INVESTIGATION OF ANTIMICROBIAL RESISTANCE IN *Staphylococcus pseudintermedius*

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ABSTRACT

Staphylococcus pseudintermedius is a coagulase positive bacterium and found on the mucous membranes of approximately 90% of healthy dogs. This organism is an opportunistic pathogen in dogs and an increasingly recognized zoonoses. As in human medicine, the emergence of antimicrobial resistance is a growing problem in companion animal practice. In the current investigation, we saught to 1. Identify the emergence of resistance among *S. pseudintermedius* colonizing healthy dogs, 2. Determine whether there are strain specific tissue tropisms among isolates causing dermatological and urinary tract infections, 3. Perform a clinical and bacteriological description of human *S. pseudintermedius* infections and 4. To describe the correlation of susceptibility of isolates to tetracycline, doxycycline and minocycline, and to describe the mechanisms of tetracycline resistance. We found that methicillin-resistant *S. pseudintermedius* colonizes 7% of healthy dogs and that since a previous surveillance study conducted in 2008, resistance has emerged in our region. *S. pseudintermedius* isolates from dermatological and urinary tract infections were genetically heterogeneous, suggesting that these organisms are true opportunists and do not possess a strain specific tissue tropism. Despite this genetic heterogeneity, antimicrobial resistance was found to be significantly higher in isolates from dermatological infections compared to those from the urinary tract. Human infections with *S. pseudintermedius* were relatively rare, comprising 0.05% of skin and soft tissue infections in a large Canadian health region. Among these human isolates, we found that all methicillin resistant isolates were the European pandemic clones ST71 and ST181. We found that tetracycline is not a satisfactory indicator for doxycycline and minocycline resistance, and that these phenotypic discrepancies could not be explained by the presence or absence of particular resistance genes. Furthermore, factors related to *tet*M (which was the most commonly identified resistance gene)

including copy number, predicted amino acid sequence or expression level, were not significantly associated with the phenotypic diversity observed. Although numerically high association were found in high MIC category on *tet*M expression comparing to low MIC category, larger studies required for further conclusions. The genotypic evidence on MICs for tetracycline and doxycycline is largely unknown and more studies are required.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BLAST	Basic local alignment search tool
CLS	Calgary Laboratory Services
CLSI	Clinical and Laboratory Standards Institute
ECOFF	Epidemiological cut-off
EUCAST	European Committee on Antimicrobial Susceptibility Testing
IQR	Interquartile range
ISCAID	International Society for Companion Animal Infection Diseases
MALDI-TOF MS	Matrix-assisted laser desorption ionization time of flight mass spectrometry
MDR	Multi drug resistant
MDRSP	Multi drug resistant *Staphylococcus pseudintermedius*
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence-typing
MRSP	Methicillin resistant *Staphylococcus pseudintermedius*
MSSP	Methicillin susceptible *Staphylococcus pseudintermedius*
PABA	Para-amino benzoic acid
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel-electrophoresis
PVL	Panton Valentin leucocidin
SCC	Staphylococcus chromosomal cassette

SNP	Single nucleotide polymorphism
SSTI	Skin and soft tissue infections
ST	Sequence type
UPGMA	Unweighted pair group method with arithmetic mean
UTI	Urinary tract infection
WGS	Whole genome sequencing

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Taxonomy, basics biochemical characteristics and identification of *Staphylococcus pseudintermedius*

Staphylococcus pseudintermedius is a Gram-positive cocci normally found as a part of the skin-associated bacterial populations of dogs (Bannoehr and Guardabassi, 2012; Rubin and Chirino-Trejo, 2011). *S. pseudintermedius* grows as grayish colonies 1-2 mm on 5% sheep blood agar and is typically associated with a double zone of hemolysis (Markey et al., 2013). Based on DNA-DNA hybridization and the recognized natural host specificity, *S. pseudintermedius* was described as a separate species in 2007 (Sasaki et al., 2007b). *Staphylococcus pseudintermedius*, *Staphylococcus intermedius*, and *Staphylococcus delphini* are collectively called the *S. intermedius* group (Bannoehr and Guardabassi, 2012). These species cannot be reliably distinguished from each other using phenotypic tests or commercial diagnostic kits (Bannoehr and Guardabassi, 2012). The morphological and biochemical characteristics of Staphylococci found in dogs have been summarized in Table 1.1.

The morphological similarity between *S. pseudintermedius* and *S. aureus* results in these organisms being misdiagnosed at human diagnostic laboratories, although they can be distinguished by a number of biochemical tests including hyaluronidase and acetoin production, polymyxin B susceptibility and carbohydrate fermentation tests such as maltose, trehalose, mannitol, galactose, lactose (Table 1.1) (Bannoehr and Guardabassi, 2012; Borjesson et al., 2015). Moreover, *S. pseudintermedius* is only positive using the tube coagulase and may be misidentified as being coagulase negative due to the poor response in the slide coagulase test and commercial latex agglutination test (Bannoehr and Guardabassi, 2012). More recently, molecular

methods including PCR amplification of species-specific target, sequencing of PCR products, and Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MOLDI-TOF MS) are being used for the identification of *S. pseudintermedius*.

Multiplex PCR assays have been developed based on the amplification of the thermoneuclease (nuc) gene which differentiates *S. pseudintermedius* from *S. aureus, S. hyicius, S. schleiferi, S. intermedius, S. pseudintermedius*, and *S. delphini* (Sasaki et al., 2010). The phylogenetic analysis of the *nuc, kat, soda,* and *gap* genes has also been used to identify different species of *Staphylococcus* (Blaiotta et al., 2010). Currently, the universal bacterial target *cpn*60 and 16S rRNA are widely used for speciation due to the slow rate of evolution of this gene (Hill et al., 2004; Wakita et al., 2002). However, MALDI-TOF MS is emerging in a trend which has replaced many of conventional bacteriological tests in current diagnostic laboratories (Murugaiyan et al., 2014; Silva et al., 2015). In MALDI-TOF MS, microbial proteins are ionized into charged molecules and the mass to charge ratio is measured by time of flight mass spectrometry (Singhal et al., 2015). The identification of a species is based on the ratio of mass and charge which is unique to each species of bacteria (Biswas and Rolain, 2013). In this method, isolated bacterial colonies are simply overlaid with matrix and are exposed to a fixed pulsed laser beam.

Table 1.1: Morphological and biochemical characteristics of coagulase positive Staphylococci species found in dogs.

Test	***S. aureus***	***S. pseudintermedius***	***S. intermedius***	***S. delphini***	***S. schleiferi***
Hemolysis	+	+	+	+	+
Tube coagulase	+	+	+	+	+
Slide coagulase	+	+/-	+	+	+
Acetoin production	+	variable	-	-	+
DNAse	+	+	+	weak +	+
Trehalose	+	+	+	-	-
Maltose	+	+	+	+	-
Mannitol	+	-	-	+	variable
Hyaluronidase	+	-	-	-	-
Pyrrolidinyl acryl amidase	-	-	-	not known	+
Beta galactosidase	-	+	+	+	variable

1.1.1 Epidemiology of *Staphylococcus pseudintermedius*

1.1.1.1 Geographical distribution of the organism reported

Staphylococcus pseudintermedius has been reported in many locations around the world, although there may be publication bias towards North America and Europe, where funding opportunities, technical skills, and advanced laboratory facilities are more readily available.4 In addition, many studies originate in Japan, South Korea, and Thailand, with more recent studies emerging from Australia and New Zealand (Saputra et al., 2017). Few studies from Africa and South America have been published. As of June 9, 2017, there were 391 papers describing *S. pseudintermedius* in PubMed (*S. intermedius* was not included). Nearly 300 of these papers highlighted the significance of *S. pseudintermedius* in domestic dogs.

Staphylococcus pseudintermedius colonizes 90% of healthy dogs (Rubin and Chirino-Trejo, 2011). The organism is most commonly found in pharynx and rectum but also been isolated from the perineum, inguinal region, nares, conjunctiva and skin (Bannoehr and Guardabassi, 2012; Iverson et al., 2015). As *S. pseudintermedius* is isolated from the pharynx, it is also frequently found in oral cavity and saliva (Iverson et al., 2015). *S. pseudintermedius* has also been found in mammary secretions perhaps explaining how pups are colonized early in life (Rota et al., 2011). The ability to recover the bacterium from a dog depends on multiple factors including whether single or multiple anatomical sites are sampled, and which specific sites are included. The rate of recovery of *S. pseudintermedius* was reported to be high from the pharynx (81.1% of colonized dogs) compared to the frequency of recovering this organism from the nares (47.7%) (Rubin and Chirino-Trejo, 2011). In one study which included only nasal swabs, Staphylococci were only recovered from only 37% of dogs (Han et al., 2016). As such, including multiple sampling sites improves recovery of the organism, with pharynx and rectum being the preferred sites for the

isolation of *S. pseudintermedius* in dogs (Rubin and Chirino-Trejo, 2011). Dogs can be described as either persistent, intermittent or transient carrier of *S. pseudintermedius* (Gomez-Sanz et al., 2013a). Persistent carriage describes a situation when *S. pseudintermedius* is carried for long period of time, while transient carriers occasionally harbour the organism.

Staphylococcus pseudintermedius is less common in cats, with colonization rates varying from 4.3 - 65% (Abraham et al., 2007; Hanselman et al., 2009). *S. pseudintermedius* has also been reported in a variety of other species such as horses, goats, wild carnivores, feral cats, cows, minks, rats, pigeons and human (Bannoehr and Guardabassi, 2012; Iverson et al., 2015; Weese, 2010). *S. pseudintermedius* is not a part of the human resident microbiota, it is therefore suggested that people acquire this organism through canine contact such as licking or handling of pets although the transmission of this organism has not been adequately studied (Gharsa et al., 2013; Walther et al., 2012).

1.1.1.2 Zoonosis/Interspecies transmission

Staphylococcus aureus colonizes 30% of the human population and causes a wide variety of opportunistic infections (Missiakas and Schneewind, 2016). In contrast, *S. pseudintermedius* is less commonly associated with disease in humans (Deurenberg and Stobberingh, 2008; Hill and Imai, 2016). Although the incidence of *S. pseudintermedius* is unknown, a number of case reports including skin and soft tissue infections, sinonasal infection, bone implant and surgical site infections have been reported (Borjesson et al., 2015; Kuan et al., 2016; Pompilio et al., 2015; Savini et al., 2013; Viau et al., 2015). People who suffer from concurrent immunosuppressive conditions are more susceptible to the secondary infections and may be overrepresented among human patients with *S. pseudintermedius* infections (Kuan et al., 2016).

Among patients with *S. pseudintermedius* infections, close association with dogs is commonly reported suggesting zoonotic transmission (Gomez-Sanz et al., 2013b). Many studies have used molecular epidemiological tools to demonstrate common strains within a household providing evidence in support of transmission from dog to human (Laarhoven et al., 2011; Lozano et al., 2017; Walther et al., 2012). Because dogs are frequently pharyngeally colonized, contaminated saliva provides ample opportunity for *S. pseudintermedius* to infect tissues damaged following dog bite wounds (Borjesson et al., 2015). Although human infections with *S. pseudintermedius* are a recognized public health risk associated with the canine contact, the magnitude of this risk has not been quantified.

1.2 Clinical infection in dogs

1.2.1 Summary of clinical significance

The clinical impact of *S. pseudintermedius* in canine practice cannot be overstated, it is very commonly encountered as a cause of infection (Saputra et al., 2017). *S. pseudintermedius* is an opportunistic pathogen, commonly isolated with wounds, abscesses, eye infections and mastitis (Bannoehr and Guardabassi, 2012). Importantly, it is the most common cause of dermatological infections, otitis externa and the second most common cause of urinary tract infections in dogs (Bannoehr and Guardabassi, 2012; Dziva et al., 2015; Windahl et al., 2014). In dogs which acquire infections following hospitalization or surgery, a nosocomial origin of the infection has frequently been suggested (Bergstrom et al., 2012; Gronthal et al., 2014; Haenni et al., 2013; Haenni et al., 2014; Walther et al., 2016; Windahl et al., 2015). Infection control strategies are practiced in veterinary clinics to minimize transmission of *S. pseudintermedius* among animals. Transmission to vulnerable patients in hospital settings readily occurs due to temporary

suppression of normal host defenses including following surgical disruption of tissues, anesthesia and stress associated with hospitalization (Bergstrom et al., 2012; Nazarali et al., 2014). A positive association between *S. pseudintermedius* infection and number of days in intensive care or surgery wards, longer overall duration of hospitalization, the severity of concurrent infection and long-term usage of antimicrobials have been identified (Gronthal et al., 2014; Singh et al., 2013; Windahl et al., 2015). Methicillin resistant isolates have been isolated from surgical devices at veterinary hospitals, highlighting the possible risk of infection associated with the use of nonsterile or improperly sterilized devices (Ishihara et al., 2010). In fact, *S. pseudintermedius* has been isolated from infections of implants following orthopedic surgery in dogs (Miedzobrodzki et al., 2010). *Staphylococcus pseudintermedius* is a biofilm forming bacterium even in wound associated infections (Han et al., 2015; Pompilio et al., 2015). The *ica*A gene which causes biofilm has been studied in *S. pseudintermedius* (Han et al., 2015). In biofilm environments, it has been reported to be highly-antimicrobial tolerant towards amoxicillin, cephalexin, clindamycin, doxycycline, marbofloxacin and combination antimicrobial protocols (Ferran et al., 2016). One study reported that only rifampicin was effective against *S. pseudintermedius* in biofilms while other antimicrobials including chloramphenicol, gentamicin, cefoxitin, linezolid, vancomycin, tetracycline, and tigecycline were ineffective in this environment (Pompilio et al., 2015).

1.2.2 Pathogenesis and virulence mechanism

Although the pathogenesis of *S. aureus* has been investigated in human and livestock, the pathogenesis of *S. pseudintermedius* in companion animals is understudied, with only a few virulence factors identified (Fitzgerald, 2009). Putative virulence factors found in *S. aureus* have

been assumed to be present in *S. pseudintermedius* although their role in disease has not been demonstrated (Fitzgerald, 2009). According to clinical experts the pathogenesis of *S. pseudintermedius* depends more upon host factors than on factors associated with the bacterium; however, there is insufficient peer-reviewed data to draw conclusions on the magnitude and role of these factors (Green, 2012). Although a number of studies have reported lists of virulence factors from *S. pseudintermedius*, the nomenclature used to describe these genes is inconsistent and many studies simply publish PCR results without depositing the sequences of DNA amplicons in databases such as GenBank.

Tissue injury, immunosuppression, and concurrent diseases are purported instigating factors of *S. pseudintermedius* infections in dogs (Bannoehr and Guardabassi, 2012). A number of putative virulence factors have been described Hill et al, 2016 have summarized virulence factors, host immune response and immunopathogenesis in *S. pseudintermedius* by extrapolating data from *S. aureus* (Hill and Imai, 2016). These factors are involved at multiple stages in the pathogenesis of infection from adhesion to immune evasion to spread the infection. Virulence proteins such as fibrinogen binding protein, fibronectin binding protein, iron-regulated surface determinants facilitate bacterial adhesion to the natural host (corneocyte) and also involved in activation of complement pathway and opsonization of the pathogen. Immunoglobins and cell-mediated immune responses are inactivated by virulence proteins including superantigen and leucocidin. The leucocidin toxin secreted by *S. aureus* inactivates mononuclear cells and neutrophils (Hill and Imai, 2016). Exfoliative toxin (Exi) digest the Dsg 1 protein in the canine skin causing acantholysis in canine skin (Iyori et al., 2011). Putative virulence proteins of *S. pseudintermedius* which have been previously published are summarized Table 1.2.

While uropathogenic *E. coli* possess specific nutrient uptake mechanisms (i.e. siderophores) and virulence factors such as virulence-associated adhesive surface fibers to survive in the hostile environment of the urinary tract in humans and companion animals, similar adaptations have not been described in *S. pseudintermedius*.(Chahales and Thanassi, 2015; Piras et al., 2015).

Table 1.2: Virulence factors found in *Staphylococcus pseudintermedius*

Virulence factor	**Function in pathogenesis**	**Reference**
Exi	Breaking down epidermis	(Iyori et al., 2011)
Enterotoxin	Toxic shock syndrome	(Tanabe et al., 2013)
SpsD, SpsO	Corneocyte adhesion	(Bannoehr et al., 2012)
SpsD, SpsL fibronectin binding protein	Adhesion to corneum, skin, and invasion	(Pietrocola et al., 2015)
BacSp222 Cationic peptide	Cytotoxic activity on eukaryotic cells	(Wladyka et al., 2015)
LukS/F-1, ExpA, Se-int, Sec_{canine}, Siet, Sea, Seb, Sec, Sed, Sei, Sej, Sek, Ser, Hlg	Invasion and peeling of skin	(Gharsa et al., 2013)
Proteases, clumping factor, protein A	Invasion, peeling of skin, inactivation of immunoglobulin	(Futagawa-Saito et al., 2006)
Sec_{canine}	Toxic shock syndrome	(Edwards et al., 1997)
Synergohymentropic	Making pores in cells	(Prevost et al., 1995)
SpsA, SpsB, SpsD, SpsK, SpsL, SpsN, NucC, Coa, and Luk-I	Invasion peeling of skin, inactivation of immunoglobulin	(Couto et al., 2016a)

1.2.3 Dermatological infections

Staphylococcus pseudintermedius is the most frequent organism identified in canine skin infections, being isolated from 80% of clinical submissions (Sindha et al., 2015). Skin infections are also referred to as pyoderma and are typically secondary to injury or concurrent disease (Beco et al., 2013a). Pyoderma can be identified using a number of tests including physical examination, skin scraping, cytological examination, skin biopsy and bacterial culture (Green, 2012). Canine pyoderma is categorized as surface, superficial and deep pyoderma based on the extent of infection in the skin (Beco et al., 2013a). Both surface and superficial pyoderma are limited to the epidermis and while deep pyoderma extends to the dermis (Beco et al., 2013a). Clinical signs depend upon the severity and site/distribution of the infection (Beco et al., 2013a). The pathological and histological findings associated with pyoderma depend upon the severity of infections. In patients with superficial pyoderma, a high number of bacterial and inflammatory cells are typically found in the stratum corneum and epidermis (Baumer et al., 2017; Beco et al., 2013a). In deep pyoderma, the bacteria spread into the dermis resulting in deeper inflammation and furunculosis followed by alopecia (Beco et al., 2013a).

Primary predisposing diseases including immunodeficiencies, primary skin allergies, parasitic infestation, metabolic diseases, endocrinopathies and long-term treatment with corticosteroids have been associated with dermatological infections (Beco et al., 2013a; Green, 2012). Hypersensitivity reactions stimulated by allergens leading to inflammatory cell infiltration create conditions favorable for the development of dysbiosis, including overgrowth of *S. pseudintermedius* (Pierezan et al., 2016). A number of risk factors have been discussed in canine skin compared to other species including thin skin structure, compact stratum corneum, the

relative paucity of intercellular lipids in the stratum corneum, the lack of a lipid squamous epithelial plug at the entrance of canine hair follicles, and a relatively high pH (Green, 2012).

Otitis externa is a common disease in dogs, with *S. pseudintermedius* being the most common bacterial cause of these infections in dogs (Bannoehr and Guardabassi, 2012). These infections are frequently polymicrobial and often complicated, as they often occur as secondary infections in animals with predisposing conditions as described above (Green, 2012). Bacterial otitis externa is not as common in cats as dogs, perhaps due to a straight ear canal and the lack of long ear pinna (Green, 2012).

1.2.4 Urinary tract infections (UTI)

Bacterial urinary tract infections (UTI) are common in dogs, with 14% of dogs visiting a veterinarian for UTI at least once in their lifetime (Ling, 1984; Thompson et al., 2011). Of dogs with a UTI, 4.5% are reported to have an unsatisfactory response to the prescribed treatment, resulting in recurrent or persistent infections (Thompson et al., 2011). Clinical signs of UTI in dogs consist of stranguria, pollakiuria, pigmenturia and hematuria (Thompson et al., 2011). Because these clinical signs are non-specific, urine culture for the isolation and identification of bacteria are the gold standard for the identification of UTIs (Brloznik et al., 2016; Dowling, 1996). The collection of urine for culture is best done by cystocentesis which allows samples to be collected while minimizing contamination with feces and the microbial communities in the lower urinary tract. The isolation of *Staphylococcus* spp. in canine diagnostic submissions varied from 11-32.8% (Windahl et al., 2014). *Staphylococcus pseudintermedius* frequently cultured from these UTI (Maaland and Guardabassi, 2011). Polymicrobial infections including both Gram positive and negative bacteria are frequently reported in canine UTI (Windahl et al., 2014). Other

staphylococci including *S. epidermidis, S. simulans, S. schleiferi ssp. schleiferi, S. aureus, S. schleiferi* ssp. *coagulans* and *S. saprophytic* have been reported as coinfected with *S. pseudintermedius* in canine UTI (Penna et al., 2010).

Urinary tract infections in dogs can be categorized as uncomplicated or complicated. Uncomplicated infections are defined as those in a structurally and physiologically normal urinary tract by non-resident organisms, while complicated infections occur in animals with anatomical, physiological and ongoing clinical manifestation or treatment failure (Weese et al., 2011). Canine UTIs are most often ascending infections from the lower urinary tract, although hematogenous spread from distant body sites is also recognized (Thompson et al., 2011).

Factors predisposing dogs to UTI can be divided into three main categories: 1. anatomical defects, 2. metabolic disorders and 3. iatrogenic. Anatomic defects may include ectopic ureters, females with recessed vulvas, persistent vaginal membranes or other causes of urine pooling which lead to ascending UTI. Metabolic diseases including diabetes mellitus and hyperadrenocorticism cause secondary immunosuppression and susceptible to various secondary bacterial infections including *S. pseudintermedius*. Other diseases such as chronic kidney disease, urolithiasis or bladder neoplasia also predispose to UTI by impairing host defense mechanisms including the disruption of mucosal integrity, presence of a nidus for bacterial growth and by affecting a dogs ability to appropriately concentrate urine (Green, 2012). Finally, iatrogenic factors such as glucocorticoid therapy, immunosuppression, complications from surgery, urinary catheterization, untreated perivulvar lesions may also predispose patients to UTI (Brloznik et al., 2016; Thompson et al., 2011)

1.2.5 Pharmacological considerations for therapy

Skin infections are the main reason for the systemic application of antibiotics in canine practice (Beco et al., 2013a). Amoxicillin, first-generation cephalosporins, tetracycline, and sulfa-trimethoprim are the first choices for treating Gram positive infections in companion animals (Prescott et al., 2002). Amoxicillin, cefalexin, cefovecin, and clindamycin were most prescribed antimicrobials for canine pyoderma in the UK (Summers et al., 2014). Selection of the appropriate antimicrobial requires the integration of patient data, antimicrobial susceptibility of the pathogen and pharmacokinetic and pharmacodynamics characteristics of the antimicrobial agent. The skin is the largest organ but has a relatively poor vascular supply compared to other organs such as the liver or kidneys (Beco et al., 2013b). In deep pyoderma, extensive necrosis and scarring with tissue debris limits penetration of antimicrobials into deep tissue, requiring the selection of drugs with good tissue penetration and the use of high doses are therefore often required (Beco et al., 2013b). Lipophilic antimicrobials such as clindamycin and doxycycline have better penetration into deep tissues compared with more hydrophilic drugs and may be good options for treating deep pyoderma (Nguyen et al., 2014). High doses are recommended with the presence of necrosis in dermatological infections in dogs (Beco et al., 2013b).

Many antimicrobials, including the β-lactams, are eliminated at least partially by renal excretion, leading to high concentrations in the urinary bladder. The extent to which a drug is excreted through the renal tubules is dependant upon the hydrophilicity of the compound. Drug families including beta-lactams, quinolone, fosfomycin, nitrofurantoin, and chloramphenicol are excreted through renal tubules and reach a bactericidal or bacteriostatic concentration in the urinary bladder (Green, 2012).

1.2.6 Treatment options for *Staphylococcus pseudintermedius* clinical infections

Superficial pyoderma is often empirically treated using non-antimicrobial options including shampoos, solutions which contain biocides such as chlorhexidine, triclosan and benzoyl peroxide (Clark et al., 2015; Loeffler et al., 2011; Valentine et al., 2012). One study found that the use of 4% chlorhexidine for treating superficial pyoderma performed similarly to systemic antimicrobial therapy (Bajwa, 2016). Oftentimes, a combination of systemically administered antibiotics and topical biocides are used for superficial canine pyoderma (Frank and Loeffler, 2012).

Guidelines for antimicrobial treatment of skin infection have been published by expert groups such as the International Society for Companion Animal Infectious Diseases (ISCAID), as well as clinical reviews describing therapeutic strategies for dermatological infections in small animals (Bajwa, 2016; Hillier et al., 2014; Loffler and Macdougall, 2007; Summers et al., 2012). These guidelines describe both topical and systemic application of antimicrobial for superficial folliculitis in dogs (Hillier et al., 2014). Topical treatment alone is only recommended in cases of surface pyoderma (Hillier et al., 2014). Moreover, the selection of systemic antimicrobials depends on the result of antimicrobial susceptibility tests, availability, safety, the cost of treatment and host-specific factors such as immunosuppression (Hillier et al., 2014).

In uncomplicated UTI, both beta-lactams and sulfa-trimethoprim are used as first-line therapy (Weese et al., 2011). When treating complicated UTI, therapy must address underlying conditions such as diabetes mellitus or hyperadrenocorticism. A thorough laboratory investigation including complete blood count, biochemical profiles, and imaging together with antimicrobial susceptibility testing may be warranted (Weese et al., 2011). A working group from ISCAID has published treatment recommendations for short-term (3-5 days) and long-term

(14 days) antimicrobial treatment strategies for both uncomplicated and complicated UTI (Dowling, 1996; Jessen et al., 2015; Weese et al., 2011). In complicated UTI, relapse (recurrent infection within 6 months after therapy) and refractory infections (microbes are positive persistently) are common necessitating additional investigation to allow the inciting cause to be identified and addressed (Jessen et al., 2015).

Identification and management of underlying conditions is an essential part of clinical success in both dermatological and UTI (Green, 2012). Moreover, clinical success is dependent upon multiple factors beyond antimicrobial susceptibility including the severity of infection and host immune status. Although variety treatment strategies have been described for uncomplicated UTIs and dermatological infections, in our region insufficient data has been published on the antimicrobial susceptibility of this organism to make evidence-based empiric therapeutic decisions.

1.3 Overview of antimicrobial resistance

Antibiotics are low molecular weight compounds which inhibit microbial growth at low concentrations (Martens and Demain, 2017). Antimicrobials have been used to treat bacterial infections for the last 70 years. At present, an estimated 17 million people die every year from bacterial infections and the burden of antimicrobial resistance is increasing (Martens and Demain, 2017). In the United States, it is estimated that there are two million infections and 23,000 deaths caused by antimicrobial-resistant organisms every year (CDC; Martens and Demain, 2017). Antibiotics are commonly prescribed drugs for bacterial infections and global antibiotic consumption has increased by 36% between 2010 and 2000 (Van Boeckel et al., 2014). A 2014 report commissioned by Wellcome Trust and the government of the UK with the aim of

estimating the emergence of antimicrobials resistance gained international attention. The authors of this report projected that mortality attributable to resistant bacterial infections will reach 10 million in 2050, which will surpass mortality caused by cancers (O'Neill, 2016).

In addition to mortality, antimicrobial resistance is also associated with increased health care costs. This has been best described in the case of *S. aureus* where the cost of treating patients with MRSA is higher than those infected with methicillin susceptible strains. In Spain, one study found that bacteremia caused by MRSA was 1.12-fold more expensive than MSSA to treat due to an additional 2.2 days of hospitalization and admission to intensive care units (Rubio-Terres et al., 2010). In the United States, the attributed mean cost per bacteremia in a patient with MRSA has been estimated to be $23,000, while MSSA infections have been estimated to cost $19,500 (Paladino, 2000). In addition, resistance associated mortality in bloodstream infections with MRSA was found to be 3 fold higher than susceptible strains (Coulter et al., 2017). In companion animal practice, the effect of resistance on mortality, length of hospitalization and cost of care has not been adequately assessed. In veterinary medicine, a similar evaluation for the contribution to increased healthcare costs and mortality of antimicrobial resistance has not been conducted.

Antimicrobial resistance can be categorized broadly as intrinsic or acquired (Giguere et al., 2013). Intrinsic resistance is defined as an innate characteristic of a bacterial species which protect against an antimicrobial due to own structural and biochemical properties, resistance to antimicrobials, even in unselected populations is ubiquitous (Leclercq, 2013). Selection of resistant organisms occurs as a natural response of bacteria to a hostile (antimicrobial containing) environment (Qekwana et al., 2017; Saputra et al., 2017). Resistance is a natural phenomenon, in *S. aureus* resistance to penicillin was reported a just a few years after its introduction,

similarly, resistance to all currently available antimicrobials already exists in nature (Lobanovska and Pilla, 2017).

1.3.1.1 The general mechanisms of antimicrobial resistance

Resistance to antimicrobials occurs by four main mechanisms: 1. decreasing the permeability of cell wall, 2. increased efflux of the drug, 3. enzymatic inactivation and 4. modification or protection of drug targets (Giguere et al., 2013).

Decreased cell permeability is a well-recognized mechanism of resistance among Gram negatives including non-fermenters such as *Pseudomonas aeruginosa* and *E. coli*, where resistance to the aminoglycosides and carbapenems has been reported to result from porin deficiencies in the cell wall (Dantas et al., 2017; Goessens et al., 2013; Mingeot-Leclercq et al., 1999). Resistance to the 3rd generation cephalosporin, ceftazidime, in *Klebsiella pneumonia* due to Ompk35 and Ompk36 deficiencies have also been reported in clinical isolates (Wassef et al., 2015).

Efflux pumps in the cell membrane can also result in resistance by reducing the intracellular antimicrobial concentration below an inhibitory level (Li, 2004). Several classes of multi-drug resistance efflux pumps have been described including major facilitator superfamily MDR efflux pump, small multidrug resistance family MDR efflux pumps, multi drug and toxin extrusion family MDR efflux pumps, resistance nodulation division family efflux pumps and the ATP-binding cassette family MDR efflux pumps (Schindler and Kaatz, 2016). Efflux-mediated resistance is common for the sulfamethoxazole, tetracycline, and fluoroquinolone drug classes (Schindler and Kaatz, 2016). In Gram-positives including *S. aureus* and *S. pseudintermedius*, efflux pumps are either chromosomal or plasmid encoded (Costa et al., 2013).

Antibiotic modification enzymes can be classified into four major families including hydrolases, group transferase, redox enzymes and lyases which inactivate the beta-lactams, aminoglycosides, chloramphenicol, and tetracyclines respectively (Schroeder et al., 2017; Wright, 2005).The β-lactamases are a very common cause of resistance to β-lactams in both Gram positive and Gram negative bacteria (Dever and Dermody, 1991). These enzymes may be excreted, or in the case of some enzymes in Gram negative bacteria remain in the periplasmic space (Dever and Dermody, 1991). The aminoglycoside group transferases (N-acetyltransferases, nucleotide transferases, and phosphotransferases) alter the structure of these drugs, inactivating them19 (Mingeot-Leclercq et al., 1999; Ramirez and Tolmasky, 2010). A similar mechanism was found for chloramphenicol where both acetyltransferase and phosphotransferase inactivating enzymes have been described (Fernandez et al., 2012).

Alteration of the antimicrobial target has been shown to confer resistance to a number of drug classes including the β-lactams, fluoroquinolones, sulfonamides, and trimethoprim (Giguere et al., 2013). Resistance can also result from the overproduction of a drug target, allowing cellular metabolic functions to continue despite the presence of an antimicrobial. One example of this is the *dfr* mediated overproduction of dihydrofolate reductase which confers resistance to trimethoprim (Brolund et al., 2010). Similarly, sulfonamide resistance can result from the hyperproduction of para-aminobenzoic acid (PABA), which competitively excludes the incorporation of sulfonamide drugs and restores folate synthesis (Then, 1982).

1.3.1.2 General mechanism of acquiring resistance

Bacteria acquire resistance by four general mechanisms: 1. mutation, 2. transformation, 3. transduction and 4. Conjugation (Giguere et al., 2013). A mutation is a random event during

DNA replication; when mutants result in resistance they may confer a fitness advantage and therefore be selected for on exposure to antimicrobials. Specific mutations leading to resistance to fluoroquinolone and tetracycline antimicrobials has been observed; single nucleotide polymorphisms (SNPs) in the *gyr*A gene are shown to result in fluoroquinolone resistance in *E.coli* (Kogaluru Shivakumaraswamy et al., 2017). Similarly, 16S rRNA mutations have been shown to lead to tetracycline resistance in *Propionibacterium acne* (Ross et al., 1998). In some cases, mutational resistance has been shown to develop as a response to long-term exposure to non-lethal antimicrobial doses; for instance, *Mycobacterium tuberculosis* undergoes a high rate of mutation leading to resistance (Andersson and Hughes, 2012).

Entire resistance genes may also be introduced into a bacterium. The three primary mechanisms of gene acquisition are transformation (acquiring free DNA from dead cells), transduction (bacteriophages), and conjugation/horizontal transfer from other bacteria (plasmids and other mobile genetic elements) (Giguere et al., 2013; Schroeder et al., 2017). Horizontal gene transfer is the most common mechanism for the acquisition of foreign resistance genes and has been shown to be particularly common in polymicrobial infections (Champness, 2007; Schroeder et al., 2017). These mobile genetic elements may contain multiple antimicrobial resistance and virulence genes and may be broadly transmissible or limited to a species (Schroeder et al., 2017). Dissemination of resistance genes in *S. pseudintermedius* has been shown to occur by transposon conjugation, transduction, and transformation (von Wintersdorff et al., 2016). One key example is the Staphylococcal Chromosomal Cassette (SCC) element which contains both virulence and resistance genes.

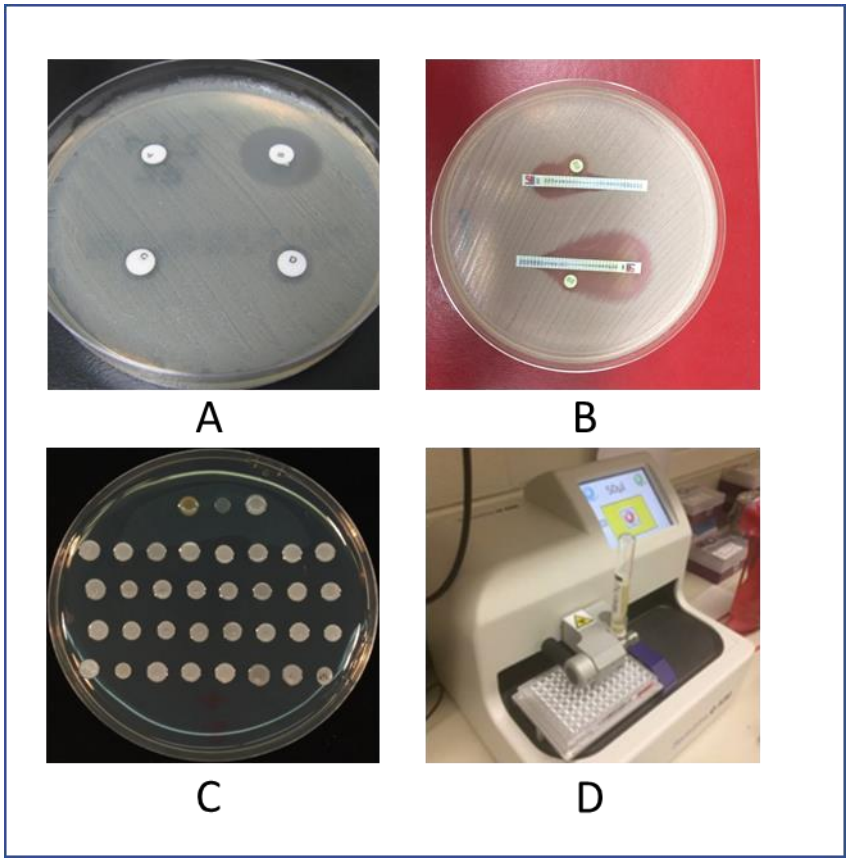

Figure 1.1: Antimicrobial susceptibility testing techniques

A: disk diffusion test, B: gradient strip test, C: agar dilution test, D: broth dilution test/micro broth

1.3.2 Summary of antimicrobial susceptibility testing

A number of laboratory methods have been developed to evaluate an antibiotic against selected microbes in vitro. Antimicrobial susceptibility testing follows a meticulously standardized protocol in the current diagnostic laboratory (Giguere et al., 2013). Standardized methods were initially introduced by Bauer et al in 1966, and have subsequently developed into our modern methods (Jorgensen and Ferraro, 2009; Matuschek et al., 2013). Standardization of protocols is critical to ensure that results are reproducible and reliable, two main international standards are recognized: The Clinical and Laboratory Standards Institute (CLSI), and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (CLSI, 2016; EUCAST, 2017). The standardization of antimicrobial susceptibility testing includes every step of the protocol including inoculum concentration, media, antibiotic disk concentration, incubation time and temperature and control bacterial strains. Test methods can be categorized as being based on diffusion or dilution (Figure 1.1).

1.3.2.1 Diffusion-based methods

The diffusion-based methods include the disk diffusion and gradient strip tests (Figure 1.1). To perform these assays, a fresh McFarland 0.5 bacterial suspension (approximately 10^8 CFU/ml) is seeded on 4 mm thick Mueller-Hinton agar. After the plate is inoculated, a disk or strip impregnated with an antibiotic is placed on the agar and incubated at 35°C for 18-24 hours (Balouiri. et al., 2016). A concentration gradient developed radially with high drug concentration immediately surrounding the disk and decreasing outwards. Bacteria grow only up to the maximum concentration they tolerate resulting in an inhibitory zone where no bacteria grow surrounding the disk. The diameter of the inhibitory zone is measured and interpreted according

to a standard set of criteria laid out by the CLSI or EUCAST (CLSI, 2010; EUCAST, 2017). The disk diffusion is the oldest method to be standardized and is the simplest test method (Jorgensen and Ferraro, 2009). The disk diffusion methods provide a qualitative description of susceptibility (categorizing isolates as susceptible, intermediate or resistant). This technique is a cost-effective means for screening the most common human and animal bacterial pathogens (Matuschek et al., 2013). This method has the advantage of not requiring special equipment, is simple to interpret and allows antimicrobial panels to be easily customized. However, this method is not suitable for certain fastidious bacterial species, limiting its application to common, aerobic pathogens (Jorgensen and Ferraro, 2009).

The gradient strip method (eg. E-test) is a hybrid diffusion-dilution assay where an antimicrobial gradient is impregnated into strips with high concentrations at one end and low at the other end (Balouiri. et al., 2016) (Figure 1.1). Agar plates (Mueller-Hinton) are inoculated as per the disk diffusion test, and following incubation the MIC is read "at the intersection of the strip and growth inhibition ellipse" (Balouiri. et al., 2016). Like disc diffusion testing this method is simple to perform and customization of antimicrobial test panels is easy. Unfortunately, the advantages of this method are offset by the relatively unreliable data generated compared to dilution based methods for determining MIC (Balouiri. et al., 2016; Jorgensen and Ferraro, 2009).

1.3.2.2 Dilution based methods

Dilution based antimicrobial susceptibility testing can be done in either and agar or broth-based media. In agar dilution, Mueller-Hinton agar plates are prepared with serial dilutions of antimicrobials and a standard bacterial inoculum (10^7) is spotted on the agar (Jorgensen and

Ferraro, 2009). The plate containing the lowest antimicrobial concentration where no growth is observed is the MIC. Although this method is technically simple to perform and has low reagent costs, it is laborious and requires large quantities of incubator space which limit its routine application in diagnostic settings.

Similar to agar dilution, broth dilution consists of a series of antimicrobial concentrations prepared in Mueller-Hinton broth. Broths are then inoculated to a standard bacterial density prepared from a 0.5 MacFarland suspension. This test can be carried out in 96 well microtitre plates (micro broth dilution) or in larger tubes (macro broth dilution). Following inoculation, the broth is incubated at 35^0C for 18-24 hours. The MIC is defined as the minimum concentration which completely suppresses the growth of bacteria, observed either as a lack of turbidity of lack of a cell pellet in each well or tube (CLSI, 2012a; Giguere et al., 2013).

1.3.2.3 Interpreting test results

Following a susceptibility test, clinical breakpoints are used to categorize isolates into one of three possible groups: susceptible, intermediate or resistant (CLSI, 2013). Because clinical breakpoints are designed to predict clinical outcome, they integrate microbiological data with the pharmacokinetic and pharmacodynamics properties of a drug (CLSI, 2012a, b; Giguere et al., 2013). The susceptible and resistant isolates has been defined by CLSI (M07-A9: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard-Ninth edition, 2012) (CLSI, 2012a). Drugs to which an organism is intermediately susceptible to may be used if the animal can be treated with a high dose, although toxicity may be limiting.

For *S. pseudintermedius*, clinical breakpoints have only been developed for ampicillin, oxacillin, cefoxitin, cephalosporins, cefazolin, cefpodoxime, pradofloxacin and doxycycline (CLSI, 2015).

These clinical breakpoints have only been described for skin and soft tissue infections, breakpoints specific to other clinical diseases have not been published in the guideline (CLSI, 2015). For other drugs, veterinary breakpoints for other staphylococci are often used for interpretation including amikacin, tetracycline, clindamycin, enrofloxacin, marbofloxacin, orbifloxacin, difloxacin and amoxicillin-clavulanate (CLSI, 2015). In the absence of veterinary breakpoints, human clinical breakpoints for *Staphylococcus aureus* are used for gentamicin, rifampin, sulfisoxazole, trimethoprim-sulfamethoxazole, vancomycin, erythromycin, chloramphenicol, quinupristin/dalfopristin, linezolid and nitrofurantoin (CLSI, 2015). For daptomycin and tigecycline, only EUCAST breakpoints for *S. aureus* are available, although these drugs are not used in companion animals (EUCAST, 2017).

When clinical breakpoints are not available, epidemiological cut-offs which are based on the population distribution of MICs can be used to identify bacteria possessing acquired resistance mechanisms. Isolates are categorized as either wild-type or resistant, isolates with MICs within normal distribution are defined as wild-type while those MICs above are defined as resistant (Morrissey et al., 2014). Importantly, the concept of epidemiological cut-off came up as the maximum possible MIC among wild isolates and considered as lack of acquired resistance in those isolates collection (Ängeby. et al., 2011). Isolates with MICs higher than ECOFF are assumed to possess some form of acquired resistance (e.g. mutational or a novel resistance gene) (Ängeby. et al., 2011). Although there is not necessarily an association between epidemiological cut-offs and clinical breakpoints of an organism, epidemiological cut-offs are useful for detecting emerging resistance (Ängeby. et al., 2011).

1.3.3 Antimicrobial resistance *Staphylococcus pseudintermedius*

1.3.3.1 Emergence of resistance

Antimicrobial resistance limits the ability of clinicians to select appropriate antimicrobials for the treatment of bacterial infections. Among *S. pseudintermedius*, resistance is emerging to the β-lactams, with resistance to penicillin reported in over 70% of *S. pseudintermedius* isolates colonizing healthy dogs and ~95% of clinical isolates (Bean and Wigmore, 2016; Kang et al., 2014; Yoon et al., 2010). A recent systemic review found that between 1980 and 2013 there was a trend of increasing resistance to penicillin and ampicillin among methicillin susceptible *S. pseudintermedius* (MSSP) (Moodley et al., 2014). Resistance to other classes including the fluoroquinolones, aminoglycosides, and chloramphenicol also increased during that period (Moodley et al., 2014). Interestingly, this review found that there was no significant difference in the frequency of resistance to non-β-lactams between methicillin resistant and susceptible isolates (Moodley et al., 2014). Alarmingly, resistance to amikacin which is used as last line therapy for treating MRSP infections in dogs is emerging and was recently identified in 37% of MRSP in the United States (Gold et al., 2014). In the Saskatoon region, MRSP was not identified in the baseline resistance surveillance study conducted in 2008 (Rubin and Chirino-Trejo, 2011). Subsequently, MRSP was identified from clinical cases in Saskatoon including a dog with necrotizing fasciitis and another with a UTI (Mayer and Rubin, 2012; Rubin and Gaunt, 2011a). Fortunately, resistance to last line of defense drugs (e.g. vancomycin or linezolid) used for treating resistant Gram-positive infections in humans has not been reported in *S. pseudintermedius*. Continued resistance surveillance targeting healthy dogs will be useful for detecting the emergence of resistance in the community. However, methodological

inconsistencies between studies including site and number of samples collected and media used to isolate *S. pseudintermedius* make the identification of global resistance trends difficult.

Methicillin resistant *S. pseudintermedius* first emerged in the late 1990s in Europe and North America and is increasingly reported globally (Bean and Wigmore, 2016; Damborg et al., 2016; Hensel et al., 2016; Kasai et al., 2016; Weese and van Duijkeren, 2010). From 2004 through 2013, the incidence of canine MRSP infections increased sevenfold at a veterinary diagnostic lab in Utrecht, the Netherlands (Duim et al., 2016). Colonization of 0-30% of healthy dogs with MRSP has been reported, and this organism has also been isolated from cats and people (Bean and Wigmore, 2016; Gomez-Sanz et al., 2011; Gronthal et al., 2015; Kjellman et al., 2015; Weese, 2010). Among diagnostic submissions, MRSP has reportedly been recovered from 5-66% of clinical submission from dermatological, otitis externa and UTI samples (Gold et al., 2014; LoPinto et al., 2015; Maluping et al., 2014; Windahl et al., 2012; Zur et al., 2016). Two main epidemic clones of MRSP are recognized, ST68 and ST71in North America and Europe respectively (van Duijkeren et al., 2011b). Both ST68 and ST71 frequently carry multiple resistance genes for tetracycline, quinolone, trimethoprim, aminoglycoside, macrolide, and lincosamide (McCarthy et al., 2015). Interestingly, MRSP in North America are reportedly often susceptible to chloramphenicol while isolates from Europe are frequently resistant (van Duijkeren et al., 2011b). More recently reports of high rates of resistance to chloramphenicol have suggested the emergence of a new MDR clone of MRSP in USA (Videla et al., 2017). Multi-drug resistant *S. pseudintermedius* (MDRSP) has also been increasing; in one study 62% of clinical isolates MRSP from dogs were MDR, resulting in very limited treatment options (Vigo et al., 2015; Zur et al., 2016). Other studies have reported that among 3%-27.5% are MDR (Detwiler et al., 2013; Garbacz et al., 2013).

Epidemiological studies have been done to identify risk factors for dogs to be infected with MRSP including previous antimicrobial therapy surgical interventions, duration of hospitalization, lack of decolonization strategy (Karanika et al., 2015; Sganga et al., 2016). Exposure to fluoroquinolones and β-lactams in the past month and long-term treatment with β-lactam drugs was identified as a risk factor for MRSP infection (Faires et al., 2010; Zur et al., 2016). Another study found that prior hospitalization and antibiotic exposure within the previous 6 months were risk factors (Nienhoff et al., 2011). The risk factors for colonization of MRSP in dogs have been studied to a limited extent; positive correlations between the number of veterinary visits, hospitalization, administration of glucocorticoids and topical antimicrobials, or being a breeding female were found (Gronthal et al., 2015; Lehner et al., 2014). In addition, Lloyd highlighted close contact with carriers or infected animals, ongoing invasive infections or surgical procedures as risk factors for MRSP colonization (Lloyd, 2010).

1.3.3.1.1 Methicillin resistance

Methicillin resistance is identified by using either cefoxitin or oxacillin susceptibility as an indicator (van Duijkeren et al., 2011a). When determining an isolates susceptibility to oxacillin, 2% NaCl must be added to the media (CLSI, 2017). For *S. pseudintermedius*, oxacillin testing is preferable to cefoxitin. MRSP isolates are frequently artifactually susceptible to cefoxitin in vitro, resulting in a high percentage of false negatives and the failure to detect methicillin resistance (Wu et al., 2016). PCR detection of *mec*A is recommended to confirm methicillin resistance identified by phenotypic tests (van Duijkeren et al., 2011a).

Gram-positive bacteria are covered by a thick layer of peptidoglycan which consists of short glycan chains of N-acetylmuramic acid and 1-4-N-acetyl glucose amine residues (Stapleton,

2002). The pentaglycine cross-bridges are cross-linked or transpeptidation occurs in the cytoplasmic membrane and which is catalyzed by the penicillin binding protein (PBP) (Stapleton, 2002). The beta-lactams inhibit transpeptidase activity by D-alanyl-D-alanyl bonds of stem peptide. However, beta-lactams have a weak affinity towards PBP2a found in methicillin resistant staphylococci (Stapleton, 2002). The serine binding site of PBP2a is less accessible to β-lactams due to a narrow-extended cleft compared to other PBPs (Peacock and Paterson, 2015).

The *mec*A gene which is the "classical gene" conferring methicillin resistance was discovered in 1986 (Becker et al., 2014). Later, *mec*C was found in MRSA playing a similar role as *mec*A among livestock and human isolates in Europe (Garcia-Alvarez et al., 2011). Furthermore, *mec*B were identified in *Macrococus caseolyticus* in Japan (Becker et al., 2014). Recently, *mec*D possessing *M. caseolyticus* from bovine and canine sources were identified although the risk of this novel methicillin resistance determinant for companion animals need to be investigated (Schwendener et al., 2017).

In Staphylococci, the *mec* family of genes encode for altered penicillin-binding proteins in the cell wall (Peacock and Paterson, 2015). The *mec*A gene was originally found in the Staphylococcal chromosomal cassette (SCC) complexes of coagulase-negative *Staphylococcus* sp. (Zong and Lu, 2010). The SCC is a mobile genetic element which facilitates integration into the chromosome and allows horizontal gene transfer (Peacock and Paterson, 2015; Sjostrom et al., 1975). Expression of *mec*A is regulated by *mec*R1 and *mec*I which are co-located in the SCC close to *mec*A gene, this complex of *mec* genes within the SCC is referred to as the "SCCmec" (Peacock and Paterson, 2015; Stapleton, 2002).

Altogether 12 SCCmec types (I – XII) have been described, SCCmec II-III, IV, V, and VII are the most common in *S. pseudintermedius* (Chanchaithong et al., 2016). Between types, the size

of the SCCmec varies between 21-67kb; different types may contain different genes for integration, chromosome recombinase and the "J region" which houses additional antimicrobial resistance genes (Peacock and Paterson, 2015). The SCCmec complex can excise from the chromosome and then reintegrate into the chromosome of another organism by excision gene called *ccr*A and *ccr*B which in SCCmec complex (Zong and Lu, 2010). Within the SCCmec complex, there is evidence of a number of mobile genetic elements including insertion sequences, transposons, integrated plasmids and unknown functional genes (Zong and Lu, 2010). The SCC elements are involved in the acquisition of multiple resistance genes in Staphylococcus including *S. pseudintermedius* (McCarthy et al., 2015; Zur et al., 2016). As an example, SCCmec type II-III are found in hospital-acquired MRSP with multiple resistance genes (Zur et al., 2016).

1.3.3.1.2 Mechanisms of resistance to tetracycline

Acquired resistance to tetracyclines has been reported by four different mechanisms: 1. the production of ribosome protection proteins, 2. efflux, 3. enzymatic inactivation of the drug and 4. mutation in genes encoding the 16S rRNA or the small subunit ribosomal protein (Chopra and Roberts, 2001). Although a large number of tetracycline resistance genes have been identified, only four genes (*tet*K, *tet*M, *te*tO, *tet*L) have been previously reported in *S. pseudintermedius* (Kadlec and Schwarz, 2012). The *tet*K and *tet*L genes encode tetracycline specific efflux pumps belonging to the major facilitator superfamily (MFS) of efflux pumps, in staphylococci, these are commonly found on plasmids (Chopra and Roberts, 2001; Khan and Novick, 1983). The *tet*K gene is found in both Gram positive and negative bacteria including *S. aureus* and *E. coli* (antibiotic resistance gene database: ardb.cbcb.umb.edu) (Adefisoye and Okoh, 2016). The *tet*L

gene has also been found among a variety of bacterial genera including *Streptococcus*, *Actinocbacillus, Lactobacillus*, and *Mannheimia* (Chopra and Roberts, 2001).

The *tet*M and *tet*O encode for the ribosomal protection proteins in Staphylococci (Kadlec and Schwarz, 2012). Both *tet*O and *tet*M are found in a wide host range including Gram-negative and Gram-positive bacteria (Chopra and Roberts, 2001). The antibacterial activity of tetracycline is due to preventing the binding of the aminoacyl-tRNA to ribosomal-mRNA complex which halts protein synthesis (Chopra and Roberts, 2001). Ribosomal protection proteins confer tetracycline resistance by binding to the aminoacyl-tRNA complex and displacing tetracycline from the ribosome (Chopra and Roberts, 2001). In *S. pseudintermedius*, *tet*M is the most commonly found tetracycline resistance gene (Kadlec and Schwarz, 2012). The *tet*M gene has been found in both the Tn916 and Tn5801 transposons which are integrative and conjugative elements (ICE) (de Vries et al., 2016). These conjugative transposons have a broad host range and have also been shown to play a role in the dissemination of chloramphenicol, kanamycin, and erythromycin resistance genes (Chopra and Roberts, 2001; de Vries et al., 2016).

The tetracyclines can be inactivated by *tet*X, *tet*34, *tet*37 and *tet*U although these genes have not been reported in Staphylococci (Thaker et al., 2010). Mutational resistance involving SNPs in the small subunit RNA and the S12 ribosomal protein which facilitate interaction between tetracycline and ribosome are also recognized (Chopra and Roberts, 2001; Nguyen et al., 2014). Multidrug resistance efflux pumps including those in the ABC, MFS, and MATE have been reported to include tetracycline as a substrate in other Gram-positive bacteria, although their role in *S. pseudintermedius* has not been studied (Schindler and Kaatz, 2016).

At present, the Clinical Laboratory Standard Institute (CLSI) recommends using tetracycline as an indicator drug to screen resistance to doxycycline, minocycline. The guideline also states that

isolates which possess intermediate resistance to tetracycline may susceptible for doxycycline (CLSI, 2017). However, because doxycycline and tetracycline have different chemical properties they may not necessarily interact with bacteria identically (Nguyen et al., 2014). Moreover, doxycycline and minocycline are lipophilic and were shown to bind with the ribosome with higher affinity than tetracycline (Nguyen et al., 2014). The correlation of resistance between these three drugs is ill-defined and the contribution of recognized resistance genes to phenotypic resistance has not been evaluated.

1.3.4 Identification of novel resistance mechanism

Next generation sequencing is an emerging technique for the identification of new antimicrobial resistance genes. The rapid advancement of techniques with decreasing analytical turnaround time and diminishing cost has increased the availability of this method to identify novel resistant determinants in bacteria. Moreover, new easier to use bioinformatic tools including “Geneious” and “CLC viewer” greatly facilitate quality checking and de-novo assembly. Genomes can be annotated using free online tools, resistance genes can be readily identified using a number of curated, searchable online databases such as CARD, ResFinder, ARDB, MEGARes and RESFAMS which already contain more than 13,000 antibiotic resistant determinants (Gordon et al., 2014; Jia et al., 2017; Kwong and McCallum, 2015; Zankari et al., 2012; Zhao et al., 2016). These tools allow researchers from the biological sciences to meaningful analyze genome sequences without advanced knowledge of computer programming. Recently, the role of WGS in the future of antimicrobial susceptibility testing was reviewed by a special committee of EUCAST with the aim of standardizing how genomic tools are used for identifying antimicrobial resistance (Ellington et al., 2017).

The advantage of WGS for the identification of new resistance genes is that it doesn't necessarily require prior knowledge of the gene's sequence as would be needed for targeted PCR based assays (Zhao et al., 2016). The identification of new genes relies on the comparison of genomes from organisms with susceptible and resistant phenotypes and it therefore essential to complement genomic approaches with classical phenotypic characterization. Furthermore, when unknown resistant phenotypes are being investigated, the next step is to identify and characterize the gene or mutation responsible for the phenotype. Once a genome is sequenced, the investigator is not limited to searching for resistance genes and mutations, but he or she can also look for variation in the genes promoter region or other nucleotide sequences which may affect expression. Once the sequence of a resistant gene is identified, the genomes of other organisms can be queried for this sequence. This strategy of resistance gene identification was exemplified by a recent study by Cavaco et al, who identified a novel linezolid-resistance gene in Enterococcus sp. in Colombia (Cavaco et al., 2017). The identification of resistance associated SNPs in the core genome has been used to forecast the emergence of resistance in bacteria. Finally, WGS is a valuable tool for detecting resistant genes from organisms which can not be cultured by conventional bacteriological methods.

Whole genome sequence collections serve as a valuable data set which can be queried when novel resistance determinants are identified. As an example, following the publication of the sequence of the mobile colistin resistance (*mcr*-1) gene, which was recently identified in *E. coli* in China, *mcr*-1 was rapidly identified in different isolate collections around the world (Liu et al., 2016).

1.4 Molecular epidemiology of *Staphylococcus pseudintermedius*

1.4.1 Molecular epidemiological tools

Molecular epidemiological tools are used to determine isolate relatedness or identify a common source of bacterial isolates in a given population at a particular time (ex. during an outbreak). Some techniques are based on DNA sequence analysis while others which compare banding patterns are indirectly based on sequence. A number of molecular tools used for characterization Staphylococcus spp. (Table 1.3) have been optimized for *S. pseudintermedius* (Solyman et al., 2013). Each technique possesses its own resolving power, cost, availability of the global database, the requirement for specialized equipment and selection of the technique is based on the requirement and purpose of the study.

There are a number of techniques specific to Staphylococci which are commonly used in molecular epidemiological investigations. *Spa* typing is one method which exploits the hypervariable region of "Staphylococcus Protein A" used for differentiating closely related strains (Sivaraman et al., 2009). The region consists of a number of polymorphic repeats, the region can be characterized using online databases available (seqnet.org, spaserver.ridom.de). The sequence is submitted to the database and assigned a *spa* type based on a comparison with previously deposited sequences. The Staphylococcus chromosomal cassette is the target region for the SCCmec typing which is classified as a hierarchical way with "types" and "subtypes". The classification is based on *ccr* and *mec* gene complexes in SCC element and has been standardized internationally. SCCmec typing can be combined with other characterization methods including *dru* typing. The *dru* typing method is focused on direct repeat unit-variable-number tandem repeat which is found in SCCmec element close to IS431(dru-typing.org). These 40 base repeat sequences are searched in database and combination of repeats are taken (dru-

typing.org). *dru* typing is a sequence-based method which has the advantage of interlaboratory reproducibility and relatively low cost (Kadlec et al., 2015). Since SCCmec is found in MRSP, both SCC *mec* typing and *dru* typing are only limited to characterize methicillin resistant isolates (Chanchaithong et al., 2014). These methods have been successfully used in investigations of *S. pseudintermedius* (Couto et al., 2016b; Kadlec et al., 2016). Band based techniques such as pulsed-field gel electrophoresis are also commonly used in large outbreaks, these techniques have relatively low reagent costs and due to their high-resolving power are useful for characterizing outbreaks (Rota et al., 2015). A combination of two methods, including one band based technique and one sequence-based method, together overcome the limitations of each technique, with high accuracy and reproducibility and interlaboratory comparison (Kadlec et al., 2015).

1.4.1.1 Pulse field gel electrophoresis

Pulse field gel electrophoresis (PFGE) was once the gold standard for investigating outbreak investigations, although it is currently being replaced by WGS (Salipante et al., 2015). Briefly, genomic DNA is digested using restriction enzymes, resulting in variably sized fragments which can be resolved on a gel. Single nucleotide polymorphisms at restriction sites change the size of bands by eliminating or generating novel restriction sites resulting in fewer or lesser fragments. The resulting banding pattern is compared, and its similarity indicates how closely associated two isolates are.

Performing PFGE is a labor-intensive process. In brief: bacterial DNA is digested using restriction enzymes, and fragments are resolved by gel electrophoresis. *Sma*I and *Cfr*9I are the most commonly used restriction enzymes for PFGE of *S. pseudintermedius* (Chanchaithong et

al., 2014). Following digestion, a specialized electrophoresis chamber which applies a current whose flow constantly changes direction, allowing large molecular weight DNA fragments to be separated. Electrophoresis can be done in a system such as the Bio-Rad CHEF-DRIII apparatus, following separation of DNA fragment the gel is stained for the visualization of bands with ethidium bromide. DNA fragments are then visualized under UV light and images are captured for analysis using software such as GelCompare. Banding patterns are then defined using the Dice coefficient and compared using the UPGMA algorithm to quantify the relatedness of isolates. Banding patterns are then compared, one set of criteria developed by Tenover et al., described four categories of relatedness that can be assigned to isolates: 1. indistinguishable, 2. closely related, 3. possibly related and 4. unrelated) (Tenover et al., 1995).

Because PFGE banding patterns vary with polymorphisms throughout the entire genome, rather than at a particular locus or set of loci, the resolving power of this technique is high compared to other fingerprinting methods such as *spa* typing or MLST (Ohadian Moghadam et al., 2017). However, a limitation of this method is that without highly trained, specialized labor and the use of standardized protocols, the data generated by PFGE cannot be readily compared between laboratories (Salipante et al., 2015). *Staphylococcus pseudintermedius* isolates have been compared with PFGE to identify transmission between dogs, and from dogs to humans (Chanchaithong et al., 2014; Gomez-Sanz et al., 2013c; Rota et al., 2015). PFGE is often performed in combination with other techniques including multi-locus sequence typing (MLST), SCCmec typing and spa typing (Chanchaithong et al., 2014).

1.4.1.2 Multilocus sequence typing (MLST)

Multilocus sequence typing schemes have been developed for many bacterial species and data is available in open databases hosted many websites such as www.mlst.net, mlst.warwick.ac.uk and pubmlst.org. This method is based on comparing the sequences of 'housekeeping' genes unique to each bacterial species, for *S. pseudintermedius* a seven-housekeeping gene (*ack, cpn60, fdh, pta, purA, sar, tuf*) scheme has been developed (Solyman et al., 2013). Each unique allelic sequence is assigned a number, and the allelic profile defines the sequence type. As of April 12, 2017, 733 sequence types have been assigned in *S. pseudintermedius* (Solyman et al., 2013). MLST provides an accurate, unambiguous, portable information useful for studies of bacterial evolution and global epidemiology (Solyman et al., 2013). However, the high cost of sequencing cost and relatively low resolving power are important limitations of this method. For MLST, each gene is PCR amplified followed by sequencing; allelic profiles are generated following submission of data to the appropriate database. Allelic profiles can be compared manually or utilizing algorithms such as eBURST, and relationships between isolates can be displayed as a dendogram or minimum spanning trees.

MLST has been widely used for *S. pseudintermedius*, ST71 and ST68 have been reported to be epidemic clones in Europe and North America respectively (Perreten et al., 2010). More recently ST71 has been found more broadly and is now considered to be a globally epidemic clone (Gronthal et al., 2014). At present, there are no descriptions of ST68 in Western Canada although it is widely reported in the United States. The dissemination of these epidemic clones across borders is no doubt facilitated by the ease of international movement of people and animals. Globally, five major MRSP clonal complexes were identified by international study including isolates from all over the world in *S. pseudintermedius* (Pires Dos Santos et al., 2016).

Interestingly the SCCmec element of those clusters differed, suggesting that multiple recombination seem to be common in the genome of *S. pseudintermedius* genome (Pires Dos Santos et al., 2016).

1.4.1.3 Whole genome sequencing (WGS)

Recent technological advancements have made whole-genome sequencing a popular molecular epidemiological tool. DNA sequencing can be done using a number of technologies commonly described the first generation and next-generation sequencing. First generation sequencing technologies are relatively low throughput, allowing at most several hundred fragments to be sequenced simultaneously. These techniques include those developed by Maxam and Gilbert, and Sanger and were used extensively early in the era of genome sequencing (Choudhuri, 2014).

Next generation technology is high-throughput and massively parallel allowing tens of thousands to billions of DNA fragments to be sequenced simultaneously allowing large amplicon libraries or fragmented whole genomes to be quickly sequenced. Bacterial genomes have been sequenced using a variety of platforms including Roch 454, Illumina and ABI SoliD (Choudhuri, 2014).

Roche 454 was the first next-generation sequencing and commercially launched in 2005, which is single molecule improvement to the standard pyrosequencing (Choudhuri, 2014). In this method, sequencing libraries are amplified by emulsion PCR. Fragments or amplicons of up to 800-1000 base pairs are sequenced following ligation of adapters which act as a universal priming sequence for both amplification/sequencing. In summary, this technique consists of DNA fragmentation and adapter ligation, one fragment-one bead complex formation, fragment amplification by em-PCR, purification and sequencing by synthesis (Choudhuri, 2014).

Illumina is the most widely used platform currently and introduced in 2008 (Choudhuri, 2014). Although both Illumina and Roche are described as sequencing by synthesis, Illumina utilizes fluorescent reversible terminator chemistry which eliminates issues with homo polymers as described in Roche 454 (Choudhuri, 2014). The technique consists of DNA library preparation, fixing to flow cells, bridge amplification, cluster generation and sequencing by synthesis (Choudhuri, 2014). The fluorescent is detected by CCD camera (Choudhuri, 2014). Furthermore, once DNA library is prepared, double-stranded DNA denatured and binds to the flow-cell channel by hybridizing adapters. After that, DNA clustered are made and the double-stranded bridge is formed followed by denatured into a single strand. Only forward strand remains in flow cell channel all reverse strands are washed away. The sequencing by synthesis occurs in those forward strands clustered, fluorescently labeled nucleotides are added individually and detected.

The ABI SOLiD platform is based on the principle of sequencing by oligonucleotide ligation and detection (Choudhuri, 2014). In summary, this method consists of DNA library preparation, the formation of bead-fragment complexes, fragment amplification, purification, bead immobilization and sequencing by ligation (Choudhuri, 2014). A series of ligation cycles occurs (ligation, cleavage, and detection) and templates are reset for the following cycles by removing extension products, the fluorescent dye is used for detection of ligated base combinations (Choudhuri, 2014). Sequenced genomes can then be assembled de-novo, or mapped to a reference genome. The rapid advancement of sequencing technology has reduced the duration and costs of sequencing, while the initial human genomic project took years to complete at a cost of billions, the same data could now be generated in a matter of days for several thousand dollars (Collins et al., 2003).

WGS has applications in new pathogen identification, the design of diagnostic tests, molecular epidemiological investigations and improves our ability to estimate evolutionary time frames within bacterial populations (Lynch et al., 2016). Furthermore, identification of microbial organism which defined as slow-growing organism including non-tuberculous Mycobacterium and unusual disease presentations such as neuro leptospirosis occur by WGS (Kwong and McCallum, 2015). In addition, for patients with infections with a pathogen which is undetectable using routine tests (e.g. genetic mutations preventing primer binding, novel pathogens, organisms which are not culturable) can be captured by WGS; the identification of a Swedish variant of Chlamydia trachomatis which was not detected using conventional methods is an illustrative example (Unemo M, 2010). The source of a cholera outbreak in Haiti 2010 was identified as peacekeeping forces from Nepal using a WGS approach to confirm and clarify the results of a PFGE based investigation (Chin et al., 2011; Oslen R. J., 2012). Recently, single nucleotide polymorphism (SNP) analysis of sequences of Yersinia pestis isolates from around the world allowed investigators to conclude that this organism evolved from a common ancestor in China and radiated globally with explorers and merchants (Morelli et al., 2010).

Next-generation sequencing is a rapidly developing technique in molecular epidemiology facilitating an improved understanding of the source, transmission route, and even mechanisms of disease pathogenesis in given population (Lynch et al., 2016). As a surveillance tool, Whole genome sequencing has some advantages over other techniques including unparalleled resolving power, less bias towards specific regions of the genome, generation of objective and portable data (compared to bands) and that it is increasingly technically easy to do and not terribly laborious. SNPs in the core genome can be used as a typing tool, and have shown promise in forecasting new or re-emerging bacterial strains (Gordon et al., 2014; Kwong and McCallum,

2015; Pumina et al., 2015; Zhao et al., 2016). Altogether, WGS facilitate origin of resistance, virulence progression and spreading of bacterial lineages in a population, the most influential tool in current molecular epidemiology (Price et al., 2013).

As a molecular epidemiological tool, WGS is now considered the gold standard for identifying and monitoring the evolution of multi drug resistant organisms (Pumina et al., 2015). Beyond the ability to identify resistant genes, WGS allows the user to identify antimicrobial resistance gene complexes, the location of resistance genes in the genome or on mobile genetic elements such as a plasmid, genome cassettes, integrons, IS elements and transposons (Gillings, 2014). That information may assist understanding resistance gene transfer or horizontal gene transfer of resistant determinants in the bacterial population (de Vries et al., 2016).

Further characterization of mobile genetic elements such as plasmids facilitates understanding the source of resistance and relationship among resistance determinants. As an example, clusters of infections with organisms containing a novel plasmid which carries bla_{NDM} was identified in *E. coli* and *Klebsiella pneumoniae* isolated from multiple locations including the community and healthcare settings hospitals; such outbreaks are not analyzable using traditional molecular epidemiological tools which are limited to a single bacterial species (Khong et al., 2016).

WGS is currently used in outbreak investigation of clinical disease including spreading and transmission of pathogens in hospitals (Popovich and Snitkin, 2017). In addition, resistance gene complexes such as genome cassettes (such as Staphylococcus chromosome cassettes), introns, transposons and mobile genetic element can be identified when sequencing the genome (de Vries et al., 2016).

Table 1.3: Summary of molecular techniques which are commonly used in Staphylococci for epidemiological characterization

Molecular technique	**Band / sequence**	**Resolution power**	**Database**
Random amplification polymorphism (RAPD-PCR)	band	high	-
Amplified fragment length polymorphism (AFLP)	band	high	-
Pulse field gel electrophoresis (PFGE)	band	high	www.cdc.gov/pulsenet www.harmony-microbe.net
Multilocus variable number tandem repeat (MLVA)	band	high	www.mlva.net
Staphylococcus protein A (*spa* typing)	sequence	high	www.spaserver.ridom.de
Staphylococcus cassette chromosome (SCCmec typing)	sequence	low	-
Direct tandem repeat unit (*dru* typing)	sequence	low	dru.typing.org
Multilocus sequence typing (MLST)	sequence	low	pubmlst.org
Whole genome sequencing (WGS)	sequence	highest	-

OBJECTIVES

1. To describe the antimicrobial susceptibility of *S. pseudintermedius* colonizing healthy dogs by conducting a surveillance study.

2. To compare the antimicrobial susceptibility and relatedness of *S. pseudintermedius* isolated from canine urinary and dermatological infections.

3. To determine the disease incidence, antimicrobial resistance profiles, and sequence types of strains of *S. pseudintermedius* causing human infections in a large Canadian health region.

4. To compare the susceptibility of *S. pseudintermedius* to tetracycline, doxycycline, and minocycline and to describe the mechanisms of tetracycline resistance.

2 ANTIMICROBIAL SUSCEPTIBILITY OF *Staphylococcus pseudintermedius* COLONIZING HEALTHY DOGS IN SASKATOON, CANADA

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Citation

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Author Contributions

Conceived and designed experiment: MARP MCG JER. Performed the experiment: MARP. Analyses the Data: MARP JER. Wrote the paper: MARP JER.

2.1 Abstract

This study reports antimicrobial susceptibility of *Staphylococcus pseudintermedius* carried healthy dogs in Saskatoon and describes changes in antimicrobial resistance since a 2008 study. One hundred healthy dogs presenting to the wellness services at the Western College of Veterinary medicine were screened for *S. pseudintermedius* by culturing rectal and pharyngeal swabs. *Staphylococcus pseudintermedius* was identified biochemically and antimicrobial minimum concentration was determined by broth microdilution. Methicillin resistance was confirmed by polymerase chain reaction (PCR) and sequencing of *mec*A gene. Of 221 *S. pseudintermedius* isolates from 78 dogs, 7 were methicillin resistant. No resistance to the fluoroquinolones, nitrofurantoin, tigecycline, vancomycin, quinupristin-dalfopristin, linezolid or daptomycin was identified. Of the 78 positive dogs, isolates resistant to penicillin were found in 78%, ampicillin 61%, and tetracycline 26% while resistance to oxacillin, erythromycin, clindamycin, trimethoprim + sulfamethoxazole, chloramphenicol, and gentamicin was found in <10% of dogs. Compared to the 2008 study, the frequency of resistance to all drugs increased, and the frequency of colonization with pan-susceptible isolates decreased from 46% to 30%.

2.2 Introduction

Staphylococcus pseudintermedius (recognized as distinct from *S. intermedius* in 2005) is a Gram-positive bacterium which colonizes the skin and mucosal surfaces of up to 90% of healthy dogs (Bannoehr and Guardabassi, 2012; Devriese et al., 2005; Rubin and Chirino-Trejo, 2011). Clinically, *S. pseudintermedius* is the most common cause of pyoderma and otitis externa, the second most common cause of urinary tract infections and is frequently implicated in nosocomial infections in dogs (Ball et al., 2008; van Duijkeren et al., 2011b). The ubiquity of canine *S.*

pseudintermedius infections in the community and the frequency of empiric treatment by veterinarians highlights the importance of antimicrobial resistance surveillance to inform evidence-based empiric therapeutic selection.

The emergence of antimicrobial resistance is a great challenge to antimicrobial therapy for animals and humans. The propensity of staphylococci to adapt to the selection pressure of antimicrobial use has been recognized since the first description of penicillin-resistant *S. aureus* in the 1940s (Barber, 1947). Resistance to penicillin among staphylococci, including companion animal *S. pseudintermedius* isolates, is most commonly due to the production of staphylococcal β-lactamase, conferred by the *bla*$_Z$ gene (Fuda et al., 2005; Malik et al., 2007). *Staphylococcus pseudintermedius* has historically remained remarkably susceptible to antimicrobials, but since 2006 there has been a dramatic worldwide increase in the frequency of methicillin resistance (Moodley et al., 2014; van Duijkeren et al., 2011b). Methicillin resistance, which is rapidly emerging among *S. pseudintermedius* in dogs and common among *S. aureus* in human is a serious threat to the efficacy of the most frequently used antibiotics, the β-lactams (Perreten et al., 2010; Prescott et al., 2002; Weese and van Duijkeren, 2010). Methicillin resistance conferred by the *mec*A and *mec*C genes results in the production of altered cell wall proteins with a low affinity for β-lactam drugs; leading to resistance to all β-lactam antimicrobials currently licensed for use in veterinary medicine including the penicillin, cephalosporins, and carbapenems (Becker et al., 2014). Because methicillin resistance is not the product of β-lactamase production, the addition of β-lactamase inhibitors such as clavulanic acid does not restore susceptibility. Furthermore, methicillin resistance in *S. pseudintermedius* is often associated with multidrug resistance, further limiting the treatment options available to veterinarians (Moodley et al., 2014; van Duijkeren et al., 2011a).

In the late 2000's there was an explosive increase in the incidence of MRSP associated with two lineages of *S. pseudintermedius*, sequence type (ST) 71 in Europe and ST68 in North America (Bardiau et al., 2013; Perreten et al., 2010). Among healthy dogs in North America and Europe 0-4.5% have been found to carry MRSP, while up to 66% of clinical *S. pseudintermedius* isolates have been reported to be methicillin resistant (Gingrich et al., 2011; Griffeth et al., 2008; Hanselman et al., 2007; Hanselman et al., 2009; Kawakami et al., 2010; van Duijkeren et al., 2011a). In Saskatoon, *S. pseudintermedius* carried by healthy dogs and those causing infections have historically been remarkably susceptible; a 2008 study failed to identify any animals carrying methicillin resistant *S. pseudintermedius* (MRSP) (Ball et al., 2008; Rubin and Chirino-Trejo, 2011). Since 2009, reports of canine infections with MRSP in Saskatoon including urinary tract infections and necrotizing fasciitis suggest the emergence of resistance in this region (Mayer and Rubin, 2012; Rubin and Chirino-Trejo, 2011). The objective of this study was to determine the antimicrobial susceptibility profiles of *S. pseudintermedius* colonizing healthy dogs in Saskatoon, and identify changes in the frequency of resistance since the 2008 investigation.

2.3 Material and Methods

2.3.1 Sample Collection

Between June and September 2014, 100 clinically healthy dogs presenting to the wellness service of the Veterinary Medical Centre at the Western College of Veterinary Medicine were included (Table 2.1). Pharyngeal and rectal samples were collected using sterile swabs with Stuart transport media (Becton Dickinson and Company, Sparks, MD) as previously described (Rubin and Chirino-Trejo, 2011). Pharyngeal samples were collected by gently rolling a sterile

swab across the pharynx for 1-3 s, and rectal swabs were collected by gently inserting a second swab 3cm into the dog's rectum and rotating for 1-3 s. All samples were processed within 4 hours of collection. This study was approved by the University of Saskatchewan animal research ethics board (protocol #20130135).

2.3.2 Culture and Susceptibility Testing

All swabs were directly plated on "CHROMagar" Staph aureus (CHROMagar, Paris, France), and Mueller-Hinton agar + 4μg/mL oxacillin. Plates were then incubated overnight at 35ºC and up to 5 *S. pseudintermedius*-like colonies (mauve color) were sub-cultured to Columbia agar with 5% sheep blood (Becton, Dickinson). Isolates were identified based on colony morphology (small, creamy grey to white, round colonies with a smooth margin and double zone of hemolysis on blood agar) and biochemically using the catalase and tube coagulase tests using rabbit plasma, the production of acetoin and hyaluronidase and the fermentation of mannitol, maltose and trehalose (Rubin and Chirino-Trejo, 2011; Winn et al., 2006). Since the carriage of genetically diverse *S. pseudintermedius* strains by individual dogs has been recognized, 3 isolates per animal were saved for future testing to increase the likelihood of detecting resistant organisms (Paul et al., 2012). Bacteria were stored at -80ºC in trypticase soy broth + 15% glycerol. For dogs carrying *S. pseudintermedius* at both sites, 2 pharyngeal and 1 rectal isolate were saved.

Antimicrobial minimum inhibitory concentrations were determined by broth microdilution using the GAPLL1F Sensititre panel (Thermo Fisher Scientific, Oakwood Village, Ohio, USA). Tests were conducted according to the Clinical and Laboratory Standards Institute (CLSI) and manufacturer's guidelines (CLSI, 2012a). A panel of drugs including: penicillin (PEN),

ampicillin (AMP), oxacillin (OXA) (with 2% NaCl), erythromycin (ERY), clindamycin (CLI), tetracycline (TET), tigecycline (TGC), trimethoprim + sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LEV), moxifloxacin (MOX), gentamicin (GEN), chloramphenicol (CHL), rifampin (RIF), nitrofurantoin (NIT), vancomycin (VAN), linezolid (LZD), daptomycin (DAP) and quinupristin + dalfopristin (QDA) was used. For quality control, *S. aureus* ACTCC 29213 and Enterococcus faecalis ACTCC 29212 were used (CLSI, 2013). Antimicrobial MICs were categorized as susceptible or resistant using CLSI breakpoints for all drugs except tigecycline and daptomycin for which the EUCAST interpretive criteria were used (CLSI, 2013, 2014; EUCAST, 2014). Isolates were considered to be MRSP when resistant to oxacillin (MIC ≥ 0.5µg/ml); genotypic resistance was confirmed by PCR and sequencing of the mecA and mecC genes using previously described primers (Stegger et al., 2012). Isolates resistant to erythromycin and susceptible to clindamycin were tested for inducible clindamycin resistance using the D-test as described by the CLSI (CLSI, 2014).

Table 2.1: Characteristics of sampled dog (n = 100)

Age	2.5 months - 12 years (median = 3 years)
Gender*	
Intact male	18
Neutered male	33
Intact female	14
Neutered female	32
History of antimicrobial use, past 6 months	
Yes	9
No	91

* Information on gender was not recorded for 1 dog.

2.4 Results

Of the 100 dogs tested, *S. pseudintermedius* was isolated from 78. A total of 221 isolates were collected, including single isolates from 5 dogs, 2 isolates from 3 dogs, and 3 isolates from 70 dogs. For dogs in which <3 isolates were initially identified, all isolates were saved. No *S. pseudintermedius* was isolated from Mueller-Hinton agar with 4µg/ml oxacillin, all isolates were recovered from CHROMagar *Staph aureus*. Antimicrobial susceptibility testing revealed phenotypic diversity among multiple isolates from individual dogs. Of the 78 positive animals, isolates with varying susceptibility profiles were grown from 30, while phenotypically homogeneous isolates were grown from 48. Consequently, the frequency of resistance among the overall isolate collection was lower than the percentage of animals carrying isolates expressing any particular resistance phenotype; for example, if 1 of 3 isolates carried by a dog was resistant to tetracycline, that dog is considered to carry tetracycline resistant isolates (Table 2.2). No resistance to ciprofloxacin, levofloxacin, moxifloxacin, nitrofurantoin, rifampin, tigecycline, vancomycin, quinupristin + dalfopristin, linezolid or daptomycin was identified. The most common resistance profile was penicillin + ampicillin resistance (n=70, 31.7%) followed by pan-susceptibility (n=67, 30.3%) (Table 2.3). Methicillin resistant isolates (n=8) were identified in 7 (9%) of *S. pseudintermedius* carrying dogs. Resistance to trimethoprim + sulfamethoxazole, chloramphenicol and gentamicin was less common (Table 2.3). All oxacillin resistant isolates possessed the *mec*A gene while *mec*C was not identified. None of the 5 erythromycin resistants, clindamycin-susceptible isolates were inducibly clindamycin resistant.

Fifteen multidrug-resistant isolates (MDR; resistance to 3 or more drugs classes) were identified, all were methicillin susceptible *S. pseudintermedius* (MSSP) (Table 2.3). Notably, one isolate was resistant to PEN, AMP, ERY, CLI, CHL and GEN.

Table 2.2: MIC distribution of isolates and the percentage of animals colonized with resistant isolates in 2008 and 2014

Drug (µg/ml)	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	% Resistant Isolates 2014 (n=221)	% Animals 2014 (n=78)	% Animals 2008 (n=153)
PEN		75	6	8	15	12	6	11	42	46				63.3	73.0	39.9
AMP			82	32	26	37	26	11	3	4				48.4	61.5	9.8
OXA				213	2	1			5					3.6	9.0	0
ERY				134	73	3			11					5.0	9.0	3.3
CLI					214	1		6						2.7	5.1	2.6
TET							174	1			46			20.8	25.6	23.5
TGC		70	142	9										0	0	0
SXT					210	3		1	7					3.6	3.8	0
CIP						221								0	0	0
LEV				214	4	1	2							0	0	0
MOX				221										0	0	0
GEN							218	1	1		1			0.5	1.3	0
CHL								16	175	28	2			0.9	2.6	0
NIT											221			0	0	0
RIF					220	1								0	0	0
VAN				3	204	14								0	0	0
LZD						138	81	2						0	0	0
DAP					221									0	0	0
QDA					219	2								0	0	0

Antimicrobial minimum inhibitory concentration (MIC) distribution for *Staphylococcus pseudintermedius* isolates (n=221) for penicillin (PEN), ampicillin (AMP), oxacillin (OXA) (with 2% NaCl), erythromycin (ERY), clindamycin (CLI), tetracycline (TET), tigecycline (TGC), trimethoprim + sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LEV), moxifloxacin (MOX), gentamicin (GEN), chloramphenicol (CHL), rifampin (RIF), nitrofurantoin (NIT), vancomycin (VAN), linezolid (LZD), daptomycin (DAP) and quinupristin + dalfopristin (QDA).Cells corresponding to concentrations tested are outlined in black while the resistance breakpoint is shaded. The number of isolates inhibited at each concentration is noted in each cell.

Table 2.3: Summary of resistance profiles of *S. pseudintermedius* isolates (n=221)

Resistance profile	Number of isolates
Pan-susceptible	67
PEN + AMP	70
PEN	27
TET	13
PEN + AMP + TET	13
PEN + AMP + OXA	6
PEN + AMP + SXT + TET	6
PEN + TET	4
PEN + AMP + TET + ERY + CLI	4
PEN + AMP + TET + ERY	3
PEN + AMP + SXT	2
PEN + AMP + OXA + TET	1
PEN + AMP + OXA + ERY	1
TET + ERY	1
PEN + ERY + CLI	1
PEN + AMP + TET + CHL	1
PEN + AMP + ERY + CLI + CHL + GEN	1

Resistance profiles of *S. pseudintermedius* isolates (left column), and a number of isolates with each profile (right column). Penicillin (PEN), ampicillin (AMP), oxacillin (OXA) (with 2% NaCl), erythromycin (ERY), clindamycin (CLI), tetracycline (TET), trimethoprim + sulfamethoxazole (SXT), gentamicin (GEN), chloramphenicol (CHL).

2.5 Discussion

Compared to the previous resistance surveillance study targeting *S. pseudintermedius* from healthy dogs presenting to the wellness service at our institution in Saskatoon in 2008, a higher frequency of resistance to specific antimicrobials, and resistance to more drugs including MRSP was identified. Furthermore, only 30% of the dogs carried pan-susceptible isolates compared to 46% in 2008 (Rubin and Chirino-Trejo, 2011). Differences in sample collection between the present investigation and that done in 2008 (the inclusion of a single isolate per dog in 2008 versus 3 presently, and the inclusion of nasal swabs in the 2008 investigation) preclude statistical comparisons between studies. However, the higher frequency of resistance including MRSP in 2014 is consistent with local clinical observations suggesting the emergence of resistance and with global MRSP trends. The frequency of carriage of healthy dogs with MRSP (7%) was higher than previously described elsewhere in North America or Europe (≤4.5%) perhaps reflecting the continued emergence of MRSP following previous studies (Hanselman et al., 2009; van Duijkeren et al., 2011a). This frequency was lower than that reported in Asia, where up to 45% colonization has been reported in Thailand, Japan and Hong Kong (Chanchaithong et al., 2014; Epstein et al., 2009; Sasaki et al., 2007a). The inclusion of Mueller-Hinton agar with 4μg/ml oxacillin did not improve our ability to recover MRSP despite the identification of 5 isolates with oxacillin MICs of >4μg/ml.

Risk factors for dogs to be infected with or carry MRSP have not been adequately characterized. There is conflicting evidence describing an association between infection with MRSP versus MSSP and previous antimicrobial administration (Lehner et al., 2014; Weese et al., 2012). Hospitalization and surgical procedures have also been positively associated with MRSP

colonization (Nienhoff et al., 2011; Weese et al., 2012). In the present investigation, none of the 7 MRSP positive animals were treated with antimicrobials in the previous 6 months, and all were clinically healthy suggesting community acquisition of the MRSP. Further study is clearly required to define risk factors associated with MRSP in dogs.

A total of 15 (6.8%) of isolates from 7 (9.0% of colonized dogs) were MDR, higher than in 2008 where only 1 dog carried MDR *S. pseudintermedius* (Rubin and Chirino-Trejo, 2011). In contrast to the literature, MDR was more frequently identified among MSSP than MRSP (Detwiler et al., 2013). The most common resistance profile among MRSP, including 6 of 8 isolates, was simply β-lactam resistance. MDR among MRSP is a serious threat to the ability of veterinarians to treat their patients. In 2009, a community associated urinary tract infection caused by MRSP resistant to the β-lactams, macrolides, fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole, chloramphenicol, and rifampin was reported in an otherwise healthy, neutered male Pug dog (Rubin and Gaunt, 2011a). Elsewhere, MRSP resistant to all antimicrobials licensed for use in companion animals have been described, highlighting the critical role of culture and susceptibility testing to guide therapy (Detwiler et al., 2013; Perreten et al., 2010; van Duijkeren et al., 2011a). Differences in clinical outcome for human patients infected with methicillin resistant versus susceptible staphylococci have not been observed, although the typically superficial nature of staphylococcal infections (pyoderma and otitis) may mask differences which have been seen in invasive MRSA vs. MSSA infections in people (Ott et al., 2010; Weese et al., 2012). More studies are needed to define risk factors associated with MRSP infection so that appropriate empiric treatments can be applied pending laboratory guided therapy.

Although ill-defined, antimicrobial resistant *S. pseudintermedius* is also a public health risk; human infections have been reported (Talan et al., 1989a). Because *S. pseudintermedius* is not part of the normal microbiota of humans, the carriage has been reported to be sporadic, colonization or infection with this organism is likely zoonotic (Paul, 2015; Paul et al., 2011). Presumptive transmission of *S. pseudintermedius* from dogs to humans working closely with them (veterinary staff) and pet owners have been reported; 3.9% - 13% of such people have been found to carry this organism (Chanchaithong et al., 2014; Paul et al., 2011; Walther et al., 2012). The frequency of human *S. pseudintermedius* infections may be under-appreciated due to its morphological and biochemical similarity to *S. aureus* leading to misidentification in diagnostic labs. The introduction of highly discriminatory identification methods such as MALDI-TOF MS which readily differentiates *S. pseudintermedius* and the closely related *S. intermedius* and *S.delphini* from *S. aureus* is helping to identify this previously under-recognized zoonosis (Silva et al., 2015).

Antimicrobial resistance appears to be emerging among *S. pseudintermedius* colonizing healthy dogs in Saskatoon, Canada. Although we presume that infections with MRSP are encountered with increasing frequency in our region, these data are not available. Culture and susceptibility testing should be encouraged to aid in the identification of MRSP in veterinary patients and to guide antimicrobial therapy. Practitioners should be aware of methicillin resistance when prescribing empiric antimicrobial therapy. Methicillin resistance should be suspected when empiric β-lactam therapy fails to cure *S. pseudintermedius* infections or for isolates demonstrated to be resistant to potentiated penicillins such as amoxicillin + clavulanic acid. Further studies to

describe the susceptibility of clinical isolates in this region would be complementary to this investigation.

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2.7 Transition Statement

The first study was designed to identify *S. pseudintermedius* colonizing healthy dogs and to evaluate the trend of antimicrobial resistance compared to the baseline study which was done in 2008. The results suggested that the frequency of resistance to penicillin and tetracycline has increased in Saskatoon, and that healthy dogs may now be colonized with MRSP, which was not seen in the baseline study. MRSP is often multidrug resistant and therapeutic options are limited for veterinarians. Dermatological and urinary tract infections are commonly caused by *S. pseudintermedius*. Although strain specific infections are seen in some bacterial species (such as uropathogenic *E. coli*), this has not been investigated in *S. pseudintermedius*. Therefore, we were interested to determine if dermatological and urinary tract infections are caused by unique strain type of *S. pseudintermedius* and whether differences in phenotypic antimicrobial susceptibility between them could be identified. We also investigated the emergence of antimicrobial resistance including MRSP in clinical isolates from *S. pseudintermedius* in dogs.

3 ANTIMICROBIAL RESISTANCE IN *Staphylococcus pseudintermedius* ISOLATES FROM UTI AND DERMATOLOGICAL INFECTION IN DOGS, SASKATOON, CANADA

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Authors contributions:

Conceived and designed experiment: MARP MCG JER. Performed the experiment: MARP JER.

Analyses the Data: MARP JER. Wrote the paper: MARP JER MCG.

3.1 Abstract

Staphylococcus pseudintermedius is an important pathogen in dogs; it is the most common cause of skin infections and the second most common cause of urinary tract infections. The ostensibly different environments of the urinary bladder and skin led us to hypothesize that isolates would cluster genetically by the site of infection (uro- and dermato- pathotypes). A collection of clinical urinary (n=50) and dermatological (n=51) isolates from unique patients were collected from a regional diagnostic laboratory. The antimicrobial susceptibility of isolates was determined by broth micro-dilution, and the relatedness of isolates was compared by pulse-field gel-electrophoresis and multi-locus sequence typing. DNA fingerprinting revealed that the isolates were genetically heterogeneous; 6 of 7 clusters identified by pulsed-field contained isolates from both infection types. Isolates from dermatological infections were significantly more likely to be resistant to erythromycin, clindamycin, and chloramphenicol than urinary isolates. Although not significant, methicillin resistance was numerically more common among dermatological than urinary isolates. These results indicate that opportunism is a more important factor than strain specific tissue tropism to the site of *S. pseudintermedius* infection in dogs. Although confirmatory studies are required, we propose that differences in the susceptibility of isolates from dermatological and urinary infections may be related to the clinical course of these diseases and the recurrent therapy.

3.2 Introduction

Staphylococcus pseudintermedius colonizes up to 90% of healthy dogs and is the most common cause of skin infections and the second most common cause of urinary tract infections in dogs (Rubin and Chirino-Trejo, 2011). Although there are many studies describing dermatological

isolates of *S. pseudintermedius*, there are few investigations into those from urinary infections; the published literature is limited to case reports and a description of the susceptibility of diagnostic isolates (Penna et al., 2010; Rubin and Gaunt, 2011b; Windahl et al., 2012). The skin and urinary tract are ostensibly dramatically different environments imparting unique obstacles to bacterial growth. While several virulence factors associated with dermato-pathogenesis including exfoliative toxin and fibronectin binding proteins have been demonstrated, the requisite virulence factors for *S. pseudintermedius* to cause urinary infections have not been identified (Iyori et al., 2011; Iyori et al., 2010; Pietrocola et al., 2015; Wladyka et al., 2015). It is unknown if isolates causing urinary and dermatological infections have unique virulence factor profiles, or if there are differentiable uro- or dermato-pathogenic strains. In *S. aureus* the association between strain and virulence genes is well recognized. Panton-Valentine Leucocidin (PVL) is associated with USA300, a community-acquired MRSA strain, which is recognized as a cause of skin and soft tissue infections and necrotizing pneumonia (Otto, 2010).

The emergence of antimicrobial resistance, including methicillin resistance, in *S. pseudintermedius*, is increasingly challenging the treatment of infections. Methicillin resistance conferred by the *mec*A gene results in resistance to the entire β-lactam class of antimicrobials which are the most commonly used drugs in companion animals (Prescott et al., 2002). Since 2006, there has been a dramatic worldwide increase in the frequency of MRSP among clinical canine infections (Moodley et al., 2014; van Duijkeren et al., 2011a). The literature has been heavily biased towards dermatological isolates where the emergence of MRSP in canine dermatological infection is well documented (Kadlec and Schwarz, 2012; Kadlec et al., 2011; Vincze et al., 2010). Although there are fewer reports, multi drug resistant MRSP from UTIs have also been described (Penna et al., 2010). This emergence has been associated with specific

clones, ST68 in North America and ST71 in Europe (Perreten et al., 2010). In our region, the frequency of MRSP colonization of healthy dogs increased from 0% to 9% between investigations conducted in 2008 and 2014 respectively (Priyantha et al., 2016). Anecdotal observations from companion animal clinicians suggest that resistance is more problematic among dermatological than urinary *S. pseudintermedius* isolates.

The objectives of this study were therefore to compare the antimicrobial susceptibility profiles and relatedness of *S. pseudintermedius* isolates from clinical urinary and dermatological submissions to a regional veterinary diagnostic laboratory. We hypothesized that resolvable uro- and dermato-pathotypes would be identifiable and that antimicrobial resistance would be more common among dermatological than urinary isolates.

3.3 Material and methods

Staphylococcus pseudintermedius isolates were collected from clinical submissions to Prairie Diagnostic Services, a regional veterinary laboratory located in Saskatoon, Canada. A total of 101 sequentially isolated *S. pseudintermedius* from dermatological (n=51) and urinary (n=50) submissions were gathered over a two-year period beginning in November 2013. Laboratory records were reviewed to ensure that duplicate isolates from the same patient were excluded from the study. Bacteria were stored at -80^{0}C in trypticase soy broth with 15% glycerol prior to analysis.

Antimicrobial minimum inhibitory concentrations were determined using the Sensititre system (GPALL1F plate format) including penicillin (PEN), ampicillin (AMP), oxacillin with 2% NaCl (OXA), chloramphenicol (CHL), erythromycin (ERY), clindamycin (CLI), gentamicin (GEN), tetracycline (TET), tigecycline (TGC), sulfamethoxazole + trimethoprim (SXT), levofloxacin

(LEV), ciprofloxacin (CIP), moxifloxacin (MOX), rifampicin (RIF), nitrofurantoin (NIT), vancomycin (VAN), daptomycin (DAP), linezolid (LZD) and quinupristin + dalfopristin (QDA). Antimicrobial susceptibility tests were conducted and interpreted according to the CLSI guidelines (2013; 2014). For daptomycin and tigecycline, EUCAST resistance breakpoints for *S. aureus* were used (EUCAST, 2014). *S. aureus* ACTCC 29212 and *E. feacalis* ACTCC 29212 were included for quality control. All Isolates were screened for *mec*A using previously published primers (Stegger et al., 2012).

The relatedness of isolates was determined using pulsed-field gel electrophoresis using a previously published protocol (Mulvey et al., 2001). Salmonella Branderup was used as a molecular weight ladder as described by PulseNet (http://www.cdc.gov/pulsenet). A CHEF DR-III apparatus (Bio-Rad) was used for electrophoresis using switch times of 5.3 to 34.9 for 18 h at 6.0 V/cm gel images were digitized and analyzed with GelCompare (Applied Maths, Austin, TX). Banding patterns were compared using the Dice algorithm and dendrograms were constructed using UPGMA. According to the Tenover criteria, isolates were considered to be related if they were at least 80% similar and Clusters were defined as groups 3 or more related isolates (Tenover et al., 1995). Methicillin resistant isolates were further characterized by MLST to provide an additional objective, a sequence based measure of relatedness (Solyman et al., 2013).

Differences in the frequency of categorical antimicrobial susceptibility (susceptible vs. resistant) and multi-drug resistance (≥ 3 classes) between dermatological and urinary isolate collections were compared using Fisher's exact test. Statistical analysis was done using the freely available online calculator GraphPad (http://graphpad.com/quickcalcs/contingency2/). A p-value of ≤ 0.05 was considered statistically significant.

3.4 Results

Overall, isolates were resistant to between 0 and 6 drug classes (median 1), with the most common phenotype identified being pan-susceptibility (Table 3.1). No resistance to nitrofurantoin, rifampin, vancomycin, linezolid, daptomycin, tigecycline or quinupristin + dalfopristin was identified. Although numerically more urinary than dermatological isolates was pan-susceptible (18 vs. 16 respectively), this difference was not significant ($p = 0.67$). The opposite trend was seen for multidrug resistance which was observed in numerically more dermatological than urinary isolates (7 and 2 respectively), this difference approached significance ($p = 0.09$). With the exception of penicillin and trimethoprim + sulfamethoxazole, resistance to all other drugs was more frequently identified in dermatological than urinary isolates (Table 3.2). For erythromycin, clindamycin, and chloramphenicol these differences were significant while for oxacillin resistance (MRSP) this difference approached significance. All oxacillin resistant isolates possessed *mec*A, while this gene was not identified among any oxacillin susceptible isolates. Finally, a greater diversity of resistance phenotypes was found among dermatological than urinary isolates (Table 3.1).

Of the 101 isolates tested by PFGE, one was indigestible by Sma1 and was excluded from subsequent analyses. Mutually exclusive clusters of urinary and dermatological isolates were not identified. Isolates were generally unrelated, 7 clusters including 25 isolates (13 dermatological and 12 urinaries) were identified; 6 clusters contained isolated from both types of infections. The eight methicillin resistant isolates didn't cluster together by PFGE, this was corroborated by MLST which revealed that eight unique sequence types (Table 3.3). While isolates SP36 and SP56 only differ at a single locus and isolates SP56 and SP60 differ at two loci, all other isolates

were no more similar than triple locus variants of each other. Importantly, the European pandemic clone ST71 was identified in Saskatoon, Canada.

Table 3.1: MIC distribution of *S. pseudintermedius* isolated from canine dermatological (n=51) and urinary tract (n=50) infections

Drug	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	R %	Site	P value
PEN		22		1	1			3	3	21			56.8	Derm	0.8
		20		2	2	2	6	1	5	12			60.0	UTI	
AMP			22	5	4	5	3	3		9			47.0	Derm	1.00
			20	7	8	6	3	3	1	2			43.1	UTI	
OXA				43		1	1		6				15.7	Derm	0.06
				49					1				1.9	UTI	
ERY				39	4				9				17.6	Derm	0.02
				43	6				1				1.9	UTI	
CLI					42			9					17.6	Derm	0.02
					49			1					1.9	UTI	
TET							34	1	4		12		23.5	Derm	0.81
							36		4		10		20.0	UTI	
SXT					45		2		4				7.8	Derm	1.00
					44		2		4				8.0	UTI	
GEN							47	1	2	1	1		3.9	Derm	1.00
							49			1			1.9	UTI	
CHL							0	14	27	3	7		13.7	Derm	0.01
							1	20	21	8			0	UTI	
CIP						49		3					5.8	Derm	0.62
						49			1				1.9	UTI	
LEV				44	2	2			3				5.8	Derm	0.62
				48		1			1				1.9	UTI	
MOX				48				3					5.8	Derm	0.62
				49				1					1.9	UTI	

PEN: penicillin, AMP: ampicillin, OXA: oxacillin, ERY: erythromycin, CLI: clindamycin, TET: tetracycline, SXT: sulfa-trimethoprim, GEN: gentamicin, CHL: chloramphenicol, CIP: ciprofloxacin, LEV: levofloxacin, MOX: moxifloxacin, Derm : clinical isolates from dermatological infection, UTI: clinical isolates from urinary tract infection. The dark vertical line indicates the clinical breakpoint for the given antimicrobial agent.

Table 3.2: Antimicrobial susceptibility profiles of *S. pseudintermedius* isolated from dermatological (n=51) and urinary tract (n=50) infections in dogs.

Susceptibility Profile	**Dermatological Isolates**	**Urinary Isolates**
Pan susceptible	16 (31.3%)	18(36%)
PEN	4 (7.8%)	5 (10%)
TET	4 (7.8%)	2 (4%)
PEN + AMP	13 (27.4%)	15 (30%)
PEN +TET	0	1(2%)
PEN +SXT	0	1 (2%)
TET + CHL	1 (2%)	0
ERY + CLI	1	0
PEN + AMP + TET	0	6 (12%)
PEN + AMP + SXT	1 (2%)	0
PEN + AMP + TET + SXT	0	1(2%)
PEN + TET + ERY + CLI + CHL	1	0
PEN + AMP + ERY + CLI + TET + CHL	3 (5.9%)	0
MRSP	1 (2%)	0
MRSP + TET	1 (2%)	0
MRSP + SXT	1 (2%)	0
MRSP + ERY + CLI	1 (2%)	0
MRSP + CHL + ERY + CLI + GEN + SXT + CIP + LEV + MXF	1 (2%)	1 (2%)
MRSP + CHL + ERY + CLI + TET + SXT + CIP + LEV + MXF	1 (2%)	0
MRSP + CHL + ERY + CLI + GEN + TET + CIP + LEV + MXF	1 (2%)	0

Penicillin (PEN), ampicillin (AMP), oxacillin (OXA) (with 2% NaCl), erythromycin (ERY), clindamycin (CLI), tetracycline (TET), trimethoprim + sulfamethoxazole (SXT), gentamicin (GEN), chloramphenicol (CHL).

Table 3.3: Summary of multilocus sequence typing data (MLST) from MRSP isolates found in the study.

Isolate ID	Infection Site	Allelic profile							ST
		ack	*cpn*60	*fdh*	*pta*	*pur*A	*sar*	*tuf*	
SP04	Skin	17	2	2	44	46	1	2	644
SP18	Skin	2	10	2	44	18	1	2	645
SP36	Urine	3	9	1	2	22	2	1	684
SP51	Skin	16	13	8	48	11	1	2	669
SP56	Skin	3	9	1	2	1	2	1	71
SP60	Skin	2	9	4	2	1	2	1	646
SP72	Skin	2	8	2	1	11	1	2	292
SP110	Skin	12	13	2	1	11	2	2	670

3.5 Discussion

Staphylococcus pseudintermedius is one of the most common causes of infections in dogs and yet we know little about the organism factors contributing to pathogenesis. We hypothesized that the unique environment of the skin and urinary bladder would select for different "pathotypes" of *S. pseudintermedius* which we attempted to resolve using DNA fingerprinting techniques. We were surprised to find that in our collection, urinary and dermatological isolates did not cluster independently by PFGE; these results were corroborated by MLST. These data suggest that opportunism is a more important factor than strain specific tissue tropism to the site of *S. pseudintermedius* infection. Interestingly, MLST also revealed that one isolate was ST71, which is reportedly a clone more common in Europe (Perreten et al., 2010). A recent report of human *S. pseudintermedius* infections from a neighboring region of Canada also identified 4 of 6 MRSP isolates as ST71 while not finding any ST68 (Somayaji et al., 2016). In North America, there is a substantial geographic bias against our region in published studies of *S. pseudintermedius*, but our results suggest that ST71 is perhaps less geographically limited than previously thought.

Significant differences in the frequency of antimicrobial resistance were found between urinary and dermatological isolates including a nearly significant association of methicillin resistance with dermatological isolates. DNA fingerprinting revealed that these differences are not attributed to strain type.

One possible explanation for this difference is the clinical course of dermatological vs. urinary tract infections in dogs and how these syndromes are treated. The appearance and integrity of the skin are influenced by systemic factors including nutritional status, hormone levels, perfusion and systemic organ function (Outerbridge, 2013). Endocrine diseases offer a particularly pointed example of an underlying disease that manifests as a dermatological disease (Outerbridge, 2013).

While urinary tract infections may also occur with some endocrinopathies, they are frequently subclinical, making them more difficult to identify (Forrester et al., 1999). Dog owners more readily identify skin lesions caused by pyoderma and the resulting pruritus, causing them to seek treatment.

While uncomplicated UTIs are relatively straightforward to identify, endocrinopathies are challenging to diagnose, possibly resulting in repeated treatment of recurrent pyoderma and additional selection pressure for development antimicrobial resistance. Additionally, uncomplicated UTI are often successfully cleared with shorter courses of antimicrobial therapy compared to pyoderma, particularly deep pyoderma, which may mitigate compliance-related treatment failure.

3.6 Conclusion

We found that *S. pseudintermedius* causing dermatological and urinary infections do not form mutually exclusive genetic clusters suggesting that site of infection is opportunistic rather than associated with strain-specific tissue tropism. Furthermore, we argue that differences in antimicrobial susceptibility between dermatological and urinary isolates can be explained by differences in antimicrobial usage resulting from differences in the clinical course of the disease. Further studies are required to understand the pathogenesis of canine *S. pseudintermedius* infections and the factors leading to the development of antimicrobial resistance.

3.7 Acknowledgements

We would like to thank Prairie Diagnostic Services for providing us with clinical *Staphylococcus pseudintermedius* isolates. We would also like to thank the Companion Animal Health Fund for a grant supporting this study and partial stipend support for MARP. Thanks to Cherise Hedlin and Michelle Sniatynski for technical assistance.

3.8 Transition Statement

Antimicrobial resistance is emerging in *S. pseudintermedius* isolates from dogs. As common house pets, humans and dogs have close contact providing ample opportunity for the transmission of organisms. *Staphylococcus pseudintermedius* is infrequently described as a cause of infection in people in the literature. Consequently, many physicians and diagnostic laboratories are not aware of *S. pseudintermedius*. We suspected that *S. pseudintermedius* has been under-recognized as a cause of human infections and is often misdiagnosed as *S. aureus*. We were interested to understand the incidence of *S. pseudintermedius* in human diagnostic submission and to describe the antimicrobial susceptibility of *S. pseudintermedius* from clinical infections and understanding of human clinical infections. Furthermore, we wanted to identify existing *S. pseudintermedius* epidemic clones which causes human infections in our region.

Additionally, as there are differences in clinical oxacillin resistance breakpoint for *S. aureus* and *S. pseudintermedius*, we suspect that methicillin resistance may be underreported. Therefore, our goal was to identify and characterize *S. pseudintermedius* from human clinical infection and understanding molecular epidemiology in a large Canadian health region.

4 HUMAN INFECTIONS DUE TO *Staphylococcus pseudintermedius*, AN EMERGING ZOONOSIS OF CANINE ORIGIN: REPORT OF 24 CASES

Copyright

Citation

Somayajii R, Priyantha MAR, Rubin JE, Deirdre C. Human infections due to Staphylococcus pseudintermedius, an emerging zoonosis of canine origin: report of 24 cases. Diagnostic Microbiology and Infectious Diseases. 2016.85(4):471-476.

Author Contributions

Conceived and designed experiment: RS, MARP, CD, JER. Performed the experiment (Clinical analysis): RS, CD. Performed the experiment (Bacteriology): MARP, JER Analyses the Data: RS, MARP, CD, JER. Wrote the paper: RS, MARP, CD, JER.

4.1 Abstract

Staphylococcus pseudintermedius has been recently identified as a novel species within the genus Staphylococcus and is commonly associated with infections in dogs. Currently, there are few reports of human infections due to this bacterium. The objective of the study was to use a population-based approach to describe the characteristics of human *Staphylococcus pseudintermedius* infections in a large Canadian healthcare region. all adult cases aged ≥18 years identified at a large regional laboratory from April 1, 2013, to April 1, 2015, who had at least one positive culture for *Staphylococcus pseudintermedius* were retrospectively reviewed. A combination of phenotypic methods, mass spectrometry (i.e., MALDI-TOF), and *cpn*60 sequencing were used to identify *Staphylococcus pseudintermedius*. Chart review was conducted, and cases were analyzed descriptively.

Twenty-seven isolates of *S. pseudintermedius* from 24 human cases were included for analysis. 58.3% were male with median age of 61 years (IQR 55-70.5). Most patients [22 (92.1%)] had confirmed contact with dogs at the time of infection. *Staphylococcus pseudintermedius* was isolated in 18 cases (75.0%) of skin and soft tissue infections (SSTI), and 2 invasive cases (8.3%) including a prosthetic joint and bloodstream infection. The other 4 patients were colonized (skin – 3; lung – 1). Methicillin resistance was identified in 3 cases with 6 total isolates (22.2%); multi-drug resistance was also demonstrated commonly. In Conclusion, *Staphylococcus pseudintermedius* is most commonly associated with SSTIs in humans. Transmission probably occurs from a pet dog. Species-level identification of *S. pseudintermedius* is important due to the high prevalence of antibiotic resistance, particularly to methicillin.

4.2 Introduction

Staphylococcus pseudintermedius is a novel coagulase-positive Staphylococcus species identified in the last decade (Devriese et al., 2005). Although this organism has been commonly associated with skin and soft tissue infections (SSTIs) in dogs, few human cases have been described (Chuang et al., 2010; Savini et al., 2013; Stegmann et al., 2010; Van Hoovels et al., 2006). This bacterium, however, appears to cause a similar infection spectrum as *S. aureus* in humans (Bannoehr and Guardabassi, 2012; Paul et al., 2012). Approximately 90% of healthy dogs are colonized with *S. pseudintermedius*, and frequent sites of isolation include the pharynx, rectum, nares, and skin (Rubin and Chirino-Trejo, 2011; van Duijkeren et al., 2011b). As an opportunistic pathogen, *S. pseudintermedius* is the leading cause of pyoderma and otitis externa and is also the most commonly isolated pathogen in urinary tract infections (Rubin and Chirino-Trejo, 2011). Methicillin resistance is rapidly emerging in *S. pseudintermedius* (Bannoehr and Guardabassi, 2012; Beever et al., 2015) with a higher incidence amongst clinical isolates in dogs (Beck et al., 2012; Gold et al., 2014), and this may also have important treatment implications in human infections (Bannoehr et al., 2009; Kadlec and Schwarz, 2012; van Duijkeren et al., 2011a).

The Staphylococcus genus is comprised of 38 species and 17 subspecies of which half are native to humans. *S. pseudintermedius* is a coagulase-positive staphylococcal species and is difficult to distinguish from *S. aureus* on the basis of morphologic characteristics. Although genetic sequencing of the *cpn*60 or *sod*A genes readily identifies *S. pseudintermedius* (Sasaki et al., 2007b), this is currently not a cost-effective modality in clinical practice. However, implementation of mass spectrometry instrumentation [i.e. matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)] in our regional clinical microbiology

laboratory has allowed rapid and accurate identification of clinically relevant bacterial species including *S. pseudintermedius* (Silva et al., 2015). Since the implementation of Vitek MS MALDI-TOF, several *S. pseudintermedius* cases from ambulatory patients have been diagnosed, which would have previously been unrecognized or misidentified using phenotypic methods. As *S. pseudintermedius* has been primarily associated with infections in companion animals, acquisition of this organism in humans suggests a zoonotic transmission event from dogs.

With a paucity of literature, little is known regarding the incidence and clinical characteristics of *S. pseudintermedius* infection in humans. It was therefore of interest to perform a population-based study to describe the epidemiology and clinical and microbiological characteristics of *S. pseudintermedius* infections in our large Canadian healthcare region.

4.3 Material and methods

4.3.1 Laboratory setting

Calgary Laboratory Services (CLS) is a large regional diagnostic laboratory that provides comprehensive testing services to an urban population of ~1.5 million people. All clinical microbiology testing is performed in a single centralized facility, on hospitalized, ambulatory and long-term care patients. Clinical samples are promptly transported to CLS following collection. Detailed instructions about proper specimen collection requirements are available in the CLS Guide to Services found at https://www.calgarylabservices.com/lab-services-guide/specimen-collection/(CLSGT).

4.3.2 Patient and bacterial specimen selection

A retrospective review of all adult patients aged ≥ 18 years with at least one culture positive for *S. pseudintermedius* was included. Subjects were identified when *S. pseudintermedius* was isolated from clinical specimens submitted to the CLS Microbiology laboratory during the study period between April 1, 2013, and April 1, 2015. All clinical specimens analyzed by CLS were done as part of the routine clinical care of patients. Demographic and clinical data including age, sex, comorbidities, and details of the nature and treatment of the infection was obtained through detailed chart review.

4.3.3 Ethics

This study was approved by the Conjoint Health Research Ethics Board (CHREB), Alberta Health Services and University of Calgary (REB13-0953). Informed consent was obtained from each patient case included in the study.

4.3.4 Microbiology Methods

All *S. pseudintermedius* isolates grew well on blood agar plates under aerobic incubation at 35^{o}C within 24-48 h. The colonies were creamy white and non-haemolytic or demonstrated small zones of β-haemolysis. Colony Gram-stain showed small clusters of Gram-positive cocci that resembled staphylococci. All isolates were catalase and slide coagulase positive. They were subsequently identified using a commercial mass spectrometry system (Vitek MS®, ACQ Software R2 version 1.4.2b, bioMérieux, St.-Laurent, Que.) using Myla Version 3.2.0-4 in accordance with the manufacturer's instructions. Ethanol-formic acid extraction was performed

on all isolates using the protocol provided by the manufacturer. A quantity of 1 µL of the extracted supernatant was placed on the steel target plate, dried and overlaid with 1 µL of the matrix. The target plate was then loaded into the Vitek MS instrument for analysis.

Molecular identification of the *S. pseudintermedius* isolates was done by sequencing of the cpn60 universal target using previously established methods (Hill et al., 2004). BLAST search against the SmartGene-Integrated Database Network System (IDNS) for bacteria indicated the most closely related species, and the overall identity score for all isolates was ≥ 99.9% with 0-2 mismatches (15, 16). Antibiotic susceptibility testing was conducted in accordance with Clinical Laboratory Standards Institute (CLSI) guidelines, M100-S24 (2014). Of note, the oxacillin resistance breakpoint for *S. pseudintermedius* (0.5 µg/ml) is set lower than that of *S. aureus* (4 µg/ml) (2015). Antimicrobial susceptibility profiles were initially determined by the automated Vitek 2 system (bioMérieux) and confirmed by broth microdilution using the Sensititre system (Thermo Fisher Diagnostics (Trek Diagnostics), Ohio, USA). The following drugs were tested on all of the isolates: penicillin, ampicillin, oxacillin, erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, gentamicin, ciprofloxacin, moxifloxacin, levofloxacin, rifampicin, vancomycin, daptomycin, linezolid, and Synercid (quinupristin + dalfopristin). All isolates were screened for the *mec*A gene by PCR using previously published primers, and the identity of amplicons was confirmed by sequencing (Stegger et al., 2012). Additionally, *S. pseudintermedius* isolates were characterized using a previously described multi-locus sequence typing method (Solyman et al., 2013).

4.3.5 Statistical analysis

Parametric and non-parametric variables were reported as means with standard deviations (SD) and medians with interquartile ranges (IQR) respectively. Patient demographic and clinical data were analyzed in a descriptive manner. Prevalence of MRSA in soft tissue and invasive infections was compared using X-squared analysis. Annual incidence rates for *S. pseudintermedius* infections were calculated utilizing epidemiologic data from CLS. Sequences generated for MLST were concatenated using the online analysis tool found on the PubMLST database (www.pubmlst.org). Study outcomes were established prior to data collection. Hypotheses were two-sided with α significance of 0.05. Clinical data were analyzed using STATA 13.1 software (College Station., Texas).

4.4 Results

A total of 24 patient cases and 27 isolates were included in the analysis. 58.3% were male with median age of 61 years (IQR 55.0 - 70.5 years). Patient characteristics and culture results are summarized in Table 4.1 and 4.2. *S. pseudintermedius* was most commonly associated with skin and soft tissue infections (18/24, 75.0%), and patients were primarily managed as outpatients (21/24, 87.5%). Diabetes mellitus (29.2%), peripheral vascular disease (20.8%), and cardiovascular disease (16.7%) were the most prevalent co-morbidities affecting the cohort. Two patients developed invasive *S. pseudintermedius* infections including a fistula-associated bloodstream infection and a monomicrobial prosthetic joint infection. *S. pseudintermedius* was a component of a polymicrobial milieu in the majority of clinical presentations (22/24, 91.7%). The other four patients, who had *S. pseudintermedius* isolated, were considered to be colonized and not clinically infected with this organism based on our review. Of the clinical cases where

data was available, 95.4% (21/22) of patients had a pet dog during the study period in temporal relation to cultures; one patient was bitten by a dog and incurred a soft tissue infection. The incidence of bloodstream infection with *S. pseudintermedius* was 6.95*10E-4% in the study period (~71900 cultured/year); 30% of these cultures were positive for *S. aureus*. The incidence of true infection with *S. pseudintermedius* was 20/24 resulting in a rate of 83.3%. In contrast, the rate of blood culture positivity for *S. aureus* was 0.0055% per year in the 2013-15 periods. Similarly, for SSTI or wound infections where cultures were done, the incidence of *S. pseudintermedius* was estimated at 0.05% for the study period (~21090 cultures/year); 30% of these cultures were positive for *S. aureus*.

Comprehensive assessment of the 27 *S. pseudintermedius* isolates was conducted. Most of them [23 (85.1%)] were from the skin and soft tissue samples collected most commonly from the lower limbs (n = 18). Three of the isolates were associated with invasive infections as previously described in the clinical cases (Table 4.1 & Table 4.2). On antimicrobial susceptibility testing, seven (26%) of the isolates were pan-susceptible. No inducible clindamycin resistance or vancomycin resistance was detected. A total of 6 methicillin-resistant *S. pseudintermedius* (MRSP) isolates (22.2%) corresponding to 3 patients were identified. These isolates were implicated in SSTI (4/6) and a prosthetic joint infection (2/6). Of note all MRSP isolates were resistant to multiple drug classes; two isolates were identified to be resistant to the β-lactams, tetracycline, macrolides, aminoglycosides, sulfonamides and the fluoroquinolones. All MRSP were positive for the *mec*A gene, which was not detected from MSSP isolates. The prevalence of MRSP in SSTI compared to invasive infections was similar (p = 0.14). MLST revealed that 16 of 27 isolates belonged to new sequence types not previously described. Methicillin-resistant

isolates belonged to ST71 (n=4) and ST181 (n=2). Within this collection, isolates were generally unrelated. With the exception of the ST71 and ST181 isolates, no isolates were more similar than least double locus variants of each other.

Table 4.1: Demographic and clinical characteristics of patients

ID	AGE & SEX	PROFILE	SITE	DIAGNOSIS	ID – CULTURE	TREATMENT	OUTCOME	MRSP
1	63F	Lymphoma, CHF, HTN	Neck	SSTI	GPB *S. pseudintermedius*	Cefazolin 2 grams IV q8h + Metronidazole 500 mg PO BID * 10 days	Discharged home	No
2	59M	Venous stasis	Left leg	SSTI	Group C Streptococcus, *Acinetobacter baumanii*, CoNS *S. pseudintermedius*	Cephalexin 500 mg PO QID * 10 days	Managed as outpatient	No
3	59M		Umbilicus	SSTI	CoNS *S. viridans* *S. pseudintermedius*	Fucidin 2% cream to area BID * 10 days	Managed as outpatient	No
4	53M	ESRD (hemodialysis), lymphedema, smoker	Vascular	Bacteremia + Fistula Wound infection	*S. pseudintermedius* (blood) *S. aureus* (from wound)	Vancomycin IV (at dialysis) * 4 weeks	Managed as outpatient	No
5	34M	Dog bite	Skin	SSTI	*Pasteurella multocida* CoNS *S. pseudintermedius*	Wound sutured Ceftriaxone/clindamycin IV * 4 days followed by amoxicillin-clavulanate 875 mg PO BID * 7 days	Non-adherence requiring repeat assessment, sutures removed 1 week later. No change to antibiotics. Managed as outpatient	No
6	54M	DM, CAD, OA, ex-smoker, thoracic surgery; preceding wire removal	Sternum	SSTI/abscess	CoNS *S. pseudintermedius* *P. acnes*	OR for sternal wire removal + cloxacillin 500 mg PO QID * 7 days	Discharged home	No
7	75M	Ex-smoker	Lung	Nodule – r/o infection	Oropharyngeal flora *S. pseudintermedius*	Amoxicillin-clavulanate 875 mg PO BID * 5 weeks	Managed as outpatient; non-infectious etiology	No
8	65M	GI bleeding	Left knee (soft tissue)	SSTI	*S. pseudintermedius*	Cephalexin 500 mg PO QID * 10 days	Managed as outpatient	No
9	60F		Left foot	R/O infection	*S. pseudintermedius*	Expectant management	Managed as outpatient; no infection diagnosed	No
10	61M		Left leg	SSTI	Enterococcus spp *S. pseudintermedius*	Doxycycline 100 mg PO BID * 10 days	Managed as outpatient	No
11	67M	DM, PVD (stenting), smoker	Right foot (1st toe)	SSTI	Staphylococcus lugdensis *S. pseudintermedius*	Cephalexin 500 mg PO QID * 10 days	Managed as outpatient	No
12	40M		Right foot	SSTI, R/O fungal infection	GNEB *Aerococcus viridans* *S. pseudintermedius*	Fucidin 2% cream daily * 2 weeks + Terbinafine 250 mg PO daily * 4 weeks	Managed as outpatient	No
13	49M	DM, prior right foot infections (great toe fusion history)	Left foot	SSTI	*S. pseudintermedius*	Clindamycin 300 mg PO QID * 10 days	Managed as outpatient	No
14	61F		Left foot (1st toe)	R/O infection	GNB (failed to grow) CoNS *S. pseudintermedius*	Expectant management	Managed as outpatient, no infection diagnosed	No
15	82M	Afib, DVT, obesity	Right foot	SSTI	*S. pseudintermedius* CoNS	Amoxicillin 500 mg PO TID * 9 days	Managed as outpatient	Yes (3 isolates)
16	74F	CVA, RA, OP	Left foot (1st toe)	SSTI	*S. aureus* Group B Streptococcus	Cephalexin 500 mg PO QID * 7 days	Managed as outpatient	No

					S. pseudintermedius			
17	80F	DM, renal transplant, OA, R total knee arthroplasty	Right knee (prosthesis)	Prosthetic joint infection	*S. pseudintermedius*	Right knee aspiration + Did not tolerate vancomycin or linezolid; Tigecycline 50 mg IV q12h * 3 months	Repeat admission in 2014 requiring partial prosthesis removal + intravenous antibiotics	Yes (2 isolates)
18	74F	ESRD on HD, DM, Afib, HTN, PVD with bilat leg amputation, GIB, chronic pain	Right knee stump	SSTI	GNEB *S. pseudintermedius*	Vancomycin IV * 4 weeks + wound care	Managed as outpatient	No
19	67F	OA	Left leg	SSTI	*S. pseudintermedius*	Levofloxacin 500mg PO daily * 7 days	Managed as outpatient	Yes
20	56F	OA	Right leg	SSTI	*S. pseudintermedius*	Ceftriaxone 2g IV daily + metronidazole 500mg PO BID * 3 days followed by levofloxacin 500mg daily + metronidazole 500mg PO BID * 14days; followed by amoxicillin-clavulanic acid 875mg PO BID * 7 days	Managed as outpatient	No
21	84M	Cardiomyopathy with ICD, COPD, OA, asthma	Right arm	R/O infection	*S. pseudintermedius*	Expectant management	Managed as outpatient, no infection diagnosed	No
22	67F	DM, COPD, PVD, CAD, HTN, OP, gout, CVA	Right leg	SSTI, R/O fungal infection	*S. aureus* (MSSA) *S. pseudintermedius*	Cephalexin 500mg PO QID * 5 days	Managed as outpatient	No
23	52M	Dyslipidemia	Ear	Otitis externa	Group B Streptococcus, *S. pseudintermedius*	Cephalexin 500mg PO QID * 10 days	Managed as outpatient	No
24	58F	DM on insulin, obesity, HTN, left heel ulcer, neuropathy	Left heel	SSTI	*S. anxious* *E. faecalis* *S. pseudintermedius*	Tissue debridement + antibiotics: fluconazole 100mg PO daily * 3 days; followed by cefazolin 2g IV q8h + metronidazole 500mg PO BID * 5 days; followed by ciprofloxacin 500mg PO BID * 3 weeks; followed by cefazolin 2g IV q8h * 5 days	Managed as outpatient	No

Abbreviations: Afib – atrial fibrillation; CAD – coronary artery disease; CHF – congestive heart failure; COPD – chronic obstructive pulmonary disease; CVA – cerebral vascular accident; DM – Diabetes mellitus; DVT – deep vein thrombosis; ICD – implantable cardiac defibrillator; HTN – Hypertension; OA – osteoarthritis; OP – osteoporosis; PVD – peripheral vascular disease; RA – Rheumatoid arthritis; SSTI – skin and soft tissue infection; GPC – Gram positive cocci; GNB – Gram negative bacilli; GNEB – Gram negative enteric bacilli; MSSA – methicillin-sensitive Staphylococcus aureus; CoNS – coagulase negative *S. aureus*; spp – species; IV – intravenous; PO – per oral; BID – twice daily; TID – three times daily; QID – Four times daily ,ESRD – end stage of renal diseas

Table 4.2: Summary of site of infection, antimicrobial resistant phenotypes, and multilocus sequence types of clinical *Staphylococcus pseudintermedius* isolates in Calgary.

	SITE OF INFECTION	ANTIMICROBIAL SUSCEPTIBILITY PROFILE	ALLELIC PROFILE	ST
1	Neck /cellulitis	Pan-Susceptible	3-2-6-1-11-1-2	504
2	Superficial wound/leg	AMP, PEN, TET, ERY, CLI	1-9-1-1-3-1-1	505
3	Drainage umbilicus	Pan-Susceptible	5-26-2-1-7-5-2	506
4	Arterial line blood	AMP, PEN	1-64-5-1-5-1-2	513
5	Sterile fluid bursa	MRSP, TET, ERY, CLI, GEN, SXT, LEV, MXF, CIP,	3-11-2-24-20-1-1	181
6	Skin	AMP, PEN	5-24-2-20-25-2-2	512
7	Sternum	Pan-Susceptible	6-11-1-1-8-2-1	217
8	Bronchial washing	AMP, PEN	15-24-2-1-1-1-2	223
9	Superficial wound/ knee	AMP, PEN	1-13-4-1-7-1-2	507
10	Superficial wound/ foot	Pan-Susceptible	3-2-2-1-8-4-1	508
11	Superficial wound/ leg	AMP, PEN, ERY, CLI, CHL,	4-65-3-1-11-1-1	514
12	Superficial wound/ toe	AMP, PEN, TET	1-7-1-20-11-1-2	327
13	Superficial wound/ foot	AMP, PEN	13-2-1-1-13-1-2	200
14	Superficial wound/ foot	AMP, PEN	5-9-2-1-1-1-2	515
15	Superficial wound / toe	AMP, PEN, GEN	1-10-4-8-7-1-2	476
16	Superficial wound / foot	MRSP, ERY, CLI, GEN, SXT, LEV, MXF, CIP	3-9-1-2-1-2-1	71
17	Superficial wound / toe	AMP, PEN	3-2-2-4-10-2-2	509
18	Left knee synovial fluid	MRSP, TET, ERY, CLI, GEN, SXT, LEV, MXF, CIP	3-11-2-24-20-1-1	181
19	Right stump sores	AMP, PEN, TET	4-9-1-44-11-1-1	516
20	Superficial wound/ leg	MRSP, ERY, CLI, SXT, LEV, MXF, CIP	3-9-1-2-1-2-1	71
21	Drainage right leg	AMP, PEN	5-7-7-2-1-1-2	510
22	Superficial wound / arm	Pan-Susceptible	3-7-4-2-7-1-1	511
23	Superficial wound / leg	PEN	5-7-3-1-21-1-2	517
24	Ear infection	Pan-Susceptible	22-66-14-2-7-1-1	518
25	Superficial wound / foot	MRSP, ERY, CLI, SXT, LEV, MXF, CIP	3-9-1-2-1-2-1	71
26	Deep wound/foot	Pan-Susceptible	2-7-2-1-1-4-2	519
27	Superficial wound / leg	MRSP, ERY, CLI, GEN, SXT, LEV, MXF, CIP	3-9-1-2-1-2-1	71

AMP - ampicillin; PEN - penicillin; MRSP - methicllin resistant, TET - tetracycline; ERY - erythromycin; CLI - clindamycin; CHL - chloramphenicol; GEN - gentamicin; SXT - trimethoprim + sulfamethoxazole; LEV - levofloxacin; CIP - ciprofloxacin; MXF - moxifloxacin. Allelic profiles: *ack, cpn60, fdh, pta, purA, sar* and *tuf*.

4.5 Discussion

Although *S. pseudintermedius* is a recently identified species, it has likely been present for far longer and is an important pathogen of zoonotic origin. This study reflects the single largest collection to date of human *S. pseudintermedius* clinical cases and isolates in the literature. Staphylococcus intermedius was originally described in 1976 but it underwent reclassification upon identification of *S. pseudintermedius* in 2005 (Devriese et al., 2005). Since this time, isolates in the *S. intermedius* group were divided into one of three clusters: *S. intermedius*, *S. pseudintermedius*, and *S. delphini* (Sasaki et al., 2007b). *S. pseudintermedius* is a normal inhabitant and frequent opportunistic pathogen of canines and other companion animals. Of the 24 clinical cases, 91.7% had confirmed contact with dogs temporally with infection reflecting the zoonotic transmission characteristics of this species.

Studies of *S. pseudintermedius* have demonstrated it to be a leading cause of soft tissue and ear infections, as well as infections of other body tissues or cavities in canine and feline species (Abraham et al., 2007; Griffeth et al., 2008). In support of this, *S. pseudintermedius* has a number of important similar phenotypic characteristics to *S. aureus*. Notably, *S. pseudintermedius* characteristics include coagulase, protease, DNase, β-haemolysin, and exfoliative toxin production which all support the pathogenic potential of this species (Fitzgerald, 2009). Similarly, in our series, *S. pseudintermedius* was most commonly implicated in skin and soft tissue infections in humans. To note, in our case series of 4 patients were diagnosed as having non-infectious etiology. The majority of infections did not require hospitalization and were managed with short courses of oral antibiotic therapies. In two cases with MRSP isolates, no specific MRSP-directed therapy was used in the management. Given the

polymicrobial nature of the cultures in most of the clinical cases, the virulence of human *S. pseudintermedius* infections in human may differ than in those of companion animals, where this organism is often the primary pathogen. However, since two invasive infections occurred in this series, *S. pseudintermedius* certainly has the potential to be virulent in the human host.

MRSP has been an emerging concern in veterinary medicine in recent years (Weese and van Duijkeren, 2010), and similar to *S. aureus*, methicillin resistance is mediated by the mecA gene which encodes production of a modified penicillin-binding protein (PBP). The mecA gene is located on a mobile element of the bacterial chromosome called the 'staphylococcal chromosomal cassette' (SCCmec) and is readily transferred between different Staphylococcal species. Almost a third of *S. pseudintermedius* isolates in our case series [22.2% (6/27)] had isolates which demonstrated methicillin resistance in addition to being multi-drug resistant to other antibiotic classes including macrolides, sulfonamides, and fluoroquinolones. Four of the MRSP isolates had oxacillin MICs of 0.5μg/ml and 1μg/ml below the *S. aureus* oxacillin breakpoint of 4 μg/ml in accordance with standard criteria to prevent misclassification as methicillin susceptible isolates. In our case series, *S. pseudintermedius* was most commonly a component of a polymicrobial environment and frequently with other Staphylococcal species. As up to 90% of healthy dogs may be colonized with *S. pseudintermedius* (Rubin and Chirino-Trejo, 2011; Talan et al., 1989a; Talan et al., 1989b), if the human transmission is common, a proportion of methicillin resistance observed in *S. aureus* and another coagulase-negative Staphylococcal species may reflect the transfer from *S. pseudintermedius*. However, as the absolute incidence *S. pseudintermedius* was very low the pathogencity, potential for inter-species

transfer, and the clinical impact in monomicrobial and polymicrobial infections requires further study.

Our MLST typing revealed that all MRSP isolates had sequence types commonly identified in Europe (ST71 and ST181), and the North American associated MRSP (ST68) was not identified despite being previously reported in dogs in Canada (Kjellman et al., 2015; Perreten et al., 2010). Sequence types 71 and 181 are widely associated with multidrug resistance; ST71 specifically has been associated with resistance to macrolides, sulfonamides and the fluoroquinolones in Europe (Moodley et al., 2013). Fortunately, resistance to drugs active against methicillin resistant isolates such as vancomycin, daptomycin, linezolid or quinupristin + dalfopristin has not yet been reported. We too did not observe this in our series. The diversity of strains identified among MSSP indicated that these infections were likely community acquired and are epidemiologically unrelated.

4.6 Conclusion

In conclusion, the introduction of highly discriminatory laboratory methods has allowed *S. pseudintermedius* to be increasingly recognized as a potential zoonosis of canine origin. In the largest case series to date of human infections with *S. pseudintermedius*, we demonstrated that it is most commonly a member of a polymicrobial environment, likely transmitted from companion animals, and implicated in skin and soft tissue infections. *S. pseudintermedius* was most commonly associated with mild-moderate SSTI similar to those described in the literature for canine species. Certainly, as it has virulence characteristics similar to that of *S. aureus*, the potential for invasive infections exists. Rising methicillin resistance in *S. pseudintermedius* and

the ability for transfer between Staphylococcal species may have significant implications for rising antimicrobial resistance and infection management.

4.7 Transition Statement

The emergence of antimicrobial resistance including MRSP, which is often multi drug resistant, limits the ability of clinicians to use currently available antimicrobials for therapy. Veterinarians are therefore increasingly reliant on non-beta-lactam drugs including chloramphenicol, tetracycline, and trimethoprim+ sulfamethoxazole for treating resistant *Staphylococcus pseudintermedius* infections. Surprisingly, in our previous studies, we have not seen an isolate which is resistant to all three drug classes, indicating that reasonable treatment options are still available for multi drug resistant infections. At present, tetracycline is used as an indicator for doxycycline and minocycline which are more commonly used drugs in dogs. Although we know that the tetracyclines differ in their chemical structure and binding affinity to the drug target, the difference in susceptibility to drugs within this class have not been evaluated. Furthermore, the reliability of tetracycline as an indicator of resistance to second generation tetracycline has not been evaluated. There are multiple mechanisms of tetracycline resistance although these are ill-defined in *Staphylococcus pseudintermedius*. Therefore, the next experiments were designed to understand the mechanism of resistance to tetracyclines in *S. pseudintermedius* and to evaluate the possibility doxycycline or minocycline to treat infections resistance to tetracycline.

5 INVESTIGATION INTO THE PHENOTYPIC AND GENOTYPIC TETRACYCLINE RESISTANCE IN *Staphylococcus pseudintermedius*

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Author Contributions

Conceived and designed experiment: MARP JER. Performed the experiment MARP, CF, JER

Analyses the Data: MARP JER. Wrote the paper: MARP JER.

5.1 Abstract

Antimicrobial resistance including methicillin resistance is emerging in *S. pseudintermedius*, non-β-lactams such as tetracycline, chloramphenicol, and sulfa-trimethoprim are therefore widely used in companion animals. The second generation tetracyclines such as doxycycline and minocycline, are the primary tetracyclines used in companion animal practice. The Clinical and Laboratory Standards Institute recommends tetracycline as an indicator drug for doxycycline and minocycline in antimicrobial susceptibility testing. For *S. pseudintermedius*, neither the reliability of tetracycline as an indicator drug nor the contribution of recognized resistance genes to phenotypic resistance has been determined. Our objectives were to determine the reliability of tetracycline as a surrogate drug for detecting resistance to doxycycline and minocycline and to describe the association between resistance genes and resistance phenotypes.

Antimicrobial susceptibility testing was performed for tetracycline, doxycycline, and minocycline in 521 *S. pseudintermedius* isolates and MICs were determined for a subset of 70 isolates by agar dilution. Tetracycline resistance genes were screened by PCR and a subset of 18 isolates representing the breadth of susceptibility and gene profiles were sequenced using the Illumina MiSeq platform. Genomes were interrogated for recognized resistance genes using online databases. Eight isolates possessing *tet*M were further characterized; *tet*M copy number and expression level were measured using an SYBR Green-based qPCR assay.

Although the majority of tetracycline resistant isolates were also resistant to doxycycline and minocycline, two remained susceptible to both doxycycline and minocycline, while a third was tetracycline and doxycycline resistant and susceptible to minocycline. All resistant isolates contained at least one recognized resistance gene; *tet*M was most commonly identified while *tet*K and *tet*L were each found in single isolates. Among isolates only possessing *tet*M, a wide

range tetracycline (2->32µg/ml), doxycycline (0.25-16µg/ml) and minocycline (0.25-16µg/ml) MICs were observed. Differences in *tet*M copy number were not significantly associated with tetracycline or doxycycline MIC, nor were differences in the expression of *tet*M. In conclusion, tetracycline may be an inadequate indicator of resistance to second generation tetracyclines and the routine inclusion of doxycycline or minocycline on antimicrobial test panels is recommended. The phenotypic diversity in susceptibility to the tetracyclines cannot be solely explained by the presence or absence of resistance genes. Additional work is clearly required to understand the mechanisms of resistance to the tetracyclines in *S. pseudintermedius*.

5.2 Introduction

Staphylococcus pseudintermedius colonizes in 90% of healthy dogs (Rubin and Chirino-Trejo, 2011). It is an opportunistic pathogen, inhabitants in mucous membranes and skin (Bannoehr and Guardabassi, 2012). Importantly, *S. pseudintermedius* is the most common cause of pyoderma, otitis externa and the second common cause of urinary tract infections in dogs (Rubin and Chirino-Trejo, 2011). Antimicrobial resistance is emerging in *S. pseudintermedius* which is a growing problem for the treatment of bacterial infections (Schwarz et al., 2016). The β-lactams are frequently prescribed by veterinarians and physicians in Canada for common infections caused by Gram positive bacteria including Staphylococci (CARSSR, 2016). Some authors have suggested that as β-lactam resistance emerges, veterinarians are becoming less likely to prescribe this class of drug in companion animals (Schnedeker et al., 2017). MRSP is often multidrug resistant and rising resistance is reported to the fluoroquinolones, aminoglycosides, macrolides, and sulfonamides (Kadlec et al., 2016). The use of last line of defense drugs such as vancomycin and linezolid, which is designated for human use, is anecdotally still rare in veterinary medicine.

As such, therapeutic alternatives are limited for veterinarians to treat resistant bacterial infections. Therefore, the effective utilization of currently available antibiotics such as tetracycline and sulfamethoxazole is particularly important in veterinary medicine as in some cases these may be the only reasonable alternatives to the β-lactams.

Tetracycline, discovered in the late 1940s, is a broad-spectrum antibiotic which is also used against atypical bacterial infections such as Rickettsiales and spirochetes, and also as an antiprotozoal (Chopra and Roberts, 2001). The first-generation tetracyclines, chlortetracycline, and oxytetracycline are used mainly in agricultural animals (Chopra and Roberts, 2001). Currently, doxycycline and minocycline are the most commonly used drugs in companion animals (Hnot et al., 2015). These two are important alternatives for treating MRSP infections in dogs, previous studies have been reported that 35-50% of MRSP remained susceptible to doxycycline and minocycline (Humphries et al., 2016; Kawakami et al., 2010). According to the CLSI, tetracycline is being used as an indicator agent for detecting resistance to the second generation tetracyclines (CLSI, 2016). As described by EUCAST, isolates susceptible to tetracycline should also be considered to be susceptible to doxycycline and minocycline while those resistant to tetracycline may remain susceptible to doxycycline and minocycline (EUCAST, 2017). The CLSI states that isolates which are intermediately resistant to tetracycline should be described as "may be susceptible for doxycycline" (CLSI, 2015). The correlation of resistance to tetracycline and doxycycline is clearly ill-defined and requires further investigation.

Resistance to tetracycline occurs by four main mechanisms. In *S. pseudintermedius* tetM is reported to be the most common. TetM is a ribosomal protection protein and acts on the amino acyl-tRNA ribosome complex and confers resistance by displacing tetracycline (Chopra and Roberts, 2001). This protein has five domains (I – V), domain IV is hypothesized to localize to

the “A” site of the 30S ribosomal subunit (Connell et al., 2003). Associations between domain III and the 30S ribosomal subunit and decoding sites have also been observed, and mutations in this region of *tet*M have been associated with elevated tetracycline MICs in *E. faecalis* (Dönhöfer A, 2012). Similarly, TetO is another ribosomal protection protein which has a similar function to TetM but is less commonly found in *S. pseudintermedius*. Efflux-mediated resistance to tetracycline is encoded by the *tet*K and *tet*L genes are frequently found on plasmids in Staphylococci (Chopra and Roberts, 2001; Roberts, 1996). These four genes are the only tetracycline resistance determinants so far identified in *S. pseudintermedius* (Kadlec and Schwarz, 2012).

Because, tetracycline acts by binding to the ribosome and interacting with the 16S rRNA and small subunit ribosomal proteins, mutations in these structures have been related to elevated tetracycline MICs (Chopra and Roberts, 2001; Gerrits et al., 2002; Nonaka et al., 2005). The small subunit ribosomal proteins S7, S3, S8, S14, and S19 also interact with tetracycline (Oehler et al., 1997). It has therefore been suggested that specific 16S rRNA SNPs, and mutations in the S7 and S12 protein sequences can affect these interactions and therefore tetracycline binding affinity (Moazed and Noller, 1987). However, mutations in these genes associated with tetracycline resistance have not been reported in *S. pseudintermedius*. Therefore, the first objective of the study was to determine the reliability of tetracycline as an indicator for detecting susceptibility to doxycycline and minocycline in *S. pseudintermedius*. The second objective was to understand the mechanism of resistance to tetracycline and to describe the association between resistance genes and phenotypic resistance in *S. pseudintermedius*.

5.3 Material and Methods

5.3.1 Antimicrobial susceptible testing and identification of resistance genes

Antimicrobial susceptibility testing was done for 521 *S. pseudintermedius* isolates from dogs for tetracycline, doxycycline, and minocycline by disk diffusion according to the method described (CLSI, 2016). The MICs were determined for a subset of 70 tetracycline susceptible and resistant isolates by agar dilution (CLSI, 2012a). The results were interpreted using the CLSI veterinary clinical breakpoints except for minocycline, the clinical breakpoint for doxycycline was used for minocycline (CLSI, 2015). These 70 isolates were also screened for *tet*K, *tet*M, *tet*O, and *tet*L by PCR using primers designed and optimized in this study (Table 5.1). PCR products were sequenced (Macrogen Inc. Seoul, Korea), the chromatogram quality of each sequence was inspected using pregap and contigs were then aligned using gap (Staden packages). The consensus sequences were examined by BLAST in the NCBI database to confirm the identity of the amplicon.

Table 5.1: The primer sequences, NCBI accession number of nucleotide sequence and products size for conventional PCR and qPCR to screen tet gene in the study.

Gene	**Primer (5' to 3')**	**Reference sequences used to design primers**	**Product size (bp)**
*tet*K	F: ATCTGCTGCATTCCCTTCAC R: GCAAACTCATTCCAGAAGCA	456769	818
*tet*M	F: ACACGCCAGGACATATGGAT R: GCAAAGTTCAGACKGACCTC	X90939	530
*tet*O	F: GATGTGTGTTCCGACAAACG R: CCATAAAGAACCCCCTCCAT	M20925	573
*tet*L	F: CTGCATTTCCAGCACTCGTA R: CGAAAGCCCACCTAAAACAA	17153	737
*tet*M*	F: TGGGCTTCCATTGGTTTATC R: TCCGTCACATTCCAACCATA	MSSP34 *tet*M sequence (in this study)	163

Primers for qPCR*

5.3.2 Whole genome sequencing

Based on the breadth of phenotypic resistance and genotypic characterization, a subset of the collection (n=18) were selected for WGS. The collection included resistant isolates with higher and lower MICs and isolates which were susceptible to tetracycline. DNA was extracted by the modified salting out method, DNA concentration was measured by QubitTM assay (Invitrogen) and quality was evaluated by running genomic DNA in 1% agarose gel (Martin-Platero et al., 2007). The WGS was run on the Illumina MiSeq platform on a V2 chip using the Nextera XT indexing kit for multiple samples.

The quality of genomes was analyzed by FASTQ and de-novo assembly by Velvet short sequence alignment (Table 5.3). Assembled genomes were annotated in RAST-OMGE using *S. pseudintermedius* ED 99 as a reference (NCBI Accession number: CP002478). The genomes were interrogated for recognized resistance genes using the online resistant gene databases ResFinder (genomicepidemilogy.org) and CARD (Comprehensive Antimicrobial Resistance Database – card. macmaster.ca) (Jia et al., 2017; Zankari et al., 2012). The nucleotide sequences of 16S rRNA were examined for the mutations comparing to the sequences from susceptible isolates in this study. In addition, sequences of small subunit proteins were investigated once aligned with susceptible isolates (CLC Genomic Workbench 9.5.1).

5.3.3 Phylogenetic analysis

*tet*M sequences were compared to each other (n=9) and to a set of previously published reference sequences from NCBI GenBank. Full-length amino acid sequences (639 aa), were aligned using MUSCLE, and a phylogenetic tree was constructed by maximum likelihood methods in MEGA 7 (Kumar et al., 2016).

5.3.4 Optimization of qPCR for *tet*M and quantification of *tet*M copy number

Primers for qPCR targeting *tet*M were designed based on the *tet*M sequence of the MSSP34 sequenced in this study. Primers from three previously published reference genes were selected as *gyrB, rho,* and *recA* (Crawford et al., 2016). The PCR protocol four genes were optimized in this study (Table 5.1). The purified PCR products were cloned into the pGEM+6 plasmid according to the manufacturer's instructions and transformed into the competent *E. coli* as described (pGEM®-T Easy Vector Systems: Promega). The competent *E. coli* were grown in LB agar (100mg/L of ampicillin) and the plasmid was extracted by standard extraction kit EZ98-10 Spin column Plasmid DNA MiniPrepskit, Bio Basic International, Version 20.0 Rev3/23/2015: Molecular Biology Kit) and confirmed the size of plasmid running in 1% gel. The qPCR protocols were optimized for *tet*M and three reference genes. The DNA was serially diluted from 10^0 to 10^{-10}, a standard curve was run for *tet*M and three reference gene and primer efficiency were calculated (Table 5.4).

Nine *S. pseudintermedius* isolates were run in the optimized qPCR was based on the standard curve for all four genes in Biorad master mix (iQ SYBR Green Supermix, 170-8882: BIO-RAD) using known positive controls (Protocol: 95^0C for 3 minutes, 40 cycles of 95^0C for 10 Seconds, 55^0C for 10 Seconds,72^0C for 30 Seconds and 95^0C for 10 Seconds). However, for *gyr*B, an annealing temperature of 65^0C was used.

To measure *tet*M copy, *Staphylococcus pseudintermedius* were cultured on 5% sheep blood agar incubated for 24 hours at 350C. A 100µl of McFarland 0.5 were inoculated into a 10 ml of TSB with 2% dextrose and incubated for 4 hours at 35^0C in shaking incubator (220rpm). DNA was extracted from 1 ml using the modified salting out method and the concentration of DNA was

measured by Nanodrop (NanoDrop: ND-2000c: Thermo Fisher) and QubitTM assay (Martin-Platero et al., 2007). To determine the *tet*M copy number, extracted DNA was used as a template for qPCR reactions, for *gyr*B and *rec*A gene, an additional 100-fold dilution was made prior to amplification due to the high copy number. The qPCR was run for *tet*M and three reference genes in SYBR green method (iQ SYBR Green Supermix, 170-8882: BIO-RAD). The final copy number of each gene was calculated considering all the dilution factors.

5.3.5 *tet*M gene expression

The *tet*M gene expression was then measured in 8 isolates selected to represent a diversity of minimum inhibitory concentration to tetracycline and doxycycline, each isolate was tested once and no replicates were included. Expression was measured following 150 minutes of exposure to three concentrations of tetracycline and doxycycline (0µg/ml, 0.01µg/ml, and 1µg/ml: based on MICs for tetracyclines of the isolates) as seen in Figure 5.1. RNA was extracted using the ALL PREP DNA/RNA MINI KIT (Qiagen) with modification of following steps. 1 ml of broth culture was centrifuged and the cell pellet was resuspended in 100 ml of TES for enzymatic digestions (lysozyme, lysostaphin, mutanolysin) as described by modified salting out method. A proteinase K digestion was then done in RLT plus buffer, the enzymatic mixture was inactivated by incubating at 80^0C for 15 minutes. The extracted RNA was purified using the DNA-free kit as described (Life Technologies, AM2235,1000U). The quality of RNA was tested by Nanodrop (NanoDrop: ND-2000c) and the Agilent RNA 6000 Nano reagent by Bioanalyzer (Agilent 2100 Bioanalyzer). RNA was normalized according to the Nanodrop reading (NanoDrop: ND-2000c: Thermo Fisher) prior to the cDNA synthesis using the iScript cDNA Synthesis kit (BIORAD;1708891). Quantification of *tet*M cDNA transcripts was then done using the same

SYBR Green-based qPCR assay (iQ SYBR Green Supermix, 170-8882: BIO-RAD). Transcripts of the three reference genes were also quantified.

5.3.6 Statistical analysis

The reference genes were evaluated using Normfinder and GeneKeeper prior for the statical analysis. The results of *tet*M copy number and gene expression were determined relative to *rec*A. The final copy numbers were calculated as a ratio to *rec*A, as the reference or control for gene fold calculation. The association of *tet* gene on different MICs category were analyzed by the chi-square test. The gene copy number and *tet*M gene expression on MICs for tetracycline and doxycycline were analyzed by Mann-Whitney test using SPSS 21.

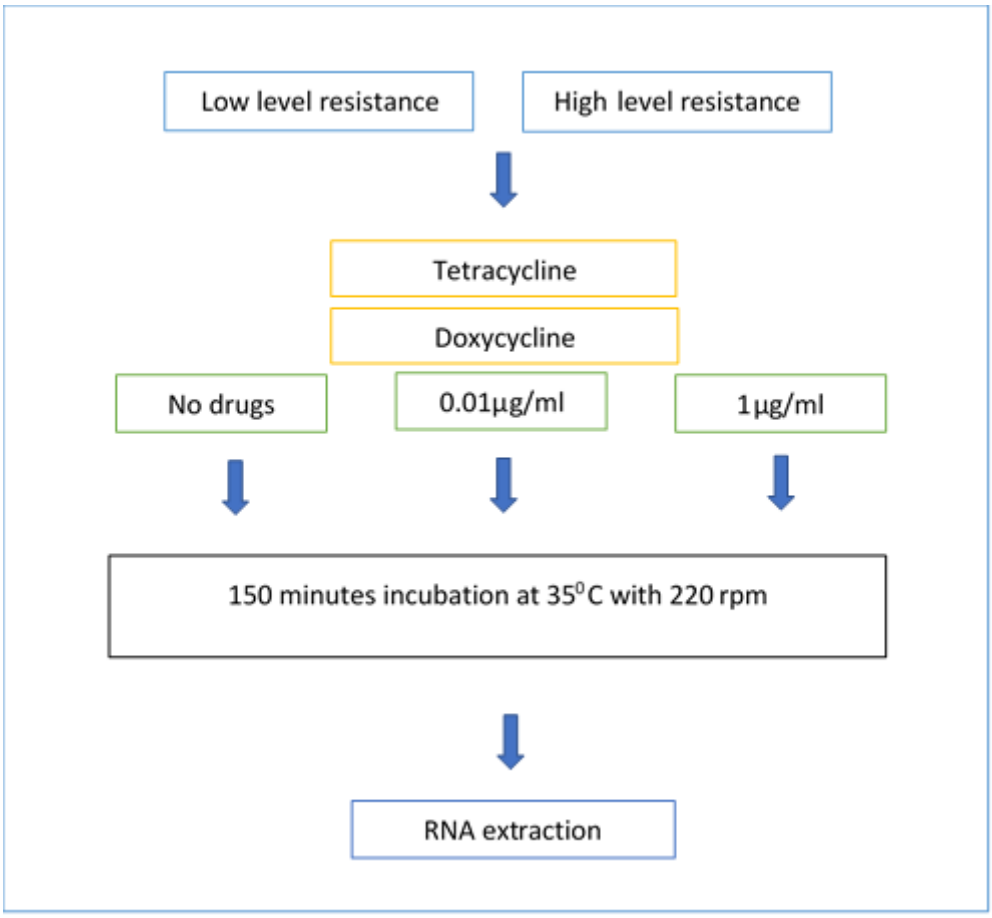

Figure 5.1: Flow diagram for the *tet*M gene expression study in different concentration of tetracycline and doxycycline in *S. pseudintermedius*.

Two groups of tetracycline resistant isolates, those with high (MICs ≥8 µg/ml) and low (MICs ≤4 µg/ml) MICs to both tetracycline and doxycycline were compared. Expression of *tet*M was measured at two drug concentrations (0.01µg/ml and 1 µg/ml) and a no antibiotic control. Expression was measured after exposure to each experimental condition for 150 minutes at 35^{0}C for 220 rpm shaking incubator.

5.4 Results

A great diversity of resistance phenotypes for tetracycline, doxycycline, and minocycline were found in this study. Two isolates (MRSP03, MSSP16) were resistant to tetracycline and susceptible to doxycycline and minocycline (Table 5.2). One isolate (RSP63A1c) was resistant to both tetracycline and doxycycline and susceptible to minocycline (Table 5.2). All isolates which phenotypically resistant to tetracycline possessed at least one resistance gene, while no resistance genes were identified in fully susceptible isolates except in SP16 which possessed a sequence with >80% similarity to tet38 (Table 5.2). The results of whole genome sequencing were consistent with PCR assays and additional resistance genes were not identified by exploring in online resistance databases. The *tet*M gene was most commonly identified and was present in all resistant isolates except for RSP63A1c where the only *tet*L was found. Another isolate, MRSP11, possessed both *tet*K and *tet*M (Table 5.2). No isolates containing *tet*O were identified. Among isolates only possessing *tet*M, a wide range of MICs for tetracycline (2->32µg/ml), doxycycline (0.25-16µg/ml) and minocycline (0.25-16µg/ml) were observed.

Resistant isolates were divided into two MIC categories: 1. low level of resistant and 2. high-level resistant (Table 5.2). For tetracycline, isolates with MICs ≤4µg/ml were classified as low-level resistant and MICs ≥8 were classified as high-level resistant. For doxycycline, isolates with MICs ≤4µg/ml were classified as low-level resistant while those with MICs ≥8µg/ml were classified as high-level resistant. To investigate the diversity of resistance to tetracyclines, we first examined *tet*M protein sequences and two distinct clusters were found (Figures 5.2). MRSP03, MSSP16, MSSP18, and MSSP15 were highly similar (Cluster 1) and MSSP11, MSSP34, MSSP08, RSA09A1a and MRSP 11 formed a second group (Cluster 2). One outlier isolate, SP03, was dissimilar from all other sequences identified in our collection. Interestingly,

all previously published TetM sequences from *S. pseudintermedius* also fell into these two clusters; interestingly both clusters included isolates with high and low MICs to tetracycline and doxycycline (Figure 5.2). The TetM sequence from SP03 was divergent from the other isolates included in this study and clustered with previously published TetM sequences from *E. feacium.* When the promoter region of *tet*M was examined, no association with tetracycline or doxycycline MIC category was identified (Figure 5.6).

A significant association between *tet*M copy number and MIC for tetracycline were not found (Figure 5.3). Furthermore, no significant differences in fold changes in *tet*M expression were identified between low MIC and high MIC categories for either tetracycline or doxycycline (Figures 5.4 & 5.5). Interestingly, *tet*M expression level was concentration-dependent increasing significantly following exposure to higher concentrations of doxycycline (P = 0.02) and insignificantly with exposure to higher concentrations of tetracycline (P = 0.172).

When the ribosomal small subunit protein sequences were examined, a silent mutation was observed in S12 was found in one isolate in this collection, while the sequences of the S3, S7, S8, S14, and S19 proteins were identical. A similar observation was made for the16S rRNA sequences, all isolates had the same nucleotide sequence at positions which were previously shown to confer resistance in *E. coli* and P. acne (Table 5.5) (Oehler et al., 1997; Ross et al., 1998).

Finally, the *mgr*A and *sav*1866 genes which have been previously reported as regulatory genes and efflux-mediated resistance for tetracycline respectively were identified in both resistant and susceptible isolates (Dawson and Locher, 2007; Truong-Bolduc et al., 2005). No meaningful association was found between the presence of these genes and resistance to tetracycline in *S. pseudintermedius*.

Table 5.2: Summary of MICs for tetracyclines and resistance gene found by PCR and WGS followed by exploring in the online antimicrobial resistance databases.

Isolate ID	**MICs by agar dilution test (µg/ml)**			**Genes detected by conventional PCR**	**Resistance gene identified in whole genome**
	tetracycline	**doxycycline**	**minocycline**		
RSA100A1a	0.12	<0.03	<0.03	-	-
RSA05A1b	0.12	<0.03	<0.03	-	-
SP16	0.12	<0.03	<0.03	-	*tet*38
RSA89A1b	0.12	0.06	<0.03	-	-
RSA08A1b	0.12	0.06	<0.03	-	-
SP53	0.25	0.06	0.06	-	-
MRSP03	2	0.25	0.25	*tet*M	*tet*M
MSSP15	4	1	0.5	*tet*M	*tet*M
MSSP16	2	0.25	0.06	*tet*M	*tet*M
MSSP11	8	8	0.5	*tet*M	*tet*M
MSSP18	16	4	0.5	*tet*M	*tet*M
RSA67A1a	32	4	0.5	*tet*M	*tet*M
RSP09A1a	32	8	4	*tet*M	*tet*M
RSP63A1c	32	4	0.06	*tet*L	*tet*L
MSSP08	>32	8	8	*tet*M	*tet*M
MSSP34	>32	8	8	*tet*M	*tet*M
MRSP11	>32	16	2	*tet*M, *tet*K	*tet*M, *tet*K
SP03	>32	16	16	*tet*M	*tet*M

Isolates with MICs in the susceptible range are coloured in green. Isolates with MICs indicating resistance are highlighted either yellow (low-level resistance) or red (high-level resistance).

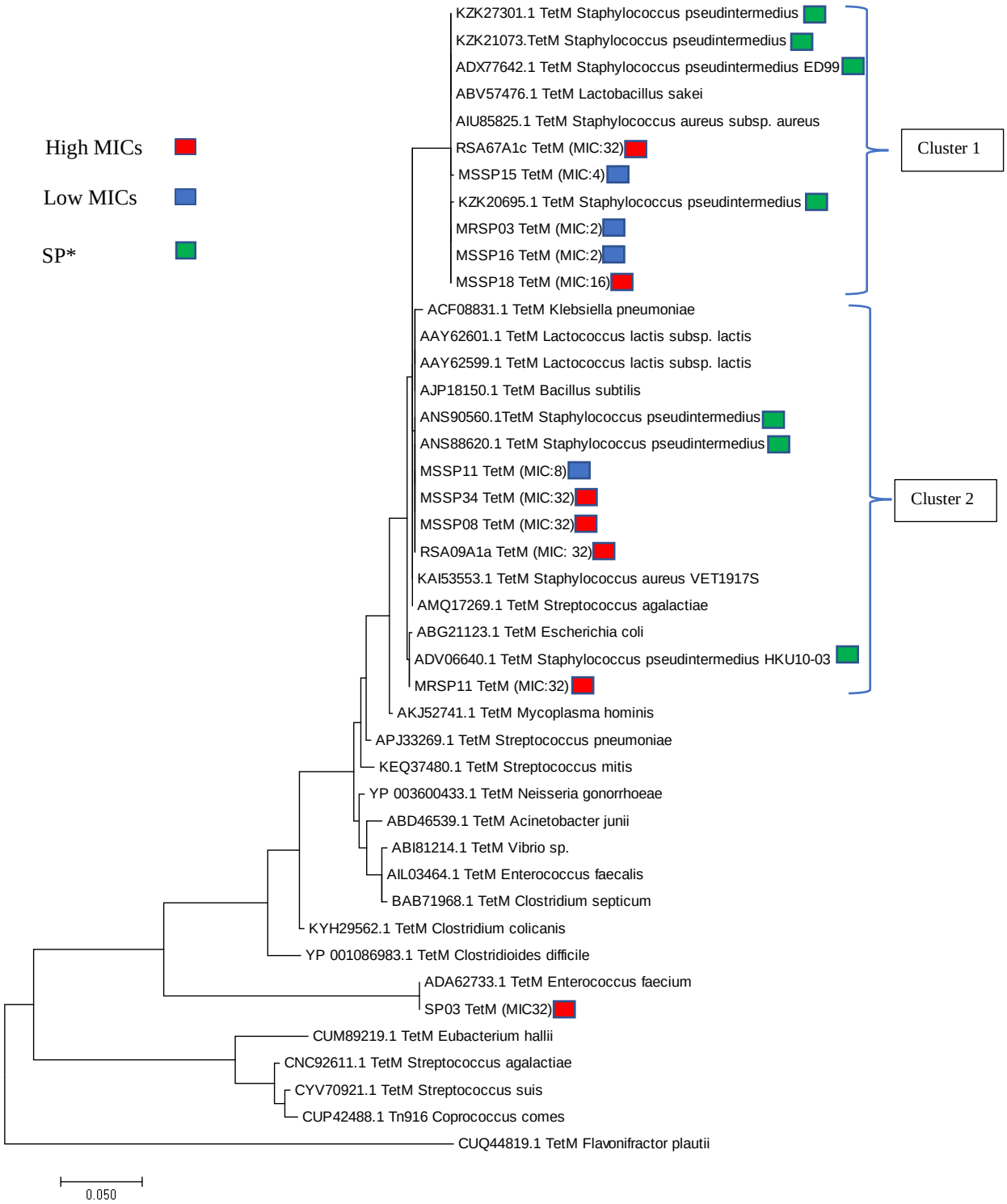

Figure 5.2: Phylogenetic tree of TetM protein sequences from in this study with published protein sequences in selected bacterial species in NCBI (MIC category was displayed according to the tetracycline).

* *S. pseudintermedius* TetM sequences which were retrieved from NCBI GenBank.

Table 5.3: Summary of WGS of *Staphylococcus pseudintermedius* isolates in this study (n=18).

ID	**Assembly size**	**GC content%**	**Contigs**	**Contigs N50**	**scaffolds**	**Scaffolds N50**
MSSP18	2500648	37.62	83	58578	75	67540
MSSP34	2575334	37.51	92	62957	84	68481
RSA100A1a	2517592	37.68	52	125796	41	125796
MSSP11	2399811	37.87	98	56695	75	74150
RSA63A1c	2497917	37.69	56	117925	47	127519
RSA67A1a	2489950	37.62	78	67706	67	96944
RSA89A1b	2548371	37.53	64	127844	47	156805
RSA05A1b	2599913	37.34	99	125626	81	125626
RSP09A1a	2551544	37.62	63	90312	50	123064
SP16	2384965	35.86	50	117709	34	125619
MRSP03	2610195	37.58	141	46522	118	56143
MSSP08	2617347	37.5	103	71264	77	94379
SP03	2096021	39.62	63	91911	45	118713
SP53	2427959	37.72	92	54637	80	62668
MSSP15	2546900	37.43	78	70554	64	116468
MSSP16	2780662	37.22	94	57797	81	72248
MRSP11	2767609	37.25	125	62978	113	64561
RSA08A1b	2545616	37.52	69	83399	58	89055

Table 5.4: Summary of primer efficiency and coefficient of correlation (R^2) for tetM and the three reference genes used for the standard curve for qPCR.

Name of the gene	**protocol**	**R^2**	**Primer efficiency %**
*tet*M	In this study	0.990	95.7
*gyr*B	In this study	0.996	102.5
rho	In this study	0.994	96.2
*rec*A	In this study	0.994	94

Table 5.5: Comparison of the specific nucleotide locations in *S. pseudintermedius* into the published information in *E. coli* and Propionibacterium to determine association for the resistance to tetracycline.

The organism	**Nucleotide position in the published**	**Nucleotide position in examined in this study**	**Reference**
E. coli	G693	G693	Oehler et al, 1997
	A892	C892	
	G890	G890	
	U1052	G1052	
	C1054	G1054	
	G1300	G1300	
	G1338	C1338	
P.acne	U1052	G1052	Ross et al,1998
	C1054	G1054	
	G1058	G1058	

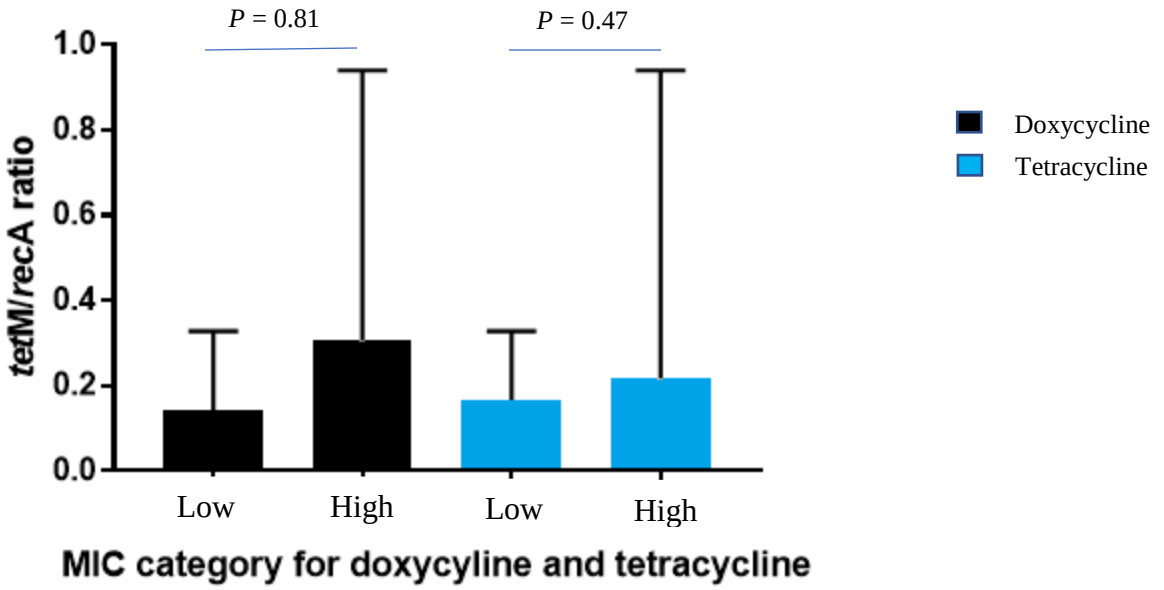

Figure 5.3: Comparison of *tet*M copy number between isolates with high and low doxycycline and tetracycline MICs.

The qPCR based quantification of *tet*M relative to the *rec*A gene. Low (n=4) and high (n=5) level resistant isolates are compared. Isolates with doxycycline MICs of ≥4µg/ml and tetracycline MICs of ≥8µg/ml were considered to be high-level resistant.

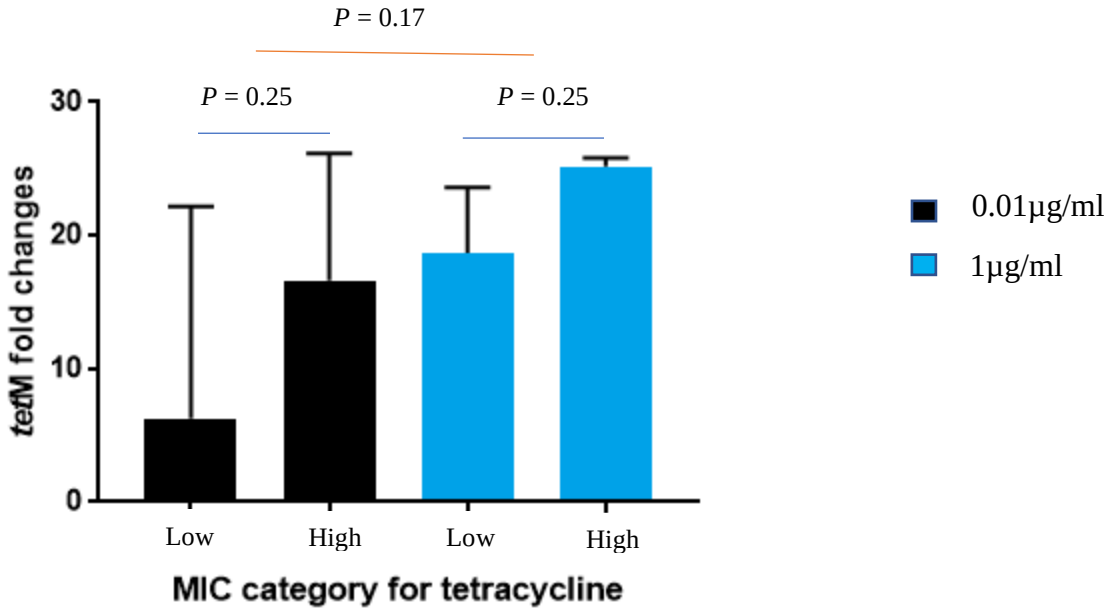

Figure 5.4: The *tet*M gene fold changes at different concentration tetracycline.

The qPCR based quantification of *tet*M relative to the *rec*A gene. Isolates with low (n=4) and high (n=4) level resistance were included and *tet*M expression was compared between these isolates on exposure to different tetracycline concentrations. Expression of *tet*M in isolates with low MICs (P = 0.15) and high MICs (P =0.56) did not differ significantly between tetracycline concentrations.

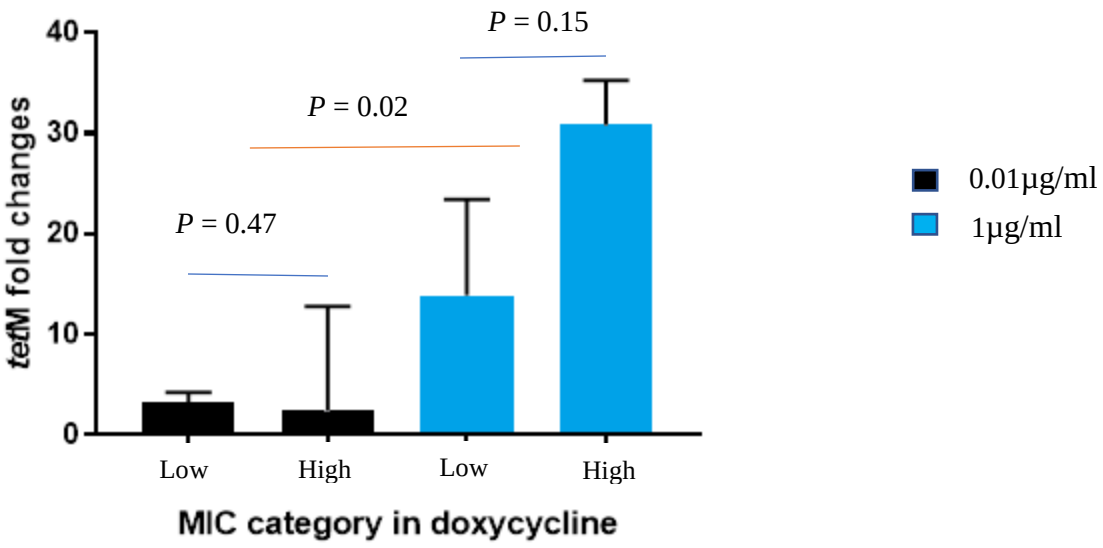

Figure 5.5: The *tet*M gene fold changes at different concentration doxycycline.

The qPCR based quantification of *tet*M relative to the *rec*A gene. Isolates with low (n=4) and high (n=4) level resistance were included and *tet*M expression was compared between these isolates on exposure to different doxycycline concentrations. The fold changes expression between low MICs were P = 0.02 and high MICs were P =0.04 at different tetracycline concentration. Expression of *tet*M in isolates with low MICs (P = 0.02) and high MICs (P =0.04) differed significantly between doxycycline concentrations.

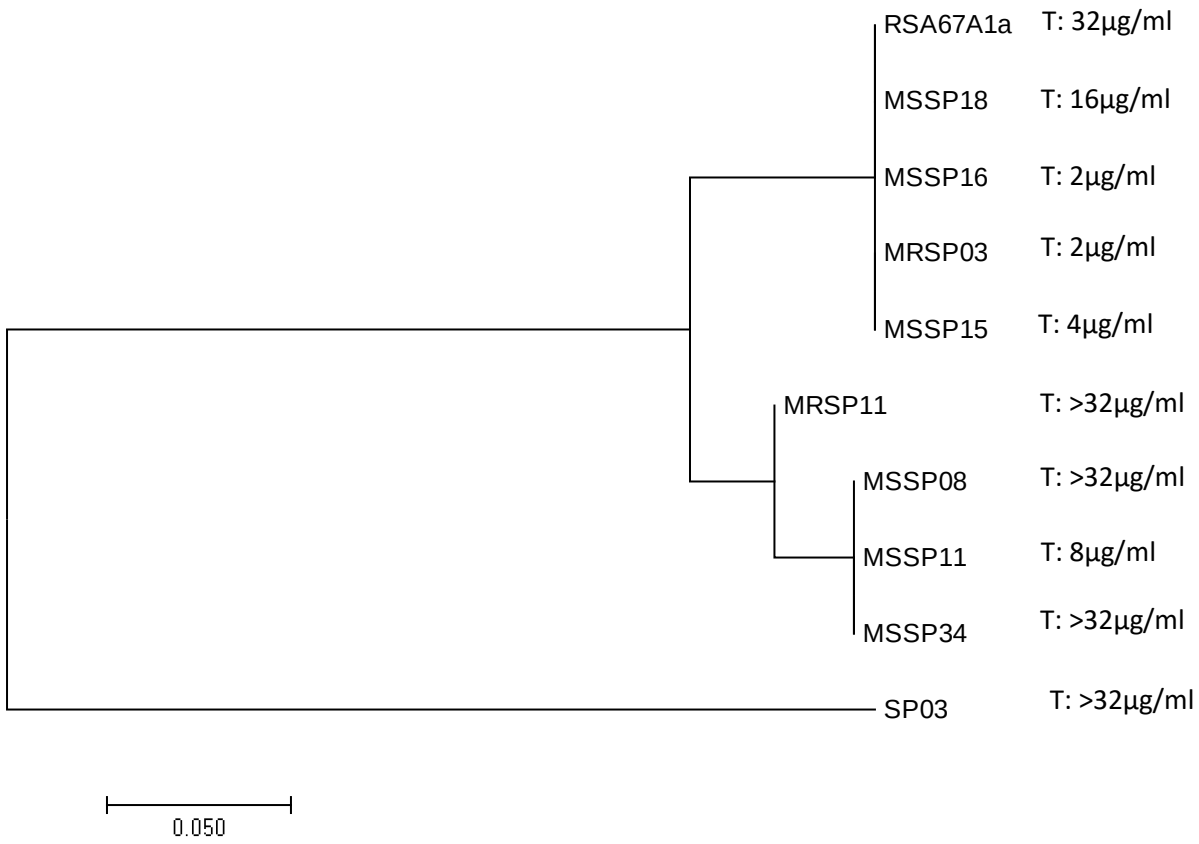

Figure 5.6: Phylogenetic tree of the promoter region (flank region) of *tet*M gene with MIC for tetracycline.

The sequences of the *tet*M promotor region (n=10) were extracted from whole genome sequences. These sequences formed two clusters, while SP03 was an outlier. Each cluster contained isolates with both high and low tetracycline MICs, which are indicated on the right.

5.5 Discussion

Doxycycline and minocycline are the most commonly used tetracyclines in companion animals, while chlortetracycline and oxytetracycline are primarily used in agriculture (Chopra and Roberts, 2001). Although phenotypic resistance is being shown frequently, resistance mechanisms for the tetracyclines (including doxycycline, minocycline) have not been thoroughly investigated in Gram positive bacteria including *S. pseudintermedius*. The literature was limited to screening for genes by PCR/microarray and reporting simple presence/absence without a detailed description of the accompanying phenotype (Kadlec and Schwarz, 2012). Resistance to tetracycline does not concur always with doxycycline and minocycline in *S. pseudintermedius* and intermediate resistance to tetracycline may be susceptible for doxycycline in Staphylococci (CLSI, 2015). Minocycline has an additional C7-dimethylamino group on ring D which is shown noncovalent interaction with C1054 in the h34 helix of 16S rRNA, was shown 20 times more affinity to the ribosome (Nguyen et al., 2014). Second generation tetracycline is more lipophilic in nature than the parent compound, these differences may result in improved pharmacodynamic interactions and more potent antimicrobial activity (Nguyen et al., 2014). Tetracycline resistant isolates may, therefore, remain susceptible to doxycycline and minocycline which could remain effective treatment options for MRSP infections in dogs (Humphries et al., 2016).

Consistent with previous studies *tet*M was the most commonly tetracycline resistance gene in this collection (Kadlec and Schwarz, 2012). Although all isolates possessing *tet*M were resistant to tetracycline, they were not always resistant to doxycycline and minocycline. A significant difference in *tet*M expression was not observed for tetracycline or doxycycline between high and low MIC categories in our collection of *S. pseudintermedius*.

The ribosomal protection protein TetM has been reported more than 50 different bacterial species, including Gram-positives, negatives and anaerobic bacteria (Connell et al., 2003; Dönhöfer A, 2012; Taylor and Chau, 1996). Diversity in the sequence of previously published TetM sequences was found, we were curious to see if these differences might be associated with phenotypic variation. Therefore, we constructed a TetM phylogenetic tree to compare these sequences to tetracycline and doxycycline MICs to see if an obvious association exists in *S. pseudintermedius*. Interestingly, we did not identify co-clustering of isolates by MIC category with TetM sequence suggesting that sequence diversity may not be responsible for the observed phenotypic diversity (Figure 5.2). However, SP03 did not cluster with rest of the sequence of the study or other *S. pseudintermedius* sequence deposited in GenBank. This particular sequence was most closely related to a *tet*M sequence from an E. faecium suggesting that in the case of this organism *tet*M may have been acquired from some other bacterial species. The high sequence diversity of *tet*M has been reported previously, this gene is reported to have a mosaic structure as a result of selection pressure on mobile genetic elements (Oggioni et al., 1996). Previous studies have found that both the carboxyl-terminal end and domain IV of TetM interact with the 23S and 16S rRNA, mutations in these TetM structures may, therefore, be associated with alterations in the level of resistance conferred by this protein (Dönhöfer A, 2012). The mutations at specific nucleotides in 16S rRNA which previously found in *E. coli* and Propionibacterium acne were not identified in this study (Table 5.4) (Roberts, 1996). Ross et al reported that G1058 to C mutation which resistance to tetracycline in Propionibacterium, (Taylor and Chau, 1996). However, mutations at these sites were not found in *S. pseudintermedius* in this study.

One isolate possessing the *tet*L efflux pump was resistant to both tetracycline and doxycycline but not minocycline; consistent with previous studies which demonstrated that minocycline is not

a substrate for this efflux pump (Nguyen et al., 2014). With one exception, no tetracycline resistance genes were found among fully susceptible isolates. The exception was one isolate possessing a gene with 86% identity to tet38, this putative resistance gene was identified by interrogating the genome of SP16 using the CARD database. This finding may indicate that the gene we identified may be a precursor of the tet38 resistance gene, or perhaps an orthologous gene which has since lost its function with respect to tetracycline resistance. In *S. aureus*, tet38 encodes for efflux pump which confers tetracycline resistance (Truong-Bolduc et al., 2005). Overall, the presence or absence of resistance gene was not predictive of phenotypic resistance to doxycycline and minocycline in the study, isolates possessing the same genes with different MICs were identified. Further studies are therefore required for the explanation.

Our attempts to attribute phenotype to other *tet*M factors including gene copy number failed to yield conclusive results. While there was no significant or apparent association with MIC and gene copy number, a non-significant but numerically apparent association was observed between *tet*M expression and MIC. The presence of *tet*M associated with resistance to tetracycline was observed in this study. However, *tet*M expression level was not significantly associated with MIC category for tetracycline and doxycycline. Although *tet*M expression level was not significantly associated with resistance, it was numerically higher in high MIC category isolates than low category isolates. The results suggest that expression may play a role although more studies are required to confirm this non-significant observation. A significant association between *tet*M expression and exposure to a different concentration of doxycycline was observed, and although a similar trend was seen for tetracycline these differences were not significant. Alternatively, it is possible that factors other than *tet*M are contributing to tetracycline and doxycycline MICs in the *S. pseudintermedius* studied.

Investigations of the relationship between gene expression and antimicrobial resistance phenotype are complicated and difficult to interpret in prokaryotes due to various reason including lack of reference genes, effects of antibiotics on other metabolic functions which may impact gene expression directly or indirectly. Moreover, normalization of reference genes is a well-known limitation in bacterial transcriptional studies (Dundas and Ling, 2012; McMillan and Pereg, 2014). Meaningful interpretation of target gene expression is limited by variability in reference gene expression (McMillan and Pereg, 2014). No single reference gene has been validated for gene expression studies in bacteria (Dundas and Ling, 2012). We therefore selected three reference genes which were originally optimized for biofilm studies (Crawford et al., 2014). All three genes were initially included in this study, and one was selected post hoc for interpretation based on Normfinder and GeneKeeper. Furthermore, small sample size and the lack of reference genes for antimicrobial resistance gene expression studies were a limitation for the significant finding of this study. However, the study was not focused on translation, post-transcriptional and translational modification, therefore further investigation must be required.

5.6 Conclusion

These results demonstrate that 116tetracycline is an inadequate indicator of resistance to other tetracyclines, we, therefore, recommend that diagnostic laboratories perform antimicrobial susceptibility testing for doxycycline and minocycline individually rather than solely relying on tetracycline as an indicator. Furthermore, phenotypic diversity in susceptibility to the tetracyclines cannot be solely explained by the presence or absence of resistance genes in the genome of *S. pseudintermedius*. The *tet*M gene is the common tetracycline resistance gene found in *S. pseudintermedius*. Although a significant difference in *tet*M expression was not found

between isolates with high and low tetracycline and doxycycline MICs, high-level resistant isolates did have numerically higher expression and additional, larger studies to confirm this observation are therefore recommended. Furthermore, proteomic studies would be helpful to clarify the presumptive differences in TetM in high and low MIC categories. The genotypic evidence on MICs for tetracycline and doxycycline is largely unknown and more studies are required.

6 DISCUSSION AND CONCLUSION

The emergence of antimicrobial resistance in *S. pseudintermedius* was investigated in healthy dogs. Strain-specific infections and antimicrobial resistance was also examined in *S. pseudintermedius* from dermatological and urinary tract infections. Additionally, we investigated the incidence of *S. pseudintermedius* among human diagnostic submissions, and characterized these isolates using phenotypic and genotypic methods. Finally, we assessed the reliability of tetracycline as an indicator for resistance to doxycycline and minocycline, and the molecular and epigenetic mechanisms contributing to tetracycline resistance were explored.

We found that *Staphylococcus pseudintermedius* colonized 80% of healthy dogs and that antimicrobial resistance increased since the last surveillance study conducted in 2008. Resistance to β-lactams and tetracycline was more common in 2014 compared to 2008, while the frequency of pan-susceptible isolates decreased. These results were consistent with the recent literature where high rates of tetracycline and β-lactam resistance were found in *S. pseudintermedius* (Gomez-Sanz et al., 2013a; Yoon et al., 2010). Moreover, MRSP was found in 7% of healthy dogs, which was higher than previously reported in North America and Europe (4.5%) (Weese and van Duijkeren, 2010). Multi-drug resistant isolates, both MSSP and MRSP, were also identified. However, resistance to other non-β-lactams including chloramphenicol, gentamicin, trimethoprim + sulfamethoxazole, erythromycin, and clindamycin remained low in the Saskatoon region. The results of this study highlight the importance of routine susceptibility testing when investigating bacterial infections involving *S. pseudintermedius*.

Dermatological and urinary tract infections caused by *S. pseudintermedius* are common in dogs. In this study, strain specific tissue tropism was not observed for the skin or urinary tract, as is seen with uropathogenic *E. coli*. Despite this lack of mutually exclusive clustering, a

significantly higher frequency of resistance to erythromycin and chloramphenicol, and a difference approaching significance was observed in dermatological vs. urinary isolates. These results suggest that *S. pseudintermedius* infections are truly opportunistic. The identification and treatment of predisposing factors leading to disease is therefore critical to successfully treat these infections.

Staphylococcus pseudintermedius is a rare zoonosis, with an incidence of 0.05% among skin and soft tissue infections in a people; compated to *S. aureus* which is isolated from 30% of these infections. *Staphylococcus pseudintermedius* may be misdiagnosed as *S. aureus* due to the lack of awareness of this veterinary pathogen in clinical diagnostic labs. Moreover, the ability to identify methicillin resistance in human diagnostic labs may be complicated by differences in the clinical breakpoints for oxacillin between these species (0.5µg/ml and 4µg/ml for *S. pseudintermedius* and *S. aureus* respectively) (CLSI, 2015). Importantly, no resistance to the last line drugs which used for Gram positive infection in humans was identified.

The emergence of MRSP and β-lactam resistance among MSSP is threatening the future of the β-lactams as effective anti-staphylococcal treatments. As a result, non-β-lactams including tetracycline have become important for treating *S. pseudintermedius* infections in dogs. Doxycycline and minocycline are prescribed for treating *S. pseudintermedius* infections in companion animals. Because our findings indicate that tetracycline is an inadequate indicator of resistance to these compounds, we recommend that laboratories include doxycycline and minocycline in routine test panels. While we did find at least one resistance gene in all resistant isolates, the relationship between the presence of a given resistance and MIC phenotype remains incompletely defined. In the case of *tet*M, isolates possessing this gene express a great deal of phenotypic diversity which is not easily explained by *tet*M copy number or expression. The

genotypic contribution of a *tet*M gene on the phenotypic outcome for tetracycline and doxycycline need to be further investigated.

6.1 Limitations of the study

6.1.1 Isolation and identification of the organism

We collected two samples per animal and only three isolates were preserved for antimicrobial susceptibility testing. If at least one MRSP was identified from each dog it was recognized as colonized with MRSP. By increasing number of sampled sites per animal, the probability of MRSP detection would be increased leading to a truer representation of the frequency of MRSP colonization. In this investigation, animals colonized with MRSP on the skin would have been missed.

6.1.2 Interpretation of antimicrobial susceptibility

Veterinary clinical breakpoints have been published for a limited number of antimicrobials for *S. pseudintermedius*. In this investigation, some antimicrobial MICs were therefore interpreted using *Staphylococcus* genus-specific or *S. aureus* clinical breakpoints. *Staphylococcus pseudintermedius* specific breakpoints are available for minocycline, trimethoprim + sulfamethoxazole, chloramphenicol, erythromycin, gentamicin, daptomycin, linezolid, tigecycline, vancomycin or and quinupristin + dalfopristin (CLSI, 2017). Another limitation is that specific clinical breakpoints published for *S. pseudintermedius* are available for skin and soft tissue infections only. The clinical breakpoint may be different in human and dogs. As an example, in both chapter 2 and 3 tetracyclines was interpreted according to the human clinical breakpoint (≥16µg/ml). Later, the canine-specific clinical breakpoint (≥1µg/ml) were published

for *S. pseudintermedius*, applying these new breakpoints would change the calculated frequency of resistance (CLSI, 2013, 2014).

6.1.3 Limitation in gene expression study

Bacterial gene expression studies related to antimicrobial resistance are limited in the literature (Fiedler et al., 2016). The selection of a "reference gene" or "housekeeping gene" is challenging in prokaryotes, as many variables can alter the overall results of the study making interpretation difficult. In this experiment, we used three reference genes which were optimized in a previously publiched investigation targeting biofilm formation (Crawford et al., 2016). Multiple reference genes are used to provide confidence in the results, interestingly the fold changes calculated with each reference gene was not uniform in this study. Although three reference genes were previously published, the effect of tetracycline exposure on the expression of these genes is not known. It is possible that if tetracycline affects the expression of these genes, which would lead to an unaccounted-for contribution to gene expression.

6.1.4 Lack of reference database to standardize tetracycline resistance gene nomenclature

Although there are curated online databases for certain resistance genes such as β-lactamases (www.lahey.org), no database exists for tetracycline resistance genes. Therefore, the classification and nomenclature of tetracycline resistance genes are complicated and likely result in researchers not using a common ontology for these genes. Furthermore, the diversity in the sequences among these resistance genes results in a lack of consensus on whether two sequences are the same gene or should be considered different.

6.1.5 Small sample size

The expression of *tet*M was investigated in eight *S. pseudintermedius* isolates. This small sample size required the use of nonparametric statistical tests (Mann-Whitney test). Unfortunately, nonparametric tests suffer from type I error and are more likely to fail to identify a significant difference. A larger sample size would improve the power of the statistical power of this study, allowing parametric methods to be applied.

6.2 Future Directions

Although *S. pseudintermedius* is a common cause of infection in dogs, the pathogenesis of this organism in the skin and urinary tract has not been investigated extensively. Many putative virulence genes in *S. pseudintermedius* were originally identified in *S. aureus*, and the role of these virulence factors in *S. pseudintermedius* has been under-investigated. Moreover, novel *S. pseudintermedius* specific virulence factors may be present which do not have a *S. aureus* homolog. The identification of virulence profiles which facilitate the pathogenesis of dermatological infections and urinary tract infections in dogs should be studied to improve our understanding of the mechanisms of disease.

Although the PVL toxin has been detected in *S. pseudintermedius*, its role in pathogenesis has not been elucidated (Bardiau et al., 2013; Wettstein Rosenkranz et al., 2014). This is in contrast to *S. aureus* in people, where PVL is associated with community-acquired MRSA infections (Berla-Kerzhner et al., 2017).

Furthermore, an understanding of the molecular mechanism leading to the transition from colonization to infection would be very useful for understanding the pathogenesis of *S. pseudintermedius* in companion animals. The activation of virulence gene expression and

strategies to evade the host immune system has not been studied in this organism. As we know, *S. pseudintermedius* is an opportunistic pathogen but it is unknown what organism factors contribute to the development of infections.

Antimicrobial resistance is a priority area of research in veterinary medicine. Resistance surveillance is the only way to acquire information on emerging resistance in healthy and diseased populations of dogs. Monitoring the emergence of antimicrobial resistance in *S. pseudintermedius* and identifying risk factors for acquiring resistance need to be further studied. Understanding resistance emergence in healthy dogs will help us to control emerging *S. pseudintermedius* clinical infections in companion animals. In addition, estimation of the cost associated with resistant infections will help to justify prevention strategies which may increase the cost of treating of these infections in companion animals. The impact of plasmid-mediated dissemination of resistance is poorly understood in *S. pseudintermedius* although it has been observed in *S. aureus* (Fessler et al., 2017). Future antimicrobial resistance studies should focus on the role of mobile genetic elements in the epidemiology of resistance, rather than focusing on individual species. Although, the role of mobile genetic elements in the dissemination of resistance across species barriers is well recognized among Enterobacteriaceae, the frequency of transmission of resistance genese between *Staphylococcus* species is unkonwn (Berg et al., 2017).

Investigations into human infections with *S. pseudintermedius* should be a priority area of future research to enrich the understanding of interspecies transmission and the factors leading to human infections. The emergence of metabolic diseases and cancers in humans that lead to immunosuppression and secondary bacterial infections, including *S. pseudintermedius*, raises concern for increased risks of infection. This is particularly true due to patient contact with dogs

and the increasing popularity of canine-assisted therapy may increase the opportunities for interspecies transmission to occur. Therefore, a risk analysis must be carried out to understand how highly susceptible patients (such as those with immunodeficiencies) acquire infections with this potential opportunistic pathogen so that transmission can be prevented.

The mechanism of resistance in *S. pseudintermedius* for a number of commonly used, old antimicrobials including the folate synthesis inhibitors is not adequately understood. Understanding the mechanism resistance is essential to develop strategies to limit the emergence of resistance to these drugs while still utilizing them for treating bacterial infections. Addressing this neglected field will become increasingly important as resistance continues to emerge and multi-drug resistant organisms become more common.

We examined the effect *tet*M gene expression on tetracycline and doxycycline MIC. However, as this investigation only targeted expression at the level of transcription, any events occurring after this point (e.g. post-translational modification) are unaccounted for. Therefore, proteomic studies are required to understand the quantitative contribution of TetM to phenotypic resistance in this organism. Investigation of TetM expression by western blot would be helpful to understand the contribution of the TetM protein to tetracycline MIC. The effect of the primary, secondary and tertiary protein structure of TetM on phenotypic resistance has not been evaluated in *Staphylococcus* spp. The investigation into how changes in the variable region of TetM affect the MIC of an isolate to tetracycline is encouraged, we suggest that site-directed mutagenesis may be one possible approach for addressing this question. Finally, only two efflux mediated resistance mechanisms (TetK and TetL) affecting tetracycline in *S. pseudintermedius* have been identified. The role of other efflux pumps which may have tetracycline as a substrate needs to be investigated; the contribution of multi drug resistance efflux pumps, such as the small multidrug

resistance (SMR) family, multi drug and toxin extrusion (MATE), resistance nodulation division (RND) and ATP-binding cassette (ABC) on the tetracyclines has not been investigated (Schindler and Kaatz, 2016). There is evidence that these categories of efflux pumps can expel tetracyclines, although their contribution to resistance in *S. pseudintermedius* has not been evaluated. We anticipate that other efflux pumps may also affect intracellular tetracycline concentrations and that understanding the affinity of these efflux pumps for tetracyclines may explain some of the unaccounted for diversity in tetracycline susceptibility observed in this study.

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