CARRIAGE OF VIRULENCE FACTORS AND ANTIMICROBIAL RESISTANCE GENES IN *STAPHYLOCOCCUS PSEUDINTERMEDIUS* FROM DOGS

A Dissertation

by

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ABSTRACT

Staphylococcus pseudintermedius is the most common microorganism isolated from canine pyoderma and opportunistic infections. Prevalence of methicillin-resistant *S. pseudintermedius* (MRSP) has increased and multi-drug resistance has become common. A total of 734 *S. pseudintermedius* isolates collected from dogs presented to the Veterinary Medical Teaching Hospital from 2007 to 2012 were studied. Isolates were analyzed for antimicrobial resistance and virulence gene carriage.

With the emergence of methicillin resistance, veterinarians have begun to use antimicrobials such as amikacin, to treat life-threatening MRSP infections. The most widespread mechanism of amikacin resistance is drug inactivation by aminoglycoside modifying enzymes (AMEs). The most prevalent gene detected here was *aph(3′)- IIIa* found in 75% (24/32) of isolates followed by *aac(6′)/aph(2″)* and *ant(4′)-Ia* in 12% (4/32) and 3% (1/32), respectively. There was a significant association between amikacin and methicillin resistance. Since AMEs can be transferred from one bacteria to another, amikacin resistance may represent a new nosocomial and zoonotic threat.

Clindamycin is an alternative to β-lactam antimicrobial therapy for canine pyoderma. Inducible and constitutive resistance to clindamycin can occur. Approximately forty *erm* genes encoding methylases involved in clindamycin resistance have been reported, with *ermB* most commonly found among *S. pseudintermedius*. We found eight of 608 isolates tested, positive for inducible clindamycin resistance by D-test and PCR detection of *ermB*.

A vaccine against staphylococcal pyoderma would reduce the reliance on antimicrobial drugs. Staphylococcal cell-wall associated proteins (CWAPs) involved in colonization of the host are attractive potential vaccine targets. Eighteen CWAPs encoded by *sps* genes have been described in *S. pseudintermedius*; however, four vary in occurrence. Isolates were analyzed by polymerase chain reaction (PCR) for the presence of *mecA*, SCC*mec* type I-VI, and *spsF, spsO, spsP,* and *spsQ*. There was a significant association between methicillin resistance and carriage of *spsP* and *spsQ*. *spsP* and *spsQ* may be viable vaccine targets.

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NOMENCLATURE

AACs	Aminoglycoside acetyltransferases
APHs	Aminoglycoside phosphotransferases
ANTs	Aminoglycoside nucleotidyltransferases
AMEs	Aminoglycoside modifying enzymes
AR	Amikacin-resistant only
ARMR	Amikacin and methicillin-resistant
ccr	Cassette chromosome recombinases
CLSI	Clinical and Laboratory Standards Institute
CWAPs	Cell wall-associated proteins
D-Test	Double-disk diffusion test
MIC	Minimum inhibitory concentration
MLS	Macrolide, lincosamides, streptogramin B
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MSSP	Methicillin-susceptible *Staphylococcus pseudintermedius*
MR	Methicillin-resistant only
OR	Odds ratio
PBP2a	Penicillin binding protein 2a
PCR	Polymerase chain reaction
S.	*Staphylococcus*
SD	Standard deviation

SCC*mec*	Staphylococcal cassette chromosome *mec*
ST	Sequence type
VMTH	Veterinary medical teaching hospital

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CHAPTER I

INTRODUCTION

Background

Staphylococcus pseudintermedius is the most frequent causative agent of canine pyoderma and is also associated with opportunistic infections in dogs (i.e., otitis externa, urinary tract infections, and surgical and non-surgical wound infections) (1,2). Many dogs harbor *S. pseudintermedius* in their nares and elsewhere on their skin without having clinical evidence of disease and may serve as a potential source of infection for other animals and humans as well (1,3,4). Most *S. pseudintermedius* are susceptible to β-lactam antibiotics such as amoxicillin but some are resistant to these drugs either due to the production of a β-lactamase, encoded by *bla*, or acquisition of an altered penicillin binding protein that has a low affinity for all β-lactam antibiotics, encoded by *mecA*. As a result of acquisition of these genes, β-lactam antibiotics are rendered ineffective and the bacteria become resistant (1,5,6). Both *bla* and *mecA* can be transmitted between *Staphylococcus* spp. so dogs colonized with *S. pseudintermedius* carrying these genes may serve as reservoirs of antibiotic resistance (7,8).

Empirical treatment of staphylococcal infections such as pyoderma and secondary post-operative infection typically involves β-lactam antibiotics such as penicillins and cephalosporins (9). In recent years isolation of methicillin-resistant *S. pseudintermedius* (MRSP) from canine skin has become a common occurrence raising a number of questions about the diagnosis, treatment, and prevention of these infections. As prevalence rates of MRSP increase, veterinarians have used other classes of

antimicrobial drugs to treat infections; however, resistance to these drugs has also increased. Studies from Europe have demonstrated that 90% of MRSP isolates were resistant to clindamycin, erythromycin, and trimethoprim, 70% were resistant to both chloramphenicol and gentamicin, and 57% were resistant to just chloramphenicol (2,10). Resistance has begun to emerge in the United States: over the past five years, resistance to chloramphenicol has become common leading to the reliance on drugs such as amikacin to treat MRSP infection (11,12).

Amikacin is an aminoglycoside antibiotic that inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit (13). The most widespread mechanism of aminoglycoside resistance is drug inactivation by cellular aminoglycoside modifying enzymes (AMEs) encoded on the chromosome, a plasmid, or carried on a transposable element (14-17). They can be divided into three classes: aminoglycoside acetyltransferases (AACs), aminoglycoside (phosphotransferases (APHs), and aminoglycoside nucleotidyltransferases (ANTs) (18). In *S. aureus*, the *aac(6')/aph(2")* gene is the most frequently encountered aminoglycoside resistance gene followed by *ant(4')-Ia* and *aph(3')- IIIa* (18,19). Each gene encodes the following enzyme respectively: AAC(6')/APH(2"), ANT(4')-I, and APH(3')-III (18).

Clindamycin is a lincosamide that reversibly binds to the bacterial 50S ribosomal subunit thereby inhibiting protein synthesis (20). In some cases, staphylococci may appear to be susceptible to clindamycin when tested *in vitro*, but the infected patient may fail to respond to therapy despite being treated with what seems to be an appropriate drug concentration for an appropriate duration. Lincosamides bind to the same or closely related binding sites in the bacterial ribosome as macrolides such as erythromycin.

Resistance to macrolides, lincosamides, and streptogramin B antibiotics (MLS_B phenotype) can occur through acquisition of a methylase enzyme that removes a methyl group from an adenine residue in the 23S ribosomal RNA component of the 50S subunit of the ribosome (21-23). Removal of this methyl group alters the site to which the antimicrobial drug binds altering its efficacy. Approximately forty *erm* genes that encode methylases have been reported in different bacterial genera, with *ermA, ermB,* and *ermC* the most commonly found among staphylococci (24). In *S. aureus*, *ermA* and *ermC* confer erythromycin resistance in 94-98% of isolates (25). In *S. pseudintermedius, ermB* is primarily responsible for MLS_B resistance, but its expression can be constitutive or inducible (11). Routine antimicrobial susceptibility testing can detect constitutive MLS_B resistance but fails to detect inducible resistance (26). Inducible clindamycin resistance can result in treatment failure and should be suspected in isolates that are erythromycin-resistant but clindamycin-susceptible on *in vitro* antimicrobial susceptibility testing.

Several studies have measured the prevalence of MRSP. In Canada, MSRP was detected in only 2.1% of dog patients (5). Morris *et al*. in Pennsylvania showed 6.2% carriage prevalence in dogs in 2010 (1). However in Japan, MRSP has been detected in even greater numbers among inpatient (46.2%) and outpatient (19.4%) dogs in a veterinary teaching hospital (5). Risk factors for carriage include recent antibiotic treatment (within the last six months), recent corticosteroid treatment, prior hospitalization, and veterinary contact within the last 4 weeks. Dogs treated with antimicrobial drugs were more likely to carry MRSP (12.6%) as compared to 2.3% of untreated dogs (10).

In order to determine the relatedness of *S. pseudintermedius* isolates, a combination of strain typing methods are used including pulse-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), s*p*a typing, *mecA* PCR, and SCC*mec* typing (27). PFGE is laborious and is commonly used although no standardized protocol exists (2). SCC*mec* typing is a standard method and is used throughout the literature either alone or in combination with other testing methods (2,5,8).

SCC*mec* identified as the Staphylococcal Cassette Chromosome *mec* is a mobile genetic element that is characterized by a combination of *mec* and *ccr* gene complexes (2,5). SCC*mec* typing is a molecular tool to examine the epidemiology of methicillin-resistant staphylococci (5). SCC*mec* types vary in different parts of the world. There is still little known about the population genetics of MRSP but the general consensus is that in Europe SCC*mec* II-III on strain ST71 dominates while in North America SCC*mec* V on strain ST68 is much more commonly isolated (5,8,28). Within North America, most of the research on *mec* typing has been done in Canada and the northeastern United States and Tennessee. To the best of our knowledge, no data has been published evaluating regional clonality of MRSP in Texas.

Finally, the colonization or carriage of *S. pseudintermedius* on the skin requires adherence to epithelial surfaces which typically requires tight attachment between host and bacterial proteins. The bacterial proteins involved are usually proteins found on the outer surfaces of the bacteria such as the outer membrane or cell wall. A recent study in *S. pseudintermedius* identified 18 cell wall associated proteins (CWAPs) that are involved in attachment to host proteins (29). This study demonstrated that there is variation in carriage of four of these 18 proteins; specifically SpsF, SpsO, SpsP, and

SpsQ are not found in all isolates of *S. pseudintermedius* (29). Studies evaluating the differences in the carriage of these proteins between isolates collected from dogs with pyoderma or other types of staphylococcal infection or between isolates that are MRSP and methicillin-susceptible *S. pseudintermedius* (MSSP) have not been published. Determining which virulence factors are required for colonization and infection may allow the development of novel strategies to reduce skin infection, including development of targeted therapeutics, and the rational design of staphylococcal vaccines.

In conclusion, these data suggest that mechanisms of antibiotic resistance in *S. pseudintermedius* are complex and widespread. New avenues of treatment will need to be explored in order to combat this ever-growing threat to the canine patient population. We hypothesized that the four CWAPs that show variation in *S. pseudintermedius* would be associated with strains associated with skin infection or strains that were resistant to antibiotics (i.e., methicillin-resistant). We further anticipated that these strains would be more likely to contain the SCC*mec* type V than other SCC*mec* types since the SCC*mec* type V has been shown to be the common type in North America. We also hypothesized that *S. pseudintermedius* strains would carry one or more of the three amikacin resistance genes previously discovered in *S. aureus* and that gene carriage would be associated with strains that are more resistant (i.e., methicillin-resistant). Finally, we anticipated that isolates carrying inducible clindamycin resistance would be present within our collection of isolates and resistance would be due to the carriage of the resistance gene *ermB*.

Purpose and objectives

The purpose of this study was to determine the epidemiology and microbiology of *Staphylococcus pseudintermedius* isolates among canine patients admitted to the Texas A&M Veterinary Medical Teaching Hospital. The objectives of this research included:

- To determine the carriage of specific cell-wall associated proteins in *S. pseudintermedius* isolates collected from dogs with skin infection, dogs with other types of infections, and dogs without clinical infection presented to the VMTH.
- To determine whether carriage of these CWA proteins differed between *S. pseudintermedius* isolates that were MRSP or MSSP.
- To determine the incidence of amikacin resistance in *S. pseudintermedius* isolated from dogs presented to the VMTH.
- To identify the amikacin resistance genes carried by these strains and to describe their antimicrobial susceptibility.
- To determine whether there was any difference in the carriage of amikacin resistance genes between isolates that were MRSP and MSSP.
- To determine whether amikacin resistance in *S. pseudintermedius* was related to a prior history of drug exposure with or without the presence of methicillin resistance.
- To determine the incidence of inducible clindamycin resistance in *S. pseudintermedius* isolated from dogs presented to the VMTH.
- To determine if *ermB* was carried by strains that exhibited a positive D-Test
- To determine if there was a history of prior antibiotic exposure in strains that demonstrated a positive D-Test.

CHAPTER II

LITERATURE REVIEW*

Isolation of MRSP from canine skin has become a common occurrence. The increased prevalence of MRSP in dogs in recent years (30) has raised a number of questions about the diagnosis, treatment, and prevention of these infections. Concerns about transmission of methicillin-resistant staphylococci can impact interactions between humans and their pets and affect patient care in veterinary practices (30). Unfortunately, in some situations, owners have been advised to remove pets from their households or even euthanize them because of concerns regarding transmission of these organisms (31). For these reasons, it is important that veterinarians understand the difference between MRSA and MRSP and know how to interpret positive culture results for these organisms in samples collected from dogs. Understanding staphylococcal infections has become increasingly important and challenging in recent years. The purpose of this report is to describe recent discoveries and advancements in our understanding of staphylococcal infections, particularly MRSP infection, in dogs and to summarize the available information regarding potential zoonotic transmission of these agents.

Staphylococci of importance in human and veterinary patients

Staphylococci are gram-positive, facultative, anaerobic cocci and are indigenous flora of the skin and mucous membranes of healthy dogs (32). Staphylococci cause opportunistic

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infections characterized by exudative lesions with local tissue inflammation (32). Skin infections are the most common type of staphylococcal infection, although bacteremia and life-threatening systemic disease (i.e., toxic shock syndrome) can occur (33). Microscopic examination of lesion exudates reveals cocci in clusters, pairs, or short chains, and neutrophils (34).

More than 50 species and subspecies of *Staphylococcus* have been described (35). Historically, the ability of staphylococci to clot plasma was considered predictive of virulence, and staphylococci with this capacity were described as coagulase positive (36). Previously, coagulase-positive staphylococci isolated from dogs were classified as either *Staphylococcus aureus* or *Staphylococcus intermedius*. In 2005, all *Staphylococcus* isolates from dogs previously identified as *S. intermedius* were reclassified as *Staphylococcus pseudintermedius,* a species in the *S. intermedius*–related group, on the basis of growth characteristics and biochemical features (37). First described in 1999 (38) and expanded in 2005, the *S. intermedius*–related group consists of closely related coagulase-positive staphylococci that differ in their host specificity (39,40). The group includes *S. pseudintermedius,* predominantly isolated from dogs, *S. intermedius,* isolated from pigeons, and *Staphylococcus delphini*, which has been found in a variety of animals including dolphins, mink, cattle, and horses (37,39,41).

Subsequent to this reclassification, *S. pseudintermedius* has been considered the predominant cause of superficial pyoderma as well as a leading cause of otitis and of opportunistic infections at surgical sites in dogs (4,28,42-46). Two studies (47,48) demonstrated that *Staphylococcus schleiferi subsp schleiferi*, which is coagulase negative, is also frequently isolated from dogs with skin disease. Although *S. aureus* is a

leading cause of infections in humans, it is less commonly isolated from dogs than is *S. pseudintermedius* (43,46,49). Infection with *S. pseudintermedius* typically results from a disruption affecting normal cutaneous homeostasis or from an underlying disease process (40). *S. pseudintermedius* can be readily cultured from samples collected from the nose, oral cavity, intestinal tract, urogenital tract, groin, and perineal regions of healthy dogs (49). In healthy dogs, reported frequencies for recovery of the organism were 8 of 69 (12%) (49), 54 of 150 (36%) (50), and 6 of 43 (14%) (51) from the nares, and 14 of 69 (20%) (49), 27 of 74 (36%) (50), and 14 of 43 (33%) (51) from the anal mucosa. Additionally, *S. pseudintermedius* is cultured more frequently from swabs of the rostral nares and anal mucosa of dogs that live in multidog households than from dogs that do not; this may reflect normal canine social behavior, such as sniffing the perianal region, which could facilitate transfer of staphylococci between animals (50).

Methicillin resistance

Antimicrobial resistance in staphylococci

Historically, infections with staphylococci were associated with high morbidity and mortality rates in humans (32). With the discovery of penicillin, this changed. Penicillin and other β-lactam antimicrobials act by binding to and inhibiting the transpeptidase required for crosslinking of peptidoglycan in bacterial cell walls (52). With β-lactam drug treatment, the peptidoglycan layer weakens and the bacteria are killed by increased osmotic pressure and cell rupture (52). Some staphylococci produce a β-lactamase enzyme that destroys the β-lactam ring of penicillins and related antimicrobials, rendering them ineffective (32,52). Methicillin was developed as a β-lactamase–resistant antimicrobial. Within a year after the introduction of methicillin in 1960, the first MRSA

strains were identified (53). Methicillin was quickly discarded as a therapeutic drug because of adverse effects; however, β-lactam–resistant staphylococci are still referred to as methicillin-resistant (54). Methicillin-resistant staphylococci are resistant to all β-lactam antimicrobials including cephalosporins, penicillins, and amoxicillin-clavulanate combinations (55). Many MRSP isolates are resistant not only to β-lactam drugs but also to one or more macrolides, lincosamides, trimethoprim-sulfonamide combinations, or fluoroquinolones (48,55). Chloramphenicol, tetracyclines, aminoglycosides, and rifampin remain drugs to consider for treatment of MRSP infection, however, culture and susceptibility testing should always be performed to evaluate susceptibility to these drugs (55).

Methicillin resistance genes

Penicillin binding protein 2a is a transpeptidase that has a low affinity for all β-lactam antimicrobials (5,6,11,56-58). This protein is encoded by the *mecA* gene, and staphylococci that carry *mecA* are resistant to all β-lactam drugs (1,5,6,56). The evolutionary origin of *mecA* is unknown; however, the authors of 1 study (59) suggested that it may have originated within the genus of *Staphylococcus* from a *mecA* homologue identified in *Staphylococcus sciuri*, a coagulase-negative organism commonly isolated from animals.

The *mecA* gene is carried on a transmissible mobile DNA element called SCC*mec* (11). The SCC*mec* can be transferred from one *Staphylococcus* isolate to another of the same or different species (8). Thus an isolate of *S pseudintermedius* that is susceptible to β-lactam drugs can become resistant to these agents through horizontal transfer of SCC*mec* from a resistant isolate, creating a new strain of MRSP (8). The

SCC*mec* contains the *mec* gene complex which consists of *mecA*, the genes that control expression of the *mecA* gene, and unique site specific recombinases called cassette chromosome recombinases (11). Typing of SCC*mec* is performed through analysis of the cassette chromosome recombinase gene complex and the *mec* gene complex. The International Working Group on the Staphylococcal Cassette Chromosome elements has defined 11 SCC*mec* types in *S. aureus* to date (60). At least five of these SCC*mec* types (II–III, III, IV, V and VII-241) as well as two non-typeable cassettes have been identified in *S pseudintermedius* (11).

Once established, MRSP strains carrying specific SCC*mec* types typically dominate in a geographical region (8). Accordingly, it becomes possible to use SCC*mec* typing in epidemiologic studies to monitor for clonal spread of antimicrobial resistance and to assess zoonotic disease patterns. In addition, MRSP strains can be further grouped via ST, which is determined through evaluation of alleles in 7 or more different loci to create an allelic profile for each isolate (11). There is still limited information available regarding the population genetics of MRSP, but in North America, strain ST68 with SCC*mec* V has been commonly isolated, whereas in Europe, strain ST71 with SCC*mec* II-III has dominated (5,8,11,28). There has been some debate about whether MRSP with SCC*mec* II-III or SCC*mec* III is the primary type found in Europe (11,27,57). The apparent differences among studies may be related to differences in the methodology used for typing (2,57).

Identification of methicillin-resistant staphylococci

Methicillin-resistant staphylococci are identified through detection of *mecA* via DNA-based tests such as PCR assays, antibody-based agglutination tests to detect penicillin

binding protein 2a, or *in vitro* antimicrobial susceptibility testing (61-63). Antimicrobial susceptibility testing includes assessment of resistance to oxacillin by means of disk diffusion or MIC testing (62,64). Evaluation of cefoxitin resistance is also used as a test for methicillin resistance in *S aureus* and coagulase-negative staphylococci, but is less reliable for testing of *S pseudintermedius* (62,65). Consequently, it is important that oxacillin, not cefoxitin, be used for identification of MRSP through antimicrobial susceptibility testing (62,65). Studies (62,65) have shown that the 2004 Clinical Laboratory Standards Institute oxacillin disk diffusion and MIC breakpoints of ≤ 17 mm and ≥ 0.5 μg/mL, respectively, predict *mecA*-mediated methicillin resistance in *S pseudintermedius* better (i.e., fewer false susceptible results) than the current 2008 criteria.

Pyoderma in dogs

Classification and diagnosis

Pyoderma is the most common skin disease in dogs and is defined as a pyogenic infection of the skin. When lesions are present, they are typically found on the ventral aspects of the abdomen and trunk, groin, muzzle, interdigital regions, and axilla (1,66). Superficial pyoderma is characterized by erythema of the skin with follicular or nonfollicular papules and vesiculopustules that give rise to yellowish exudates or crusts (43,67). Superficial pyoderma may progress to a deeper form of disease that affects structures below the epithelium. With deep pyoderma, the predominant lesions often manifest as erythematous, exudative, alopecic nodules, draining tracts, and surrounding friable skin with surface ulcers (68). Importantly, superficial and deep pyodermas often develop secondary to an underlying disease process. These can include immune-

mediated diseases, endocrinopathies, ectoparasitism, cornification defects, adverse drug reactions, foreign bodies, and neoplasia (69,70). In these situations, it is important not only to treat the bacterial infection but also to properly diagnose and treat the inciting cause of secondary pyoderma (69). Management of the bacterial infection consists of topical or systemic treatments or combinations of these (12).

Pyoderma caused by methicillin-resistant staphylococci is clinically indistinguishable from that caused by methicillin-susceptible strains (67). However, pyoderma in dogs with methicillin-resistant staphylococcus infection can manifest as visible lesions 2 to 3 weeks after the initiation of empirical treatment, with bacteria detected on repeated cytologic examination. When empirical treatment fails, culture and antimicrobial susceptibility testing of samples is essential for choosing an appropriate alternate antimicrobial (32,49). Although MRSP is not more virulent than methicillin-susceptible *S pseudintermedius*, and the outcomes following appropriate treatment can be the same, proper antimicrobial selection is essential for success (67). Further, when an animal with a prior history of infection with methicillin-resistant staphylococci develops a subsequent infection or another pet in the same household develops an infection, a sample should always be collected for culture and susceptibility testing rather than initiating empirical treatment (12).

Careful collection of material is essential when obtaining samples for culture from the skin. Aspiration of material from an intact pustule is preferred; however, in dogs with superficial pyoderma, pustules are frequently transient, leaving circular, often alopecic skin lesions with exfoliative borders (commonly referred to as epidermal collarettes) on the skin (71). Results of some studies (71,72) have indicated that

epidermal collarettes are a characteristic of superficial pyoderma in dogs. In 1 study (71), bacteriologic culture of *S. pseudintermedius* from epidermal collarette swabs was successful for 18 of 22 dogs with superficial pyoderma, with 81.8% sensitivity and 100% specificity. Biopsy of the skin can also be performed to obtain sufficient tissue samples for diagnostic testing, particularly in cases of deep pyoderma when bacteria have infiltrated the underlying dermis (73).

Treatment

First-time cases of canine pyoderma are typically treated empirically, on the basis of the clinician's experience, without culture and susceptibility testing (74). Antiseptic shampoos that contain benzoyl peroxide, chlorhexidine, ethyl lactate, triclosan, or salicylic acid are commonly used for treatment of superficial pyoderma in dogs (75). Investigators in 1 study (67) found that use of topical treatments alone resulted in clinical resolution of staphylococcal pyoderma in 17 of 26 cases, with clinical improvement in 4 of the remaining cases. These treatments are often prescribed when large areas of the body are affected or when haired skin is involved (76). A full description of effective topical treatments for staphylococcal pyoderma can be found elsewhere (76). Topical products can be used alone or in conjunction with systemic antimicrobial administration (9). Although topical treatments offer some advantages over systemic treatment, such as higher local antimicrobial concentrations, these may not always be adequate to achieve clinical resolution of pyoderma (77).

For empirical systemic treatment of pyoderma in dogs, amoxicillin-clavulanic acid or first-generation cephalosporins (i.e., cephalexin) are the drugs most commonly selected (9). Clindamycin has been recommended as an appropriate alternative choice on

the basis of its favorable safety profile, clinical efficacy, and distribution into the skin (9,12). Cefovecin and cefpodoxime proxetil are third-generation cephalosporins that are convenient for use in dogs because they do not require frequent administration. However, these drugs are only recommended as first-line agents in situations where owner compliance is a concern, because they have the potential to select for both methicillin-resistant staphylococci and extended spectrum β-lactamase producing organisms (9). Although effective against many staphylococcal isolates, trimethoprim-sulfonamide combinations should be avoided for long-term use because of potential adverse effects such as keratoconjunctivitis sicca, blood dyscrasias, and hypothyroidism (78). In dogs with recurrent infection, a sample should be collected from a lesion for culture and susceptibility testing to guide proper drug selection (12).

Treatment for superficial pyoderma should be continued for 1 week past the resolution of clinical signs; this would typically require ≥ 3 weeks of treatment (9). Because of the increased depth and severity of lesions in dogs with deep pyoderma, a minimum treatment period of 4 weeks should be considered, with an endpoint of 2 weeks past the resolution of clinical signs (9). It is important that the patient be reexamined and that cytologic evaluation of aspirates and impression smears be repeated during the course of treatment to assess the patient's response. Otherwise, if infection is detected after the end of the treatment period, it becomes difficult to determine whether the patient has been reinfected or the original treatment protocol has failed because of an incorrect antimicrobial choice or premature discontinuation of an appropriate drug regimen. Overall prognosis is good if the underlying cause can be identified and corrected or well-controlled to prevent recurrent infection (69). As previously

mentioned, conditions that predispose dogs to pyoderma can hinder successful management of the infection if not properly addressed (12).

When infection with methicillin-resistant staphylococci has been identified, proper drug choice is essential. Topical treatments can be provided but should only be used as adjuncts to systemic antimicrobial treatment administered at the correct dosage on the basis of the dog's current body weight and given for the prescribed period (12). A methicillin-resistant *Staphylococcus* infection should not be treated with β-lactams, cephalosporins, or amoxicillin-clavulanic acid as these are ineffective against the bacteria (11,32,55). Fluoroquinolones are often a poor choice for long-term treatment, because susceptible strains quickly develop resistance to this class of drugs (48,55,79,80). Development of resistance against trimethoprim-sulfonamide combinations and clindamycin has also been reported (55). In addition, it is important to note that inducible clindamycin resistance can occur, wherein an isolate appears susceptible *in vitro*, but resistance is induced during treatment of the patient, resulting in treatment failure (81). Specialized laboratory testing can detect inducible clindamycin resistance when an isolate is susceptible to clindamycin but resistant to erythromycin (81).

Currently, aminoglycosides (i.e., gentamicin or amikacin), tetracyclines (i.e., doxycycline or minocycline), rifampin, and chloramphenicol are considered potentially therapeutic choices for treatment of methicillin-resistant staphylococci with multiple drug resistance if MIC testing confirms susceptibility to 1 or more of these agents (55). All drug choices require evaluation of patient factors that would contraindicate selection of a drug. In particular, well-known, serious adverse effects may occur with each of

these drugs. Aminoglycosides can have nephrotoxic effects (12,55). Doxycycline has been reported to rarely cause renal or liver injury and esophageal lesions, especially in cats (55). The most common adverse effect of rifampin is hepatotoxicity, but gastrointestinal disturbances and orange-red discoloration to the urine, tears, and sclera have also been noted (12,55). In dogs, chloramphenicol can cause gastrointestinal upset and weight loss and uncommonly results in liver toxicosis, bone marrow suppression, weakness, and neurologic tremors (12). Additionally, this drug can have serious adverse effects such as aplastic anemia and bone marrow suppression in humans, and therefore requires special handling (12,55). Chloramphenicol can also have adverse interactions with several classes of drugs, because it interferes with the cytochrome P450 pathway and thus decreases clearance of other drugs metabolized by this pathway (55).

Additional factors specific to treatment of methicillin-resistant staphylococcal infection should also be considered when choosing an appropriate antimicrobial agent. Rifampin should not be used alone because resistance develops rapidly under these circumstances (28,82,83). In a 2011 study (82) of dogs with MRSP infection, resistance to rifampin emerged rapidly, even when the drug was used in combination with other antimicrobials. Chloramphenicol has been an important agent for treatment of MRSP infection in dogs, because historically MRSP has been susceptible to this drug. However, in Europe, chloramphenicol resistance in MRSP has become widespread (11,12). This is of particular concern because MRSP has only been reported since 2007 in Europe, and the finding suggests that chloramphenicol resistance has developed rapidly (84). Resistance to this drug occurs through inactivation by a type A chloramphenicol acetyltransferase, which can be transferred among bacterial strains (57,85). Finally,

despite the potential efficacy of vancomycin and linezolid against methicillin-resistant staphylococci (55), these drugs should not be used in veterinary medicine because of their importance for treating human MRSA infections (86).

Zoonotic potential of MRSA and MRSP

Several species, including humans, dogs, cats, horses, pigs, poultry, and some exotic animals, can serve as carriers for, and as sources of infection with, various strains of *Staphylococcus* (1,5,11,56). There are several misconceptions among clients and veterinarians regarding the implications of infection with MRSA or MRSP in dogs. It is also important to understand the difference between colonization with (i.e. carriage of) an organism and infection. Discriminating between MRSA and MRSP and understanding the applicable terminology is important for diagnostic reasons, for establishing the correct treatment protocol, and for protecting public health.

MRSA

Staphylococcus aureus is a leading cause of nosocomial infection in humans and is found in approximately 30% of healthy individuals in the United States (87). The US CDC defines a person as being colonized or having bacterial carriage when the bacteria is present without causing disease in the individual (88). This is in contrast to infection, in which an individual has clinical signs of disease. Fortunately, only a small proportion (< 2%) of healthy or asymptomatic individuals in the general population of the United States carry MRSA (87). Colonization can be transient or can become persistent, particularly in cases of repeated exposure. A person who lives in close contact with an MRSA-infected person can become persistently colonized with the same MRSA strain for months to years, and during this time they can serve as a source of secondary

transmission to other individuals (89,90). The degree of risk appears to be related to closeness of exposure, because the risk of colonization for a spouse or child of a patient with MRSA is almost 7.5 times as great as that for a casual associate such as a friend or roommate (89).

Staphylococcus aureus colonization in dogs is possible, but it is not common because the organism is not normally a component of the indigenous bacterial flora of dogs (58). The reported prevalence of *S. aureus* infection in dogs has ranged from 2 of 24 (8.3%) (51) to 6 of 59 (10%) (49), whereas that of *S. aureus* carriage in healthy dogs has ranged from 2 of 43 (4.7%) (51) to 6 of 50 (12%) (49). Prevalence of MRSA is lower, with the organism isolated from 1 of 59 (1.7%) infected dogs in 1 study (49) and rates of carriage in healthy dogs as low as 0% found in studies that evaluated 50 dogs (49) and 200 dogs (91). Specific host and environmental conditions must be met for a dog to be exposed, become colonized, and serve as a potential reservoir for the organism while remaining apparently healthy. It has been suggested that MRSA infection or colonization in dogs results from transmission of the organism by humans (5,32,49,92). When identified in dogs, the MRSA strain most frequently isolated is USA100, which is a common cause of human hospital-acquired MRSA infections (1,31). Dogs used in pet therapy programs were shown to become contaminated with MRSA during hospital visits, suggesting that these animals could potentially serve as a means of transfer of MRSA from one human patient to another, thus spreading infection throughout a hospital (93).

Interestingly, an association has been made between isolation of MRSA and employment within the veterinary profession (5,94). Rates of MRSA carriage in small

animal hospital personnel (i.e., clinicians and technical staff) have been reported to range from 27 of 417 (6.5%) (95) to 59 of 341 (17.3%) (94), values that exceed the previously mentioned estimate of MRSA carriage in < 2% of healthy asymptomatic people in the United States (87). Carriage of MRSA in veterinary personnel may be attributable to contact with infected or colonized patients, particularly animals such as horses, in which *S aureus* is commonly part of the indigenous bacterial flora (5). To decrease the potential for transmission, veterinarians and veterinary staff should be educated about this occupational health risk and should consistently practice preventative measures, especially appropriate hand hygiene (96).

MRSP

Staphylococcus pseudintermedius is the most commonly encountered *Staphylococcus* species in the canine population and, unlike *S. aureus*, is part of the indigenous bacterial flora in dogs (97). In a study (10) in 2011 in which samples from dogs were collected with swabs in the waiting room area of a small animal hospital, investigators identified factors potentially associated with recovery of MRSP from dogs, including antimicrobial or corticosteroid treatment within the 6 months prior to culture, previous hospitalization, and entering a veterinary facility within the 4 weeks prior to culture. Results of the same study (10) revealed that 49 of 390 (12.6%) dogs treated with antimicrobials tested positive for MRSP, compared with 9 of 386 (2.3%) dogs that did not receive these drugs. This suggests that antimicrobial administration may potentially select for carriage of MRSP in dogs and supports the need for proper timing and selection of antimicrobial treatments.

Unlike *S. aureus*, *S. pseudintermedius* is not a commensal organism in humans (98). Although infection does occur in humans, it appears to be uncommon and is usually associated with zoonotic transmission from a canine host (1,3,99-101). Our current understanding is that the overall importance of *S. pseudintermedius* as a zoonotic pathogen is less than that of MRSA (30). In humans, *S. intermedius* (*S. pseudintermedius* would have been identified as *S. intermedius* prior to 2005), infection was described in a few hospitalized patients in 1997 with a low prevalence (2/3,397 [0.06%]) (98). In a 2010 study (1) that included several regions across the United States, the prevalence of MRSP carriage in veterinary dermatologists and their technical staff was found to be 9 of 171 (5.3%). This was slightly lower than the 16 of 258 (6.2%) carriage rate in healthy dogs in the same study (1). Investigators in another study (101) showed that 6 of 13 owners of dogs with deep pyoderma carried antimicrobial-resistant strains of *S pseudintermedius* identical to those recovered from their own dogs; the strains recovered from each dog-owner pair were distinct among the different households. This raises concerns that horizontal transfer of resistance genes may occur between antimicrobial-resistant *S. pseudintermedius* and pathogenic strains of staphylococci carried by humans (101). At present, *S. pseudintermedius* rarely causes disease in humans, and the risk of transmission of MRSP from a pet to the owner should be evaluated on an individual basis (101).

Infection control measures

In-hospital practices

Treatment of patients infected with methicillin-resistant staphylococci must incorporate prevention of nosocomial and zoonotic transmission. Consistent attention to hand

hygiene (i.e., using gloves, washing hands with soap and water after touching a patient (3,102), the use of alcohol pouches to clean hands when water is not available (102) has been repeatedly shown to be a protective factor against transmission, because staphylococcal infections are often spread through direct skin contact (5,32,56). Several studies (5,32,103,104) have shown that hospital personnel and equipment can serve as routes for transmission of infection. Investigators of a 2010 study (103) found that 66 of 100 (66%) pens, 44 of 80 (55%) stethoscopes, 60 of 126 (47.6%) cell phones, and 37 of 130 (28.5%) white coats used by physicians in a human hospital were contaminated with various bacteria. *Staphylococcus* spp were most commonly found, comprising 122 of 436 (28%) isolates, and 9 (7.4%) of these were identified as MRSA.

Staphylococci can survive for long periods in the environment. One study (104) showed that staphylococci were able to survive on a variety of fabric types and plastic materials in a human hospital, sometimes for > 90 days. In 1 veterinary teaching hospital, evaluation of environmental surface samples via DNA sequencing and PCR assay revealed that various cages, the top surfaces of a CT scan stand, a stand in a cat ward, and floors of the intensive care unit and MRI room were contaminated with MRSP (5). Routine disinfection of hospital surfaces and equipment as well as the use of stringent hand hygiene practices are critical to prevent or minimize the spread of infection. Most disinfectants are effective when applied to clean surfaces, and some quaternary ammonium compounds have been shown to retain antimicrobial activity for up to 48 hours (105).

In addition to appropriate use of disinfectants and hand hygiene, the handling of infected patients should be limited to veterinary staff directly involved in their care to

minimize the potential for transfer of organisms to other patients or staff members. Personal protective equipment should be worn to prevent contamination of clothing or body surfaces and transmission of bacteria to other patients or coworkers (106). This includes washable attire such as laboratory coats and disposable items such as gowns, gloves, or masks (106). Any patient with wounds should have those areas covered with a dressing to reduce environmental contamination, and soiled dressings should be disposed of properly (32) (i.e., in a trash bag kept near the patient's cage and used solely for their contaminated waste or autoclaved as biological waste). Proper treatment and containment of the infection are the ultimate goals. Ideally, every hospital should have a formal written manual that delineates infection control procedures and guidelines, and an individual staff member should be assigned the task of ensuring that the program is understood and followed (106).

Owner recommendations

It has been shown that MRSP and MRSA can be transferred among humans and pets in households (56,99). Dogs known to have MRSP infections should not be allowed to share a bed with their owners, because this provides an opportunity for close contact and potential transfer of the organism (32). Similarly, humans with known MRSA infections should not allow a pet to lick their wounds or share their bed (32). Personal hygiene and environmental disinfection are vital in maintaining appropriate infection control in the home environment. Staphylococci can survive for days on fabric, vinyl, and plastic (107), and dust particles can preserve these organisms and also serve as source of contamination or infection (108). Cleaning and disinfection are appropriate measures for disrupting transmission and reinfection. However, the most important infection

prevention measure is to consistently practice proper hand hygiene after handling a patient or contaminated material (106,109).

Conclusions

Because they are uniquely adapted commensal organisms, staphylococci are likely to remain a cause of opportunistic infection in humans and animals. Staphylococcal infections can range from simple skin infections and dermatologic disorders to severe systemic bacteremias that can cause multiorgan failure and death (33,42,51). For dogs with pyoderma, there are several key points that remain at the heart of successful treatment. Any underlying, predisposing conditions must be corrected or controlled as well as possible to provide the best opportunity for clinical resolution. Because pyoderma caused by antimicrobial-susceptible staphylococci is clinically indistinguishable than that caused by antimicrobial-resistant strains (67), patients that receive empirical treatment must be reevaluated during the treatment period to assess clinical response. When empirical treatment fails or infection reoccurs, culture and antimicrobial susceptibility testing of a sample should be performed to guide selection of an appropriate alternate antimicrobial drug. Finally, despite the potential efficacy of vancomycin and linezolid against methicillin-resistant staphylococci, these drugs should not be used in veterinary medicine because of their importance for treating human MRSA infections. Breaking the transmission cycle between humans and animals requires diligent hand hygiene and careful disinfection of the surfaces or materials on which staphylococci survive and proliferate.

Additional research is needed to improve our understanding of *S. pseudintermedius* infection in dogs. Debate remains about whether there are differences

between MRSA and MRSP infections in dogs with regard to severity of clinical signs and outcome. In general, it is thought that dogs are not preferred carriers of *S. aureus* and infection or colonization of dogs by MRSA results from transmission from humans (1,5,32). It is also thought that humans are not preferred carriers of *S. pseudintermedius.* Although this suggests that the potential for colonization or infection of the owner is generally low when a pet is identified as having an MRSP infection, this may not be the situation for immunocompromised owners (1,4), and each case should be evaluated individually.

CHAPTER III

CELL-WALL ASSOCIATED PROTEINS

Introduction

Staphylococcus pseudintermedius is the most frequent bacterial agent associated with pyoderma and secondary skin infection in dogs (1,2). Many dogs harbor *S. pseudintermedius* in their nares, oral cavity, and skin without clinical evidence of disease (1,3,4,42,100). Physical barriers, including intact epithelium and healthy mucous membranes, are generally sufficient to prevent infection with *S. pseudintermedius*. However, any condition that weakens the host's ability to mount an immune response or that compromises these epidermal barriers can result in opportunistic *S. pseudintermedius* infections. This commonly occurs in dogs that have endocrinopathies (such as diabetes mellitus or hypothyroidism) (67), cornification disorders (110), or compromised immunity (111).

Empirical treatment of staphylococcal infections such as pyoderma and secondary post-operative infection typically involves β-lactam antibiotics, such as cephalexin (9). Staphylococci that carry the penicillin binding protein 2a (PBP2a), encoded by the *mecA* gene, have a low affinity for all β-lactam antimicrobials and are considered methicillin-resistant (5,6,11,56-58). Methicillin-resistant *S. pseudintermedius* (MRSP) are increasingly resistant to other antimicrobials thus reducing treatment options for dogs with MRSP infections. In Europe, 90% of MRSP isolates are resistant to clindamycin, erythromycin, and trimethoprim, 70% are resistant to chloramphenicol and gentamicin, and 57% are resistant to chloramphenicol alone (2,10). In the United

States, resistance to fluoroquinolones and chloramphenicol in MRSP has become common, reducing antimicrobial options for treatment (11,12).

Colonization or carriage of *S. pseudintermedius* on the skin requires adherence to epithelial surfaces, typically through tight attachment between host and bacterial proteins. The bacterial proteins involved are usually proteins on the outer surfaces of the bacteria, such as the outer membrane or cell wall proteins. A recent study identified 18 cell-wall associated proteins (CWAPs) in *S. pseudintermedius* that are involved in attachment to host proteins (29). This study also showed that four of these 18 proteins (SpsF, SpsO, SpsP, and SpsQ) were present in only some isolates of *S. pseudintermedius* (29). It is not known whether there is any difference in the carriage of these proteins between isolates collected from healthy, asymptomatic dogs and dogs with clinical infection, or between isolates that are MRSP and MSSP. Determining which virulence factors are required for colonization and infection may allow development of novel strategies to reduce skin infection, including development of targeted therapeutics and rational design of staphylococcal vaccines.

The objectives of this study were 1) to estimate the prevalence of the four variable CWAPs isolated from *S. pseudintermedius*; 2) to determine whether an association exists between these CWAPs and methicillin resistance; 3) to estimate the prevalence of methicillin-resistant *S. pseudintermedius* (MRSP); and, 4) to describe the susceptibility of MRSP to clindamycin, aminoglycosides, chloramphenicol, and fluoroquinolones.

Materials and methods

Bacterial isolates

A total of 374 canine *S. pseudintermedius* isolates collected at the Texas A&M University Veterinary Medical Teaching Hospital (VMTH) between September 2010 and February 2012 were available for use in this study. All isolates were stored at -80^{o}C at the time of collection and revived for this study by inoculating onto trypticase soy agar supplemented with 5% sheep blood (blood agar plates) (BD Diagnostic Systems, Franklin Lakes, NJ, USA). With the Institutional Animal Care and Use Committee's (IACUC) approval, client-owned animals were swabbed for culture either upon entry into the hospital or when clinically warranted and divided into two populations based on health status (infected vs uninfected). Isolates from animals presented to the canine orthopedic surgery service that had no evidence of infection (i.e., clinically normal skin) were placed into the uninfected category for this study. Upon entry into the hospital, samples from the nares and perineum were collected separately from these patients using sterile swabs. Isolates derived from dogs that were presented for a clinical illness that required a bacterial culture for disease diagnosis were placed into the infected category. Specimens from this population were collected from the site of infection.

Descriptive statistics

Records of the 294 canine patients from which the 374 isolates had been obtained were analyzed retrospectively. Characteristics including breed, age, sex, presenting complaint, and concurrent diseases or diagnoses were recorded along with date of admission, date of discharge, and whether the dog was an inpatient or an outpatient. Culture and results

of antimicrobial drug minimum inhibitory concentration (MIC) testing were documented as was the number of days from hospital entry to time of culture.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing on all isolates was performed according to the Clinical Laboratory Standards Institute (CLSI) guidelines (112) using commercially available systems (TREK Sensititre, TREK Diagnostics, Cleveland, OH, USA) and confirmed by oxacillin disk diffusion test on Mueller Hinton agar (BD Diagnostic Systems, Franklin Lakes, NJ, USA). As isolates were tested for antimicrobial susceptibility as part of patient care, the drugs tested varied (i.e., an isolate from a skin culture was tested with a different set of antimicrobial drugs than an isolate from a urine culture). The breakpoints provided in the CLSI veterinary standard (112) were used to determine whether an isolate was susceptible or resistant to an antimicrobial drug.

mecA *PCR*

Staphylococcus pseudintermedius isolates were tested for the presence of *mecA* by PCR using previously described primers (113) (M1, M2; Table 1) and methods (114) in the clinical microbiology laboratory of the VMTH at the time of original isolation. *Staphylococcus aureus* ATCC 43300 (methicillin-resistant) (ATCC, Manassas, VA, USA) and *S. aureus* ATCC 21923 (methicillin-susceptible) were used as control strains.

Table 1. Oligonucleotides used during this study.

Primer (Constructed on)	Protocol	Significance	Sequence, Reading 5'-3'	BP Size	Reference
M1	*mecA*	*mecA*	TGGCTATCGTGTCACAATCG	310 bp	113
M2	*mecA*		CTGGAACTTGTTGAGCAGAG		113
mA1 (*mecA*)	SCC*mec* PCR 1	*mecA*	TGCTATCCACCCTCAAACAGG	286 bp	115
mA2 (*mecA*)	SCC*mec* PCR 1		AACGTTGTAACCACCCCAAGA		115
α1 (*ccrA1*)	SCC*mec* PCR 1	SCC*mec* type I	AACCTATATCATCAATCAGTACGT	695 bp	115
α2 (*ccrA2*)	SCC*mec* PCR 1	SCC*mec* type II	TAAAGGCATCAATGCACAAACACT	937 bp	115
α3 (*ccrA3*)	SCC*mec* PCR 1	SCC*mec* type III	AGCTCAAAAGCAAGCAATAGAAT	1791 bp	115
βc(*ccrB1, ccrB2, ccrB3*)	SCC*mec* PCR 1	α1, 2, 3 reverse primer	ATTGCCTTGATAATAGCCITCT		115
α4.2 (*ccrA4*)	SCC*mec* PCR 1	SCC*mec* type IV	GTATCAATGCACCAGAACTT	1287 bp	115
β4.2 (*ccrB4*)	SCC*mec* PCR 1		TTGCGACTCTCTTGGCGTTT		115
γR (*ccrC*)	SCC*mec* PCR 1	SCC*mec* type V	CCTTTATAGACTGGATTATTCAAAATAT	518 bp	115
γF (*ccrC*)	SCC*mec* PCR 1		CGTCTATTACAAGATGTTAAGGATAAT		115
mI6 (*mecI*)	SCC*mec* PCR 2	SCC*mec* type II or III*	CATAACTTCCCATTCTGCAGATG	1963 bp	115
IS7 (IS*1272*)	SCC*mec* PCR 2	SCC*mec* type I or IV	ATGCTTAATGATAGCATCCGAATG	2827 bp	115
IS2(iS-2) (IS*431*)	SCC*mec* PCR 2	SCC*mec* type V	TGAGGTTATTCAGATATTTCGATGT	804 bp	115
mA7 (*mecA*)	SCC*mec* PCR 2	mI6, IS7, IS2 reverse primer	ATATACCAAACCCGACAACTACA		115
spsF-F	Sps PCR	*Sps* F	AGTGGAAGCAACAGTTGAACGC	539 bp	29
spsF-R	Sps PCR		TGGACCTACTTGGCTACCACCA		29
spsO-F	Sps PCR	*Sps* O	GGTAGTGTATCAGTGCTAATAGGAGC	604 bp	29
spsO-R	Sps PCR		TTGACAAATCAGTAGCTGATGCAT		29
spsP-F	Sps PCR	*Sps* P	CAGGAGGACTAGGGTAATGTTCC	277 bp	29
spsP-R	Sps PCR		GCAAAACTTGGCGTGTTTACAAG		29
spsQ-F	Sps PCR	*Sps* Q	CCGCTCTATTTTTAGGTTAATC	593 bp	29
spsQ-R	Sps PCR		GCGCTTCATCGAAACTTGGCGCAGG		29
R20		*Sps* sequencing	CAGCTATGACCATGATTACG	165 bp +	
U19			GTTTTCCCAGTCACGACGT	*Sps* gene bp	

*SCC*mec *typing*

MRSP isolates were inoculated on blood agar plates and incubated at 37°C for 24 h. A single colony was used to inoculate 10 ml of LB broth and incubated at 37ºC overnight. DNA was purified from the broth culture using a DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions for gram-positive bacteria. DNA concentration was quantified using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

SCC*mec* typing was performed by PCR to distinguish between SCC*mec* types I to VI. SCC*mec* PCR 1 amplification was carried out as a multiplex PCR in a 50 μL reaction volume that consisted of 1X storage buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DDT, 0.5% Polysorbate 20, 0.5% Nonidet P-40, 50% glycerol), 3.2 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (Life Technologies, Grand Island, NY, USA), 10 pmol of each previously described primer (115) (mA1, mA2, α1, α2, α3, βc, α4.2, β4.2, γR, γF, mI6, IS7, IS2, mA7; Table 1), 1.25 U of TaKaRa Ex Taq DNA polymerase (Clontech Laboratories, Otsu, Shiga, Japan), and 100 ng of purified bacterial DNA. The PCR assays were performed using an Applied Biosystems 2720 Thermal Cycler (Life Technologies, Grand Island, NY, USA) with an initial 2 min denaturation step at 94°C, followed by 30 cycles consisting of a 2 min denaturation step at 94°C, a 1 min annealing step at 57°C, a 2 min extension at 72°C, and a final extension step for 2 min at 72°C (115). SCC*mec* PCR 2 amplification was carried out as a multiplex as described for SCC*mec* PCR1 with the alteration of the

annealing temperature to 60°C (115). PCR products were visualized using UV light and documented with a digital imaging system (Fluoro Chem, Alpha Innotech, Santa Clara, CA, USA) after electrophoresis on a 1% (wt/vol) agarose gel (Phenix Research Products, Candler, NC, USA) containing 0.1 μL GelRed Nucleic Acid Gel Stain 10,000X (Biotium, Inc., Hayward, CA, USA) per ml of gel. Two molecular weight ladders, 100 bp and 1 kb plus, (Life Technologies, Grand Island, NY, USA) were used to compare PCR product sizes. *S. aureus* ATCC 43300 (ATCC, Manassas, Virginia, USA), S. aureus ATCC 21923, and *S. aureus* ATCC 25932 were used as controls. All primers were synthesized by Sigma-Genosys, The Woodlands, TX, USA.

sps *gene PCR and gel electrophoresis*

PCR amplification of the *sps* genes was carried out as single PCR reactions using 50 μL mixtures that consisted of 1X storage buffer (10 mM KCl, 0.01mM EDTA, 0.1mM DTT, 5% glycerol, 0.01% Triton X-100), 2 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate (Life Technologies, Grand Island, NY, USA), 20 pmol of each previously described primer (29) (spsF-F, spsF-R, spsO-F, spsO-R, spsP-F, spsP-R, spsQ-F, spsQ-R; Table 1), 2.5 U of Econo Taq polymerase (Lucigen, Middleton, WI, USA), and 1 colony of bacteria grown on blood agar plates. The PCR assays were performed using an Applied Biosystems 2720 Thermal Cycler with an initial 2 min denaturation step at 95°C, followed by 30 cycles consisting of a 20 s denaturation step at 95°C, a 20 s annealing step at 50°C, a 2 min extension at 72°C, and a final extension step for 3 min at 72°C (29). PCR products were visualized using UV light and documented with a digital imaging system after electrophoresis on 1% (wt/vol) agarose gel containing 0.1 μL

GelRed Nucleic Acid Gel Stain 10,000X per ml of gel. A 100 bp molecular weight ladder was used compare product sizes (Life Technologies; Grand Island, NY, USA).

Confirmation of sps *gene PCR product sequence*

sps gene PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Purified DNA fragments were cloned using the pT7Blue Perfectly Blunt Cloning Kit (Novagen, EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions and transformed into chemical competent *E. coli* NovaBlue cells (Novagen, EMD Millipore, Billerica, MA, USA). These cells were plated on LB agar (116) with 40 μg/mL x-gal (Roche, Indianapolis, IN, USA) and 100 μg/mL carbenicillin (Teknova, Hollister, CA, USA). White colonies were tested using primers R20 and U19 for the presence of an appropriately sized insert. Colonies that were positive for the insert by PCR were inoculated into 4 mL of LB broth for overnight culture at 37^{o}C and plasmid DNA was isolated with a QIAprep Spin MiniPrep Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Plasmid DNA was quantified using a NanoDrop ND-1000 Spectrophotometer. DNA sequencing was then performed using the R20 primer. The resultant sequences were compared with the known sequence for these genes from ED99 available in GenBank as NC_017568.1 (40).

Statistical analysis

Data were analyzed using mixed-effects logistic regression to determine the association between methicillin resistance status (MRSP versus MSSP) of the isolate and individual variables of the dog from which they were isolated such as age and breed. Because some dogs contributed more than 1 isolate, dog was modeled as a random effect. All variables

associated with MRSP at a significance level of $P < 0.1$ were included in a multivariable random effects logistic regression modeling approach. A purposeful step-wise elimination process was used in which all variables were included in the model. Variables were eliminated from the model if their coefficient did not remain significantly associated with disease. All pair-wise interactions were examined for retained variables. The association between MRSP and other variables was summarized as the odds ratio (OR), and the 95% confidence limit (95% CI) for the OR, estimated using maximum likelihood methods. Models were fit using the glme function of S-PLUS statistical software (Version 8.2, TIBCO, Inc., Seattle, WA). Model fit was assessed by examining diagnostic plots of residuals. Comparisons of categorical variables summarized as simple proportions were made using chi-squared analysis using S-PLUS. Significance level for all statistical analyses was $P < 0.05$.

Results

Age

The ages of the dogs from which MRSP was isolated were similar (median, 6 years; range, 0.2 to 15 years) to those of dogs with MSSP (median, 6 years; range, 0.5 to 16 years The proportion of MRSP isolates that were from dogs > 5 years of age (59%; 41/69) was similar ($P = 0.7460$) to that for MSSP isolates (56%; 172/305).

Breed

There were 79 distinct breeds of dogs reported. Isolates were most common from dogs of the following breeds: Labrador Retriever ($n = 56$ isolates); German Shepherd Dog ($n = 31$); mixed-breed ($n = 33$); Golden Retrievers ($n = 17$); Bulldog-type ($n = 17$); Miniature Schnauzer ($n = 12$); Boxer ($n = 10$); and Yorkshire Terrier ($n = 9$). However,

breed was not significantly associated with MRSP. Breed was also examined using the AKC breed groupings of Sporting, Hound, Working, Terrier, Toy, Non-Sporting, and Herding, with an additional group creating for mixed-breed. Although there was no significant difference in carriage of MRSP between groups, dogs in the Toy group tended to be less likely to have MRSP ($P = 0.0549$; Table 2).

Sex

There was no significant difference in the proportion of isolates that were cultured from female dogs versus male dogs ($P = 0.7200$), and the odds of an isolate coming from female dogs (relative to male dogs) were not significantly greater ($P = 0.6186$) for the MRSP group than the MSSP group (OR = 0.9; 95% CI, 0.5 to 1.5).

Patient hospitalization

There was no significant association between MRSP and whether isolates came from dogs that were inpatients or outpatients. The proportion of isolates that came from inpatients was similar among MRSP isolates (57%; 39/69) and MSSP isolates (54%; 166/305; $P = 0.8556$), and the odds of an isolate coming from an inpatient dog (relative to outpatient dogs) were not significantly greater ($P = 0.75$) for the MRSP group than the MSSP group (OR = 1.1; 95% CI, 0.7 to 1.9). Most isolates (81%; 304/374) were collected from dogs on the day of admission (days in hospital = 0). The proportion of isolates that were collected after day 0 was significantly ($P < 0.0001$) greater for the MRSP isolates (36%; 25/69) than for the MSSP isolates (15%; 45/305; Table 2); the maximum value was 18 days for the MRSP and 34 days for the MSSP.

Table 2. Variables significantly associated with the MRSP colonization status of individual dogs (*n* = 294) from which isolates of *Staphylococcus pseudintermedius* (*n* =374) were obtained.*

Variable	MRSP	MSSP	Odds Ratio (95% CI)	*P*-value
AKC group				
Mixed breed	8/69 (12%)	25/305 (8%)	1	NA
Sporting	22/69 (32%)	69/305 (23%)	0.9 (0.4 to 2.4)	0.9939
Hound	(3/69 (4%)	28/305 (9%)	0.3 (0.1 to 1.4)	0.1323
Working	10/69 (14%)	35/305 (11%)	0.9 (0.3 to 2.6)	0.8343
Terrier	9/69 (13%)	30/305 (10%)	0.9 (0.3 to 2.8)	0.9076
Toy	4/69 (6%)	44/305 (14%)	0.3 (0.1 to >1.0)	0.0549
Non-Working	4/69 (6%)	25/305 (8%)	0.5 (0.1 to 1.9)	0.3045
Herding	9/69 (13%)	49/305 (16%)	0.6 (0.2 to 1.7)	0.3084
Samples collected after day of admission (day 0 of hospitalization)				
No	44/69 (64%)	260/305 (85%)	1	NA
Yes	25/69 (36%)	45/305 (15%)	**3.3 (1.8 to 5.9)**	**<0.0001**
Disease status				
Healthy	13/69 (19%)	168/305 (55%)	1	NA
Diseased	56/69 (81%)	137/305 (45%)	**5.5 (2.8 to 10.1)**	**<0.0001**
Source (Including nasal and perineal)				
Nasal or perineal skin	13/69 (19%)	172/305 (56%)	1	NA
Other skin (pyoderma, wounds, etc…)	32/69 (46%)	79/305 (26%)	**5.4 (2.7 to 10.8)**	**<0.0001**
Urine	12/69 (17%)	25/305 (8%)	**6.4 (2.6 to 15.5)**	**<0.0001**
Other non-skin sources	12/69 (17%)	29/305 (10%)	**5.5 (2.3 to 13.2)**	**<0.0001**
Source (skin excluding nasal and perineal isolates)				
Pyoderma	17/32 (53%)	35/79 (44%)	3.1 (0.8 to 11.8)	0.1051
Wounds	12/32 (38%)	25/79 (32%)	3.0 (0.8 to 12.3)	0.1219
Other skin	3/32 (9%)	19/79 (24%)	1	NA

*Bolded items were significantly associated with MRSP isolates and underlined items had approached the threshold of statistical significance associated with isolates from MSSP.

Isolate source

There were 22 distinct anatomic sites or tissues from which the isolates were cultured. The most common site of origin of isolates was the skin (n = 296) followed by urine (n = 37), bone (n = 9), bladder stones or bladder mucosa (n = 8), and other sources (n = 41). The 296 isolates that came from skin sources included 104 from the perineum, 81 from the nares, 52 from pyoderma, 37 from wounds, 8 from ears, 8 from incisional infections, 5 from tumors, and 1 from an implant infection that resulted in a draining tract through the skin. Because the proportion of isolates that were MRSP was the same for both nasal (7%; 6/81) and perineal isolates (7%; 7/104), and because these isolates were collected primarily from dogs with healthy skin examined by the orthopedic service, the nasal and perineal categories were collapsed. For analysis, the other skin sources were regrouped to pyoderma, wounds, and other skin sources. There was a significant association between source and MRSP (Table 2). All other skin sources were significantly (P < 0.0001) more likely to yield MRSP than nasal or perineal isolates, with similar magnitude of ORs compared to MSSP. However, when restricting analyses to the 111 isolates from skin sources other than nasal and perineal, there was no significant association of MRSP with skin source. Isolates that were methicillin-resistant were significantly (P < 0.0001) more likely to be associated with isolates from dogs with disease (Table 2).

Antimicrobial resistance

Of the 374 isolates in this study, 69 (18%) were methicillin-resistant based on oxacillin resistance as measured by microbroth dilution (MIC ≥ 0.5 μg/ml), disk diffusion test (zone of inhibition ≤ 17mm), and PCR for the *mecA* gene (Figure 1). Aside from β-

lactam drugs, MRSP isolates were more likely to be resistant to antimicrobial drugs than MSSP isolates (Figure 1, P = <0.0001). There were only three drugs to which more than 50% of the MRSP isolates were susceptible: amikacin (70%), chloramphenicol (55%), and gentamicin (52%) (Figure 1).

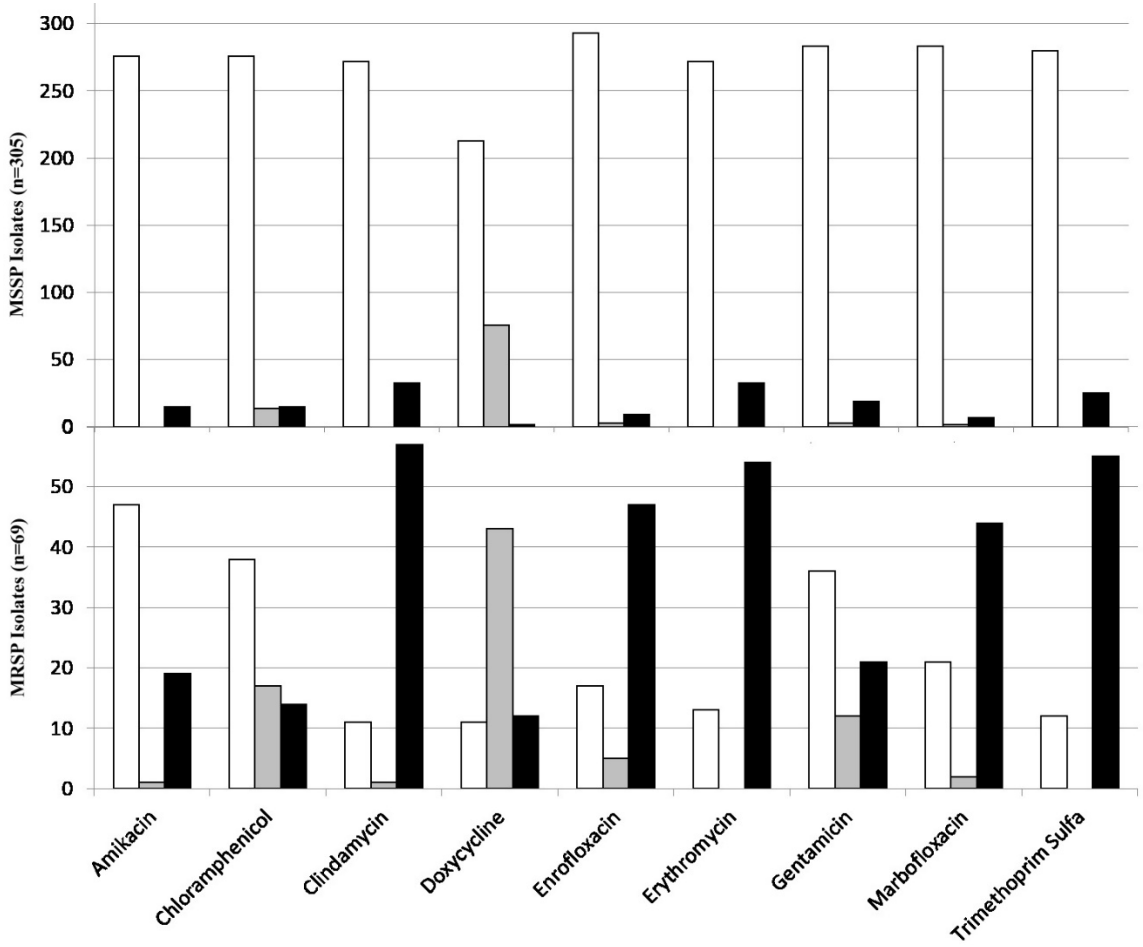

Figure 1. Antimicrobial resistance patterns associated with MRSP and MSSP isolates of *Staphylococcus pseudintermedius* (N= 374).[a] Bar graphs compare isolates of MSSP (top row; n = 305) and MRSP (bottom row; n = 69) that are susceptible, intermediate, or resistant to nine non-β-lactam antibiotics. Color differentiation is as follows: black = resistant isolates; grey = intermediate isolates; white = susceptible isolates. Antimicrobial susceptibility of MRSP and MSSP to all nine of these drugs was statistically significant with a *P*-value <0.0001. [a]MIC breakpoints from the Clinical and Laboratory Standards Institute were used to determine category (112).

*SCC*mec *type*

The 69 MRSP isolates were SCC*mec* typed by PCR. The SCC*mec* typing PCR used in this study can distinguish SCC*mec* types I-VI. Currently, there are 11 established SCC*mec* types (117). Of the 69 isolates, 44 (64%) were type V, nine (13%) were type III, six (9%) were type IV, five (7%) had an unknown PCR product pattern that could not be typed by the multiplex PCR utilized in this study, and 1 MRSP isolate (1%) was from a healthy dog that was *mecA* positive but had no visible SCC*mec* PCR products despite repeated PCR attempts. In addition, there were four isolates that had multiple SCC*mec* types. For each of these four isolates, the isolate contained SCC*mec* type V in addition to another SCC*mec* type.

sps *genes-single genes*

Isolates were screened by PCR to determine whether they carried any of the genes encoding the four CWAPs of interest. Of the 374 isolates, 70 (19%) carried the *spsF* gene, 35 (9%) carried the *spsO* gene, 64 (17%) carried the *spsP* gene, and 72 (19%) carried the *spsQ* gene (Table 3). Of these genes, only *spsP* and *spsQ* were significantly associated with methicillin resistance (Table 3). The proportion of *spsP*-bearing isolates was significantly ($P = 0.0006$) greater among MRSP isolates (32%; 22/69) than among MSSP isolates (14%; 42/305). The odds of an isolate having *spsP* (relative to another *sps* gene) were significantly greater ($P = 0.0005$) for the MRSP group than the MSSP group (OR = 2.9; 95% CI, 1.6 to 5.4). The proportion of *spsQ*-bearing MRSP isolates (35%; 24/69) was also significantly greater ($P = 0.0006$) than the proportion of *spsQ*-bearing MSSP isolates (16%; 48/305). The odds of an isolate having *spsQ* (relative to another

Table 3. *sps* genes associated with methicillin-resistant *Staphylococcus pseudintermedius* (*n* = 374).*

***sps* Gene**	**MRSP**	**MSSP**	**Odds Ratio (95% CI)**	**P-value**
Individual Genes				
spsF	15/69 (22%)	55/305 (18%)	1.2 (0.7 to 2.5)	0.4755
spsO	5/69 (7%)	30/305 (10%)	0.7 (0.3 to 1.9)	0.5069
spsP	22/69 (32%)	42/305 (14%)	2.9 (1.6 to 5.4)	**0.0005**
spsQ	24/69 (35%)	48/305 (16%)	2.8 (1.6 to 5.1)	**0.0005**
Two Genes				
spsF and *spsO*	2/69 (3%)	11/305 (4%)	0.8 (0.2 to 3.7)	0.7725
spsF and *spsP*	2/69 (3%)	10/305 (3%)	0.9 (0.2 to 4.1)	0.8716
spsF and *spsQ*	4/69 (6%)	11/305 (4%)	1.6 (0.5 to 5.3)	0.4072
spsO and *spsP*	0/69 (0%)	5/305 (2%)	**Incalculable	ND
spsO and *spsQ*	0/69 (0%)	6/305 (2%)	**Incalculable	ND
spsP* and *spsQ	20/69 (29%)	37/305 (12%)	3 (1.6 to 5.5)	**0.0007**
Three Genes				
spsF and *spsO* and *spsP*	0/69 (0%)	3/305 (1%)	**Incalculable	ND
spsF and *spsO* and *spsQ*	0/69 (0%)	3/305 (1%)	**Incalculable	ND
spsF and *spsP* and *spsQ*	2/69 (3%)	9/305 (3%)	1.0 (0.2 to 4.8)	0.9815
spsO and *spsP* and *spsQ*	0/69 (0%)	3/305 (1%)	**Incalculable	ND
All Genes				
spsF and *spsO* and *spsP* and *spsQ*	0/69 (0%)	2/305 (1%)	**Incalculable	ND

*Those marked in bold were significantly associated with methicillin-resistant isolates.
**Odds ratio was incalculable because no MRSP isolates carried these gene combinations.
ND = Not Determined

sps gene) were significantly greater ($P = 0.0005$) for the MRSP group than the MSSP group (OR = 2.8; 95% CI, 1.6 to 5.1).

sps *genes-pairwise comparisons*

Some isolates carried more than one of the four genes. There was no difference in rates of carriage of multiple genes between MRSP and MSSP except for isolates carrying both *spsP* and *spsQ* (Table 3). The proportion of isolates positive for both *spsP* and *spsQ* was significantly ($P = 0.0009$) greater among MRSP isolates (29%; 20/69) than among MSSP isolates (12%; 37/305); and the odds of an isolate having both *spsP* and *spsQ* (relative to isolates having neither gene or having only *spsP* or only *spsQ*) were significantly greater ($P = 0.0007$) for the MRSP group than the MSSP group (OR = 3.0; 95% CI, 1.6 to 5.5).

sps *genes-multiple genes*

It was only possible to calculate ORs of differences between carriage of 3 or more CWAP genes in MRSP and MSSP for the combination of *spsF*, *spsP*, and *spsQ* as none of the other gene combinations were present in MRSP isolates, making the OR incalculable (Table 3). Eleven isolates were positive for the *spsF, spsP*, and *spsQ* genes. The proportion of isolates positive for these 3 genes was similar ($P = 0.7104$) between MRSP isolates (3%; 2/69) and MSSP isolates (3%; 9/305); and the odds of an isolate having *spsF, spsP*, and *spsQ* (relative to isolates not having all 3) were not significantly greater ($P = 0.9815$) for the MRSP group than the MSSP group (OR = 1.0; 95% CI, 0.2 to 4.8).

sps *genes vs. source*

There was no association between any individual *sps* gene or combination of genes and isolation of *S. pseudintermedius* from pyoderma or wounds although isolates with more than two genes came predominantly from dogs with pyoderma (12%; 6/52) as compared with dogs with wounds (3%; 1/37) or other skin sources (5%; 1/22). The nasal and perineal skin isolates were excluded from analysis because they were collected from dogs presenting to only one hospital service which resulted in a confounding effect.

Discussion

There were a number of clinically relevant findings from this study. The first is that there was a strong association between methicillin resistance in *S. pseudintermedius* isolates and infection (Table 2).This was true for skin, urine, and other sites of infection. Among the MRSP isolates tested here, we found that 70% (47/67) were susceptible to amikacin and 55% (38/69) were susceptible to chloramphenicol, but fewer were susceptible to other drugs such as marbofloxacin (31%; 21/67) and enrofloxacin (25%; 17/69) (Figure 1). These findings may reflect the patient population of our hospital which includes both primary-care patients and patients referred to specialty services (such as dermatology and orthopedic surgery). As a referral practice, it is common to receive patients with infections that have failed to respond to empiric antimicrobial therapy. Prior antimicrobial therapy is a risk factor for establishment of carriage (10) and infections (118) with MRSP and may explain the occurrence of antimicrobial resistance among the studied isolates. Prior exposure to antimicrobial drugs was not included in all patient histories. Consequently, we were unable to evaluate whether prior antimicrobial use was associated with our finding linking methicillin-resistance and infection.

Methicillin-resistant staphylococci are identified phenotypically through antimicrobial susceptibility testing and genetically through the detection of *mecA*, a gene that confers resistance to all β-lactam drugs and that is carried on a transmissible mobile DNA element called SCC*mec* (11). SCC*mec* contains the *mec* gene complex consisting of *mecA*, the genes that control expression of the *mecA* gene, and unique site-specific recombinases called cassette chromosome recombinases (*ccr*) (11). Once established, SCC*mec* types tend to dominate specific geographical regions and can be used to perform epidemiologic studies and monitor disease patterns (8). Our finding that 64% of all isolates possessed SCC*mec* type V was consistent with other published data which showed that in North America SCC*mec* type V is the dominating clonal lineage (11). Currently, there are as many as 11 SCC*mec* types reported in the literature (117). Six of our isolates were either untypable or had banding patterns that were not recognized by the multiplex utilized in this study despite repeated PCR attempts. Because the multiplex used in this study only recognized SCC*mec* types I to VI but all isolates carried *mecA*, we hypothesize that these six isolates are among SCC*mec* types VII to XI. Sequencing of the SCC*mec* of these isolates would resolve the question of type but was not undertaken in this study.

Increased prevalence of MRSP and increasing prevalence of MRSP resistant to all classes of antimicrobial drugs, including aminoglycosides, have led to the search for alternatives to antimicrobial therapy including vaccines. In theory, a successful staphylococcal vaccine would prevent bacterial adherence or promote immune mediated killing of the bacteria (119,120). Such vaccines would naturally target outer membrane proteins of the bacteria. For *S. pseudintermedius*, surface antigen expression and

variation was recently described among the cell-wall-anchored proteins SpsD and SpsO (121). Antibodies to SpsD and SpsL were identified in canine pyoderma patients, indicating that these bacterial proteins induce a humoral immune response (29). In this study, there was a significant association with *spsP* and *spsQ* with MRSP and of MRSP with infection. Together, these findings suggest a role for the proteins encoded by these genes during infection. SpsP and SpsO may offer an attractive target for future staphylococcal vaccines against MRSP. It was not surprising that *spsP* and *spsQ* were associated with each other as both genes are located adjacent to each other near *oriC* on the *S. pseudintermedius* chromosome and have been shown previously to have 67 to 90% sequence identity in any pairwise alignment (29).

This study is limited by its retrospective nature. Patient histories were obtained through examination of the medical records. Some cases may have had previous cultures or prior antimicrobial treatment that were not noted in the records or not identified during the study period. Another limitation of the study was the sampling strategy. The healthy control population consisted of dogs admitted to the orthopedic service that had specimens collected from the nares and perineum for a separate study. Almost all nasal and perineal samples in the present study were from this population; thus, skin source was strongly associated with disease status. Because isolation of MRSP was strongly associated with disease status, it appeared that MRSP was strongly associated with skin sources other than nasal and perineal; however, when swabs from the nasal and perineal region were excluded, swabs of sites of infection or pyoderma were not significantly associated with MRSP (Table 2). Future studies should use unbiased sampling design to avoid problems of selection bias and confounding.

In this study, MRSP was strongly associated with clinical disease. While there was no significant difference between *sps* gene and source in this study it is interesting that the majority of isolates with multiple genes were from dogs that had pyoderma. In addition, *spsP* and *spsQ* were significantly associated with methicillin resistance. This new information may impact the development of *S. pseudintermedius* vaccines or alternative therapies for staphylococcal infections in dogs.

CHAPTER IV

AMIKACIN RESISTANCE GENES*

Introduction

Staphylococcus pseudintermedius is the most common bacterial agent isolated from canine pyoderma and wound infections in dogs (1,2). Treatment of staphylococcal infection typically involves therapy with β-lactam antibiotics such as penicillin and cephalosporins. Resistance to this class of antimicrobial drug has increased in recent years, associated with the rise in methicillin resistance in *S. pseudintermedius* (55). In addition to being resistant to β-lactam antimicrobials, methicillin-resistant *S. pseudintermedius* (MRSP) strains are increasingly resistant to other antimicrobials (15). A multi-center study from Europe and North America showed that MRSP isolates are commonly resistant to virtually all antimicrobial drug classes approved for use in dogs with 90% of MRSP isolates resistant to ciprofloxacin, clindamycin, erythromycin, kanamycin, streptomycin, and trimethoprim, while 57% were resistant to chloramphenicol (2,10). Decreased susceptibility of MRSP to other antimicrobials has left relatively few options for therapy. Data for North America are limited but many North American MRSP isolates were previously susceptible to chloramphenicol, rifampin, and amikacin. We have isolated MRSP that are resistant to chloramphenicol and have begun to identify isolates resistant to amikacin at our hospital (Table 4).

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Table 4. Amikacin-resistant and chloramphenicol-resistant *Staphylococcus pseudintermedius* isolates collected from the Texas A&M Veterinary Medical Teaching Hospital (VMTH), 2010-2012.

	2010	**2011**	**2012**
Total *S. pseudintermedius* isolates	186	292	212
Amikacin-resistant *S. pseudintermedius* isolates*	5	30	37
Percentage of amikacin-resistant *S. pseudintermedius* isolates	2.7%	10.3%	17.5%
Chloramphenicol-resistant *S. pseudintermedius* isolates**	8	48	45
Percentage of chloramphenicol-resistant *S. pseudintermedius* isolates	4.3%	16.4%	21.2%

*Isolates with an amikacin minimum inhibitory concentration ≥ 64μg/ml were considered resistant.
**Isolates with a chloramphenicol minimum inhibitory concentration ≥ 16μg/ml were considered resistant.

Aminoglycosides like amikacin inhibit bacterial protein synthesis by binding to the 30S ribosomal subunit (13). The most widespread mechanism of aminoglycoside resistance is drug inactivation by cellular aminoglycoside modifying enzymes (AMEs) encoded on the chromosome, a plasmid, or carried on a transposable element (14,17). They can be divided into three classes: aminoglycoside acetyltransferases (AACs), aminoglycoside (phosphotransferases (APHs), and aminoglycoside nucleotidyltransferases (ANTs) (18). In *S. aureus*, the *aac(6′)/aph(2″)* gene is the most frequently encountered aminoglycoside resistance gene followed by *aph(3′)- IIIa* and *ant(4′)-Ia* (18,19).

The purpose of this study was to determine which genes encoding amikacin modifying enzymes are present in amikacin-resistant *S. pseudintermedius* isolates collected from canine patients using a previously described multiplex polymerase chain

reaction (PCR) assay (18), and to see if an association exists between amikacin resistance, methicillin resistance, and resistance to non-β-lactam antibiotics. Although amikacin resistance has been noted in *S. pseudintermedius* and studies of aminoglycoside resistance in *S. aureus* have been published, no studies have assessed aminoglycoside resistance gene carriage in *S. pseudintermedius* (14,18,19,122). *Staphylococcus pseudintermedius* is commonly associated with canine pyoderma and post-operative wound infections (1,2). Amikacin resistance in *S. pseudintermedius* has significant repercussions for the treatment of canine MRSP infections particularly as resistance to other classes of antimicrobial drugs, like fluoroquinolones, limits antimicrobial choices for treatment. Understanding which aminoglycoside resistance mechanisms are present in *S. pseudintermedius* is crucial to the development of strategies to prevent resistance to this last line of therapy.

Materials and methods

Bacterial isolates

A total of 422 canine *Staphylococcus pseudintermedius* isolates collected from the Texas A&M Veterinary Medical Teaching Hospital (VMTH) between 2010-2012 were available for study. All isolates were cultured from patient specimens by technicians in the VMTH Clinical Microbiology Laboratory according to the standard operating procedures of the laboratory. Antimicrobial susceptibility was performed according to the CLSI standards (63) using a commercially available system (TREK Sensititre, TREK Diagnostics, Cleveland, OH, USA). In this study, isolates with intermediate susceptibility based on the CLSI interpretive criteria were considered to be resistant to the antimicrobial drug tested (63).

To identify methicillin resistance, every isolate was evaluated by three tests; oxacillin microbroth dilution test, oxacillin disk diffusion test, and PCR for detection of *mecA*. The oxacillin breakpoints used to confirm methicillin-resistance were minimum inhibitory concentration (MIC) $\geq$ 0.5 μg/ml and disk diffusion zone of inhibition $\leq$17 mm. These breakpoints were adopted by the Clinical Microbiology Laboratory following recognition that the CLSI Standards published in 2008 failed to identify some MRSP isolates (62,65). All three tests were performed on every isolate. Any isolate in which at least two of the three tests indicated methicillin resistance was deemed MRSP. PCR was performed using previously described primers (113) and methods (114), with *S. aureus* ATCC 43300 and *S. aureus* ATCC 21923 used as positive and negative controls, respectively (ATCC, Manassas, Virginia, USA). Disk diffusion and microbroth dilution tests were performed in accordance with the CLSI Performance Standards (63). Amikacin susceptibility of staphylococcal isolates was not routinely tested in the Clinical Microbiology Laboratory prior to 2010. As such, only isolates collected between October 22, 2010 and December 31, 2012 were included in this study. From this inclusion period, two datasets were generated. Dataset 1 included 422 isolates that were tested for amikacin resistance. This dataset was used to determine the prevalence of amikacin resistance and the association of amikacin resistance with resistance to other antimicrobial drugs. A second dataset included only the 32 amikacin-resistant isolates from dataset 1 that were available for further testing. The isolates in the second dataset were analyzed by PCR for the presence of resistance genes that encode aminoglycoside modifying enzymes.

Amikacin disk diffusion

Isolates not tested for amikacin resistance at the time of initial culture (i.e., antimicrobial susceptibilities originally performed on isolates from urine) were screened for amikacin resistance by disk diffusion. The disk diffusion tests were performed according to the Clinical and Laboratory Standards Institute (CLSI) Performance Standards (63). Isolates with a zone of inhibition ≤ 14 mm were considered resistant to amikacin.

DNA isolation and purification

Of the 422 initial isolates, 32 of the total 53 amikacin-resistant isolates were available for genetic testing. All isolates were stored at -80^{o}C at the time of collection and revived by inoculating them onto trypticase soy agar supplemented with 5% sheep blood (blood agar plates) (BD Diagnostic Systems, Franklin Lakes, NJ, USA) and incubated at 37°C for 24 hr. A single colony was used to inoculate 10 ml of L-broth (LB) which contained 10g/L tryptone, 5 g/L yeast extract, and 10 g/L $NaCl_2$ and incubated at 37^{o}C overnight. The LB components were supplied by BD Diagnostic Systems, Franklin Lakes, NJ, USA (tryptone and yeast extract) and Mallinckrodt Chemicals, St. Louis, MO, USA ($NaCl_2$). DNA was purified from the broth culture using a DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions for Gram positive bacteria. DNA was quantified using a NanoDrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Polymerase chain reaction

Previously published primers for *aac(6')/aph(2")* (5′-GAAGTACGCAGAAGAGA-3′; 5′-ACATGGCAAGCTCTAGGA-3′), *aph(3')-IIIa* (5′-AAATACCGCTGCGTA-3′; 5′-CATACTCTTCCGAGCAA-3′), *ant(4')-Ia* (5′-AATCGGTAGAAGCCCAA-3′; 5′-

GCACCTGCCATTGCTA-3′) and *mecA* (5′-CCTAGTAAAGCTCCGGAA-3′; 5′-CTAGTCCATTCGGTCCA-3′) were used to generate PCR products of 491, 242, 135, and 314 base pairs respectively (Sigma-Aldrich, St. Louis, MO, USA) (18). PCR amplification was carried out as previously described using 50-μL mixtures containing 0.2 μM forward and reverse primers, 1× Taq buffer, 3 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, 1 U of Ex Taq DNA polymerase, and inoculation with 2 μl of purified chromosomal template DNA. All PCR reagents were supplied by Takara Bio Company, Otsu, Shiga, Japan. The PCR assays were performed using an Applied Biosystems 2720 Thermal Cycler (Life Technologies, Grand Island, NY, USA) with an initial 5 minute denaturation step at 95ºC, followed by 30 cycles consisting of a 2 minute denaturation step at 95°C, a 30 second annealing step at 58°C, a 30 second extension at 72°C, and finishing with a final extension step for 7 minutes at 72ºC as previously described (18). PCR products were visualized using UV light and documented with an digital imaging system (Fluoro Chem, Alpha Innotech, Santa Clara, CA, USA) following electrophoresis on 1% (wt/vol) agarose gel (Phenix Research Products, Candler, NC, USA) containing 0.1 μl GelRed Nucleic Acid Gel Stain 10,000× (Biotium, Hayward, CA, USA) per ml of gel. The 1 kb Plus™ molecular weight ladder (Invitrogen, Grand Island, NY, USA) was used for comparison of product size (Figure 2).

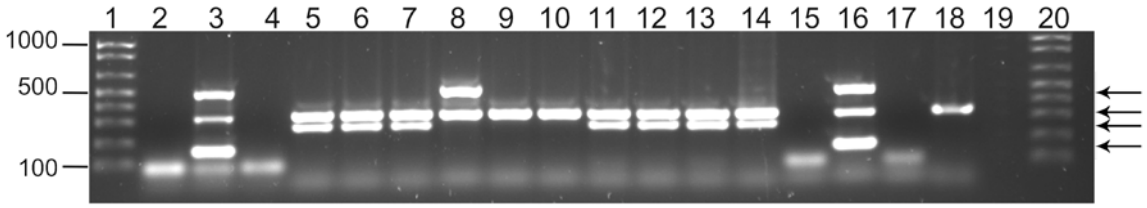

Figure 2. Multiplex PCR for aminoglycoside modifying enzyme genes and *mecA*. The molecular size marker used in lanes 1 and 20 was the 1 Kb Plus DNA ladder (Invitrogen, Grand Island, NY). Template DNA used in the multiplex PCR was as follows: lanes 2 and 15, *S. aureus* ATCC 29213; lanes 3 and 16, *S. aureus* ATCC 43300; lanes 4, 17, and 19, no template DNA as a negative control; lanes 5 to 14, and 18, clinical *S. pseudintermedius* isolates (lane 5- isolate 13-089; lane 6-isolate 24-089, lane 7-isolate 29-086, lane 8-isolate 30-027, lane 9-isolate 30-076, lane 10-isolate 30-077, lane 11-isolate 31-094, lane 12-isolate 32-006, lane 13-isolate 32-010, lane 14-isolate 35-079; lane 18, isolate 30-077). Black arrows from top to bottom correspond to PCR products *aac(6')/aph(2'')* (predicted 491 bp), *mecA* (predicted 314 bp), *aph(3')- IIIa* (predicted 242 bp), and *ant(4')-Ia* (predicted 135 bp), respectively.

Statistical analysis

Data were summarized using cross-tabulations and analyzed using chi-squared or Fisher's exact tests, and logistic regression based on the binary outcome of amikacin resistance and binary outcomes of susceptibility for each of the other antimicrobials. Results of logistic regression were summarized as odds ratios (ORs) and 95% confidence intervals for the ORs, estimated using maximum likelihood methods. Analysis was performed at the level of isolate and ignored the fact that some isolates originated from the same dog. Significance was set at $P < 0.05$ and all analyses were performed using S-PLUS statistical software (Version 8.2; TIBCO, Inc., Seattle, WA).

Results

A total of 422 isolates were collected from 413 dogs. Of these 422 isolates, 369 (87%) were susceptible to amikacin and 53 (13%) were resistant to amikacin based on the CLSI interpretive criteria for amikacin (63). Of the 422 isolates, 338 (80%) were MSSP and 84

(20%) were MRSP. All of the MRSP were positive for *mecA* by PCR. There were 316 isolates (75%) that were both MSSP and susceptible to amikacin, 53 (13%) that were MRSP but susceptible to amikacin, 22 (5%) that were MSSP but resistant to amikacin, and 31 (7%) that were MRSP and amikacin-resistant. The odds of an isolate being MRSP were significantly greater for isolates that were amikacin-resistant rather than amikacin-susceptible (Table 5). Resistance to each of the other antimicrobials tested was significantly associated with resistance to amikacin (Table 5). Because amikacin resistance was significantly associated with methicillin resistance, it was unclear whether resistance to the other antimicrobial drugs was associated with amikacin resistance alone or due to the association of amikacin resistance with methicillin resistance. Of the 422 *S. pseudintermedius* isolates, there were 106 isolates that were resistant to methicillin only (MR; $n = 53$), amikacin only (AR; $n = 22$), or both amikacin and methicillin (ARMR; $n = 31$). Among these 106 resistant *S. pseudintermedius* isolates, those that were resistant to both amikacin and methicillin were significantly more likely to be resistant to chloramphenicol, clindamycin, enrofloxacin, erythromycin, gentamicin, marbofloxacin, and trimethoprim/sulfonamide combination than the isolates that were either methicillin-resistant or amikacin-resistant alone (Table 6). Of the antimicrobials examined, only resistance to doxycycline and rifampin were not more likely among *S. pseudintermedius* isolates resistant to both amikacin and methicillin relative to isolates that were only resistant to 1 drug (Table 6). Isolates with an MIC of ≥ 8 μg/ml were considered resistant to doxycycline.

Table 5. Association of resistance to amikacin with resistance to other antimicrobials for isolates of *Staphylococcus pseudintermedius* (*n* = 422), expressed as odds ratios derived by logistic regression analysis.

Antimicrobial agent(s)	**No. of amikacin-susceptible isolates (%)**	**No. of amikacin-resistant isolates (%)**	**Odds Ratio (95% CI)**	***P*-value**
Oxacillin				
S	316 (93%)	22 (7%)	1 (NA)	
R	53 (63%)	31 (37%)	8.4 (4.5 to 15.6)	<0.0001
Chloramphenicol				
S	325 (92%)	30 (8%)	1 (NA)	
R	44 (66%)	23 (34%)	5.7 (3.0 to 10.6)	<0.0001
Clindamycin				
S	298 (96%)	11 (4%)	1 (NA)	
R	71 (63%)	42 (37%)	16.0 (7.6 to 34.0)	<0.0001
Doxycycline				
S	251 (95%)	13 (5%)	1 (NA)	
R	117 (75%)	39 (25%)	6.4 (3.3 to 12.5)	<0.0001
Enrofloxacin				
S	328 (95%)	18 (5%)	1 (NA)	
R	41 (54%)	35 (46%)	15.6 (8.1 to 29.9)	<0.0001
Erythromycin				
S	299 (96%)	11 (4%)	1 (NA)	
R	68 (62%)	42 (38%)	16.8 (8.2 to 34.3)	<0.0001
Gentamicin				
S	348 (100%)	0 (0%)	1 (NA)	
R	21 (28%)	53 (72%)	Inestimable*	<0.0001*
Marbofloxacin				
S	331 (92%)	27 (8%)	1 (NA)	
R	37 (59%)	26 (41%)	8.6 (4.6 to 16.3)	<0.0001
Rifampin				
S	359 (88%)	48 (12%)	1 (NA)	
R	8 (62%)	5 (38%)	4.7 (1.5 to 14.9)	0.0093
Trimethoprim/Sulfa				
S	309 (96%)	14 (4%)	1 (NA)	
R	58 (60%)	39 (40%)	14.8 (7.6 to 28.9)	<0.0001

S = Susceptible, R = Resistant

*Inestimable because of complete separation (i.e., no isolate resistant to amikacin was susceptible to gentamicin); *P* value derived from *chi*-square test with continuity correction

NA = Not applicable (reference category)

Table 6. Association of resistance to amikacin and/or methicillin relative to resistance to other antimicrobials in *Staphylococcus pseudintermedius* isolates (*n* = 106)

Antimicrobial agent(s)	**No. of AR & MR (%)**	**No. of AR or MR (%)**	**Odds Ratio (95% CI)**	***P*-value**
Chloramphenicol				
S	14 (22	50 (78%)	1 (NA)	
R	17 (40%)	25 (60%)	4.9 (1.2 to 4.9)	0.0444
Clindamycin				
S	1 (5%)	21 (95%)	1 (NA)	
R	30 (36%)	54 (64%)	11.6 (1.5 to 85.7)	0.0177
Doxycycline				
S	5 (22%)	18 (78%)	1 (NA)	
R	25 (31%)	56 (69%)	1.6 (0.5 to 4.8)	0.3986
Enrofloxacin				
S	2 (5%)	35 (95%)	1 (NA)	
R	29 (42%)	40 (58%)	12.7 (2.8 to 56.9)	0.0012
Erythromycin				
S	1 (4%)	22 (96%)	1 (NA)	
R	30 (37%)	51 (63%)	12.9 (1.8 to 95.0)	0.0135
Gentamicin				
S	0 (0%)	38 (100%)	1 (NA)	
R	31 (46%)	37 (54%)	Inestimable*	<0.0001*
Marbofloxacin				
S	8 (17%)	40 (83%)	1 (NA)	
R	23 (40%)	34 (60%)	3.4 (1.3 to 8.5)	0.0112
Rifampin				
S	1 (29%)	65 (71%)	1 (NA)	
R	4 (31%)	5 (69%)	1.1 (0.3 to 3.8)	0.9163
Trimethoprim/Sulfa				
S	0 (0%)	26 (100%)	1 (NA)	
R	31 (40%)	47 (60%)	Inestimable*	0.0003*

S = susceptible, R = resistant, AR & MR = isolates resistant to amikacin and oxacillin, AR or MR = isolates resistant to amikacin or oxacillin
*Inestimable because of complete separation (No observations in one category); *P* value derived from *chi*-square test with continuity correction
NA = Not applicable (reference category)

There were 32 isolates from 32 unique dogs available for identification of amikacin resistance genes. A retrospective analysis of patient records associated with the isolates was performed. Among these 32 dogs the majority were treated on an outpatient basis (59%; 19/32) while the remainder (41%; 13/32) were hospitalized. Of the 13

isolates from hospitalized patients, 4 were MSSP. Of the 9 MRSP isolates, 4 were susceptible to marbofloxacin, clindamycin or chloramphenicol while 5 were resistant to all other drugs except rifampin. The median time of culture upon entry into the hospital was zero days (SD = 2.2 days; range 0 to 12 days) with day zero defined as entry into the hospital for either the patient appointment or clinical emergency. Most samples (69%; 22/32) were collected on day zero. Cultures were primarily taken from sources of skin disease (72%; 23/32). The majority of these samples were collected from patients with pyoderma skin lesions (47%; 15/32) followed by skin wounds (12%; 4/32). The next most common sites sampled were urine and orthopedic implants, both seen in 3/32 cultures (9%).

Of the 32 dogs, 81% (26/32) had a history of prior antimicrobial administration, of which 53% (17/26) had received antimicrobials within 6 weeks. Of the 26 dogs with a history of antimicrobial administration, 54% (14/26) had received monotherapy while 46% (12/26) had received multiple antimicrobials before their culture was taken. Only 19% (6/32) reported no history of prior antimicrobial use. There was no significant difference ($P = 1.0000$; Fisher's exact test) in whether dogs with amikacin-resistant isolates had a history of prior antimicrobial administration and whether their isolates were MSSP (82%; 9/11) or MRSP (81%; 17/21). The distribution of the three categories of prior antimicrobials (none, monotherapy, multi-drug) did not differ significantly ($P = 0.7926$; Fisher's exact test) between amikacin-resistant isolates that were MSSP and MRSP. Similarly, the proportion of dogs that received multiple drugs did not differ significantly ($P = 0.6530$) between isolates that were MSSP (27%; 3/11) and those that were MRSP (43%; 9/21). However, using logistic regression analysis, the odds of an

MRSP isolate coming from a dog with a history of antimicrobials within the preceding 6 weeks of culture was significantly (P = 0.0498) greater than for MSSP isolates (OR = 5.3; 95% CI = 1.1 to 26.6).

Among amikacin-resistant isolates, 66% (21/32) were concurrently methicillin-resistant while 34% (11/32) were methicillin-susceptible. Of the 32 amikacin-resistant isolates tested, the gene *aac(6')/aph(2'')* was present in 12% (4/32) isolates, the gene *aph(3')- IIIa* was present in 75% (24/32) isolates, and the gene *ant(4')-Ia* was present in 3% (1/32) isolates. There were four amikacin-resistant isolates in which none of these three genes were detected. Some isolates carried more than one gene. Representative PCR results are shown in Figure 2. There was no association between methicillin resistance and carriage of a specific amikacin resistance gene. The *aph(3')- IIIa* gene tended to be more prevalent among isolates that were MRSP (86%; 18/21) compared to MSSP isolates (55%; 6/11); however, this difference was not significant (P = 0.0877; Fisher's exact test). The proportion of MRSP isolates that carried the *aac(6')/aph(2'')* gene (5%; 1/21) was less than that of MSSP isolates (27%; 3/11); however, this difference was not significant (P = 0.1055; Fisher's exact test). The 1 isolate that carried the *ant(4')-Ia* gene was MRSP.

Discussion

Staphylococcus pseudintermedius is the most common bacterial pathogen associated with canine pyoderma and post-operative wound infections (1,2). Treatment of these infections typically involves β-lactam antibiotics such as amoxicillin and cephalexin. The spread of methicillin-resistant *S. pseudintermedius* (MRSP) across Europe led to the widespread use of alternative antibiotics such as chloramphenicol (2,10). This ultimately

led to resistance to chloramphenicol and virtually all classes of antibiotics approved for use in dogs (2,10). Methicillin resistance has begun to emerge in the United States and over the past 5 years, resistance to chloramphenicol has become common (Table 4). This has led to reliance on other antimicrobial drugs such as amikacin to treat life-threatening MRSP infections. During the past two years, aminoglycoside-resistant MRSP have been identified among patients in our small animal hospital (Table 4). Aminoglycosides like amikacin are not routinely used to treat staphylococcal infections due to potential nephrotoxic effects of these drugs and inconvenient route of administration (12,55). The increased prevalence of methicillin-resistant and multi-drug resistant *S. pseudintermedius* has left clinicians with few choices for antimicrobial therapy sometimes making aminoglycosides a last available choice for therapy.

Aminoglycosides are bactericidal agents that bind irreversibly to the 30S ribosomal subunit of susceptible bacteria thereby inhibiting protein synthesis (13). Drug inactivation by AMEs is the main mechanism of aminoglycoside resistance (14,17,19). In a study of *S. aureus*, the *aac(6')/aph(2'')* gene was found in 66% of resistant *S. aureus* isolates followed by *ant(4')-Ia* and *aph(3')- IIIa* genes with frequencies of 24% and 8%, respectively (14). Similar results have previously been found (18). In contrast, we found that in *S. pseudintermedius*, the most common amikacin resistance gene was *aph(3')-IIIa*, which was present in 75% (24/32) of amikacin-resistant isolates of *S. pseudintermedius* followed by *aac(6')/aph(2'')* and *ant(4')-Ia* genes with 12% (4/32) and 3% (1/32), respectively. The gene *aph(3')- IIIa* has been demonstrated in the chromosomal DNA of *S. pseudintermedius* strains as well as on transposons carried on plasmids (7,123,124). It is unclear whether *aph(3')- IIIa* is carried on the chromosome or

on a plasmid and whether or not it is part of a transposable element in the strains in our study. Understanding which resistance genes are present and how they are transmitted has important clinical ramifications for infected patients. Under antimicrobial selective pressure, antibiotic resistance genes can be transferred from one strain or species of *Staphylococcus* to another by plasmid conjugation, phage-mediated transduction, or transposon movement and could result in the widespread of antibiotic resistance among staphylococci (7).

Our study is limited by its retrospective nature. Patient histories were obtained solely through the available medical records. Some cases may have had previous cultures or antimicrobial therapy that was not noted in the records. Due to the use of an antimicrobial susceptibility testing system that did not measure amikacin MICs, amikacin-resistant isolates may have been missed prior to 2010. Additionally, the exclusion of 21 amikacin-resistant isolates from genetic testing because they were not stored at the time of isolation may have affected the prevalence of certain amikacin-resistance genes. Despite these limitations, we documented a 15% rise in aminoglycoside resistance at the VMTH over the past 2 years (Table 4) and determined that *aph(3')- IIIa* was the most common gene in isolates from patients presented to our hospital. Another limitation of our study is that without genetic fingerprinting, *spa* typing or other method to compare the genetic relatedness of the isolates, we cannot rule out the possibility that some of the isolates represent a nosocomial clone, particularly for the 5 MRSP isolates from inpatients that were resistant to all other drugs. Finally, this study found that doxycycline resistance was not more likely in isolates that were both amikacin-resistant and methicillin-resistant as compared to isolates that were either

amikacin or methicillin resistant. In 2013 a strong case was made for the adoption of canine breakpoints for doxycycline (susceptible, ≤0.125 µg/ml; intermediate, 0.25 µg/ml; resistant, >0.5 µg/ml) for *S. pseudintermedius* isolates instead of using the human breakpoints (susceptible, ≤4 µg/ml; intermediate, 8 µg/ml; resistant, >16 µg/ml). The majority of isolates from this study (250/422) had an MIC ≤2 µg/ml. It is possible that if we had used the more conservative breakpoints we would have found that doxycycline resistance was more likely in isolates that were both methicillin- and amikacin-resistant. Unfortunately we were unable to test this as the lowest concentration of doxycycline available on the commercial microbroth dilution system used by our laboratory is 2 µg/ml, well above the proposed breakpoint for canine staphylococcal isolates and not all of the isolates tested by the laboratory were available for re-testing.

This study also found a significant association between amikacin resistance and methicillin resistance (Table 5). Similarly, a study in *S. aureus* and coagulase negative staphylococci identified the presence of at least one AME gene associated with *mecA* in 72% of the methicillin-resistant staphylococci (14). One theory, aside from gene transfer from another source, as to why these genes seem to be commonly present together is that *mecA* and the AME genes may be located adjacent to each other on the bacterial chromosome (125,126). Among the isolates in this study, resistance to other drugs was more likely to be found in isolates that were both amikacin- and methicillin-resistant (Table 6). This may reflect development of amikacin resistance as a result of treatment of multi-drug resistant MRSP with amikacin but does not explain the finding of amikacin-resistant isolates that were susceptible to all other drugs. Within the 422 *S. pseudintermedius* isolates, there were 22 isolates that were amikacin-resistant but

methicillin-susceptible. In these isolates, it is possible, although unproven, that amikacin resistance may be plasmid-mediated. If this is true, the association between amikacin and methicillin resistance may change over time and future studies may find that amikacin resistance is not linked with methicillin resistance.

Aminoglycosides remain important antimicrobial drugs for the treatment of life-threatening infections in veterinary medicine even though resistance among species of staphylococci continues to be demonstrated worldwide (14,127). Since AMEs can be carried on plasmids or on transposable elements, it may be important to monitor aminoglycoside resistance in staphylococci over time as transmission of resistance between bacterial strains or species (for example from *S. pseudintermedius* to *S. aureus*) may represent a new nosocomial and zoonotic threat, particularly in the face of increased multi-drug resistant staphylococci (7,17,128).

CHAPTER V

INDUCIBLE CLINDAMYCIN RESISTANCE*

Introduction

Increased prevalence of methicillin resistance and multi-drug resistance in *Staphylococcus pseudintermedius* has resulted in greater use of clindamycin to treat canine pyoderma because of its perceived clinical efficacy and good distribution to the skin (9,12). Clindamycin is a lincosamide that reversibly binds to the bacterial 50S ribosomal subunit thereby inhibiting protein synthesis (20). In some cases, staphylococci may appear to be susceptible to clindamycin when tested *in vitro,* but the infected patient may fail to respond to therapy despite being treated with what seems to be an appropriate drug concentration for an appropriate duration. Lincosamides bind to the same or closely related binding sites in the bacterial ribosome as macrolides such as erythromycin. Resistance to macrolides, lincosamides, and streptogramin B antibiotics (MLS_B phenotype) can occur through acquisition of a methylase enzyme that removes a methyl group from an adenine residue in the 23S ribosomal RNA component of the 50S subunit of the ribosome (21-23). Removal of this methyl group alters the site to which the antimicrobial drug binds altering its efficacy. An active efflux pump encoded by the *msrA* gene also confers resistance to macrolides and streptogramin antibiotics but not lincosamides such as clindamycin (MS phenotype) (23).

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Approximately forty *erm* genes that encode methylases have been reported in different bacterial genera, with *ermA, ermB,* and *ermC* the most commonly found among staphylococci (24). In *S. aureus*, *ermA* and *ermC* confer erythromycin resistance in 94-98% of isolates (25). In *S. pseudintermedius*, *ermB* is primarily responsible for MLS_B resistance, but its expression can be constitutive or inducible (11). Mutation in the macrolide-inducible DNA sequence preceding *ermB* genes can alter resistance from inducible to constitutive (129). These mutations occur at a rate of about one in every 2×10^6 replications (130,131). Infections in which bacteria are present and dividing in purulent material in numbers greater than this are common, which means that these mutations readily occur, resulting in constitutive MLS resistance, and strains carrying the mutation will dominate within the bacterial population at the site of infection, particularly in the presence of antimicrobial selection pressure (129). Therefore, if bacteria carrying inducible MLS_B resistance are present in an infection, such mutations may result in constitutive MLS_B resistance leading to treatment failure. Routine antimicrobial susceptibility testing can detect constitutive MLS_B resistance but fails to detect inducible resistance (26). Detailed descriptions of the mechanisms of *erm* gene expression and mutations leading to constitutive MLS_B resistance have been previously published (129,130). Inducible clindamycin resistance can result in treatment failure and should be suspected in isolates that are erythromycin-resistant but clindamycin-susceptible on *in vitro* antimicrobial susceptibility testing. In this study we evaluated the frequency of inducible clindamycin resistance in *S. pseudintermedius* from patients presented to the Texas A&M Veterinary Medical Teaching Hospital (VMTH) by double

disk diffusion testing ("D-test") for inducible clindamycin resistance and the presence of *ermB* by polymerase chain reaction (PCR).

Materials and methods

A total of 608 canine *Staphylococcus pseudintermedius* isolates collected from the VMTH between 2007-2012 were screened for inducible clindamycin resistance. At the time of initial collection, all isolates were presumptively identified as *S. pseudintermedius* based on Gram stain, colony color, polymyxin B susceptibility, production of coagulase and catalase, and ability to grow on salt-mannitol agar. All isolates were tested for antimicrobial susceptibility using commercially available systems (VITEK, bioMérieux, Durham, NC or TREK Sensititre, TREK Diagnostics, Cleveland, OH) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for MIC testing (132). Isolates were screened to identify those that were intermediate or resistant to erythromycin and susceptible or intermediate to clindamycin. Those meeting the criteria were further tested for the presence of a positive D-test according to the CLSI guidelines (112). Quality control strains for antimicrobial susceptibility testing included *S. aureus* ATCC 43300, ATCC 25923, and ATCC 29213. Quality control strains for the D-test included *S. aureus* BAA-977 and BAA-976. All quality control strains were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Eight isolates met the screening criteria and underwent further testing. All eight were susceptible for clindamycin on the MIC panel; seven were erythromycin-resistant and one was intermediate to erythromycin. One isolate exhibited intermediate resistance to clindamycin; however, it was susceptible to erythromycin and was not tested further. Species identification of the eight isolates was confirmed by PCR

using primers and methods previously described (133). Bacterial DNA was purified for the *ermB* PCR using a DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions for gram-positive bacteria. PCR amplification of a 639 base pair product specific for *ermB* was performed using primers (5) and methods (134) previously described with an alteration in the annealing temperature to 46^0C followed by 1% gel electrophoresis. All primers and PCR reagents were purchased from Sigma-Genosys, Houston, TX and Takara Bio Company, Otsu, Shiga, Japan respectively. The resultant PCR product was confirmed as *ermB* by sequencing at the DNA Core Laboratory at the Texas A&M University College of Veterinary Medicine.

Results and discussion

The isolates in this study came from eight dogs that presented to the VMTH between February 2008 and April 2010. Two isolates were isolated from each of the following sites: infected tibial plateau leveling osteotomy (TPLO) implants, skin lesions, and the urinary tract (one from an infection and one from a bladder stone). One of the skin lesion isolates came from a dog with generalized demodicosis and deep pyoderma, and the second was collected from the pre-scrubbed surgical site for a torn cranial cruciate ligament repair. The remaining two isolates came from a blood culture and post-surgical lavage of the peritoneum following exploratory abdominal surgery.

Upon presentation to the VMTH, six of the eight dogs had received prior antibiotic therapy with one or more antimicrobial drugs within six weeks of entering the hospital. Five of these dogs were receiving antimicrobial therapy at the time of culture. One dog received erythromycin and another received clindamycin. In this study, all

isolates considered resistant or intermediate to erythromycin but susceptible to clindamycin *in vitro* tested positive for inducible clindamycin resistance by D-test and the presence of *ermB* associated with MLS_B resistance (Table 7, Figure 3). Two of the isolates were methicillin-susceptible (25%) while the remaining six were methicillin-resistant (75%).

Table 7. Inducible clindamycin resistance, erythromycin resistance, and oxacillin resistance in *Staphylococcus pseudintermedius*.

Isolate No.	MIC (µg/mL) with Interpretation[a]			D-test Result	PCR test for *ermB*
	Clindamycin	Erythromycin	Oxacillin		
11-001	≤0.5 (S)	≥8 (R)	≥8 (R)	Positive	Positive
11-025	≤0.5 (S)	≥8 (R)	≥8 (R)	Positive	Positive
11-033	≤0.5 (S)	1 (I)	≥8 (R)	Positive	Positive
11-064	≤0.5 (S)	≥8 (R)	≥8 (R)	Positive	Positive
12-012	≤0.5 (S)	≥8 (R)	≤0.25 (S)	Positive	Positive
17-016	≤0.5 (S)	≥8 (R)	2 (R)	Positive	Positive
18-007	≤0.5 (S)	≥8 (R)	≥8 (R)	Positive	Positive
24-014	≤0.5 (S)	≥8 (R)	≤0.25 (S)	Positive	Positive

[a]Minimum Inhibitory Concentration (MIC) for clindamycin, erythromycin, and oxacillin. Breakpoints for antimicrobials were from CLSI VET01-A4 (112). Abbreviations for interpretations were as follows: R = Resistant to antimicrobial; S = Susceptible to antimicrobial; I = Intermediate susceptibility to antimicrobial. Guidelines for D-test performance and interpretation were from CLSI M02-A11 (112).

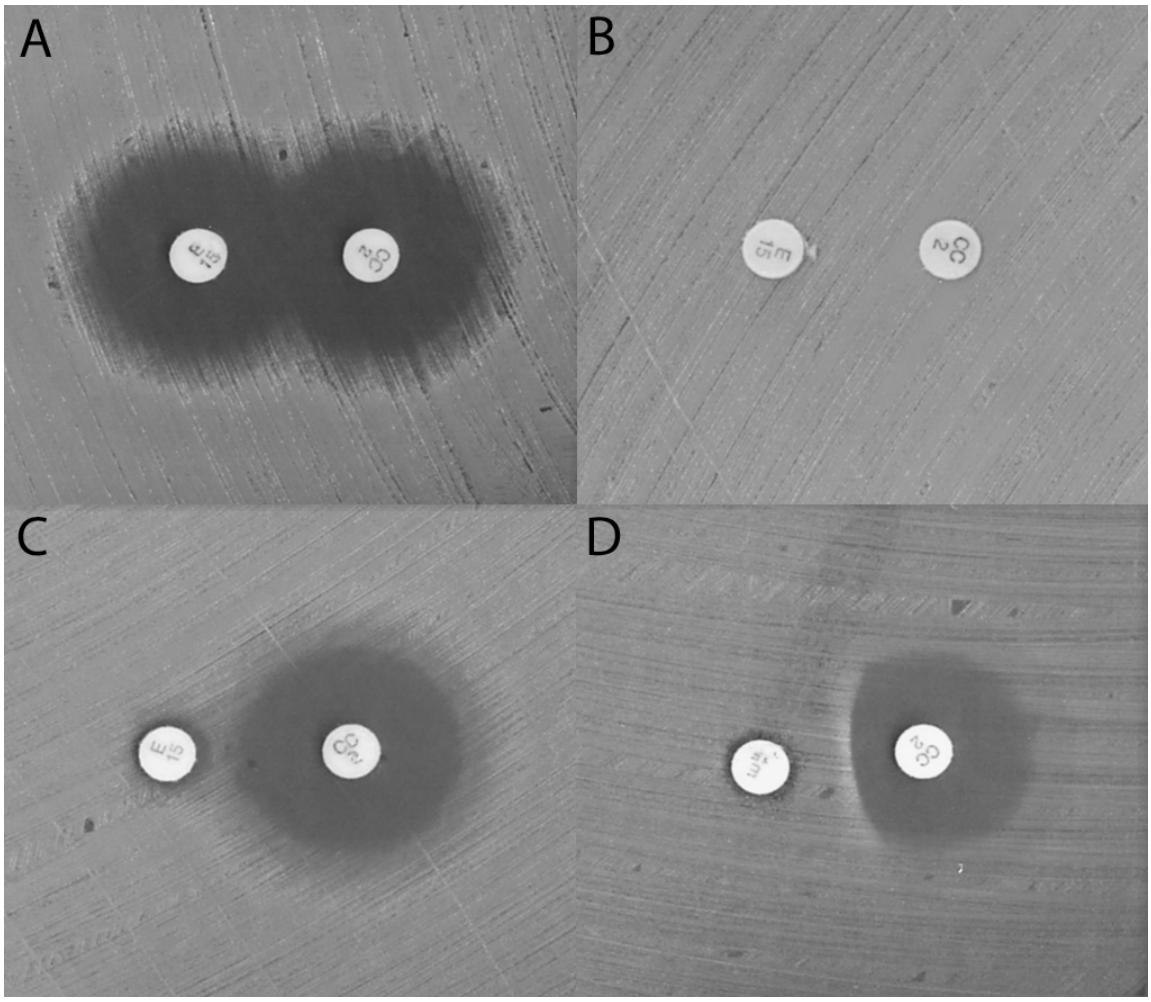

Figure 3. Disk diffusion testing for inducible clindamycin resistance of *Staphylococcus pseudintermedius* isolates. The disk labeled E15 contained15 µg of erythromycin and the disk labeled CC2 contained 2 µg of clindamycin. The disks are spaced 15mm apart; (A) ATCC 25923 *Staphylococcus aureus*, erythromycin- and clindamycin-susceptible, negative D-test. (B) *S. pseudintermedius* clinical isolate 11-012, erythromycin- and clindamycin-resistant, negative D-test; (C) BAA-976 *S. aureus*, erythromycin-resistant, clindamycin-susceptible, negative D-test; (D) BAA-977 *S. aureus*, erythromycin-resistant, inducible clindamycin-resistant, positive D-test.

S. pseudintermedius is the most common bacterial agent isolated from canine pyoderma and surgical and non-surgical wound infections (1,2). Of the eight dogs that provided isolates evaluated in this study, four had skin lesions or TPLO implant-related

surgical infections. Treatment of staphylococcal infection at these sites in dogs typically involves therapy with β-lactam antibiotics such as penicillins and cephalosporins. With increased prevalence of methicillin resistance, alternatives to β-lactam antibiotics have been sought (12,55). In addition to being resistant to β-lactam antibiotics, methicillin-resistant *S. pseudintermedius* (MRSP) strains are increasingly resistant to other antibiotics. A recent multi-center study in Europe and North America showed that MRSP isolates are commonly resistant to virtually all classes of antibiotics approved for use in dogs (2,10). Six of the isolates in this study were MRSP strains while two were methicillin-susceptible. In methicillin-resistant *Staphylococcus* from North America collected from 2006-2008, 17.7% (11/62) of *S. aureus* isolates carried inducible clindamycin resistance vs. 0% (0/46) of *S. pseudintermedius* isolates (81). In MRSP isolates from Europe and North America collected from 2004-2009 1.9% (2/103) of isolates were positive for *ermB* and displayed inducible resistance to clindamycin (11). In the study described here, the differences in inducible clindamycin resistance could be attributed to either rapid changes in antimicrobial resistance patterns or geographic differences in the occurrence of inducible resistance.

Increased methicillin resistance and inducible clindamycin resistance in *S. pseudintermedius* has significant implications for canine and human health. While *S. pseudintermedius* infection in humans is relatively uncommon, zoonotic transmission of *S. pseudintermedius* to the owner of an infected pet or veterinary staff is a potential threat (1,4). Recent studies have demonstrated that 5.3% of veterinary dermatologists and their technical staff carry MRSP and that owners of dogs with deep pyoderma can carry identical *S. pseudintermedius* strains to those carried by their infected pets (1,101).

Additionally, there is the potential for transmission of antimicrobial resistance genes from canine isolates of *S. pseudintermedius* to human isolates of *S. aureus* (135,136).

For empiric, systemic treatment of canine pyoderma, amoxicillin/clavulanate and first-generation cephalosporins are the most common first-line drugs selected (9). With increased occurrence of antimicrobial resistance, clindamycin is recommended as an appropriate, alternative choice due to its favorable safety profile, clinical efficacy, and distribution into the skin (9,12). Infections refractory to empiric therapy should be cultured and isolated bacteria tested for antimicrobial susceptibility. *S. pseudintermedius* isolates that are resistant to macrolides such as erythromycin but susceptible to clindamycin should be tested for the presence of inducible clindamycin resistance either by D-test or genetic testing. While the occurrence of such isolates is relatively low (1.32%; 8/608 in our study), failure to detect these isolates can result in treatment failures in infected patients and associated increased patient morbidity and expense for clients. In two of the cases presented here, clindamycin was used for antibiotic therapy resulting in treatment failure. Clinicians must recognize the potential for inducible clindamycin resistance and be able to recognize the potentially predictive pattern on antimicrobial susceptibility results. Performing the D-test is not a standard practice in all microbiology laboratories. The laboratory should be asked to perform this test whenever a *S. pseudintermedius* isolate is reported as susceptible (or intermediate) to clindamycin while resistant (or intermediate) to erythromycin on *in vitro* antimicrobial susceptibility tests (80).

CHAPTER VI

CONCLUSIONS

S. pseudintermedius is the most frequent causative agent of canine pyoderma and is also associated with opportunistic infections in dogs (otitis externa, urinary tract infections, and surgical and non-surgical wound infections) (1,2). Many dogs harbor *S. pseudintermedius* in their nares and elsewhere on their skin without having clinical evidence of disease and for this reason serve as a potential source of infection for other animals and humans as well (1,3,4). When empirical treatment fails or infection reoccurs, culture and antimicrobial susceptibility testing of a sample should be performed to guide selection of an appropriate alternate antimicrobial drug.

In this study, MRSP was strongly associated with clinical disease. While there was no significant difference between *sps* gene and source in this study it is interesting that the majority of isolates with multiple genes were from dogs that had pyoderma. In addition, *spsP* and *spsQ* were significantly associated with methicillin resistance. This new information may impact the development of *S. pseudintermedius* vaccines or alternative therapies for staphylococcal infections in dogs as antibiotic resistance rates are on the rise.

In recent years isolation of methicillin-resistant *S. pseudintermedius* (MRSP) from canine skin has become common and veterinarians have attempted to utilize other classes of antimicrobial drugs. Unfortunately, studies from Europe have demonstrated that 90% of MRSP isolates were resistant to clindamycin, erythromycin, and trimethoprim, 70% were resistant to both chloramphenicol and gentamicin, and 57%

were resistant to just chloramphenicol (2,10). Resistance has begun to emerge in the United States: over the past five years, resistance to chloramphenicol has become common leading to the reliance on drugs such as amikacin to treat MRSP infection (11,12). Aminoglycosides remain important antimicrobial drugs for the treatment of life-threatening infections in veterinary medicine, however, this study found a significant association between amikacin resistance and methicillin resistance. Since AMEs can be carried on plasmids or on transposable elements, it may be important to monitor aminoglycoside resistance in staphylococci over time as transmission of resistance between bacterial strains or species (for example from *S. pseudintermedius* to *S. aureus*) may represent a new nosocomial and zoonotic threat, particularly in the face of increased multi-drug resistant staphylococci (7,17,128).

Finally, the rise in MRSP cases raises a number of questions about the diagnosis, treatment, and prevention of these infections. As such it is imprudent to compound the problem by adding to patient morbidity simply due to diagnostic error. *S. pseudintermedius* isolates that are resistant to macrolides such as erythromycin but susceptible to clindamycin should be tested for the presence of inducible clindamycin resistance either by D-test or genetic testing. While the occurrence of such isolates is relatively low (1.32%; 8/608 in our study), failure to detect these isolates can result in treatment failures in infected patients and associated increased patient morbidity and expense for clients. In two of the cases presented in this study, clindamycin was used for antibiotic therapy resulting in treatment failure. Clinicians must recognize the potential for inducible clindamycin resistance and be able to recognize the potentially predictive pattern on antimicrobial susceptibility results. Performing the D-test is not a standard

practice in all microbiology laboratories. The laboratory should be asked to perform this test whenever a *S. pseudintermedius* isolate is reported as susceptible (or intermediate) to clindamycin while resistant (or intermediate) to erythromycin on *in vitro* antimicrobial susceptibility tests (80).

Because they are uniquely adapted commensal organisms, staphylococci are likely to remain a cause of opportunistic infection in humans and animals. Staphylococcal infections can range from simple skin infections and dermatologic disorders to severe systemic bacteremias that can cause multiorgan failure and death (33,42,51). Debate remains about whether there are differences between MRSA and MRSP infections in dogs with regard to severity of clinical signs and outcome. Additional research is needed to improve our understanding of *S. pseudintermedius* infection in dogs as resistance rates continue to rise rendering are current medications ineffective.

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