisms. Also, the authors chose to include only high-risk samples, so the samples used in this study represent those samples with a potentially higher likelihood of being MRSP positive. This resulted in the inclusion of the majority of canine sample submissions to the laboratory except for urine and fecal specimens. Patient data was based on information provided by the submitting veterinarian on the laboratory submission form. Most fields were routinely completed, however limited clinical histories were available in many cases. For this reason, a risk factor analysis, including antimicrobial usage, could not be performed.

This estimated MRSP prevalence in this study is similar to findings in Canada and other parts of the world. The traditional culture method did not differ significantly from any of the test methods; however, both methods that included enrichment broth did isolate more MRSP. This suggests that differences in prevalence estimates from different studies could be attributed to culture methodology. The lack of statistical

significance does not diminish the role that enrichment broth could have in detecting MRSP in a canine patient. The increased recovery of MRSP using enrichment broth culture methods does support the use of these methods on a routine basis. However, the MSAox selective agar should not be used alone because of its low sensitivity. The isolation of MRSP from any clinical sample could impact therapeutic decisions. Further work exploring alternative methods for the detection of MRSP in clinical specimens should be considered, particularly for those laboratories where molecular tools are unavailable.

Table 4.1. Patient information collected from laboratory submission forms. Age is presented in years as a median (range), while other factors are presented as a proportion.

Factor	MRSP detected	MRSP not detected	Total of MRSP
Age in years (*n* = 516)	6.1 (0.71–13.9)	6.9 (0.14–15.9)	–
Breed group (*n* = 526)			
Herding (*n* = 53)	17.0%	83.0%	13.2%
Hound (*n* = 21)	19.0%	81.0%	5.9%
Mixed (*n* = 89)	11.2%	88.8%	14.7%
Non-sporting (*n* = 55)	3.6%	96.4%	2.9%
Sporting (*n* = 186)	11.3%	88.7%	30.9%
Terrier (*n* = 33)	27.3%	72.7%	13.2%
Toy (*n* = 34)	2.9%	97.1%	1.5%
Working (*n* = 55)	21.8%	78.2%	17.7%
Sex (*n* = 542)			
Female (*n* = 242)	13.6%	86.4%	47.8%
Male (*n* = 300)	12.0%	88.0%	52.2%
Province of residence (*n* = 556)			
New Brunswick (*n* = 136)	16.2%	83.8%	31.4%
Newfoundland & Labrador (*n* = 57)	5.3%	94.7%	4.3%
Nova Scotia (*n* = 256)	10.6%	89.4%	38.6%
Ontario (*n* = 2)	50.0%	50.0%	1.4%
Prince Edward Island (*n* = 105)	16.2%	83.8%	24.3%

Table 4.2. Estimates of prevalence and sensitivity (95% CI) from model 1 at the sample level, using four testing methods for the isolation of MRSP from clinical specimens (*n* = 741).

	Prevalence	Sensitivity
Traditional	10.1% (8.1%–12.5%)	74.9% (64.2%–83.2%)
MSAox	8.9% (7.1%–11.2%)	66.4% (56.1%–75.3%)
EB with MSAox	11.2% (9.1%–13.7%)	84.0% (75.0%–90.1%)
EB with Traditional	10.8% (8.8%–13.2%)	80.8% (71.6%–87.6%)
Overall	13.4% (11.1%–16.0%)	1[a]

[a] The overall method was used as the reference for positive samples.

Table 4.3. Discordance assessment between each test method, where the value listed is the *p*-value for a statistical test that the two test methods have an equal apparent prevalence. An asterisk denotes a significant difference.

	Traditional	MSAox	EB with MSAox
MSAox	0.208	–	–
EB with MSAox	0.223	0.003*	–
EB with Traditional	0.322	0.015*	0.547

Table 4.4. Estimates of sensitivity and specificity (95% CI) at the sample level, using MSAox and EB with MSAox as a rapid screening tool for the detection of MRSP from clinical specimens (n = 741). The reference method was the overall MRSP result, where a sample was considered MRSP positive if any 1 of the 4 test methods was positive.

	Sensitivity	Specificity
MSAox at 24 h	36.6% (26.5%–48.3%)	97.9% (96.6%–98.8%)
MSAox at 48 h	69.9% (59.4%–78.7%)	82.6% (79.2%–85.6%)
EB with MSAox at 24h	80.7% (70.6%–87.9%)	83.2% (79.9%–86.1%)
EB with MSAox at 48h	89.7% (81.4%–94.6%)	72.9% (69.0%–76.5%)

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5.0 GENERAL DISCUSSION

The main aims of this thesis was to describe the situation of MRSP in Atlantic Canada both at a regional Veterinary Teaching Hospital (VTH) and a regional diagnostic laboratory, as well as to explore culture methods for the rapid detection of this pathogen. More specifically, the strain type diversity of MRSP in canine clinical samples was studied using the direct repeat unit (*dru*) typing method and their antimicrobial resistance patterns were described (Chapter 2). The persistence of MRSP in the VTH environment and its potential to cause hospital-acquired infections at a veterinary teaching hospital was investigated (Chapter 3). Selective culture methods were compared to traditional culture methods for the detection of MRSP and their use as rapid culture-based method and/or presumptive identification was evaluated (Chapter 4). The prevalence of MRSP in high-risk canine clinical specimens submitted to a regional veterinary diagnostic laboratory was estimated (Chapter 4).

5.1 Strain types of MRSP isolated from dogs in Atlantic Canada

This thesis and the literature demonstrate that the *dru* typing method is a useful tool for exploring the diversity of MRSP. Three main *dru* clusters 9a, 10h, and 11a were identified in a convenience sample of MRSP isolated from canine samples at a veterinary diagnostic laboratory, with *dru* type (dt) 11a (31%), dt10h (25%), and dt9a (18%) predominating (Chapter 2.) The distribution of these *dru* types is similar to what has been reported in North America, but more closely related to the distribution in the United States versus Ontario, Canada (Kadlec et al., 2015). *Dru* type 9a, which has been strongly associated with sequence type (ST) 71, has successfully disseminated in

Europe (90% of isolates) and makes up the majority of isolates in the US (66% of isolates) and Ontario, Canada (47%% of isolates), but is not as common in Atlantic Canada (18%) (Kadlec et al., 2015). Significant differences in the *dru* type distribution were noted between the Atlantic Canadian provinces in this thesis, and the distribution in this study was most similar to what has been observed in five US states. The five US states previously investigated, however, make up different geographic regions (West, Midwest, South, and Southwest) in the continental US, versus one region in Canada in this thesis (Kadlec et al., 2015). Notably, differences in the *dru* types were observed between Newfoundland and the Maritime provinces (NB, NS, and PEI). Cluster 10h was not present in the isolates from Newfoundland, while it was the predominant cluster in samples from PEI. Cluster 11a was the predominant cluster in both New Brunswick and Nova Scotia. The convenience sampling approach used in this chapter may have biased the results of this interprovincial comparison. Since Nova Scotia, New Brunswick and Newfoundland/Labrador have their own provincial veterinary diagnostic laboratories the submissions to AVC Diagnostic Services may not be representative of all diagnostic submissions from these provinces.

5.2 Antimicrobial resistance patterns of MRSP isolated from dogs in Atlantic Canada

Multi-drug resistance (MDR) has been frequently reported in MRSP isolated from canine specimens, and the results of this thesis present a similar phenomenon in Atlantic Canada (Chapter 2). Of the isolates studied in this thesis, greater than 70% of the isolates were resistant to ≥2 antimicrobial classes, not including the β-lactams. In

this thesis, *dru* cluster 9a had the highest proportion of MDR at 94.4%, and the significantly higher proportion of resistant isolates for each antimicrobial tested, except fusidic acid, chloramphenicol, and doxycycline. In the literature, ST71 (associated with *dru* type 9a) has been demonstrated to have increased antimicrobial resistance compared to other STs, which could partially explain its successful dissemination (Perreten et al., 2010). Resistance to other drug classes reported in this thesis is lower than reports in the literature. For example, amikacin resistance was not detected in our MRSP isolates, while over 30% of the isolates tested at a diagnostic laboratory in the US were resistant to amikacin (Gold et al., 2014). Similarly, 15% of isolates in this thesis were resistant to doxycycline, while 62%, 50%, and 35% of the isolates in Ontario, Australia and the United Kingdom, respectively, were resistant to doxycycline (Maluping et al., 2014; Siak et al., 2014; Weese et al., 2012). The likely selection bias must be noted in the isolates studied in this thesis. These samples were submitted to a regional veterinary diagnostic laboratory for bacterial culture and susceptibility testing and were often submitted for cases with difficult clinical management, particularly those non-responsive to antimicrobial therapy. Although MDR in MRSP isolates is common in this region, it can be suggested the isolates in Atlantic Canada are less resistant than those in other parts of the world. This could be explained by the low prevalence of cluster 9a isolates in the Atlantic region of Canada.

5.3 MRSP in the hospital environment and its potential to cause hospital-associated infections

This thesis further confirms the role of MRSP in a veterinary hospital setting with the potential to cause hospital-associated infections as described in the literature (Chapter 3). MRSP was detected in 12% (95% CI 6.8%–18.3%) of environmental samples after one cycle of cleaning and two cycles of disinfection, with one sample remaining positive after repeat cleaning and disinfection. Previously in the literature, MRSP has been reported in environmental samples collected from veterinary hospital areas, but it was not clear whether these areas had been cleaned and disinfected prior to collection (Ishihara et al., 2010; Youn et al., 2011). Isolates from the hospital environment and the patient associated with that area were compared using pulsed-field gel electrophoresis (PFGE). This thesis demonstrated that environmental and patient isolates were clonal, as confirmed by indistinguishable PFGE banding patterns. These results suggest that patients were in fact contaminating the hospital environment. Finding MRSP in hospital areas after cleaning and disinfection highlights the importance of diligent infection control policies and procedures to prevent hospital-acquired infections. It should be noted that some strains isolated from the hospital environment were non-clonal to the patient strain associated with that area, indicating undetected sources of MRSP contamination in the hospital, such as uncultured MRSP infections or subclinical carriers.

Evidence supporting the transmission of MRSP between two hospitalized patients was described (Chapter 3). Two patients had a *dt*9a MRSP isolated from a

surgical site infection, and the isolates had indistinguishable banding patterns on PFGE indicating that these isolates were the same clone. The patients were epidemiologically linked both spatially and temporally. They were housed side-by-side in the wards while in the hospital, and were in the hospital in the same time period but originated from different referring veterinarians from different provinces. Hospital-acquired infections of MRSP have been reported in the literature in a large outbreak at VTH in Helsinki, Finland (Grönthal et al., 2014). In the case presented in Chapter 3 and the report of the Finnish outbreak, a common source of MRSP was not identified. A thorough investigation was performed in the Finnish report and patient-to-patient transmission via hospital personnel was suggested as the likely route (Grönthal et al., 2014). In the case presented in Chapter 3, environmental samples were not collected prior to cleaning and disinfection, and MRSP was not detected after cleaning and disinfection, so an environmental source could not be confirmed or refuted. Since the same hospital personnel handled the MRSP positive patients in this case, transmission between patients was most likely via hospital personnel. This route of transmission is further supported by two studies that investigated MRSP contamination of hospital personnel clothing and cellular phones, where MRSP was isolated from 14% and 1.6% of sampled clothing and phones, respectively (Julian et al., 2012; Singh et al., 2013). This thesis and the literature suggest that hospital personnel have a potential important role in the transmission of MRSP among patients in a hospital setting.

5.4 Comparison of selective and traditional culture methods for the detection of MRSP

This thesis compared a selective culture medium, mannitol salt agar with 2µg/mL oxacillin (MSAox) with and without prior enrichment broth (EB) incubation, to traditional culture media for the detection of MRSP from canine clinical specimens deemed high-risk for MRSP (Chapter 4). It was demonstrated that the prevalence of MRSP differed between selective and traditional culture methods, although these differences were not significant. MRSP detection was greater using EB with MSAox (11.2%) and EB with traditional culture (10.8%) when compared to traditional culture alone (10.1%). Meanwhile, the MSAox on its own detected significantly less MRSP than any method that included EB (8.9%). Diagnostic test characteristics for each culture method were evaluated, where the EB methods had the highest sensitivity. To the author's knowledge, this is the first study evaluating the diagnostic test characteristics for the detection of MRSP using selective culture methods, whereas they have been well-studied for MRSA in human medicine (Denys et al., 2013; Kali et al., 2014; Stoakes et al., 2006). Studies investigating MRSA culture methods using human isolates found that mannitol salt agar (MSA) had the lowest sensitivity (Kali et al., 2014; Stoakes et al., 2006). MSA with 2µg/mL oxacillin (MSAox) was chosen as the selective medium in this study because of the performance of a similar commercial medium (ORSAB) in a previous study, the use of MSAox use in previous studies investigating MRSP isolates from dogs, and the ease and the low cost of preparing the medium (Horstmann et al., 2012). When MSAox and EB with MSAox were evaluated as a rapid screening test, MSAox continued to be out-performed by methods that included an

enrichment broth step. The MSAox selective medium would not seem ideal for use as a rapid screening test, because a large proportion of positive samples would not be detected at either 24 h or 48 h of incubation. The use of enrichment broth with MSAox does increase the sensitivity of this selective medium, however, the extra day of incubation required slows the reporting time of the MRSP result. These results highlight how differences in culture methodology can influence prevalence estimates, and caution should be taken when making strong comparisons between studies investigating prevalence. The lack of statistical significance in MRSP recovery between selective and traditional culture methods does not diminish the clinical significance of MRSP. The isolation of MRSP from any clinical sample could impact clinical management for the patient. Thus enhanced detection for high-risk clinical specimens using EB and MSAox culturing methods should be considered.

5.5 Prevalence of MRSP in clinical canine specimens submitted to a veterinary diagnostic laboratory in Atlantic Canada

The prevalence of MRSP in clinical canine specimens deemed high-risk for MRSP was investigated (Chapter 4). MRSP was detected in a total of 13.4% (n = 741) of samples from dogs at high-risk for MRSP submitted to a regional veterinary diagnostic laboratory. This prevalence is likely an overestimate of MRSP in total canine submissions to the laboratory because only high-risk samples were included. The true prevalence of MRSP in all canine submissions was estimated by adjusting the prevalence using the culture method with the highest sensitivity for those samples that were not included in the study. With the adjustment, the estimated true prevalence of

MRSP in all canine submissions to a regional veterinary diagnostic laboratory in Atlantic Canada was 5.8%. The prevalence of MRSP in clinical specimens has been well documented in the literature ranging from 0-41%, including studies in Canada (Bardiau et al., 2013; Beck et al., 2012; Detwiler et al., 2013; Garbacz et al., 2013; Hariharan et al., 2014; LoPinto et al., 2014; Maluping et al., 2014; Siak et al., 2014; Wang et al., 2012; Windahl et al., 2012). The estimated MRSP prevalence in this study is similar to findings in Canada and other parts of the world.

5.6 Conclusions

- The MRSP strain type diversity in Atlantic Canada is similar to the diversity reported elsewhere in North America, with three main *dru* clusters identified: 11a, 10h, and 9a (Chapter 2).
- One successful MRSP strain, *dru* type 9a, is not as common in Atlantic Canada as in Ontario, Canada (Chapter 2).
- Multi-drug resistance in these isolates is common, especially within cluster 9a isolates, but resistance to the non-β-lactam antimicrobials is still considerably lower than reported elsewhere (Chapter 2).
- MRSP isolates are recovered from the hospital environment after cleaning and disinfection (Chapter 3).
- MRSP isolated from the hospital environment are clonal to the patient strains associated with that area, however non-clonal strains were also recovered (Chapter 3).

- Patient-to-patient transmission of MRSP in the hospital environment was documented and hospital personnel were suspected of facilitating the transfer (Chapter 3).
- Selective culture methods detected more MRSP than the traditional culture methods, although this increase was not statistically significant (Chapter 4).
- The selective medium, mannitol salt agar with 2µg/mL oxacillin, would not be suitable as a rapid culture based presumptive identification tool for MRSP (Chapter 4).
- MRSP was commonly (13.4%) isolated from canine clinical specimens submitted to a regional veterinary diagnostic laboratory deemed high-risk for MRSP (Chapter 4).

5.7 Future Directions

The results of this thesis warrant further research in other aspects important to the epidemiology of MRSP in Atlantic Canada. The *dru* typing method provides an easy approach to strain type MRSP, however offers no information to follow clonal spread. To further understand the population genetic structure of this pathogen in Atlantic Canada, MLST should be performed on the isolates studied in this thesis. This will identify common ancestors and lineages including the potential to track the spread of these clones with in the region. This information could provide insight on the origin of methicillin-resistant strains in our region. It would also be particularly interesting to investigate the relatedness of strains from the island provinces in this region, since they had significantly different *dru* type diversity when compared to the mainland provinces.

The low prevalence of *dt*9a in Atlantic Canada suggests a low prevalence of ST71 in this region. MLST would provide stronger evidence on the true prevalence of ST71 in the region.

The colonization of dogs and colonization or contamination of hospital personnel with MRSP was not evaluated in this thesis. It can be suggested that dogs colonized with MRSP play a role in the contamination of the hospital environment, as demonstrated by the detection of MRSP strains in the environment that are not clonal to the MRSP isolated from infected patient associated with that area. Limited information in the literature is available on the longitudinal carriage of MRSP in dogs. This information would help understand the risk these dogs pose to contaminating the hospital environment, and provide more evidence to strengthen infection control policies after a MRSP infection was diagnosed but resolved. Hospital personnel have been suggested to be the route of transmission in hospital-acquired MRSP. Screening hospital personnel for MRSP on their hands, clothing, and nasal passages could provide confirmation of these claims. Furthermore, monitoring personnel and patient interaction could contribute to the understanding of the transmission of MRSP from patient to humans and subsequently other patients.

The methods used in this thesis to detect MRSP in the hospital environment provided a qualitative assessment of bacterial contamination. Collecting samples from measured areas as well as culturing samples using dilution techniques could quantify the bacterial burden in the hospital setting. Quantitative culture methods would allow a

more concrete assessment of the exposure risk the environment plays in the spread of MRSP.

The presence of virulence factors in MRSP isolates was not studied in this thesis. Biofilm production has been suggested to be a virulence factor associated with the success of the MRSP dissemination worldwide. It is possible that the persistence of MRSP in the hospital environment after cleaning and disinfection is related to the isolate's ability to produce biofilms. Investigating the biofilm production potential as well as the genetic markers for biofilm production in the isolates recovered from hospital patients and from the hospital environment would provide further evidence to support this hypothesis. It would also be interesting to note from which surface in the hospital environment where each isolate was recovered. The ability of MRSP to form stronger biofilms on some surfaces compared to others could instigate changes to the routine cleaning protocols in a hospital setting.

The results in thesis were based on convenience samples collected at a regional veterinary diagnostic laboratory, which likely contributed to a selection bias in reported conclusions. To gain a more accurate understanding of the dissemination of MRSP in Atlantic Canada, primary veterinary care facilities should be included for patient and sample recruitment. As veterinary practitioners become increasingly more aware of MRSP in the canine population, it is likely that submissions to the diagnostic laboratory will decrease, as rationale for empirical therapy will change.

5.8 References

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