Molecular Epidemiology and Genetic Analysis of *Staphylococcus* species in Companion Animal Medicine

by

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Abstract

In human medicine, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the leading causes of bacterial infections in the United States (191). In veterinary medicine, an increased number of multidrug-resistant *Staphylococcus* spp. in dogs, horses, and swine have been reported (36, 220, 295). Methicillin-resistance in *S. aureus* is determined by the addition of the penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene (216, 217). The gene is carried on a large heterologous mobile element called the staphylococcal cassette chromosome mec (SCC*mec*) (114). To date, eleven SCC*mec* types have been identified in human MRSA clones, and several in staphylococci from animal origin (266). In horses and dogs, MRSA is frequently reported. Additionally, methicillin-resistant strains of *Staphylococcus pseudintermedius* (MRSP) are frequently diagnosed in dogs (220, 271, 295). The epidemiology of methicillin-resistant staphylococci from animal origin requires more investigation because of its zoonotic potential and to optimize preventative measures (14, 66, 87, 151). Using traditional microbiological methods and molecular techniques, an investigation of methicillin resistant *Staphylococci* sp isolated from equine and canine species was undertaken. Special emphasis was given to determining the most sensitive diagnostic method for recognition of methicillin-resistance (MR) in canine isolates due to the differing response of methicillin-resistant strains of *S. schleiferi* subspecies *coagulans* (MRSC) and *S. pseudintermedius* (MRSP) to cefoxitin (FOX) as compared to MRSA. From this investigation, it was hypothesized that the differences in the reaction of MRSP and MRSC to cefoxitin, when compared with MRSA, were based on

variations in their penicillin-binding proteins. Thus, an analysis of the PBPs from *S. pseudintermedius* and *S. schleiferi* subsp *coagulans* was performed. Additionally, molecular epidemiology typing methods were used and the presence of three genes associated with virulence factors (*S. intermedius* exfoliative toxin [SIET], LUK-I and panton-valentine leukocidin [PVL]) were investigated in canine *Staphylococcus* sp). Results from the pulse field gel electrophoresis of the equine MRSA indicated a highly clonal population, with a predominance of the clone USA500, followed by USA100. Typing using multiplex PCR identified a preponderance of SCC*mec* type-IV, though the SCC*mec* type-II was also detected. In comparison, the type-V cassette was present in 66% (113/171) of the canine isolates, followed by type IV at 13% (22/171). Interestingly, 11% (18/171) of the MRSC were SCC*mec* type-V and all the MRSA isolates were type II. While comparing diagnostic methods, the sensitivity and specificity of oxacillin for the detection of MR strains was 83% and 93%, respectively. However, using FOX, the sensitivity was 20% and the susceptibility was 100%. By the means of PCR, the genes that encode PVL were detected in 4% of the canine MRSA, but were not detected in *S. pseudintermedius* or *S. schleiferi* subsp *coagulans.* In contrast, the SIET and LUK-I genes were detected in the three staphylococci, *S. aureus, S. pseudintermedius* and *S. schleiferi* subsp *coagulans.* Using sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a different pattern of PBPs was found in *S. pseudintermedius* (5 proteins) and *S. schleiferi* subspecies *coagulans* (3 - 4 proteins), when compared with *S. aureus* (4 – 5 proteins). A competitive binding assay demonstrated a decreased affinity of cefoxitin towards the PBPs of MRSP as compared to MRSA. From the results of these summarized studies, it is clear that more investigation directed towards understanding the different mechanisms of resistance against

antimicrobial drugs by staphylococci from animal origin are required to address the epidemiology of *Staphylococcus* sp in veterinary medicine.

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List of Abbreviations

CA – MRSA	Community-acquired MRSA
ET	Exfoliative toxins
FOX	Cefoxitin
HA – MRSA	Hospital-acquired MRSA
LT	Leukotoxin
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSC	Methicillin-resistant *Staphylococcus schleiferi* subspecies *coagulans*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
OX	Oxacillin
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PCR	Polymerase chain reaction
PVL	Panton-valentine leukocidin
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

I. Literature review

Staphylococcus species and host specificity

Staphylococci are common bacteria isolated from the skin and mucosal microflora of mammals and birds. Although, staphylococci are considered part of their normal flora. Many staphylococcal species are considered opportunistic pathogens able to cause serious infections of the skin, tissues and body cavities (187). Members of the *Staphylococcus* genus are Gram - positive cocci, usually organized in clusters, catalase producers and aerobic or facultative anaerobes (187). Currently, 43 *Staphylococcus* species have been identified within the genus. The cell wall structure includes peptidoglycans and teichoic acid and the nucleotide Guanine + Cytosine content of deoxyribonucleic acid (DNA) is 30 – 39 mol%. Whole genome sequencing has been executed for different *Staphylococcus aureus* strains and the single genome has a size of 2.8 to 2.9 Mbp (14). Myriads of mobile genetics elements have been associated with *S. aureus* and most of them are plasmids related with the development of antimicrobial resistance (14, 220, 250).

Staphylococcus aureus is a well-known microorganism, identified as early as 1880 as a cause of mild to severe infections in humans (187). Skin infections are the most common presentation of the disease in people, although complicated clinical cases are also commonly described (14). Usually, severe infections are associated with food-borne disease, soft-tissue infection, pneumonia, toxic shock syndrome, infection of plastic implants, osteomyelitis, among others (187). Additionally, *S. aureus* has the potential to become resistant to β-lactams and other

classes of antimicrobials (82, 214). The anterior nares are the niche of *S. aureus*, for that reason people can be considered carriers of multidrug resistant strains, of which some are identified as persistent carriers by repeated isolation from the nares (14). Spread of *S. aureus* may occur from the nose to the skin and other body areas by direct contact. Usually, personnel or patients that are in contact with hospital environments have a high *S. aureus* nasal carrier rate of multidrug resistant *S. aureus* (187). Nosocomial infections are transmitted to patients primarily by medical personnel with contaminated hands or fomites (14). Nosocomial infections due to *S. aureus* have become increasingly important since this microorganism has developed resistance against multiple antimicrobials in hospital environments. Nosocomial infections caused by multidrug resistant *S. aureus* are known as hospital-acquired MRSA infections (2, 40). In particular, *S. aureus* strains that are resistant to β-lactam drugs are often described as methicillin resistant, which will be discussed further (14, 43). In human medicine, the acquisition of infection by methicillin resistant *S. aureus* (MRSA) was first linked to hospital environments or the use of antimicrobial therapy, and termed hospital-acquired MRSA (HA-MRSA). But by the mid-1990s, reports began to appear in the United States of MRSA infections occurring in patients without identifiable risk factors (healthy and no contact with hospitals environments), and were designated community-acquired MRSA infection (CA-MRSA) particularly among children (36,56,65,79).

At the present time, *S. aureus* is one of the leading causes of nosocomial infections, and a high proportion of these infections are caused by MRSA (54). In the United States, the Active Bacterial Core Surveillance (ABCs)/Emerging Infections Program Network) reported 8,987 cases of invasive MRSA from July 2004 through December 2005, of which 2,389 (26.6%) were hospital-onset infections; 5,250 (58.4%) were community-onset infections; 1,234 (13.7%) were

community-associated infections; and 114 (1.3%) could not be classified. The same study reported 1,598 in-hospital deaths among patients with MRSA infection during the surveillance period (134). Although MRSA is still considered an important threat, results from a CDC study published in 2009 indicated that invasive (life-threatening) MRSA infections that began in hospitals declined 28% from 2005 through 2008. The same study reported a 17% decrease in invasive MRSA infections that were diagnosed before hospital admissions (community onset) in people with recent exposures to healthcare settings (29). The trend of reduced MRSA is correlated with successful prevention efforts by groups of health care facilities that have implemented programs designed to improve central line insertion and care practices (29). Different studies have found that strategies for infection control precautions, such as hand hygiene, active surveillance culturing, decontamination of the environment and equipment, and institution of contact precautions with infected and colonized patients for MRSA, have contributed to reduction in the rate of MRSA infections in hospital environments (96, 188, 298).

In humans and animals, staphylococcal infections can be from exogenous or endogenous origin. Many are associated with immunocompromised patients (allergic, endocrine or metabolic conditions), trauma, or repeat infections (187, 220). *S. aureus* in animals is a commonly reported isolate. As in humans, *S. aureus* infections in animals are commonly associated with dermatitis and other skin lesions (220). In cattle, sheep, and goats mastitis of *S. aureus* origin is frequently reported. In addition, *S. aureus* is associated with botryomycosis mainly in horses (scirrhous cord) and pigs. In poultry, bumble foot, arthritis and septicemia are frequently attributed to *S. aureus*. In dogs and cats, pyoderma and suppurative lesions are also associated when *S. aureus* is reported (68, 220, 250).

Although, *S. aureus* is the clinically most important *Staphylococcus* species associated with a wide range of human and animal diseases, other members of the *Staphylococcus* genus are also clinically important in veterinary medicine (220, 250). There are animals species considered as host specific for some *Staphylococcus* sp. Also, staphylococci strain specificity by some hosts has been reported. (68, 220). A summary of the most important *Staphylococcus* species that are associated with animal diseases is presented in Table 1.

Members of the *Staphylococcus intermedius* cluster, (*S. intermedius, S. pseudintermedius* and *S. delphini*) have been associated with disease in different animal species (220, 295). For years, *Staphylococcus intermedius* (now reclassified as *S. pseudintermedius*) has been described as the most common cause of bacterial infections in the skin of dogs, producing otitis externa and pyoderma (10, 58). Canine infections caused by *S. pseudintermedius* are characterized by erythema, vesicle formation, pustules, exudates, yellow-brown crusts and the disappearance of the keratinized layer of the epidermis (184, 250). In addition, infections of other body sites and cavities can occur; such as mastitis, endometritis, cystitis, wound infection and osteomyelitis (45, 183, 220). *Staphylococcus delphini,* another group member of the *S. intermedius* cluster, has been isolated from skin infections of horses, cows, mink, and pigeons (238). This species was named after being isolated from dolphins that were living in an aquarium and suffering from multiple suppurative skin lesions (281). Some authors have suggested that *S. delphini* may be commonly misidentified as *S. intermedius* and could be more clinically important than considered at present (68, 238). The third member of this cluster, *S. intermedius,* has been recovered from the nares of horses and the upper respiratory tract of pigeons (10, 220).

S. schleiferi subsp *coagulans* is another cause of canine pyoderma and otitis externa. However, this organism is not isolated from dogs suffering first-time pyoderma and is frequently

found in animals with recurrent chronic diseases (72, 112, 168, 183, 184). *S. schleiferi* subsp *schleiferi* has also been associated with dogs suffering recurrent pyoderma. Both species are considered opportunistic pathogens in the canine, because it is believed that they are part of the normal flora of the canine skin (72, 94, 136). There is a lack of information related with the ecology, epidemiology and pathogenesis of *S. schleiferi* (94).

Staphylococcus hyicus has been occasionally isolated from cows associated with mastitis, (20, 220) from horses with dermatitis, (59) and chickens with exudative dermatitis or tenosynovitis (132). However, the most important presentation of *S. hyicus* is in swine, where it is associated with the skin disease, exudative dermatitis (Greasy-Pig Disease) (68, 250). The disease in swine is highly contagious, and in piglets under 3 weeks of age, death may occur within 12 – 24 hours. Anorexia, depression and fever, together with an extensive and greasy-dermatitis are observed in affected animals (220, 250). The primary virulence factor of *S. hyicus* is exfoliation-inducing exotoxin (218). The *S. hyicus* exfoliative toxin (SHET) exhibits a close relation to comparable exfoliative toxins produced by *Staphylococcus aureus* isolated from Staphylococcal scaled skin syndrome infections in humans (120, 121, 239). In an unpublished study at the Auburn University Large Animal Clinic (AULAC) *S. hyicus* was the predominant Gram - positive microorganism isolated from *Stomoxys calcitrans* collected from dairy cows.

With the exception of *S. schleiferi* subsp *schleiferi,* which is coagulase negative, and *S. hyicus,* which is coagulase variable, all the other staphylococci discussed above are coagulase positive microorganisms (14). Originally, only coagulase positive staphylococci were considered pathogenic. Since the 1970s, coagulase negative staphylococci have been implicated as a cause of human infections (65).

Staphylococci are considered normal flora and are considered opportunistic pathogens (14, 135). Opportunistic staphylococcal infections occur when the conditions are suitable, such as the occurrence of injuries or medical practices involving surgery, implantation of artificial prostheses, or immunosuppressive therapy (135). While it is true that there is some host specificity within the various species of staphylococci, members of the *Staphylococcus* genus can colonize and infect more than one animal species (68, 220). Staphylococci are considered ubiquitous. They are spread widely throughout different ecosystems, and subsequently cross-species contamination of staphylococci are common (135, 220). Staphylococcal strains tend to display high host specificity (135). Transmission between animal species and between animals and humans is of significant importance (68, 220). Kloos (1980) has proposed that when host specificity is high, the contaminating species will usually be eliminated within several hours or days, unless defense barriers are broken (135).

The ability of the staphylococci to adapt to different hosts has been demonstrated in cases when *Staphylococcus* species that commonly infect animals have been reported in humans. *S. hyicus* was found in a wound infection producing bacteremia after a donkey bite (201), and also in an immunocompromised patient (35). *Staphylococcus schleiferi* subsp. *coagulans* has been reported associated with wound infections in humans and also in a case of disseminated disease (104). It has been suggested that similarities in phenotypic and microbiological characteristics between *S. schleiferi* subsp. *coagulans* and *S. aureus* have resulted in frequent mistakes in identification using common laboratory methods (32, 143). The first case of *S. pseudintermedius* in humans was reported in 2006, in a patient with bacterial infection after a surgical procedure (274). Recently, this microorganism was also associated with a case of community–acquired

pneumonia (147). For years, *S. pseudintermedius* has been considered a dog-bite pathogen and zoonotic transmission between pets and owners has been reported (89, 98, 109, 257, 274).

Most common antimicrobials used in veterinary medicine

In the last century, the development of antimicrobials has had a dramatic effect on human and veterinary medicine based on the use for treatment and prevention of bacterial diseases (182). The use of antimicrobial drugs in veterinary medicine varies depending on the type of animal (companion versus production) and the number of animals to be treated and the type of production environment. Treatments may be given on an individual basis (companion animals, dairy cattle, horses and sows) and given by oral or parenteral methods. However, in most cases when large groups of animals have to be treated, as in poultry or swine production, they are applied via the water or feed (245).

β-lactam antibiotics are the most commonly used parental antimicrobials in human and veterinary medicine. The β-lactam includes penicillins, cephalosporins, cephamycins, carbapenems, monobactams and β-lactamase inhibitors (85). Together, these drugs share a common structural element, the β-lactam ring, and they have bactericidal effects (85, 220). The bactericidal result is associated with the effects of the drug on the actively growing cells. Their mode of action stems from binding to bacterial cell receptors known as Penicillin Binding Proteins (PBPs) (220, 250). The bound complex (β-lactam ↔ PBPs) prevents the synthesis and incorporation of peptidoglycan into the cell wall of susceptible bacteria and the result is death of the bacterium (85). The spectrum of action of β-lactam drugs is broad and includes Gram - positive and Gram - negative pathogens (250). Resistance to β-lactam antibiotics is mainly mediated by a large number of hydrolase enzymes called β-lactamases. These enzymes cleave

the β-lactam ring which inactivates the antimicrobial drug (37, 85). The second common mechanism that bacteria have to resist against β-lactams is related with structural changes within the PBPs, which could include the acquisition of PBPs or modification by mutations in the PBPs. The alterations of the PBPs mainly generate a reduced affinity to β-lactams (100). In Gram - negative bacteria different mechanisms of resistant to β-lactams have been described. These mechanisms consist of alterations on the outer membrane that reduces the β-lactam uptake or export by multidrug transporters (210, 245).

Glycopeptides are a group of antibiotics effective against Gram - positive bacteria. Glycopeptides are not effective against Gram - negative bacteria because the outer membrane confers intrinsic resistance to glycopeptides (220). This class of drug also inhibits the synthesis of cell wall peptidoglycan by binding the C-terminal (acyl-D-alanyl-D-alanine amino acid) of the cell wall precursor, pentapeptide. This binding prevents the addition of new units to the peptidoglycan (85). Vancomycin is the most commonly used glycopeptide, and the only one used in veterinary medicine (206). However, the use of this drug in veterinary medicine has been questioned, because in humans it is considered the "last resource" drug in cases of β-lactam resistant *S. aureus* (85).

There are a number of classes of antibacterials that inhibit protein synthesis. Aminoglycosides have a bactericidal effect on susceptible bacteria by interfering with ribosomal protein synthesis (85, 300). This group of antibacterials binds to the 30S ribosomal subunit having an effect in different steps of protein synthesis, resulting in the formation of non-functional proteins (85, 220). Amikacin and gentamicin are frequently used in veterinary medicine (206, 300). These drugs express an excellent activity against Gram - negative aerobes. Members of the *Enterobacteriaceae* and some *Mycobacterium* and *Mycoplasma* species are also

susceptible (85, 300). Resistance to aminoglycosides is mainly mediated by enzymatic inactivation, specifically by aminoglycoside-modifying enzymes (N-acetyltransferases, O-adenyltransferases or O-phosphotransferases) (248).

Tetracyclines are also antimicrobials that inhibit bacterial protein synthesis. Tetracyclines enter the bacterial cell by an active uptake process and bind to the 30S ribosomal subunit in the mRNA translation complex which prevents binding of the cell wall aminoacyl-tRNA to the mRNA-ribosome complex interaction (86, 245). Tetracyclines are broad spectrum antibiotics, which have an effect against Gram - positive and Gram - negative bacteria. In addition, tetracyclines are used in cases of *Mycoplasma sp, Ehrlichia sp, Anaplasma sp, Chlamydia* sp and *Rickettsia* sp infections (85). The two most common mechanisms of resistance are the active efflux pump mediated by the inducible genes *tetK* and *tetL* (located on plasmids) and the chromosomally encoded *tetM* (85). Tetracycline has been commonly used in feed for prophylactic medication (220). In small animals, the use of tetracyclines by oral administration is limited, therefore the use of doxycycline or minocycline is recommended (206). Tetracyclines are widely used in food animal medicine, for a variety of diseases such as tick borne infections, chlamydiosis, foot rot, mycoplasmosis, and bordetellosis (250).

Chloramphenicol is a bacteriostatic antibiotic which binds to the 50S ribosomal subunit and, similar to tetracyclines, prevents the linking of amino acids to the growing polypeptide chain (220). It is effective against a wide variety of Gram - positive and Gram - negative bacteria. In dogs and cats, chloramphenicol has the disadvantage of a narrow margin of safety and frequent administration is required to maintain adequate blood concentrations (206). Chloramphenicol is commonly used in horses (300). The use of this drug in food animals is prohibited in the United States (85). Thus, florfenicol was developed as an injectable alternative

for use in cattle and pigs. Florfenicol is an analog of chloramphenicol with a similar mechanism of action, but lacks the associated bone marrow suppression found with the use of chloramphenicol. Florfenicol has been used exclusively in veterinary medicine (85, 205). Resistance against chloramphenicol and its analogs is plasmid-mediated or chromosomally-based (220). Enzymatic inactivation of the chloramphenicols is mediated by the inactivation of the drug by a specific acetyltransferases. The acetyltransferases (Cat) are capable of transferring acetyl groups to the C1 and C3 positions of the chloramphenicol molecule, and the acetylated chloramphenicol molecules cannot inhibit bacterial protein biosynthesis (186). Another described mechanism of resistance is a decreased intracellular chloramphenicol absorption and accumulation resulting from mutations causing reduced expression of a major outer membrane transport protein (186). The specific chloramphenicol exporters as encoded by genes *cmlA* and *cmlB* are also associated with the development of resistance against chloramphenicol (86, 245).

Macrolides are another group of antimicrobials that cause inhibition of protein synthesis through binding to the 50S ribosome subunit resulting in inhibition of translocation and protein synthesis (85). Erythromycin, oleandomycin, triacetyl-oleandomycin (troleandomycin), carbomycin, spiramycin, tylosin, rosamicin, azithromycin, clarithromycin, dirithromycin and others are members of this group (4, 85). The spectrum of activity of the macrolides covers mainly aerobic Gram - positive cocci and bacilli. However, these drugs are not active against most aerobic, enteric, Gram - negative bacilli (4).

Erythromycin is one of the most common macrolides used in veterinary medicine, and a major indication is for the treatment of *Rhodococcus equi* pneumonia (300). In addition, erythromycin is the most active macrolide against *Staphylococcus* sp, and is effective against *Clostridium perfringens* and *Corynebacterium diphtheriae* (4). Although macrolides are not

very active against most Gram - negative bacilli, modest activity against *Haemophilus sp, Bordetella pertussis, Pasteurella multocida,* and *Neisseria gonorrhoeae* have been reported (4, 124). Macrolides have also exhibited moderate activity to some intracellular and atypical organisms such as *Chlamydia* sp, *Ureaplasma* sp, *Leptospira sp, Mycobacteria* sp, *Campylobacter* sp, and *Mycoplasma pneumonia* (4, 85, 124).

The use of erythromycin in large animals is limited, because the irritating effects when it is injected intramuscularly and the toxic effect in ruminants if given orally (85). Other alternative macrolids, such as tylosin and tilmicosin, are used exclusively in veterinary medicine (85, 250). Tylosin has been used in ruminant, swine and poultry medicine. Tilmicosin is recommended in cases of bovine respiratory disease, however, it can be lethal when applied intramuscularly to horses or swine (250). Resistance to macrolides is chromosomal-dependent or plasmid-mediated, and involves the alteration of the binding site on the 50S ribosomal subunit (220).

Lincosamides are bacteriostatic drugs that interfere with the bacterial protein synthesis by binding the 50S subunit of the 70S ribosome (85). Drugs in the lincosamide group have a moderate spectrum of action. In veterinary medicine clindamycin, lincomycin and pirlymicin are the most commonly used (250). Clindamycin is primarily used in small animals for treatment of anaerobic or staphylococcal soft tissue infections (250). Lincomycin is commonly used in swine to control dysentery, erysipelas and mycoplasmosis (85, 250). Finally, pirlymicin is used as an intramammary infusion to treat mastitis in cows for Gram - positives (*Staphylococcus* sp and *Streptococcus* sp) (85). The mechanism of resistance against lincosamides is due to a mutation of the binding site (similar to resistance found in macrolides). The resistance mechanism is

related with an interference with methylation of the 23s binding site. If this mutation occurs, then the bacteria are resistant to both the macrolides and lincosamides (85, 245).

There are other antimicrobial classes that inhibit the nucleic acid synthesis; sulphonamides, rifampin, quinolones, novobiocin and nitroimidazoles belong to this group (85). The quinolones inhibit the nucleic acid synthesis by blocking the DNA gyrase, the action of type II topoisomerases, and the topoisomerase IV.(131, 230, 254) The DNA gyrase is a tetrameric enzyme composed of two A and B subunits encoded by *gyrA* and *gyrB*, respectively. The main function of this enzyme is to catalyze the negative supercoiling of DNA (110). Quinolones have a wide spectrum of activity and have been extensively used therapeutically. Antibiotics of the fluoroquinolone class include enrofloxacin, ciprofloxacin, orbifloxacin, marbofloxacin, norfloxacin, danofloxacin and several others (85). Enrofloxacin is the most common used fluoroquinolone in veterinary medicine. This drug is approved for use in small animals and cattle in the US and is occasionally used in an extra-label manner to treat infections in horses (300).

Fluoroquinolones have been successfully used against Gram - negative aerobes such as members of the *Enterobacteriaceae* and *Pseudomonas aeruginosa*, and it is also a good alternative to treat *Mycoplasma* sp and *Rickettsia* sp infections (85, 300). These drugs are also active against intracellular microorganisms and although less effective against Gram - positive aerobes, many isolates of *Staphylococcus* sp are susceptible (300). Bacterial resistance to fluoroquinolones is associated with multiple mechanisms. One of these mechanisms is the mutational alteration of the target structures mainly involving genes *gyrA* and *gyrB* (coding for DNA gyrase), and *parC* and *parE* (coding for DNA topoisomerase IV) (44). Also, efflux systems are used for Gram - positive and Gram - negative bacteria as a mechanism which confers

fluoroquinolone resistance (85, 245). Finally, the decreased drug uptake in Gram - negative bacteria is also associated to fluorquinolones resistance. This mechanism consists of MAR-mediated down regulation of OmpF porin production (221).

Trimethoprim and sulfonamides are usually used in a synergistic combination. These drugs act by blocking different enzymatic steps in tetrahydrofolate biosynthesis (245). Sulfonamides are synthetic bacteriostatic and are structural analogs of *p*-aminobenzoic. Their mechanism of action involves the competitive inhibition of the conversion of *p*-aminobenzoic acid (PABA) to dihydropteroate which bacteria need for folate synthesis and ultimately purine and DNA synthesis (85). Trimethoprim inhibits the dihydrofolate reductase enzyme and exerts a synergistic action with sulfonamides which competitively inhibits incorporation of PABA into folic acid (85, 300). These drugs have a broad spectrum activity against many Gram - positive and Gram - negative aerobes. In some cases, *Pseudomonas* sp, *Mycoplasma* sp, and many isolates of *Klebsiella* sp are resistant (300). Acquired resistance is plasmid mediated or chromosomally-based, and some bacteria are intrinsically resistant (220). Acquired resistance may be due to plasmid-encoded dihydropteroate synthetase or dihydrofolate reductase (DHFR) enzymes, which make the bacteria resistant to sulfonamides and trimethoprim, respectively (245). Intrinsic resistance to both, sulfonamides and trimethoprim, can be related to outer membrane impermeability (*Pseudomonas aeruginosa*), use of exogenous folates (enterococci, lactobacilli), and also the DHFR enzymes of several bacterial genera (*Clostridium*, *Neisseria*, *Brucella*, *Bacteroides* and *Moraxella*) which exhibit a low affinity for trimethoprim (85, 221).

Rifampicin is a bactericidal antibiotic that acts by inhibiting the bacterial β-subunit of RNA polymerase, the enzyme that catalyzes transcription of RNA to DNA (85). The efficient intracellular penetration has made this drug valuable for treating intracellular bacterial infection

in humans and animals (206). Gram - negative bacteria are relatively impervious to this enzyme, therefore most are resistant. Rifampin is one of the most active known antimicrobials against *Staphylococcus aureus, Streptococcus* sp *Rhodococcus equi*, *Mycobacterium* sp and *Corynebacterium* sp. Rifampicin resistance is mediated by mutations in the *rpoB* gene encoding the β-subunit of RNA polymerase (106).

Mechanisms of antimicrobial resistance and methicillin- resistance in *Staphylococcus aureus* and other *Staphylococcus* species

One of the most important aspects of staphylococcal infections in human and veterinary medicine is the potential for *Staphylococcus* sp to express resistance to antimicrobials. The acquisition of antimicrobial resistance against β-lactam drugs in *S. aureus* is explained basically by two mechanisms, the production of β-lactamases and the expression of an additional Penicillin-Binding Protein (PBP). β-lactamases are enzymes that hydrolyze penicillin and are encoded by the *blaZ* gene in the *S. aureus* genome (85). The expression of penicillin-binding protein 2 (PBP2' or PBP2a) is encoded by the *mecA* gene. The *mecA* gene is carried by a mobile genetic element, the staphylococcal chromosomal cassette *mec* (SCC*mec*). These are the primary mechanisms causing resistance against the β-lactam antibiotics in MRSA (118, 158, 187, 252).

β-lactamases have evolved from PBPs, (37) which are enzymes that participate in the cell wall synthesis and cell septation in different bacteria (84, 258). Through an evolutionary process PBPs have developed covalent modifications stimulated by penicillin and other β-lactam antimicrobials. The result is the origination of a diverse number of β-lactamases (37). Some *S. aureus* strains are capable of producing increased levels of β-lactamases. These strains are

considered hyper-producers of β-lactamases and phenotypically are in the borderline between susceptible and resistant (172). As mentioned previously, the production of β-lactamases is encoded by the *blaZ* gene, which is controlled by the *blaR1 – blaI* element located upstream of the structural gene *blaZ* (99). The *blaR1* is a sensor-transducer and *blaI* is a repressor. In the presence of β-lactams, *blaR1* binds the drug and a transmembrane signal results in the removal of the *blaI* from the DNA binding site. This mechanism induces the transcription of the structural and regulatory genes by autoregulation (99, 215). In the presence of β-lactams, BlaR1 is continuously synthesized. Once the extracellular concentration of β-lactams decreases, *blaR1* is auto-down regulated and the intracellular concentration of uncleaved BlaI increases and is available to bind DNA specific sites (302).

The *mecA* gene expression is mainly regulated by the *mecR1-mecI* element, located upstream of *mecA* in the SCC*mec* (99, 252). However, both *mecI – blaI* repressors can block the transcription of *mecA* or *blaZ.* The blocking occurs by the binding of homodimers to palindromic sites within the promoter/operator regions of these genes (99, 153, 215). The functioning of the *mecR1 – mecI* element is similar to the autoregulation of the *blaR1 – blaI* system (215). In the presence of β-lactams, the *mecR1* leads to proteolytic autocleavage of its cytoplasmic domain. The autocleavage generates a signal, which is followed by cleavage of the cognate repressor (*blaI* or *mecI*, respectively) and the induction of transcription of *blaZ* or *mecA* (153, 302). The product of the transcription of the *mecA* gene is the PBP2a which has low affinity for β-lactam antibiotics (216, 224).

In addition to the β-lactams, many MRSA strains exhibit resistance against other antimicrobial classes. This microorganism has developed mechanisms of resistance against virtually all classes of available Antimicrobials. For this reason, they are now defined as multi-

drug resistant *S. aureus* (MDRSA) (215). In human medicine, the glycopeptide vancomycin has been the drug of choice in complicated cases of MRSA. Vancomycin is one of the few antibiotics that have remained effective against MRSA (215). However, after 40 years of use, a vancomycin resistant strain with reduced affinity to vancomycin was reported by the Clinical and Laboratory Standard Institute (108). The organism was categorized as vancomycin intermediate resistant *S. aureus* (VISA). VISA has become prevalent worldwide (46). The mechanism of resistance has been related with changes in the cell wall (46, 215). Cui *et al.,* (2006), concluded that clogging of the cell wall occurs by vancomycin molecules, which causes the occurrence of a disruptive anomalous diffusion of vancomycin through the VISA cell wall. They also described that the thickened cell wall of VISA could protect ongoing peptidoglycan biosynthesis in the cytoplasmic membrane from vancomycin inhibition, allowing the cells to continue producing nascent cell wall peptidoglycan and thus making the cells resistant to vancomycin. These two mechanisms prevent vancomycin from reaching its true target in the VISA strains (46).

Three related erythromycin ribosomal methilase genes, *ermA, ermB* and *ermC* alter the ribosomal target site and confers resistance of *S. aureus* against macrolides, lincosamides and streptogramin B (149). In *S. aureus* the *erm* genes are carried in transposons or plasmids. When the genes are expressed and methylase is produced an *in vitro* resistance against macrolides, lincosamides and streptogramin B is expressed (200). Aminoglycosides, specifically gentamicin, have been used to treat serious staphylococcal infections. Kelamni *et al.,* (2008) examined 6 MDRSA and reported the existence of an aminoglycoside-modifying enzyme (AME) coding genes in 3 of the 6 isolates. In addition, 2 isolates possessed a bifunctional AME coding gene *aac*(6')-*aph*(2''), and *aph*(3')-III. One of the strains showed high-level resistance to gentamycin and streptomycin, and the second one, a methicillin susceptible (MS) *S. aureus* (MSSA)

possessed *aph* (3')-III and exhibited low level resistance to gentamycin, streptomycin, and kanamycin (129).

The resistance of *S. aureus* against trimethoprim-sulfamethoxazole has been associated with a single amino acid substitution in the plasmid-mediated or chromosomal-based DHFR gene (49, 50). Although, it varies worldwide, in general the resistance against trimethoprim-sulfamethoxazole is low in the industrialized world and higher in developing countries. Pappas *et al.,* (2009) have indicated that trimethoprim-sulfamethoxazole is one of the numerous overlooked therapeutic options for MRSA decolonization and is recognized as potentially effective in preventing MRSA infections in certain settings (207). In *S. aureus*, the resistance against tetracycline is mediated by the *tetK* and *tetM*. The *tetK* gene mediates the active efflux pump and is typically located on plasmids (265). The chromosomally encoded *tet*M confers resistance to almost all tetracyclines (21).

The resistance against fluoroquinolones in *S. aureus* is mainly associated with mutations within the *gyrA* and *gylA* genes. The *gyrA* gene encodes for one of the subunits of the DNA gyrase, and *gylA* for one of the topoisomerase IV subunits. Mutations at these loci reduce drug binding to the target proteins (230, 259). A second described mechanism of resistance against fluoroquinolones is related with the NorA efflux pumps in *S. aureus*. NorA is an ATP-dependent efflux pump capable of pumping out hydrophilic quinolones like enoxacin or norfloxacin, but not affecting the hydrophobic quinolones such as sparfloxacin (231). *S. aureus* has also developed resistance against rifampin which is mediated by multiple mutations of the *rpoB* gene (299).

There have been several studies published investigating the mechanisms of resistance against antimicrobials exhibited by *S. hyicus* were investigated. A number of small plasmids

carrying antibiotic resistance genes were detected in a study comparing isolates of *S. hyicus* from diseased and healthy pigs. (296) Also a plasmid of 11.5 kbp, mediating tetracycline and macrolide/lincosamide resistance, was found more often in isolates from diseased pigs, but genes coding for virulence factors were not found to be located on this plasmid (244).

In 2005, it was shown that the Cfr protein represents a methyltransferase which modifies the 23S rRNA (128). This Cfr-mediated modification affects the binding of phenicols to the bacterial ribosome. Additionally, the binding of another four different antimicrobial classes, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A were affected (159). The *cfr* gene was detected in staphylococcal isolates of animal origin; *S. hyicus*, *Staphylococcus warneri*, and *Staphylococcus simulans*. The *cfr* gene was located either on the 17.1-kb plasmid type pSCFS1 or on the 35.7-kb plasmid type pSCFS3 (128). Most recently, Kehrenberg *et al.*, (2007), reported the detection of the entire co-integrate structure on plasmid pSCFS6, and concluded that it might explain a potential IS21-558-mediated transfer of the resistance genes *cfr* and *lsa*(B). This occurrence could facilitate the further dissemination of the multidrug resistance *cfr* gene (127).

The resistant to tetracycline was investigated in staphylococcal species from animal origin (*S. aureus*, *S. hyicus*, *S. intermedius*, *S. lentus*, *S. sciuri*, and *S. xylosus*) (246). The *tetL* genes were originally detected in all the examined staphylococcal species. In the same study, the *tetO* gene was found in a single *S. intermedius* isolate (246). Resistance against macrolides has been related with the occurrence of the different *erm* genes among animal staphylococci. The *ermC* genes were frequently seen in *S. hyicus* and other porcine staphylococci, while *ermB* genes were the dominant *erm* genes in canine *S. intermedius* isolates (61). Genes of the class *ermF* have so far only been detected in a few *S. intermedius* isolates from pigeons (42).

Resistance to gentamicin, kanamycin and tobramycin is mediated by the *aadA-aphD* gene, also known as *aac* (6‘)-*aph* (2‘‘), which encodes a bifunctional enzyme with acetyl- and phosphotransferase activity. This gene is associated with the composite transposon Tn4001 (227). It has been determined that plasmid and chromosomal loci of this transposon in staphylococci ocurred as the result of conjugative transfer events between enterococci and *S. aureus*, *S. intermedius* and *S. hyicus* isolates (196). In *S. intermedius* isolates from pet animals, Tn*5404*-like elements carrying the *aphA-3* (also referred to as *aph [3’]-IIIa*), *sat4*, and *aadE* (also referred to as *ant [6]-Ia*) genes were detected, which expressed resistance to kanamycin/neomycin, streptothricin, and streptomycin, respectively (23).

In a study in which *S. intermedius* isolates from canine origin were characterized, it was observed that all multiresistant strains produced β-lactamases. In this study, it was postulated that the resistance determinants were plasmid-localized (214). However, in other studies, only small numbers of plasmids could be found in *S. intermedius* isolates (93, 297).

The mechanism of resistance against fluoroquinolones in *S. intermedius* and *S. schleiferi* subsp *coagulans* was studied by Intorre, *et al.* (2007) (113). Nucleotide sequencing of the quinolone-resistance determining regions of the *gyrA* and *grlA* genes identified that dichotomous resistance to fluoroquinolones was associated with the occurrence of one alteration in GyrA-84 and one in GrlA-80 in *S. intermedius*, while in *S. schleiferi* the same pattern of resistance was observed in isolates showing these changes only in *gyrA* gene region (113). Descloux *et al.*, (2008) also revealed mutations in the *gyrB*/*gyrA* and *grlB*/*grlA* loci involved in fluoroquinolone resistance (55).

MRSA SCC*mec* and its molecular epidemiology human, horses and dogs

As previously discussed, in human medicine the acquisition of infection with methicillin resistant *S. aureus* was first related with hospital environments and the use of antimicrobial therapy, which was defined as hospital-acquired MRSA (HA-MRSA) (236). In the mid-1990s, reports of MRSA in the community began to appear in the United States, particularly among children. These reports were not hospital related and were defined as community-acquired MRSA infections (CA-MRSA) occurring in patients without identifiable risk factors (158, 187). There have been several different methods described for the epidemiological typing of MRSA (253). Using a popular method, SCC*mecA* typing, eleven different forms of this mobile unit have been identified (114, 266). SCC*mec* types-I, II and III are commonly associated with HA-MRSA strains, whereas types IV, V, and VI are generally characteristic of CA-MRSA (235, 253, 262). The first CA-MRSA clones where SCC*mec* type-IV, which were smaller than the cassettes described in HA-MRSA clones. In addition, most of the CA-MRSA strains with the SCC*mec* type-IV were only resistant to β-lactam antibiotics and contained the genes (*lukS-PV* and *lukF-PV*) for Panton-Valentine leukocidin (PVL) toxin (190). An example of an important CA-MRSA clone is the USA300 in the United States, which was reported by the Centers for Disease Control and Prevention. USA300 represents a major health problem and most of the USA300 clones carry the PVL genes (171, 190, 261).

The SCC*mec* type-I, II and III were first described by Ito *et al*. using 38 epidemic MRSA strains isolated from 20 countries. The type-I was isolated in 1961 in the United Kingdom (UK), and become disseminated worldwide in the 1960s. SCCmec type II was found in 1982 in Japan, and this New York/Japan clone has spread around the world. Finally, the MRSA strain harbouring SCCmec type III was discovered in 1985, New Zealand (115).

MRSA strains that carried the SCC*mec* type-V were first described in a community from Australia and were characterized as CA-MRSA (116). The SCC*mec* type-VI was found and characterized using the prototype of the "pediatric" MRSA clone (sequence type 5), which was dominant in a pediatric hospital in Portugal in 1992, and also detected in Poland, Argentina, Colombia, and the United States (234). In 2005, a CA-MRSA found in Taiwan was considered a variant of the type-V, which was described as PVL-positive and classified as SCC*mec*V_T (26), but later in 2008 was reclassified as SCCmec type-VII (107). Zhang *et al.*, (2009) reported the finding of SCC*mec* type-VIII which was identified in a Canadian MRSA epidemic strain (303). SCC*mec* type-IX and X, have been described recently in CC398 MRSA strains (154). The origins of the CC398 strain were isolated from livestock, particularly pigs (154). Finally, the SCC*mec* type XI has been proposed by Cuny *et al.,* who described a new cassette in the human MRSA clone CC130 (48). The MRSA CC130 strain has been reported from the UK and Denmark in cattle and humans (48).

MRSA from equine origin have been investigated multiple times, and the SCC*mec* type – IV has been considered most prevalent (290). Previous studies have reported the clone USA500 or Canadian MRSA-5 as predominant in horses and horse personnel in some geographical areas (6, 41). This clone is characterized by the possession of the SCC*mec* type-IV and the lack of PVL genes. In addition, it is hypothesized that this clone is a derivative of a human epidemic clone which only accounts for a small percentage of human infections (5, 6, 41, 156, 182, 198). Some authors have hypothesized that the human USA500 clone has become somewhat adapted to horses (47, 182, 288, 291). Anderson *et al.*, have mentioned that it is impossible to infer the origin of MRSA in horses colonized by the USA100 clone (6).

The SCC*mec* has been investigated in canine MRSA and types II, III and IV have been reported (12, 25, 94, 163, 222). The SCC*mec* type-III and -IV have been described in MR *S. schleiferi* subsp *coagulans* (MRSC) from canine origin (73, 226), whereas in MR *S. pseudintermedius* (MRSP) a variant labeled SCC*mec* type II-III has been reported (55, 237). In addition, the SCC*mec* type-III and type-V also have been identified in MRSP (73, 180, 222, 233, 237).

The typing of the SCC*mec* together with PFGE has been used for epidemiological surveillance documenting the evolution of human MRSA infections. Using these techniques MRSA clones were classified as HA-MRSA and CA-MRSA (171, 261). However, in evaluating such data, it is important to understand that the epidemiological definitions pertaining to MRSA infections in humans have changed in recent years (36, 253). Currently, an infiltration of classical CA-MRSA strains in healthcare settings has been demonstrated in the USA and in other countries (202, 253). Also, there is a recognized increase in the number of MRSA from livestock, primarily identified in swine and veal calves (139, 278). Most of the reported MRSA cases in livestock have been associated with certain clonal MRSA strains, the sequence type (ST) or clonal complex (CC) 398 (268). The MRSA ST398 has also been reported in horses, poultry and companion animals (194, 269). Additionally, zoonotic transmission of the MRSA ST398 clone has been demonstrated (87, 289).

Thus, a new MRSA epidemiological classification system has been created in human medicine. Hospital-acquired MRSA encompasses the classical scenario, where the patient acquires the infection in a healthcare setting, with an onset at least 48 hours after admission. Different risk factors may be involved, such as prolonged hospital stay, hospitalization in intensive care unit, prolonged antibiotic treatment, among others (29, 236). The CA-MRSA,

which emerges in the community, is developed by patients that lack the risk factors associated with HA-MRSA. However, some risk factors could be related, such as close contact in military settings, day-care centers, sports settings and prisons institutions (236). Two new definitions are currently in use, healthcare-associated MRSA (HCA-MRSA) and livestock-associated MRSA (LA-MRSA). The former is associated with outpatients, infected or colonized by HA-MRSA strains, and associated with previous hospitalizations, receiving home nursing, attending centers for dialysis and/or diabetes (13). Finally, LA-MRSA is currently related with the clonal spread of the MRSA ST398, which has been mainly detected in Europe, although infections have also have been reported in the USA and Asia (178). Usually MRSA ST398 is associated with asymptomatic colonization, however clinical cases ranging from soft tissue and skin infections to endocarditis, pneumonia, and necrotizing fasciitis, have been described (103, 142, 165, 203, 289).

Penicillin-Binding Proteins in *Staphylococcus species*

Penicillin binding proteins (PBPs) are enzymes that participate in the cell wall synthesis and cell septation in different bacteria. These enzymatic proteins are anchored in the cytoplasmic cell membrane and are involved in the last steps of cell wall peptidoglycan synthesis. The natural substrate of the PBPs is a highly conserved serine, specifically the C-terminal D-Ala-D-Ala. This portion is excised from the disaccharide-pentapeptide building block of the cell wall. When a β-lactam antimicrobial is present, the PBP active site forms an ester with the carbonyl group of the β-lactam ring. The β-lactam formed ester is then hydrolyzed at a slow rate in a manner which renders the PBP nonfunctional (169).

Most bacterial species possess multiple PBPs. The PBPs are differentiated based on their enzymatic function, molecular weight, and affinity for β–lactam antibiotics. Alterations of PBPs have been found to mediate high-level β-lactam resistance in staphylococci, enterococci, and pneumococci (169, 306) Based on molecular weight there are two groups of PBPs, low-molecular-weight and high-molecular-weight. Usually each bacterial species has a set of PBPs that are targeted by β-lactam antibiotics, each with a different degree of affinity (166). The low-molecular-weight PBPs are not considered essential for the bacteria cell growth and usually have transpeptidase or transglycosilase function. High-molecular-weight PBPs are divided in two classes, A and B. Each class is composed of two domains, a transpeptidase functional activity (C-terminal domain) and a second domain with a poorly defined function. It has been suggested that the class A high-molecular-weight PBPs have transglycosilase activity (N-terminal domain) (169, 306). In *S. aureus*, the loss of the PBPs catalytic activity impairs the ability of the bacterium to control cell wall integrity.

Most bacteria have a set of PBPs composed of four to eight PBPs (166). Therefore by convention, the PBPs are differentiated according to molecular weights and designated numerically in order of decreasing molecular mass (166, 306). In addition, the genes that encode the PBPs are specific to each bacterial species and are considered to be unique. Although, it is evident by nucleotide sequencing that loci of PBPs have a high degree of homology among different bacterial species, they are considered unique (166). It has been previously documented that the production of an altered PBP and may result in the development of acquired resistance against β-lactam antimicrobials (215).

Four native PBPs have been described in *S. aureus*, designated as 1, 2, 3 and 4 based on molecular weight. In addition, most MRSA strains express the PBP2a, which has low affinity

for β-lactam antibiotics (216, 224). In *S. aureus*, the PBP2 is particularly important because this protein is bifunctional (transglycosilase and transpeptidase activity) and plays an important role in the cross-linking and elongation of peptidoglycans that constitute the bacterial cell wall. As mentioned previously, when β-lactam drugs acylate the transpeptidase active site of PBP2, there is an inhibition of the peptide cross-linking which produces a bactericidal effect. However, when *S. aureus* is carrying the *mecA* gene, and PBP2a is expressed, the transpeptidase activity of this protein together with the transglycosilase activity of the remaining PBP2 cooperates to enable the cell-wall synthesis and bacterial growth in the presence of β-lactam antibiotics (169, 193, 216).

As described, the PBPs in *S aureus* have been well studied. In contrast, information relative to PBPs in common staphylococci from animal origin is limited. In 1985, Canepari *et al.* studied the PBPs pattern of *Staphylococcus* species from animal origin (34). Identical three-band PBP patterns (molecular weights: PBP1, 85Kd; PBP2, 82Kd and PBP3, 79 Kd) were identified within *S. intermedius* strains examined using radiolabeled-penicillin. In *S. simulans* a three-band pattern was also observed (molecular weights: PBP1, 83 Kd; PBP2, 80Kd and PBP3, 79 Kd) (34). In the same study, identical two-band PBP patterns (PBP1, 85; PBP2, 79 Kd) were displayed in two *S. delphini* and only one PBP (79 Kd) was detected in the membrane preparation of *S. hyicus* strains tested (34). Varaldo *et al*, (280) investigated the PBPs in two *S. hyicus* species and identified that *S. hyicus* subsp. *hyicus* strains had only one PBP (79 Kd) while the *S. hyicus* subsp *chromogenes* strains had three distinct PBPs (PBP1, 84 Kd; PBP2, 82 Kd and PBP3 79 Kd) (280).

Historically, the identification of PBPs was based on the observed electrophoretic pattern exhibited by proteins labeled with radioactive penicillin (169, 219, 306). Typically, bacterial

membranes or whole bacteria were incubated with ^{3}H- or ^{14}C-labeled penicillins and separated through sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on their molecular mass. Competitive assays have been used to obtain PBP binding affinity data for individual β-lactam compounds as well as the kinetics of binding in the study of PBP–β-lactam interactions (219, 306). This traditional technique has some disadvantages in that it is time consuming, it involves accumulation of hazardous materials and it is not commercially available (79, 305). In 1999, Zhao *et al.*, (305) introduced the use of Bocillin FL (Boc-FL) which is a commercially synthesized fluorescent penicillin and available as a labeling reagent for PBPs (Molecular Probes, Inc., Eugene, Oregon.). The use of Boc-FL to label PBPs also requires electrophoretic separation by SDS-PAGE followed by the detection with a FluorImager or under UV light (79, 305).

Although the expression of PBP2a by the SCC*mec*A is considered the most important element associated with β-lactam resistance, there are studies that demonstrate that other PBPs are involved in the production of different levels of resistance against β-lactams. Memmi *et al.*, (2008), demonstrated that PBP4 is essential for β-lactam resistance in community-acquired MRSA. PBP2a lacks transglycosilase activity. As a consequence, cell wall biosynthesis in MRSA is completely dependent on the cooperative function of the transpeptidase domain with the transglycosilase domain or with other PBPs (175). PBPs 1, 2 and 3 have each been characterized as essential and antibacterial activity has been attributed to drug binding by these PBPs (82, 169).

Different degrees of methicillin resistance can be found in MRSA and it has been suggested that additional factors are involved in the expression of high-level methicillin resistance (18, 52, 141). Some of those factors are expressed during cell wall synthesis and regulation or play a role

in the stress response. Some of the most studied resistant mechanisms are the factor essential methicillin genes (*fem genes*). It is suggested that approximately 30 *fem* genes participate in the cell wall synthesis (18, 215). The most studied are *femA* and *femB.* The *femA* gene has been considered essential for the expression of complete oxacillin resistance in *S. aureus* (1, 138, 155, 252). Myriads of genes such as *sgtB, murZ, tcaA, psrA, htrA, fmt,* and *vraS* are expressed when *S. aureus* is exposed to different classes of antibiotics. The finding of the *fem* genes, as well as the other genes, has led to the identification of the "cell wall stimulon" (267). The cell wall stimulon includes genes encoding proteins involved in peptidoglycan biosynthesis in *S. aureus* and other bacteria which are down or up regulated in response to challenge with cell wall-active antibiotics (267). The conclusion from the numerous investigations is that the response of *S. aureus* to cell wall active antibiotics is multifactorial (158, 169).

Molecular typing

It is well known that MRSA is a world-wide phenomenon. Human infections by MRSA are difficult to treat, costly and in some cases become complicated ending in patient death. Initial nosocomial infections identified as HA-MRSA required epidemiological studies to understand the origin and significance of such outbreaks (62). Later, the appearance of CA-MRSA required the improvement and/or development of molecular techniques to distinguish isolates that were epidemiologically related from those that were unrelated. Effective methods of MRSA control are needed and to develop such it is important to fully understand the epidemiology of MRSA infections (63, 284).

Various molecular techniques have been developed to investigate MRSA disease. In this review, only the most commonly used methods will be discussed. All techniques have strengths and weakness. Some are only proper to investigate local outbreaks. More sophisticated techniques involving complex algorithms and statistical analysis are required to compare worldwide MRSA clones and establish an informational database (63).

Multilocus Sequence Typing:

Multilocus Sequence Typing (MLST) is the reference method for defining the core genetic population structure of *S. aureus.* The technique consists of the analysis of the standard polymorphism that can be found in the nucleotide sequence of seven housekeeping genes (64). Each new sequence is identified as a new allele, and the combination of the 7 alleles forms the allelic profile or sequence type (ST) (63, 253). Nucleotide sequence of both strands is analyzed to generate an allelic profile. For example, the sequence type (ST) 1 has the allelic profile 1-1-1-1-1-1-1 in the *S. aureus* scheme. A web-based database is available for forming the basis of the analysis, www.mlst.net.

The current MRSA nomenclature is based mainly on MLST and the SCC*mec* type. When 5 of the 7 MLST housekeeping genes have identical nucleotide sequences, MRSA isolates are grouped within a single clonal complex (CC). The ST with the largest number of single locus variants (SLV) is the ancestor of a CC. Furthermore, subgroup founders can be described as SLVs or double locus variants (DLV) of a founder of a CC that has become prevalent in a population and may be diversified to produce its own set of SLVs and DLVs (57, 65). MLST is valuable as an established method with a standard nomenclature. However, it is expensive, time consuming and laborious.

Pulse field gel electrophoresis

Pulse field gel electrophoresis (PFGE) has long been considered the gold standard for molecular epidemiological studies of *Staphylococcus aureus* and numerous other bacterial species. This technique is adequate for the study of local outbreaks and also for long-term MRSA surveillance. Fragments of DNA cut using restriction enzymes such as *Sma*I are separated under conditions of alternating polarity allowing for the resolution of large DNA fragments and making a DNA fingerprint useful for discrimination between strains (187). PFGE has a high discriminatory power. However, there are problems with reproducibility among laboratories. In addition, PFGE is time consuming and there is no standard nomenclature for analysis of PFGE results. Additionally, some MRSA strains, like the livestock-associated ST398, are non typable by *SmaI* PFGE (111).

Spa typing

In contrast to MLST in which 7 housekeeping genes are analyzed and to PFGE where the whole bacterial chromosome is used, Spa typing involves the analysis of only a single-loci. The variations in the polymorphic region X of the *S. aureus* protein A (Spa) gene are used to differentiate MRSA clones. The *spa* gene consists of a number of 24-bp repeats and the diversity is attributed to deletions and duplications and rarely to point mutations (74). An advantage of Spa typing over MLST is that it can be used to investigate both the molecular evolution and hospital outbreaks of MRSA (140). The technique of Spa typing is rapid and results can be reported using a standardized nomenclature (253).

Staphylococcal cassette chromosome typing

The typing of the SCC*mec* element is also employed in molecular epidemiological characterization of MRSA. The SCC*mec* is comprised of the *mecA* regulon, the recombinases (*ccr* genes) and the junkyard regions (J regions). Three major classes define the *mecA* complex:

class A containing the complete *mecA* regulon (*mecI-mecR1-mecA*), and classes B and C containing the *mecA* regulatory genes disrupted by insertion sequences ΨIS1272-ΔmecR1-mecA and IS431-ΔmecR1-mecA, respectively (125). The ccr gene complex can be constituted by two genes (*ccrA* and *ccrB*) or by a single gene (*ccrC*). Based on allelic variations in the *ccr* region, a series of allotypes has been defined. The *ccr* gene complex identified in *S. aureus* includes type 1 (carrying *ccrA1B1*), type 2 (carrying *ccrA2B2*), type 3 (carrying *ccrA3B3*), type 4 (carrying *ccrA4B4*) and type 5 (carrying *ccrC*). The J regions (J1, J2 and J3) which in some cases carry additional antibiotic resistance determinants also have variants. The variations in the J regions (within the same *mec–ccr* combination) are used for defining SCC*mec* subtypes. The International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) published in 2009, the "Guidelines for Reporting Novel SCC*mec* Elements" (114). The aim of the document was to establish a consensus on a uniform nomenclature system for SCC elements and to establish guidelines for the identification of SCC elements for epidemiological investigations (114). The greatest advantage of this technique is the standard nomenclature for the identification of the SCC*mec* elements. However, the low throughput, high cost and no standardized protocol are some of the disadvantages that this technique have (253).

Virulence Factors

The pathogenesis of the disease caused by *S. aureus* has been widely studied. The induction of a staphylococcal infection requires minor trauma or immunosuppression, together with the existence of virulence factors (71, 220). Virulence factors of *S. aureus* have been

extensively studied and classified according to their role in the infection process. *S. aureus* virulence factors may be structural or secreted products (90, 187).

The structural virulence factors are represented by numerous surface proteins called "Microbial surface components recognizing adhesive matrix molecules" (MSCRAMMS). These groups of proteins are anchored to the peptidoglycan by a covalent bond and participate in the adherence to different host tissues proteins such as collagen, fibronectin, or vitronectin (71, 90, 209). Another important structural component is the protein A (Spa) which binds to the FC portion of IgG having an antiphagocytic effect. In addition, Spa modulates platelet aggregation and hence it may be involved in the induction of endovascular disease. Spa also sensitizes B cells increasing their recognition of TLR2 ligands and the binding of tumor necrosis factor receptor 1, which has been associated with staphylococcal pneumonia (71). Most *S. aureus* strains produce a microcapsule that is considered an important virulence factor. This capsule plays an important role in the protection from the innate defenses including complement and the prevention of killing by neutrophils or macrophages. The *S, aureus* capsule is a potent activator of human CD4+ T cells (71).

As mentioned previously, other virulence factors are secreted into the extracellular medium. Numerous enzymes, such as lipase, esterase, elastase, staphylokinase, deaxiribonuclease, hyaluronidase and phospholipase, contribute to host tissue destruction. Some of these secreted proteins are considered superantigens. The most commonly studied are enterotoxins (SE), toxic shock syndrome toxin-1 (TSST) and exfoliative toxins (ET), which are mainly involved in toxin-mediated disease and sepsis (158, 187). These superantigens can produce a sepsis-like syndrome by initiating a "cytokine storm." Some strains also produce epidermolysins or ETs capable of causing scalded skin syndrome or bullous impetigo (218). In

addition, *S. aureus* produces cytotoxins, such as β-haemolysin, α-toxin and panton-valentine leukocidin (PVL). These cytotoxins can cause leukocyte, monocyte and erythrocyte destruction by the formation of pores in the cell membrane (71, 90). Other virulence factors such as catalase, coagulase and clumping factor are also involved in staphylococcal pathogenesis (187).

The regulation of the virulence factors is important in the pathogenesis of the staphylococcal disease and the expression is coordinated by global regulators. The expression of regulators constitutes the virulence regulon (71). Three systems have been well characterized. The accessory gene regulator (*agr*), the staphylococcal accessory element locus (*sae*) and the staphylococcal accessory regulatory locus (*sar*). In addition, there are many other systems and proteins that participate in the regulation of virulence factors (71). The most studied mechanism is the *agr* which is a quorum-sensing system that plays a critical role in the regulation of staphylococcal virulence (164, 212). *Agr* encodes a two-component signaling pathway whose activating ligand is an *agr*-encoded autoinducing peptide (AIP). It has been determined that a polymorphism in the amino acid sequence of the AIP and of its corresponding receptor divides *S. aureus* strains into four major groups. Within a given group, each strain produces a peptide that can activate the *agr* response in the other group member strains. In contrast, the AIPs belonging to different groups are usually mutually inhibitory (117).

A coordinated effect of the genes that constitute the virulence regulon is crucial during the pathogenesis of *S. aureus*. Endothelial cells and also epithelial cells, osteoblasts and neutrophils are infected playing an important role in the infection process (70). Phagocytes take the bacteria up into a plasma membrane-derived vacuole (phagosome) which undergoes maturation into endosomes and lyposomes with degradative properties. Staphylococci may escape from the endosomes and replicate in the cytoplasm of the cells (70). Some of the

virulence factors mentioned above participate in this process. In addition, small-colony variants may contribute to persistent and antibiotic-resistant infections (71, 92, 286).

The most important virulence factors of *S. hyicus* are related with the expression of exudative epidermitis-inducing exotoxins and exfoliative toxins (ET). Two different ET have been identified in *S. hyicus*. SHETA which is synthesized under chromosomal control and SHETB synthesized under plasmid control (120, 121, 239). Because *S. pseudintermedius* is highly prevalent in dogs, (295) some of its virulence factors have been studied. The production of enzymes (coagulase, protease and thermonuclease) and also the expression of toxins (hemolysins, exfoliative and enterotoxins) have been demonstrated in this *Staphylococcus* species (76, 80). Specifically, Leukotoxin (Luk-I); exfoliative toxins (ET): ETA, ETB, ETC, and SIET; and enterotoxins: SEA, SEB, SEC, SED, SECcanine are among the most important virulence factors of pathogenic *S. pseudintermedius* (68, 263).

The leucotoxin, Luk-I, was first described in *Staphylococcus intermedius* (78). This microorganism was thought to be the major staphylococcal pathogen of dogs and cats, but it is now considered that *S. intermedius* strains isolated from these hosts belong to the specie *S. pseudintermedius* (58, 237, 238). Characterization and nucleotide sequence analysis have shown that similar to PVL, Luk-I is encoded as *luk*I operon with two cotranscribed genes, *luk*S and *luk*F, encoding LukS and LukF. Luk-I demonstrated a strong leukotoxicity on various polymorphonuclear leukocytes, but only a slight hemolytic activity on rabbit erythrocytes (78, 158). An important ET produced by *S. intermedius* and *S. pseudintermedius* is the *S. intermedius* exfoliative toxin (SIET) (148, 264). This ET causes erythema, exfoliation, and crusting in dogs with pyoderma (264). The production of ET is associated with the pathogenesis of skin diseases

of staphylococcal origin. Moreover, other virulence factors such as fibronectin binding protein may be involved in skin colonization (80).

The description of virulence factors in *S. schleiferi* subsp *coagulans* is sparse. Like *S. aureus* and *S. pseudintermedius, S. schleiferi* subsp *coagulans* produces β-hemolysins and is adherent to glass (146). In addition, this microorganism is considered coagulase positive. In 2005, Roberts *et al.*, reported the lack of PVL genes in 40 *S. schleiferi* subsp *coagulans* strains isolated from companion animals (226). Because of the increasing reports of MRSC isolated from companion animals, and because this microorganism has been associated with human clinical diseases, it is crucial to study its ecology, mechanisms of antimicrobial resistance and virulence factors.

In conclusion, the mechanisms of antimicrobial resistance and contributing virulence factors of *S. aureus* have been well characterized. Recently, studies have demonstrated the evidence that CA-MRSA clones have infiltrated healthcare settings worldwide which is considered an epidemiological change in the ecology of MRSA (253). In addition, multiple cases of human staphylococcal infections from animals have been reported. Currently, the clonal spread of the ST398 strain has caused alarm in the scientific community (53). From these, it is apparent that molecular typing is an indispensable tool to study the epidemiology of staphylococci. Also, it is apparent that it is necessary to investigate the epidemiology and pathogenesis of other potential zoonotic *Staphylococcus* species. The final goal should be to study the evolution of this genus in veterinary medicine, to establish more appropriate control measures, and finally to alleviate humans and animals affected with staphylococcal diseases.

II. Staphylococcal cassette chromosome *mec* types and antimicrobial susceptibility patterns among methicillin-resistance *Staphylococcus aureus* isolates from Horses

Summary

Methicillin resistance in *Staphylococcus* aureus is determined by the function of the penicillin-binding protein 2a (PBP2a) encoded by the mecA gene. The *mecA* gene is carried on a large heterologous mobile element called the staphylococcal cassette chromosome mec (SCC*mec*). To date, eleven types of SCCmec types have been identified (266). In the past five years, methicillin resistant *S. aureus* (MRSA) has been recognized as an emerging pathogen in veterinary medicine. However, the epidemiology of staphylococcal diseases in domestic animals is not well understood. In this study, sixty-eight equine MRSA isolates, collected from July 1998 to March 2011 by the Auburn University College of Veterinary Bacteriology and Mycology Diagnostic Laboratory, were analyzed phenotypically and compared based on their antimicrobial susceptibility and pulse-field gel electrophoresis (PFGE) patterns. Detection of the PBP2a was also performed. A multiplex PCR designed to detect and differentiate six different types of SCC*mec* was executed. In horses, the USA500 SCC*mec* type IV has been documented to be common (47, 182, 294). Results of PFGE and SCC*mec* multiplex PCR indicated that the MRSA isolates were highly clonal and was characterized as predominantly USA500, type IV strains. However, a relevant outcome of this investigation was the finding of the previously characterized MRSA USA100 clone in 11% (7/63) of the tested isolates. In addition, this study

supports that adequate biosafety methods (ie, improvements in hand hygiene by personnel with animal contact) in veterinary environments will reduce nosocomial outbreaks of MRSA in equine medicine.

Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most important antimicrobial-resistant pathogens of humans and animals, worldwide. Commonly, *S. aureus* is responsible for mild diseases. However, life threatening cases are reported frequently and with the development of increased antimicrobial resistance by *S. aureus*, treatment options for clinical cases have become limited. MRSA has been reported as one of the most important nosocomial causes of infection in human and veterinary hospitals; where patients with open wounds, invasive devices, and debilitated immune systems are at greater risk of infection than the general public (6, 28, 40, 56, 133, 134, 183, 293).

Infection and colonization with MRSA in domestic animals has become of interest in the medical community because of reports of possible zoonoses or reverse zoonoses. MRSA has been described in different members of the animal kingdom (mammals, reptiles and birds), being particularly important in chickens, cats, dogs, pigs and horses (8). Studies have identified MRSA colonization rates of up to 11% in horses on private farms or upon admission to veterinary hospitals (6, 12, 30, 182, 269, 273, 282, 294). Other studies have identified higher rates, such as 43% from one farm (182). In horses, *S. aureus* is considered to be an opportunistic pathogen. Most *S. aureus* clinical cases are associated with joint, surgical incisions and skin infections, but other kind of infections have also been reported, such as metritis, pneumonia, and bacteremia, among others.

In the genus *Staphylococcus*, resistance to β-lactams is mediated by two mechanisms; the production of β-lactamases and, more importantly, the expression of penicillin-binding protein 2 (PBP2' or PBP2A). The PBP2a is encoded by the *mec*A gene which is carried by a mobile genetic element called staphylococcal cassette chromosomal *mec* (SCC*mec*) (114, 118, 158,

187). The production of β-lactamases can be overcome by the use of β-lactamase inhibitors such as clavulanate or sulbactam. However, resistance to methicillin and all β-lactams is due to PBP2a which has a lower affinity for the β-lactam antimicrobials than the normal PBP such that these antimicrobials are ineffective. Because the SCC*mec* generally contains extra insertion sequences that allow for incorporation of additional antimicrobial resistance markers, MRSA strains are called multidrug resistant; exhibiting resistance against other antimicrobial groups, such as erythromycin, clindamycin, aminoglycosides, fluorquinolones, rifampin and co-trimoxasole (75, 126, 130).

Typing of MRSA strains is used in epidemiological studies to define ancestral lineages and to identify epidemiologically related isolates (253, 261). The most important techniques in typing MRSA strains are molecular based; multilocus sequence typing (MLST), pulse field gel electrophoresis (PFGE), staphylococcal protein A (Spa) and SCC*mec* typing (9, 14, 74, 140). MLST consists of sequencing seven housekeeping genes. Each new sequence is a new allele and the combination of the 7 alleles forms the allelic profile or sequence type (ST) (64). PFGE has long been considered the gold standard for molecular epidemiological studies of *Staphylococcus aureus* and numerous other bacterial pathogens. Fragments of DNA digested using restriction enzymes are separated under conditions of alternating polarity allowing for the resolution of large DNA fragments making a DNA fingerprint useful for discrimination between strains (185). In contrast, only a single-locus is analyzed in *spa* typing. Variations in the polymorphic region X of the *S. aureus spa* gene are used to differentiate isolates. The *spa* gene consists primarily of a number of 24-base pair repeats and the diversity in the *spa* region is attributed to deletions and duplications, rarely to point mutations (140).

In addition to the previously described methods, typing of the SCC*mec* element is also employed in molecular epidemiological studies of MRSA. The SCC*mec* is comprised of the *mecA* regulon, the recombinases (*ccr* genes) and the junkyard regions (J regions) (114). Three major classes define the *mecA* complex: class A, containing the complete *mecA* regulon (*mecI-mecR1-mecA*), and classes B and C, containing the *mecA* regulatory genes disrupted by insertion sequences, ΨIS1272-ΔmecR1-mecA and IS431-Δ*mec*R1-*mec*A, respectively (125, 177). The *ccr* gene complex can be constituted by two genes (*ccrA* and *ccrB*) or by a single gene (*ccrC*). Based on allelic variations in *ccr*, a series of allotypes has been defined (125). The *ccr* gene complex identified in *S. aureus* includes type 1 (carrying *ccrA1B1*), type 2 (carrying *ccrA2B2*), type 3 (carrying *ccrA3B3*), type 4 (carrying *ccrA4B4*) and type 5 (carrying *ccrC*) (125). The J regions (J1, J2 and J3), which in some cases carry additional antibiotic resistance determinants also have variants (177, 199). The variations in the J regions (within the same *mec–ccr* combination) are used for defining SCC*mec* subtypes (114, 176, 177). To date, eleven different types of SCC*mecA* elements have been identified in *S. aureus*, type-I to Type-XI (253, 266).

In human medicine, MRSA are differentiated in clinical cases as hospital-acquired MRSA (HA-MRSA) or community-associated MRSA (CA-MRSA) (14). Commonly, SCC*mec* types-I, II and III are found in HA-MRSA. The SCC*mec* types-IV to VIII are associated with CA-MRSA. The first CA-MRSA clonal isolates were SCC*mec* type-IV, which were smaller than the cassettes described in HA-MRSA clonal isolates (204). In addition, most of the CA-MRSA strains with the SCC*mec* type-IV were only resistant to β-lactam antibiotics and contained the genes (*lukS-PV* and *lukF-PV*) for Panton-Valentine leukocidin (PVL) toxin. Moreover, the type-IV has variations in the J regions (mainly J1), whereby type-IV can be subtyped from type-IVa through IVg.

Different studies have demonstrated a separation of some *S. aureus* lineages according to host-specificity (123). Various strains have the capacity to infect different hosts, but the great majority of clonal isolates are associated only to a single host species (275). MRSA strains from dogs and cats share a similar genetic background to human nosocomial MRSA. Baptiste *et al.* suggested that common strains indicated a spread from humans to animals based on the finding of MRSA identical to human EMRSA-15 in dogs and hospital staff. Therefore, Baptiste *et al.* concluded that the MRSA identified in dogs and cats did not represent a new emergence of MRSA clonal isolates (12). In contrast, MRSA strains isolated from horses have been suggested to represent the possible emergence of new MRSA clones (47, 182, 291). Because *S. aureus* has the potential of zoonotic transmission, and different MRSA clones have disseminated widely among animal species, it is important to investigate the existence of new clones, and to maintain epidemiological surveillance of resistance patterns and clonal relationships.

The aim of this study was to use microbiological and molecular techniques to characterize MRSA strains isolated from clinical submissions collected from horses and submitted to the university veterinary diagnostic laboratory. Characterization was done using conventional microbiological techniques, detection of the PBP2a, standard PFGE, amplification of lukS/F-PV genes by PCR, *SCCmecA* typing and subtyping of the SCCmec type-IV. The relationships of the isolates were analyzed using data generated from the various methods. Results of this study provide a newly described genetic characterization of MRSA of animal origin and contribute towards the epidemiological surveillance informational database that is necessary for the understanding and characterization of MRSA in the equine population and the potential impact on human MRSA infections.

Material and methods

Bacterial strains. In this study, sixty-eight equine MRSA isolates collected from July 1998 to March 2011 by the Auburn University, College of Veterinary Medicine, Bacteriology and Mycology Diagnostic Laboratory were included. To avoid overrepresentation of isolates from an individual case submission, only the first isolate from each patient during the study period was included. Specimen sources were recorded and each isolate was processed following established laboratory protocols. After incubation for growth on bovine blood agar (4%), isolates were selected based on colony characteristics: 1 to 3 mm of diameter, white, opaque, or golden color, in addition to hemolysis. Conventional biochemical assays including catalase (3% H2O2), tube coagulase test (BBL coagulase plasmas; Becton Dickinson), acetoin production (1ml of 1.7% MRVP medium) and carbohydrate fermentation patterns (trehalose 1%; purple agar base: 3.7%, mannitol 1.2% and maltose 3.7%) were used for species identification. *S. aureus* were considered to be catalase, coagulase positive, acetoin producer and fermenter of trehalose, mannitol and maltose (187).

Antimicrobial susceptibility. Antimicrobial susceptibility testing to OXA (1µg) and FOX (30µg) was determined by the disk diffusion method using Mueller Hinton salt agar (2%) according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (43, 192). The antimicrobial susceptibility profiles of each of the isolates were determined using disk diffusion or a Mueller-Hinton broth in accordance with CLSI guidelines (43, 192). The antimicrobial agents tested were ampicillin, amoxicillin/clavulanic acid, amikacin, ceftiofur, clindamycin, chloramphenicol, cephalothin, erythromycin, enrofloxacin, gentamicin, penicillin, tetracycline, trimethoprim-sulfamethoxazole, and vancomycin, following CLSI criteria guidelines (43, 192).

Quality control strains included *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212.

DNA isolation. For the extraction of bacterial DNA, two or three colonies were obtained from 18 – 48 hours cultures inoculated on bovine blood agar (4%) or tryptic soy agar (4.1%). The individual colonies were picked and suspended in 100µl of RNAse-free distilled water. The bacterial suspension was boiled at 95°C for 7min and then centrifuged at 15,000 *g* x 1 min and the supernatant was collected. The DNA supernatant extracts were stored at -20°C until used for PCR.

Detection of the penicillin - binding protein 2a. The presence of a functional PBP2a is associated with methicillin resistance in *S. aureus*. A commercial latex agglutination test was used, according to the instructions of the manufacturer (Oxoid; Hampshire, UK) to detect the PBP2a. Results were considered positive when agglutination and clearing of the latex suspension were observed. Negative results, were characterized by failure to produce agglutination. Intermediate results were retested.

Multiplex PCR for detection of the *mecA* gene and the SCC*mec* type-IV. In order to type the SCC*mec* cassette, a multiplex PCR described by Milheirico *et al.,* was performed (177). The multiplex PCR allows the typing of the SCC*mec* type I through VI. Briefly, the PCR mixture contained 25 µl of PCR master mix preparation containing 25units/ml *taq* DNA polymerase, 200µm (each) deoxynucleoside triphosphate and 1.5mM of $MgCl_2$ (Promega® cat# M7502). Additionally, the reaction mixture contained 20 µl of a primer mix composed of 0.2 µM primers kdp F1 and kdp R1; 0.4 µM primers CIF2 F2, CIF2 R2, RIF5 F10, RIF5 R13, SCC*mec* III J1F,

SCC*mec* III J1R, SCC*mec* V J1 F, and SCC*mec* V J1 R; 0.8 µM primers *mec*I P2, *mec*I P3, dcs F2, dcs R1, *mec*A P4, *mec*A P7, ccrB2 F2, ccrB2 R2, ccrC F2, and ccrC R2. Finally, 1-2 µl of DNA template and distilled RNase free water were added to give a 50µl total reaction volume. Nucleotide primers sequences are detailed in Table 2. Cycling reaction conditions were as follows: 94°C for 4 min; 30 cycles of 94°C for 30 seg, 53°C for 30 seg, and 72°C for 1 min; and a final extension at 72°C for 4 min. PCR products (10 µl) were resolved in a 3% agarose gel in 0.5% Tris-acetate-EDTA buffer containing 0.5 µg/ml of ethidium bromide by electrophoresis at 4 V/cm for 2.5 hr and ethidium bromide staining was visualized with ultra-violet light (177). MRSA reference strains from the American Type Culture Collection (ATCC) were used as type-specific controls: SCC*mec* type-I: BAA-44; type-II: 43300/BAA-1681; type-III: BAA-39; type-IV: BAA-1756/BAA-1696; type-V: BAA-1688; type-VI: BAA-42.

For the subtyping of the SCC*mec*-type IV the protocol described by Milheirico *et al.*, (2007) was used (176). Each PCR mixture contained 25 µl of PCR master mix preparation, containing 25 units/ml *taq* DNA polymerase, 200µm (each) deoxynucleoside triphosphate and 1.5mM of $MgCl_2$ (Promega® cat# M7502). Additionally, 0.2 µM for primers J IVa F, J IVa R, J IVb F and J IVb R; 0.4 µM for primers ccrB2 F, J IVc F and J IVc R; 0.8 µM for primers ccrB2 R, J IVd F and J IVd R; 0.9 µM for primers J IVg F and J IVg R and 1.8 µM for primers J IVh F and J IVh R. Finally, 1-2 µl of DNA template and distilled RNase free water were added to give a 50µl total reaction volume. Nucleotide primer sequences are described in Table 3. Cycling conditions were as follows: 94°C for 4 min; 35 cycles of 94°C for 30 seg, 48°C for 30 seg and 72°C for 2 min; and a final extension at 72°C for 4 min. PCR products (20 µL) were resolved in 2% Seakem LE agarose (Cambrex Bio Science Rockland, USA) in 1 × Tris-acetate-EDTA buffer at 80 V for 60 min and visualized with 0.5 µg/ml of ethidium bromide staining.

PCR for detection of the lukS/F-PV genes. The presence of the lukS/F-PV genes, which encode for the PVL S/F bicomponent proteins, were determined by PCR. Briefly, the PCR mixture contained 25 µl of PCR master mix preparation (50units/ml *taq* DNA polymerase, 400µm (each) deoxynucleoside triphosphate and 3mM of $MgCl_2$ Promega® (M7502)); 0.08 µM of primers: Luk-PV-1 (5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3') and Luk-PV-2 (5'-GCATCAAGTGTATTGGATAGCAAAAGC-3') (157); 1-2 µl of DNA template and distilled RNase free water in a 50µl total reaction volume for each isolate. Cycling conditions were 94°C for 10 min; followed by 10 cycles of 94°C for 45 seg, 55°C for 45 seg, and 72°C for 75 seg; and 25 cycles of 94°C for 45 seg, 50°C for 45 seg, and 72°C for 75 seg (170). PCR products (8 µl) were resolved in a 2% agarose gel in 0.5% Tris-borate-EDTA buffer. Electrophoresis was carried out at 4 V/cm for 1 hr

and visualized with ethidium bromide. Methicillin-resistant *S. aureus* from the ATCC reference strains were used as type-specific controls: BAA-1696 (PVL positive) and BAA-1681 (PVL negative).

Pulse field gel electrophoresis (PFGE). Staphylococcal isolates were typed by PFGE using previously described protocols with some modifications (9, 185). The protocol included bacterial lysis using a combination of lysostaphin (L-7386; Sigma®), protein digestion with proteinase-K and DNA restriction digestion with *Sma*I (Biolabs, R0141S) (171, 185). A single colony of the test isolate was inoculated into 5 ml of brain heart infusion broth and incubated at 37°C for 18 - 24 hr. Two hundred microliters of the adjusted cell suspension was centrifuged at 12,000 X *g* for 5 min, and the supernatant was aspirated. The pellet was resuspended in 300 µl of Tris-EDTA (TE) buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]) and equilibrated in a 37°C

water bath for 10 min. Four µl of conventional lysostaphin stock solution (1 mg/ml in 20 mM sodium acetate [pH 4.5]) and 300 µl of 1.8% PFGE agarose (SeaKem, Gold agarose) in TE buffer (equilibrated to 55°C) were added to the cell suspension, gently mixed, and dispensed into the wells of a small disposable mold (≈100 µl each). The plugs were allowed to solidify in the refrigerator (4°C) for 10 min, then removed and placed into a tube containing at least 3 ml of EC lysis buffer (6 mM Tris HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroylsarcosine) and incubated at 37°C for 4 - 6 hr. The EC lysis buffer was decanted and 4 ml of TE buffer was added. Plugs were placed in screw-capped flasks and refrigerated at 4°C, until needed. Protein digestion was performed using 2.5 ml ESP buffer (0.5M EDTA, 1% sodium lauroylsarcosine) with a 1 mg proteinase-K / ml final concentration per plug in a 1.5 µl microcentrifuge tube. Digestion was done at 50 ºC in a water bath, overnight. The next day, the EC lysis buffer was decanted and 4 ml of TE buffer was added for washing in a shaking incubator at 37°C for 30 min. The washes with TE buffer were repeated at least two more times and the plugs were stored in 4 ml of TE buffer at 4°C.

For *Sma*I restriction enzyme digestion, a plug slice (2 x 7 x 1.2 mm) was cut and incubated with of *Sma*I restriction enzyme (20 *Sma*I Units, 12.5 µl of NEB Buffer 4 and 112.5 dH2O RNase free / plug) at 25°C for 4 to 6 hr. A solution of 1% SeaKem ® Gold (Lonza Rockland, Inc, ME, USA) agarose was prepared in 0.5 TBE from 10X Tris-borate-EDTA buffer (Gibco BRL no. 15581044) and kept in a water bath at 55°C until used. After enzymatic digestion, the plug slices were loaded directly on to the end of the comb tooth and placed on the comb holder and allowed to dry (≈10 min). Equilibrated agarose was then carefully poured into the gel casting platform. After 45 min, the comb was removed and the holes were filled with 1.8% agarose.

PFGE was performed using a contour-clamped homogeneous electric field apparatus: (CHEF Mapper, Bio-Rad, Hercules, Calif.). Running parameters were as follows: 200 V (6 V/cm); temperature setting of 14°C; initial switch, 5 seg; final switch, 40 seg; and run time of 18 hr. After the electrophoresis run was completed, the gel was stained in a 1.5 g/ml ethidium bromide solution (AMRESCO X328, 10 mg/ml; Amresco, Inc., Solon, Ohio) for 30 min in a covered container and destained in fresh distilled water for at least 45 min. PFGE gels were analyzed by visual comparison and the relatedness of PFGE types was determined by employing the software BioNumerics v. 6.6 software (Applied Maths, Kortrijk, Belgium). Cluster analysis was performed by UPGMA based on the dice similarity coefficient with optimization and position tolerance set at 1.0%. MRSA isolates were clustered using an 80% homology cut-off, above which strains were considered to be closely related and assigned to the same group. PFGE type band patterns were also interpreted according to the criteria proposed by Tenover *et al.*, (260).

Results

Epidemiological and antibiogram information. Since 1999, MRSA isolates have been collected from horse clinical specimens isolated by the Auburn University, College of Veterinary Medicine, Bacteriology and Mycology Diagnostic Laboratory. From September 1999 to March 2011, sixty-eight MRSA isolates were included in the collection. Isolates were classified as MR after Oxacillin or Cefoxitin disk diffusion susceptibility testing, using CLSI guidelines. Sixty-seven isolates were tested for PBP2a using the latex agglutination test and 66 of the MRSA isolates were positive; one isolate was oxacillin resistant, but PBP2a negative. Possibly, the

hyper production of β- lactamases is the mechanism that leads the resistant against β-lactam in the mentioned isolate (167).

The antimicrobial susceptibility testing data was obtained retrospectively. Therefore, different drug panels were used for testing some of the isolates. Most of the isolates exhibited susceptibility to Chloramphenicol (S=47/48), Clindamycin (S=44/42), Enrofloxacin (S= 49/55) and Vancomycin (S=46/47). In contrast, a high pattern of resistance was observed against Ampicillin (R= 57/58), Amoxicillin/clavulanic acid (R=41/41), Cephalothin (R= 49/52), Erythromycin (R=48/60), Gentamicin (R=56/61), Penicillin (R=66/67), Trimethoprim/Sulfa (R=59/66), Tetracycline (R=58/70) and Ceftiofur (R=66/67). Amikacin was tested in just 23 isolates, from which 1 was resistant, 15 were susceptible and 7 were considered intermediate.

The frequency of *Staphylococcus* sp isolated per year is shown in figure 1. Figure 1 demonstrates the curve showing a peak in the number of cases in 2005, with a decrease in the number of MRSA isolates from 2006 until March 2011. Regarding to the sample source of MRSA, 54% (37/68) of the MRSA isolates were collected from skin (pustules, wound swabs, and others), 12% (8/68) from bones and joint fluids, 9% (6/68) from upper and lower respiratory tract, 7% from internal body tissues and cavities, 6% (4/68) from blood, 3% (2/68) from guttural pouch, and 4% (3/68) from eye and urinary/reproductive tract, figure 2.

MRSA SCC*mec* typing. In this study, a multiplex PCR strategy for the rapid assignment of SCC*mec* types to MR staphylococci strains was used. This approach was able to properly identify SCC*mec* types-I to VI in MRSA reference strains obtained from the ATCC used as type-specific controls, figure 5. Sixty-four isolates where tested for the identification of the SCC*mec* type, of which 61 isolates (95.31%) were type IV. Surprisingly, 3 of the 64 (4.68%) equine

isolates were identified as type II. Figure 3. The SCC*mec* type-IV has been frequently documented in horses, (47) with other SCC*mec* types being uncommon (295). The SCC*mecA*-type IVd was found in all the MRSA strains carrying the type-IV. The PCR detection of the lukS/F-PV genes was performed in 49 isolates and no positive results were identified for any of the 49 tested isolates.

Results of 63 MRSA isolates tested by PFGE were analyzed after *Sma*I digestion and compared with MRSA USA-genotypes from the Center for Disease Control and Prevention (CDC), Atlanta, GA, figure 5. USA500 and USA100 were classified as the predominant clones, represented by 46% (29/63) and 11% (7/63) of the isolates, respectively. Another group was represented by 36% (23/63) of the isolates which possessed a high similarity with USA500 (similarity > 70%, but < than 80%). Three isolates representing 5% (3/63) of the isolates were not related to any of the USA clones and 2% (1/63) of the isolates were non-typable by *SmaI* PFGE analysis.

Discussion

Methicillin resistant *Staphylococcus* in horses are associated with a variety of clinical signs, with skin/soft tissue infections being the most common. Studies to determine predisposing factors to MRSA colonization in horses have shown that colonization at the time of hospital admission is a risk factor for the development of such infections (294). It is important to note that isolates included in the present study were collected from horse clinical specimens and does not represent a randomized population study sample. In this investigation, 54% (37/68) of the isolates were collected from skin, followed by 12% (8/68) and 9% (6/68) from bone/joint fluids

and respiratory tract, respectively. The remaining isolates were collected from different body sites. Similar results have been published previously (7, 47, 291).

Even though this study did not directly evaluate the preventive methods against MRSA implemented at veterinary hospitals, the time course per year of isolate collection is shown in figure 1. From 1999 to 2005, a high number of MRSA isolates where reported in horse clinical specimens (> 6 per year) with the highest numbers recovered in years 2000 and 2005; 11 and 16 cases, respectively. During these years there was considered to be a MRSA clinical outbreak. Fortunately, biosafety measures were then implemented and from 2006 to the present the number of MRSA in horses from clinical specimens processed has decreased to a level of 0 to 1 per year. Because the strict hygiene measures have been adopted in veterinary environments similar to human hospitals, the decrease in the number of cases could suggest that the origin of the infection is not nosocomial. For that reason, some authors have suggested that the report of cases not related with nosocomial infections might be associated with community acquired MRSA clones circulating in horses (162, 290, 293). The decrease of hospital-acquired MRSA transmission in human and veterinary hospitals has been the result of the identification of patients infected with MRSA strains combined with their isolation, as well as improvements in hand hygiene by personnel with animal contact (6, 40, 188, 208).

Antimicrobial therapies commonly used in horses residing in the United States include penicillin, ampicillin, cephalothin, ceftiofur, erythromycin, rifampin, tetracycline, trimethoprim/sulfa, gentamicin, amikacin, chloramphenicol, enrofloxacin, ticarcillin and ticarcillin/clavulanic acid. In some cases, amoxicillin/clavulanic acid, cefoxitin, imipenem and oxacillin have also been used (43, 86, 300). Horse MRSA strains from Canada and USA have shown resistance when tested against penicillin, ampicillin, cephalothin, oxacillin, tetracycline,

gentamicin, erythromycin, clindamycin, tetracycline, trimethoprim. In contrast, those same horse MRSA isolates exhibited a susceptible pattern when tested against amikacin, enrofloxacin, chloramphenicol, clarithromycin, vancomycin and imipenem (5, 47, 156, 247).

In 2009, Anderson *et al.*, reported that 62.5% (35/56 isolates) of MRSA from horses were resistant to erythromycin (5). However, a previous study reported that 100% (11/11) of the MRSA isolates tested where resistant against the same drug (247). In the present study, 80% (48/60) of MRSA were considered resistant against erythromycin. With the exception of cephalothin, isolates characterized in this study exhibited a similar pattern of susceptibility and resistance when compared with antimicrobials tested in previous publications. In this study, 94% (49/52) of the isolates were phenotypically resistant against cephalothin, which is consistent with a recent report by Lynn, 2011 (156). These results should be interpreted with caution because, in accordance with CLSI guidelines, a *S. aureus* isolate resistant to oxacillin should also be considered resistant to all other β-lactams. Seguin *et al.,* in 1999 reported that all MRSA isolates tested as phenotypically susceptible using cephalothin (247). With the exception of the study published by Lin *et al.,* (156) there are no recently published susceptibility patterns for equine MRSA isolates.

Previous studies have reported the clone USA500 or Canadian MRSA-5 as prevalent in horses and horse personnel in some geographical areas (5, 41). This clone is characterized by the possession of the SCC*mec* type-IV and the lack of PVL genes. In addition, it is hypothesized that this clone is a derivative of a human epidemic clone, which only accounts for a small percentage of human infections (5, 6, 41, 156, 182, 198). Some authors have hypothesized that the USA500 clone has become somewhat adapted to horses and the results of this study confirm the significant prevalence of the USA500 clone in the equine isolates characterized (47, 182,

288, 291). This is supported by the fact that 82% (52/63) of the horse MRSA isolates included in this study were classified as USA500 or highly related to this clone and they carried the SCC*mec* type-IV, but did not possess the *pvl* genes. It is important to note, that previous studies have identified the USA500 in samples collected from veterinary personnel who worked with horses (101, 182, 291). Although this clone is considered a human epidemic clone, it still has a low prevalence in people and is currently not considered to be a common MRSA clone in humans (41). Due to its appearance in the animal population, it is important to maintain surveillance for the clones that circulate in human personnel in frequent contact with animals.

Clone USA100 is common in human populations, being associated with hospital-acquired MRSA in the USA (51, 191, 262). This clone has been commonly reported in dogs and cats in North America (150, 292). The identification of USA100 has been used as a basis to support the idea that the epidemiology of MRSA in pets reflects the relationship between animals and their owners or personnel that work with animals. This fact also is used to support the hypothesis that MRSA in pets is ultimately human in origin (290). In this study, 11% (6/63) isolates were identified as USA100; this result agrees with previous studies, which also reported the USA100 clone in horses, although it is not considered common in equine (41, 51). In addition, the SCC*mec* type-II was found in three isolates, of which two were classified as USA-100 and one was non-typable by PFGE. The authors of this study agree with Anderson *et al.*, who have mentioned that it is impossible to infer the origin of MRSA in horses colonized by the USA100 clone (6).

The acquisition or origin of the USA100 in horses could have occurred from contact with horses, companion animals or from non-animal sources (6). The 64 isolates classified as SCC*mec* type –IV and considered USA500 by PFGE exhibited a resistance to trimethoprim-sulfa

and tetracycline, which is consistent with previous reports where USA500 human clones were resistant to clindamycin, erythromycin, gentamicin, levofloxacin, tetracycline, trimethoprim-sulfamethoxazole, and the β-lactams (2, 171). In contrast, the three isolates that carried the SCC*mec* type-II were susceptible against trimethoprim-sulfa and two of them were susceptible to tetracycline. McDougal *et al.* in 2003 reported that USA100 were multiresistant to commonly used therapeutic agents (171). However, in a most recent study susceptibility against gentamicin, tetracycline, and trimethoprim-sulfamethoxazole was reported (2). As mentioned, it is documented that horse MRSA strains are usually resistant against tetracycline and trimethoprim-sulfamethoxazole (5, 47, 156). The findings of USA-100 that carried the SCC*mecA* type II and exhibited susceptibility against tetracycline and trimethoprim-sulfamethoxazole may be an indication of a human clone that was transmitted to horses. It is necessary to further investigate the origin of clones other than USA500 in horses.

In conclusion, with some limitations, this study supports that adequate biosafety methods (ie, improvements in hand hygiene by personnel with animal contact) in veterinary environments will reduce nosocomial outbreaks of MRSA in equine medicine. A relevant outcome of this investigation was the finding of MRSA USA100 clone in 11% (7/63) of the tested isolates. This clone has been reported previously in horses, however it is uncommon. The MRSA USA100 is frequently reported in people and then it is possible that humans were the source of this clone. Typing methods for the molecular investigation MRSA in human and animals are essential. The results of this study, as well as the current finding of the new clone ST398 in equine nasal samples, (269) support the need for more epidemiological studies to define the behavior of community and hospital-MRSA colonization in horses.

III. Characterization of *Staphylococcus aureus, Staphylococcus pseudintermedius* and *Staphylococcus schleiferi* subspecies *coagulans* isolated from canine clinical specimens

Summary

Multidrug-resistant *Staphylococcus* species in dogs have become a serious challenge in veterinary medicine. The coagulase positive staphylococci most commonly associated with infections in the canine are the *Staphylococcus pseudintermedius* (SP), previously recognized as *S. intermedius* (both members of the *S. intermedius* group), *S. schleiferi* subspecies *coagulans* (SC) and *S. aureus* (SA). To better understand the magnitude of this problem, a total of 324 *Staphylococcus* sp from canine clinical specimens were analyzed based on the following objectives: 1) characterize canine *Staphylococcus* sp using conventional biochemical tests and molecular techniques, 2) identify epidemiological risk factors and 3) distinguish the most appropriate diagnostic method for recognition of methicillin-resistance (MR). Isolates were characterized by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), latex agglutination for penicillin-binding protein 2a (PBP2a), agar disk diffusion for oxacillin (OX) and cefoxitin (FOX) antimicrobial susceptibility testing, and multiplex PCR for SCC*mecA* typing. Thirty-nine percent (125/324) of the isolates were methicillin resistant (MR) *Staphylococcus pseudintermedius* (MRSP), 9% (28/324) MR *Staphylococcus schleiferi* subspecies *coagulans* (MRSC) and 9% (30/324) MR *Staphylococcus aureus* (MRSA). Fifty-one percent (151/297) of the isolates were collected from skin and 35 % (99/285) from dogs ≥ 9

years old. The type V cassette was present in 66% (113/171) of the isolates, followed by type IV with 13% (23/171). Interestingly, 11% (18/171) of the MRSC were SCC*mecA* type V. Based on the 2008Clinical and Laboratory Standard Institute (CLSI) criteria, the sensitivity and specificity of OXA for the detection of MR strains was 83% and 93%, respectively, but for FOX the sensitivity was 20% and the specificity 100%. In contrast, when the 2004 CLSI criteria were considered for the analysis, the sensitivity and specificity for OXA were 98% and 87.5% respectively. To our knowledge this is the first time that SCC*mecA* Type V has been reported in MRSC. Our findings confirm that cefoxitin is not appropriate for the detection of MR in canine staphylococci. The use of OXA considering the 2004 CLSI break points criteria provides a more accurate prediction of MR in canine isolates. The amplification of the *mecA* gene by PCR or the PBP2a latex agglutination test also provides improved methods for classification of MR staphylococcal strains.

Introduction

An increasing number of methicillin resistant (MR) *Staphylococcus species* have been recognized in the canine (67, 119, 173). Several species have been associated with canine diseases within this genus. *Staphylococcus pseudintermedius*, previously misidentified as *S. intermedius*, is the most prevalent bacteria associated with superficial pyoderma. It has also been associated with more severe infections of the skin and soft tissues causing opportunistic infections in other body sites. Likewise, *S. schleiferi* subspecies *coagulans* has been implicated in canine dermatitis and otitis externa (168, 184). While less prevalent in the canine, *Staphylococcus aureus* has been associated with numerous conditions, such as soft tissue infections, chronic pyoderma, post-surgical wound infection, joint invasion, urinary tract infections and death (119, 223, 250, 295).

The increase in prevalence of MR *Staphylococcus* species in dogs demonstrates the corresponding emergence of these important pathogens in the canine population. The isolation of canine MR *Staphylococcus* species generates alarm in veterinary medicine due to a myriad of factors. First, there are limitations in treatment options as MR strains are resistant to all β-lactam drugs. In addition, the genome of these MR pathogens frequently contains other genes that confer resistance to other antimicrobial classes (multi-drug resistant). Dog owners are economically impacted due to long and often expensive antimicrobial therapies and multiple visits to the veterinarian. Second, there is a possibility of zoonotic transmission. Cases of human-to-animal and animal-to-human transmission of MRSA have been documented (28, 66, 173, 276) as well as some reports confirming zoonotic transmission of *S. pseudintermedius* (237, 272, 274). Case reports of human infections with *S. schleiferi* subsp *coagulans* have also been published (143, 277). Finally, there challenges in providing rapid, yet accurate identification of a

MR isolate. Veterinary microbiology diagnostic laboratories typically utilize interpretative guidelines established by the Clinical and Laboratory Standard Institute (CLSI) for antimicrobial susceptibility testing. The CLSI recommends the use of oxacillin (OXA) and cefoxitin (FOX) break points standardized for *S. aureus* isolates to interpret the *in vitro* susceptibility of *S. pseudintermedius* and other coagulase-positive staphylococci of animal origin (43). Initial studies have demonstrated that when the 2008 OXA and FOX CLSI break points were applied, they failed to accurately identify MR in both *S. pseudintermedius* (MRSP) and *S. schleiferi* subsp *coagulans* (MRSC) (16, 17, 241).

The acquisition of antimicrobial resistance against β-lactam drugs in *S. aureus* is explained basically by two mechanisms: the production of β-lactamases and the expression of penicillin-binding protein 2 (PBP2' or PBP2A). The PBP2a is encoded by the *mecA* gene and carried by a mobile genetic element, the staphylococcal chromosomal cassette *mec* (SCC*mec*) (118, 158, 187, 252). The SCC*mec* is composed of the *mecA* gene, the recombinases (*ccr* genes) and the junkyard regions. SCC*mec* typing, which classifies SCC*mec* elements based on their structural differences, is used in epidemiological studies to discriminate MRSA strains or to define MRSA clones in combination with the genotype of methicillin susceptible (MS) *S. aureus* strain where a SCC*mec* element has integrated (114, 199). To date, eleven different types of SCC*mecA* elements have been identified in *S. aureus* (266). In canine MRSA isolates the types II, III and IV have been documented (12, 25, 94, 163, 222). The SCC*mec* type-III and -IV have been described in MR *S. schleiferi* subsp *coagulans* from canine origin (73, 226), whereas in MRSP a variant labeled SCC*mec* type II-III has been reported (55, 237). In addition, the SCC*mec* type-III and type-V have been also identified in MRSP (73, 180, 222, 233, 237).

The increase in the incidence of MR *Staphylococcus sp* in canines necessitates further epidemiological and molecular studies. Facilitating the increased accuracy in the diagnosis and recognition of the aforementioned pathogens is vital to such investigations. Consequently, the main objective of this study was to characterize coagulase positive *Staphylococcus* species isolated from canine clinical specimens from an epidemiological, microbiological and molecular point of view. Additionally, an evaluation of the laboratory methods available for the detection of MR was completed. To meet the above objectives, an examination of the methods used by veterinary clinical laboratories for the identification of methicillin resistance in staphylococci was performed; epidemiological data from canines carrying MR staphylococci isolates were analyzed, and molecular typing of the SCC*mec* by the means of multiplex PCR was carried out.

As expected, a significant frequency of MR in *Staphylococcus* species from dogs was identified. Although the number of MRSA and MRSC included in this study was low, the epidemiological data collected offers valuable information. To the author's knowledge, this is the first time that type-V SCC*mec* has been reported in MRSC. It is suggested that further investigation needs to be done to support the present epidemiological data as well gain insight on the mechanisms of antimicrobial resistance developed by canine staphylococcal species.

Material and methods

Bacterial strains. A total of 324 coagulase positive *Staphylococcus* species were collected from canine clinical specimens received in Auburn University, College of Veterinary Medicine, Bacteriology and Mycology Diagnostic Laboratory. With the exception of some of the MRSA isolates, which were collected starting in 2000, all the other isolates were collected from October 2006 through February 2010. Specimen sources were skin (biopsies and surgical wounds), ear, bone, fluid, urine and other tissues. Clinical specimens were processed following established laboratory protocols, inoculating samples in bovine blood agar (4%) which were incubated at 35 ºC for 24 – 48 hr. Isolates were selected based on colony characteristics: 1 to 3 mm of diameter, white, opaque, or golden color in the case of *S. aureus* and also the production of hemolysis were considered. Conventional biochemical assays including catalase (3% H_2O_2), tube coagulase test (BBL coagulase plasmas; Becton Dickinson), acetoin production (1ml of 1.7% MRVP medium) and carbohydrate fermentation patterns (trehalose, 1%; purple agar base: 3.7%, mannitol 1.2% and maltose 3.7%) were used for specie identification, Table 4. In this study, only one isolate of each species per patient was included. Epidemiological information, such as age, gender and source of infection were collected with some limitations. All the isolates were stored frozen at -80°C in 20% glycerol stock.

Susceptibility testing against β-lactam drugs. The CLSI has suggested that because MR *Staphylococcus* species have the potential to be resistant to all β-lactams an isolate identified as MR should be reported as resistant against all β-lactam antimicrobials. Following the CLSI guidelines a standardized agar disk diffusion test using OXA (1µg) and FOX (30µg) on Mueller Hinton salt agar (2%) was performed to identify methicillin resistant strains. Drugs were

evaluated according to the zone diameter interpretative criteria published in 2004 and 2008, CLSI documents M31-S1 and M31-A3 respectively, Table 5 (43, 192).

Detection of the penicillin binding protein 2a (PBP2a). The presence of a functional PBP2a is associated with methicillin resistance in *S. aureus*. A validated latex agglutination test was used to detect the PBP2a, according to manufacturer instructions (Oxoid; Hampshire, UK). Results were considered positive when agglutination and clearing of the latex suspension were observed. Negative results, were characterized by failure to produce agglutination. Intermediate results were retested.

DNA isolation, purification and sequencing. For the extraction of bacterial DNA, two or three colonies were obtained from 18 to 48 hour cultures inoculated in bovine blood (4%) or tryptic soy agar (4.1%). The colonies were suspended in 100µl of RNAse-free distilled water. The suspension was boiled at 95°C for 7min and then centrifuged at 15,000 *g* x 1 min. The supernatant was then used as a crude extract for the polymerase chain reaction (PCR) which was performed for SCC*mec* typing and amplification of the *ccrC* gene. To characterize the PCR products from the *mec*A and *ccrC* regions, DNA was isolated from the agarose gel after electrophoresis was performed using a QUIAquick ® gel extraction kit, and following the manufacturer's instructions. The extracted DNA was submitted for automated nucleotide sequencing. Nucleotide sequencing results were analyzed using the National Center of Biotechnology Information (NCBI) database through BLAST 2.2.24., and nucleotide sequence accession numbers: AB121219.1 for *ccr*C and NC_002952.2 for the *mec*A gene.

Restriction-fragment polymerase chain reaction (RF-PCR). Due to the inability of conventional microbiological methods to differentiate the *S. intermedius* group: *S. pseudintermedius, S. intermedius* and *S. delphini,* a restriction fragment length polymorphism-PCR (RFLP-PCR) described by Bannoehr *et al*., 2009 was performed, Table 6 (11). The PCR method amplifies the staphylococcal DNA sequence that encodes the enzyme phosphoacetyltransferase, (*pta* loci), and analyzes the presence or absence of a restriction site for the enzyme *Mbo*I. The *Mbo*I restriction site is absent in all *S. intermedius* and *S. delphini*.

According to the method of Bannoehr *at al.,* the *pta* PCR product of 320 bp is expected from a conserved region of all staphylococcal species. *S. pseudintermedius* PCR products contained a single *MboI* site, resulting in two restriction fragments of 213 bp and 107 bp, respectively (10). *S. aureus* isolates should contain a unique MboI site at different loci resulting in restriction fragments of 156 bp and 164 bp that appear as a single band after being resolved in 2% (wt/vol) agarose by electrophoresis. The RFLP-PCR profile of *S. aureus* is distinguishable from the restriction fragment profile of *S. pseudintermedius*. In contrast, *S. delphini, S. intermedius* and the *S. schleiferi* strains do not contain any *Mbo*I restriction site in the *pta* PCR amplified region (10).

SCC*mec* typing. In order to type the SCC*mec* cassette, a multiplex PCR described by Milheirico *et al.,* was performed (177). The multiplex PCR allows the typing of the SCC*mec* type I through VI. Briefly, the PCR mixture contained 25 µl of PCR master mix preparation containing 25units/ml *taq* DNA polymerase, 200µm (each) deoxynucleoside triphosphate and 1.5mM of $MgCl_2$ (Promega® cat# M7502). Additionally, 20 µl of a primer mix composed of 0.2 µM primers kdp F1 and kdp R1; 0.4 µM primers CIF2 F2, CIF2 R2, RIF5 F10, RIF5 R13,

SCC*mec* III J1F, SCC*mec* III J1R, SCC*mec* V J1 F, and SCC*mec* V J1 R; 0.8 µM primers *mec*I P2, *mec*I P3, dcs F2, dcs R1, *mec*A P4, *mec*A P7, ccrB2 F2, ccrB2 R2, ccrC F2, and ccrC R2. Finally, 1-2 µl of DNA template and distilled RNase free water were added to give a 50µl total reaction volume. Primer nucleotide sequences are detailed in Table 6. Cycling reaction conditions were: 94°C for 4 min; 30 cycles of 94°C for 30 seg, 53°C for 30 seg, and 72°C for 1 min; and a final extension at 72°C for 4 min. PCR products (10 µl) were resolved in a 3% agarose gel in 0.5% Tris-borate-EDTA buffer and 0.5 µg/ml of ethidium bromide for DNA staining and visualization with electrophoresis. Electrophoresis was carried out at 4 V/cm for 2.5 hr and ethidium bromide was visualized with ultraviolet light (177). MRSA reference strains from the American Type Culture Collection (ATCC) were used as type-specific controls: SCC*mec* type-I: BAA-44; type-II: 43300/BAA-1681; type-III: BAA-39; type-IV: BAA-1756/BAA-1696; type-V: BAA-1688; type-VI: BAA-42.

Statistical analysis. Statistical analysis was performed using statistical software (Statistical Analysis System; SAS version 9.2, SAS Institute, Cary, NC). Data were classified according to susceptibility or resistance to the antimicrobials being analyzed. Categories including the isolates origin (source, age, gender) and the isolates testing (OXA and FOX susceptibility, penicillin-latex agglutination test [PLAT]) were analyzed using the frequency procedure (PROC FREQ) and compared through the Chi-square test and Fisher exact tests. For all comparisons, values of $p< 0.05$ were considered significant. The sensitivity was calculated as the number of isolates that were resistant by the screening test (OXA / FOX), divided by the number of resistant isolates that demonstrated to carry the *mecA* gene by PCR. The specificity was calculated as the number of isolates that were susceptible by the screening test (OXA / FOX),

divided by the number of susceptible isolates that did not carry the *mecA* gene demonstrated by PCR.

Results

Species classification and epidemiological information. A total of 324 coagulase positive *Staphylococcus* species were isolated from clinical specimens collected from dogs. After microbiological identification, RFLP-PCR and susceptibility testing against FOX and OXA; 43.5% (141/324) of the isolates were classified as MS and 56.47% (183/324) as MR. The frequency distribution by species and susceptibility against methicillin was: 3.7% (12/324) MSSA; 9.25% (30/324) MRSA; 4.93% (16/324) MS *S. schleiferi* subspecies *coagulans* (MSSP); 8.64% (28/324) MRSC; 34.87% (113/324) MS *S. pseudintermedius* (MSSP) and 38.58% (125/324) MRSP.

Complete epidemiological data was not available for each isolate, for this reason there was no exact match between the total of isolates collected with the results shown below. Regarding the source of isolates, 51% (152/297) were collected from skin (skin biopsies, pustule, wound swabs, and others), 20% (59/297) from ears (ear swabs and surgical ear tissues), 10% (30/297) from bones and internal body tissues, 10% (31/297) from urine, 2% (7/297) from joint fluids and 6% (18/297) were from a non-groupable source, Table 7. To determine the influence of age, four different groups were created and analyzed by age, Table 8. As a result, 11% (31/285) and 35% (99/285) were found in animals <2 and ≥ 9 years old, respectively. In addition, 27% (78/285) were isolated from dogs in the 2 – 5 years old group and 27.01% (77/285) in the 6 – 8 years old group. Interestingly, 17 MRSA isolates were isolated from animals <2 years old,

which represents 57% (17/30) of the total of MRSA isolates. Even though this group had the higher number of isolates, no MRSA was isolated from animals ≥ 9 years old. Regarding gender, a significant difference ($p<0.05$) was observed specifically in *S. schleiferi* subspecies *coagulans.* Specifically, 65% (28/43) of the total *S. schleiferi* subspecies *coagulans* were collected from females and 35% (14/44) from males. In contrast, 59% (22/37) of *S. aureus* isolates were isolated from males and 41% (15/37) from females. Concerning *S. pseudintermedius,* no significant sex difference was found, Table 8.

PBP2a latex agglutination test. The PLAT was easy to utilize and provides a rapid result once an isolate from the suspect culture is ready to be tested in the laboratory. Using this method a total of 174 isolates were found to be MR, of which 20% (35/175) were MRSA, 16% (27/175) MRSC and 64% (112/175) MRSP, Table 9.

SCC*mec* typing. The SCC*mec* element contains the *mec* gene complex (the *mec*A gene and its regulators) and the *ccr* gene complex, which encodes site-specific recombinases responsible for the mobility of SCC*mec* (114, 126). In this study, a multiplex PCR strategy for the rapid assignment of SCC*mec* types to MR staphylococci strains was used. This approach was able to properly identify SCC*mec* types-I to VI in MRSA reference strains from the ATCC used as type-specific controls, figure 5. Using the multiplex PCR method, it was determined that of the 324 isolates included in the study, 171 carried the *mec*A gene. Also, the technique provided the identification of the SCC*mec* in MRSP and MRSC, although 9% (16/171) of those isolates were untypable for SCC*mec,* but *mecA* positive. The results of the SCC*mec* typing are summarized in Table 10. Most importantly, the SCC*mec* type-V was identified in 66% (113/171) of the canine

clinical isolates, followed by the type-IV and II, with 13% (23/171) and 10% (17/171), respectively.

Surprisingly, the SCC*mec* type-V was present in 67% (18/27) of the MRSC. The most unique feature of type V SCC*mec* is the carriage of a type 5 *ccr* gene complex composed by the *ccr*C gene and its surrounding ORFs (116). Accordingly, structural elements that correspond with type-V (*ccrC* and *mecA*) were sequenced and confirmed with published sequences in the data base of the NCBI. The *SCCmec* type-V has been previously reported in MRSA and MRSP; to our knowledge this is the first time that this element is described in MR *S. schleiferi* subspecies *coagulans.*

Disc diffusion testing for resistance to oxacillin and cefoxitin versus penicillin-binding protein 2a latex agglutination test. Results of the disc diffusion test of OXA and FOX are summarized in Table 9 using both 2004 and 2008 CLSI interpretative criteria. As indicated, when the isolates were evaluated by the 2008 criteria for resistance to FOX, 26 were identified as MRSA, 4 as MRSC and 5 as MRSP. In contrast, when testing the same isolates against OXA, 29 were identified as MRSA, 24 as MRSC and 95 as MRSP. The lack of correlation between OXA and FOX for the recognition of MR in *Staphylococcus* species has been reported previously, (16, 241) and the observations were confirmed to be significantly in this study using a larger number of isolates.

To determine the most appropriate method for detection of MR in veterinary staphylococci, a statistical analysis based on sensitivity and specificity was performed. For the analysis, PCR amplification of the *mec*A gene was considered the gold standard. Data from 299 staphylococcal isolates was used for this analysis, Table 9 and 11. Using PCR for *mec*A it was

determined that 29 *S. aureus* were MR. Using the 2008 CLSI criteria the same isolates were considered MR by OXA. In comparison, isolates of the group *S. schleiferi* subspecies *coagulans* and *S. pseudintermedius* did not have a good correlation when examined using the same criteria. By PCR, 115 isolates were identified as MRSP and 27 MRSC. Whereas, when isolates were tested using OXA susceptibility, the number of MRSP and MRSC was 95 and 24, respectively. When isolates were examined using FOX susceptibility, 26 *S. aureus* were classified as MRSA, but just 4 were considered MRSC and 5 MRSP. As mentioned previously, using PLAT, 35 isolates were recognized as MRSA, 27 MRSC and 112 MRSP. Therefore, these results confirm that FOX susceptibility testing does not adequately identify MR in either *S. pseudintermedius* or *S. schleiferi* subspecies *coagulans.*

The classification of MR using the 2004 CLSI criteria is summarized in Table 9. Using this criterion the number of MR *S. aureus* and *S. schleiferi* subsp *coagulans* identified was identical to the PCR results, 29 and 27, respectively. In contrast, 124 isolates were considered MRSP using the CLSI 2004 interpretive standards, while PCR identified only 115 of the isolates as MRSP. Ninety-four % of the isolates were identified as resistant by *SCCmecA* and PBP2a and 5% of the isolates identified as MR by PLAT were false positives when the SCC*mecA* PCR was considered the gold standard. It is interesting to note that 12 isolates belonging to the *S. pseudintermedius* group were classified as MR by OXA using 2004 criteria, but were not classified as MR by PLAT and PCR.

According to the CLSI interpretive break points published in 2008, the sensitivity of the OXA disk diffusion test for *S. aureus, S. pseudintermedius* and *S. schleiferi* subsp *coagulans* was 100%, 78.2% and 85.1% respectively. The specificity was 90.9% for *S. aureus*, 94.0% for *S. pseudintermedius* and 94.1% for *S. schleiferi* subspecies *coagulans*. In comparison, FOX

diffusion testing had a sensitivity of 89.6%, 4.35% and 14.8% for *S. aureus, S. pseudintermedius* and *S. schleiferi* subspecies *coagulans* respectively, with specificities of 100% for the three species of staphylococci. In contrast, when examining the CLSI 2004 criteria the sensitivity of OXA was 100% for the three staphylococcal species with specificity of 100% for MRSC, 90.9% for MRSA and 91% for MRSP, Table 11.

The overall sensitivity and specificity of the FOX disk diffusion test to identified MR using the 2008 criteria for the three *Staphylococcus* species was 20.4% and 100%, respectively while the values for OXA were 83.04% and 93.7%. The same parameters were calculated using the CLSI interpretive breakpoints published in 2004 for OXA, yielding an overall sensitivity of 98.25%, with a specificity of 87.5%. Although the use of PLAT is an alternative method for the detection of MR in *Staphylococcus* species, its overall sensitivity and specificity were also calculated and found to be 98.8% and 97.65%, respectively.

Discussion

Staphylococci are considered opportunistic pathogens in the canine. Of the 324 isolates recovered from canine specimens, 73% (238/324) were identified as *S. pseudintermedius*, 14% (44/324) were *S. schleiferi* subspecies *coagulans* and only 13% (42/324) were *S. aureus*. Of these staphylococcal isolates, 38% (125/324) were found to be MRSP, followed by 9% (28/324) MRSC and 9% (30/324) MRSA. The identified distribution is becoming a common trend (119, 183, 237). The increase in the number of methicillin resistant staphylococci in dogs has serious consequences not just for the canine population but also for humans due to the potential of zoonotic transmission with reports of humans infected with *S. pseudintermedius* and S. schleiferi

subspecies coagulans (257, 274). Furthermore, it has been hypothesized that horizontal gene transfer of the SCC*mec* components (*mecA* and *ccr* genes) among staphylococcal species could lead to the development of resistance to antimicrobial drugs throughout the genus (33, 158).

Although limited, some unique epidemiological findings were identified in this study. *Staphylococcus schleiferi* subsp *coagulans* was isolated from skin and ears, 37% (16/43) and 58% (25/43) respectively, but was not associated with urinary or body cavity tissue infections. This host-tissue association has been reported previously in canines infected with this microorganism (19, 69, 112). *S. aureus* was isolated primarily from bone/tissue and skin, in equal numbers 31% (12/39). An interesting finding was that, within the *S. aureus* group, MRSA strains were found in 11 of the 12 cases associated with bone/tissue and in 5 cases of joint infections (no MSSA were isolated from joints). These results differ from other studies, in which most MRSA and MSSA were found associated with pyodermas and otitis externa or interna infections, and rarely became invasive (67). However, there have been serious reports of MRSA in dogs after surgical procedures which emphasize the potential for deep and skeletal tissue infections (151, 173). The finding that more *S. aureus* isolates were associated with bone, joint fluid and tissue infections was statistically significant when compared with the sources of *S. pseudintermedius* and *S. schleiferi* subspecies *coagulans.* It is recommended by the authors to expand the investigation by increasing the number of isolates in order to validate and confirm the clinical significance of this information. Of the remaining specimens, 10% of the staphylococcal isolates were from urine, of which 94% (29/31) were *S. pseudintermedius.* The other 6% (2/31) were *S. aureus*. As expected 58% (124/215) of the *S. pseudintermedius* isolates were collected from skin.

Boost *et al*., (2008), reported higher colonization rates in female dogs infected with *S. aureus* than infected males. The result of the mentioned study indicated that colonization was significantly more frequent in female (12%) than in male dogs (6%). The authors speculated that this difference would possibly be due to hormonal factors or behavioral differences between genders (24). Similarly, the results of the present study show in the group *S. schleiferi* subsp *coagulans* a significant difference ($p < 0.05$) in relation to the gender of the infected canines, 65% (28/43) were from female and 35% (15/43) from male dogs. By contrast, in the MRSA group, 61% (20/33) and 39% (13/33) where recovered from males and females respectively. These contradictory results only indicate that the data continues to be insufficient to generate accurate conclusions regarding to gender.

In relation to age, except for the group <2 years old, the distribution was relatively homogeneous. The group <2 years old had the lowest frequency of isolates collected, 11% (31/285) of the total of which 55% (17/31) where caused by MRSA. These findings reaffirm a previous report where a trend for carriage associated with the age of the dog and MRSA was reported; higher colonization in older dogs (10%) compared to puppies (5%) and younger dogs (8%) (24). The authors of the present study are in agreement with Weese and Duijkeren in 2009 on their statement about the necessity of the development of new studies directed to the investigation of risk factors associated with staphylococcal diseases in pets (295).

Different techniques have been used to identify and characterize markers for particular epidemic clonal lineages of *S. aureus* in human medicine. Pulse field gel electrophoresis, multilocus sequence typing, *spa* and SCC*mec*A typing are the most commonly used (14). These techniques are good tools for use in *S. aureus* epidemiological investigations, including determining the ancestral lineage of related isolates and the execution of epidemiological

investigations in hospital environments (253). SCC*mecA* typing was used in this study, which permitted the identification of MRSA strains that are commonly related with nosocomial or health care associated (HA-MRSA) infections. Typically, SCC*mec* I, II and III is associated with nosocomial clones while SCC*mec* types IV, V and VII are more prevalent in the community (CA-MRSA) (14, 56, 253). As was expressed previously, in veterinary medicine it is necessary to develop epidemiological studies to identify the behavior of MRSA strains and other *Staphylococcus* species in domestic animals.

Several studies using SCC*mecA* typing have been published on MRSA strains from animal origin and others have included different *Staphylococcus* species (162, 222). The SCC*mec* type-III, -V, –VII, and two variants, the II-III (variant of the type-II) and another identified as type-V_T (variant of type-V) have been found in MRSP (26, 33, 55, 73, 233, 237). The SCC*mec* type-V_T has recently been reclassified as SCC*mec* type VII (256). Little is known about the of SCC*mecA* genetic composition in *S. schleiferi* subspecies *coagulans*. However, the SCC*mec* type-III and -IV has been identified in MRSC from dogs (73, 226). Previous studies in MRSA strains collected from canine specimens have reported strains harboring the SCC*mec* type-II, -III and -IV (25, 163, 180, 237).

Results of the SCC*mecA* typing are shown in Table 10. In this study, 66% (113/171) of the canine MR *Staphylococcus* species carried the SCC*mec* type-V which corresponds by species to 83% (94/113) MRSP, 16% (18/113) MRSC, and 1% (1/113) MRSA isolates. These findings are remarkable; not only is this the first time the SCC*mec* type-V has been reported in MRSC, but also because 67% (18/27) carried SCC*mec* type-V while only a single MRSC isolate harbored the type-IV. The SCC*mec* type-II was the most frequent cassette found in MRSA strains, which represented only10% (17/171) out of the total MR isolates examined, but was 57%

(17/29) of the MRSA group. The type-IV cassette was carried by 13% (23/171) of the total strains included in this study; 44% (10/23) MRSA, 52% (12/23) MRSP and 4% (1/23) MRSC.

Using multiplex PCR, all the SCC*mec* elements carried by MRSA isolates were typed. However, for MRSC and MRSP 9% (16/171) of the isolates were confirmed positive for the *mec*A gene, but could not be typed. Previous studies, using multilocus sequence typing, have hypothesized about a broad geographic dissemination of successful MRSP genetic lineages. They resolved that two dominant sequence types, in Northern Europe (ST71) and in the USA (ST68), contained distinct SCC*mec* elements; type- III and type-V, respectively (10). Consistent with that report, the SCC*mec* type-V was dominant in the MRSP isolates investigated in this study.

In 2004, Ito *et al.* described for the first time the SCC*mec* type-V, which is defined as a small SCC*mec* element (28 kb) that does not carry any antibiotic resistance genes besides *mecA* (116). Unlike the extant SCC*mec* types, it carries a set of foreign genes encoding a restriction-modification system that might play a role in the stabilization of the element on the chromosome. It is important to emphasize that CA-MRSA is characterized by these two structural variants of the mec element, the SCC*mec* type-IV and type-V (114, 137, 161, 177, 179). It has been speculated that those mobile genetic elements were formed in a different *Staphylococcus* species, and that *S. aureus* acquired those elements by horizontal transfer. Also it has been suggested that the *mec* gene complex and the *ccr* gene complexes (of diverse SCC elements) go through complex recombination and rearrangement processes in the genomes of coagulase-negative staphylococci. Thus, novel types of SCC*mec* elements are incessantly generated and only a fraction of them are transferred to *S. aureus* strains in the community (116). Furthermore, the fact that SCC*mec* type-IV has been continuously reported in MR *Staphylococcus* species of

animal origin has influenced researchers to hypothesize that the small size and high polymorphism of type-IV SCC*mec* may render this element to be highly transmissible to other staphylococcal species by an unidentified mechanism. Therefore, this may contribute to cats and dogs becoming a potential reservoir for community-acquired MR staphylococci (163). The incidence of the SCC*mec* type -IV in 34% (10/29) of the MRSA isolated in this study might support such occurrence.

The findings of this study are a valuable contribution to information further establishing the epidemiological significance of veterinary staphylococci in animals and humans. From the 171 mecA positive strains, the SCC*mec* type –V was found in 66% of the isolates examined, followed by 13% SCC*mec* type –IV. The SCC*mec* type -IV has been found in coagulase negative staphylococci and it is believed that these strains might be involved in the horizontal transmission of SCC*mec* type-IV and type-V to *S. aureus* (114, 115). Results of this study may support the theory that MRSP and MRSC could be involved in the horizontal transfer of the SCCmec elements. As mentioned previously, the main limitations of this study were the low number of MRSA and MRSC isolates as well as difficulties in the collection of epidemiological information to generate more sustainable conclusions.

In 2008, the CLSI issued a new guideline for the determination of *in vitro* antimicrobial susceptibility of MR *Staphylococcus* species for isolates from animal origin (43). This new document replaced the guideline published in 2004 (192). Changes related to the detection of MR in *Staphylococcus* species from human and animal origin were: 1) the use of FOX as a surrogate for OXA for coagulase positive *Staphylococcus* species*;* 2) FOX was the preferred disk when performing disk diffusion testing; 3) *S. aureus* interpretative criteria should be used for the coagulase positive veterinary *Staphylococcus* species such as *S. intermedius,* but not for the

coagulase-variable species *S. hyicus;* 4) Use of OXA for the determination of *in vitro* antimicrobial susceptibility by disk diffusion using zone diameters equivalent to those recommended for human and veterinary isolates of *S. aureus* (≥ 13 mm susceptible, 11-12 mm intermediate, ≤10 mm resistant); and 5) As an alternative method, the use of a latex agglutination test to identify isolates that are PBP2A positive (43). Recent reports demonstrated the failure of FOX to detect MR in clinical *mec*A-positive *S. pseudintermedius* and MR *S. schleiferi* subspecies *coagulans* (16, 241). Also, a failure to detect methicillin resistance in some *mec*A-positive isolates of *S. pseudintermedius* has been reported when the current interpretative criteria for OXA was evaluated (241).

In this study, the diagnostic sensitivity and specificity of cefoxitin, oxacillin and penicillin-binding protein 2a latex agglutination test were compared using amplification of the *mecA* by PCR as the gold standard. For this purpose, the previous CLSI criteria (2004) was also considered which refers to the use of OXA for detection of MR, with disk diffusion zone diameters of ≥ 13 mm as susceptible, 11-12 mm as intermediate and ≤10 mm as resistant for *S. aureus* (192). For other staphylococci the CLSI criteria (2004) indicate ≥ 18 mm susceptible and ≤ 17 mm resistant. After analyzing the results of this study, it was concluded that for *S. aureus,* the 2008 interpretive standards for OXA offered an excellent alternative (sensitivity 100%) for the recognition of MR. On the other hand, for *S. pseudintermedius* and *S. schleiferi* subspecies *coagulans* the sensitivity was 75% and 85% respectively, but when utilizing the 2004 criteria the sensitivity was 100% for all three staphylococcal species. Overall, using the 2008 criteria for OXA interpretation, the sensitivity was 83%, but when the total sensitivity was calculated using the 2004 criteria, the sensitivity increased to 98%.

Using FOX susceptibility testing (2008 CLSI), the sensitivity was low, 4.35 and 14.8 for *S. pseudintermedius* and *S. schleiferi* subspecies *coagulans,* respectively that FOX is not recommended for the detection of MR in these staphylococcal species. The PLAT was very useful, easy to perform, and once the suspect culture was ready to be tested in the laboratory the results were fast. Although, 5% of the isolates identified as MR by PLAT were false positives when SCC*mecA* detection by PCR was considered the gold standard, this technique is an excellent alternative for the identification of MR in *Staphylococcus* species yielding a sensitivity and specificity of 98.8% and 97.65%, respectively. Therefore, the authors of this study agree with the document published by Papich in 2009, (205) who reported that The Veterinary Antimicrobial Susceptibility Testing (VAST) subcommittee of the CLSI revised the current 2008 standard to indicate that oxacillin disk diffusion ($R \geq 17$ mm) and MIC breakpoints ($R \geq 0.5$ μg/ml) are the accurate indicators of *mecA*-mediated resistance in *S. pseudintermedius,* which is also recommended by other authors (16, 241).

In summary, veterinarians should be alert to the increase in multi-drug resistant staphylococci. Further epidemiological studies on *Staphylococcus* in the canine should be performed to improve detection of drug resistance and identify the changes in the staphylococcal population and its possible affect upon man. It is recommended that the PLAT or the 2004 CLSI criteria for OXA disk diffusion test interpretation be used for detection of MR in *S. schleiferi* subspecies *coagulans* and *S. pseudintermedius* isolates. The authors of this study urge CLSI to revise and publish the correct standards for interpretation of oxacillin for the detection of *mecA*-positive *S. pseudintermedius* strains. With increased discovery of methicillin resistant *S. schleiferi* subspecies *coagulans,* it is imperative to evaluate the interpretive criteria to ensure adequate detection. To our knowledge this is the first time that SCC*mec* type-V has been

reported in *S. schleiferi* subspecies *coagulans* supporting the need for further study of this species. In general, these results are an important contribution in the study of the zoonotic potential of the genus *Staphylococcus*, and the capability of these bacteria to interchange genetic material, including the SCC*mec* element, as well as other virulence factors.

IV. Epidemiological aspects and virulence factors genes (*siet, lukI* and *lukF*) in *Staphylococcus pseudintermedius, Staphylococcus schleiferi* subspecies *coagulans and Staphylococcus aureus* isolated from canine clinical specimens

Summary

In recent years a higher interest in *Staphylococcus* species has occurred due to the increased number of multidrug resistant *Staphylococcus* species reported in humans as well as in animals and the risk of subsequent zoonoses (289, 295). The most common *Staphylococcus* sp isolated from dogs are *Staphylococcus pseudintermedius* and *Staphylococcus schleiferi* subspecies *coagulans* (220, 250). Epidemiological information of the risk factors associated with methicillin-resistance (MR) in *S. pseudintermedius* and *S. schleiferi* subsp *coagulans* isolated from dogs is sparse (31, 295). The aim of this study was to analyze and display epidemiological information of *S. schleiferi* subsp *coagulans, S. pseudintermedius* and *S. aureus* isolated from canine clinical specimens. In addition, the presence of the genes *siet*, *lukI* and *lukF*, which encode for important virulence factors in the mentioned *Staphylococcus* sp was characterized. A total of 577 coagulase positive *Staphylococcus* species were collected from culture of canine clinical specimens, and epidemiological information, such as age, gender, source of infection, breed and previous antimicrobial therapy were collected with some limitations. In addition, the incidence of the genes *siet*, *lukI* and *lukF* were determined by PCR. The number of MR strains recovered represented 38.82% (224/577) of the total collected. Overall, results indicated a higher probability of finding MR *S. schleiferi* subsp *coagulans*

(MRSC) and MR *S. pseudintermedius* (MRSP) during 2009 and 2010, when compared with 2007. A significant difference ($p \leq 0.05$) indicated that isolates from dogs with a history of antimicrobial therapy have a higher risk of being MR positive, than from dogs without therapy. Genes that encode PVL were not found either in *S. pseudintermedius* or *S. schleiferi* subsp *coagulans.* Within the *S. aureus* group, the panton-valentine leukocidin PCR was done in the MRSA group with four testing positive, 12.9% (4/31). LUK-I genes were present in a total of 254 isolates and distributed as follow, 1 in MRSC, 3 in MRSA, 91 in MSSP and 159 in MRSP. The *siet* gene sequence was amplified in 5 MRSA, 27 MRSC, 8 MSSC, 118 MRSP and 90 MSSP. An important finding in this study was the discovery of the genes *siet* and *luk*I in *S. aureus* and *S. schleiferi* subsp *coagulans,* which may be related with horizontal gene transfer. Collectively, these findings support the need for further epidemiological and clinical studies concerning virulence factors in *Staphylococcus* species of canine origin.

Introduction

For decades staphylococcal disease in humans and animals has been studied by the scientific community, but in the last years a higher interest has been demonstrated because of the increased number of multidrug resistant *Staphylococcus* species reported in humans as well as in animals and because of the risk of subsequent zoonoses (220, 271). *Staphylococcus* species are considered commensal microorganisms in both, human and many animal species (66, 68, 220). In humans, *S. aureus* is the most common *Staphylococcus* species associated with human disease. Likewise, this microorganism is an important pathogen in veterinary medicine. Cattle, sheep, goats, pigs, horses, dogs, cats and poultry are frequently affected by *S. aureus* (220, 250). The pathogenesis of the disease caused by *S. aureus* has been widely studied and the induction of the infection requires minor trauma and immunosuppression, together with the existence of virulence factors (71, 220). Virulence factors in *S. aureus* have been extensively investigated and classified according to their role in the infection process. Some are structural and others are secreted products (90, 187).

The structural virulence factors are represented by numerous surface proteins called "Microbial surface components recognizing adhesive matrix molecules" (MSCRAMS). These groups of proteins are anchored to the peptidoglycan by a covalent bond, and participate in the adherence to different host proteins, such as collagen, fibronectin, or vitronectin (71, 90, 209). An important structural factor is protein A (Spa) which binds the FC portion of IgG having an antiphagocytic effect. In addition, Spa modulates platelet aggregation. Hence it may be involved in the induction of endovascular disease. Spa also sensitizes B cells by increasing their recognition of TLR2 ligands and binding tumor necrosis factor receptor 1 (TNFR1) which has been associated with staphylococcal pneumonia (71). Most *S. aureus* produce a microcapsule

that is considered an important virulence factor by providing protection from the innate defenses including complement, prevention of killing by neutrophils or macrophages and activating human CD4+ T cells.

As mentioned previously other virulence factors are secreted into the extracellular medium. Numerous enzymes, such as lipase, esterases, elastase, staphylokinase, deoxyribonuclease, hyaluronidase and phospholipase contribute to host tissue destruction. Some of these proteins are considered superantigens and the most commonly studied are enterotoxins (SE), toxic shock syndrome toxin-1 (TSST) and exfoliative toxins (ET) which are mainly involved in toxin-mediated disease and sepsis (158, 187). These superantigens can produce a sepsis-like syndrome by initiating a "cytokine storm." Some strains also produce epidermolysins or ETs capable of causing scalded skin syndrome or bullous impetigo (218). In addition, *S. aureus* produces cytotoxins, such as β-haemolysin, α-toxin and Panton-Valentine leukocidin (PVL). The cytotoxins can cause leukocytes, monocytes and erythrocytes destruction by the formation of pores in the cell membrane (71, 90). Other virulence factors such as catalase, coagulase, and clumping factor are also involved in staphylococcal pathogenesis.

The regulation of the virulence factors is important in the pathogenesis of staphylococcal disease. In *S. aureus* the accessory gene regulator (*agr*) is a quorum-sensing system that plays a critical role in the regulation of staphylococcal virulence. The *agr* controls a large set of genes, including most of those encoding cell-wall-associated and extra-cellular proteins (197). In members of the *S. intermedius* group, an *agr* locus has also been identified (60). During pathogenesis, endothelial cells are crucial in the infection process. Epithelial cells, osteoblasts and neutrophils can also be infected. Phagocytes take the bacteria up into a plasma membrane-derived vacuole (phagosome), which undergo maturation into endosomes and lyposomes with

degradative properties. Staphylococci may escape from the endosomes, replicating in the cytoplasm of the cells. Some of the virulence factors mentioned previously participate in this process. In addition, the development of genetic variants called small-colony variants during an active infection may contribute to persistent and recurrent infections (286).

In dogs, *S. aureus* is considered a transient microorganism, and *Staphylococcus pseudintermedius* is considered a commensal organism mainly isolated from the skin and mucosa (68, 295). Commonly, *S. pseudintermedius* is associated with pyoderma, severe skin and soft tissue infections, and also causes opportunistic infections in other body sites, especially in the ears (237, 271). Other staphylococcal species associated with canine disease is *Staphylococcus schleiferi* subspecies *coagulans,* which is also implicated in canine dermatitis and otitis externa (31, 184). In the canine, *S. aureus* is less prevalent than *S. pseudintermedius;* however, it has been associated with numerous conditions, such as soft tissue infections, chronic pyoderma, post-surgical wound infection, joint invasion, urinary tract infections and even death (223, 250, 295).

Staphylococcus pseudintermedius is highly prevalent in dogs and some of its virulence factors have been studied. The production of enzymes (coagulase, protease and thermonuclease) and also the expression of toxins (hemolysins, exfoliative and enterotoxins) have been identified. Specifically, Leukotoxin (Luk-I), exfoliative toxins (ET): ETA, ETB, ETC, SIET and enterotoxins: SEA, SEB, SEC, SED, SEC_{canine} are among the most important virulence factors of pathogenic *S. pseudintermedius* (68, 263). The leukotoxin, Luk-I, was first described in *Staphylococcus intermedius* (78) which was thought to be the major staphylococcal pathogen of dogs and cats. It is now believed that isolates previously identified as *S. intermedius* actually belong to *S. pseudintermedius* (58, 237, 238). Characterization and sequence analysis have

shown that, similar to PVL, Luk-I is encoded as *luk*I operon with two cotranscribed genes, *luk*S and *luk*F, encoding LukS and LukF, respectively. Luk-I exhibits a strong leukotoxicity on various polymorphonuclear leukocytes, but has only slight hemolytic activity on rabbit erythrocytes (78, 158). Another important virulence factor produced by *S. pseudintermedius* is the exfoliative toxin, SIET; this toxin causes erythema, exfoliation, and crusting in dogs with pyoderma. The production of ET is associated with the pathogenesis of skin diseases of staphylococcal origin. In addition, other virulence factors such as fibronectin binding protein may be involved in skin colonization (80).

In addition to virulence factors, it is well known that different *Staphylococcus* species have the potential to develop resistance against diverse types of antimicrobials. The development of an infection by a multidrug-resistant *Staphylococcus* species (MDRS) that is able to express a variety of virulence factors creates a challenging case for clinicians (67, 122). The most important mechanism for the development of resistance against antimicrobials by *Staphylococcus* species is the production of an additional Penicillin-binding protein (PBP), called PBP2a (252). This protein has lower affinity for β-lactams, and confers a potential resistance against all the β-lactam drugs. The PBP2a is encoded by the *mec*A gene, which is carried by a mobile genetic element named staphylococcal cassette chromosomal *mec* (SCC*mec*) (114, 118, 158, 187). The SCC generally contains additional insertion sequences that allow the incorporation of additional antimicrobial resistance markers. Therefore, the MDRS typically exhibit resistance to other antimicrobial groups, such as erythromycin, clindamycin, aminoglycosides, fluoroquinolones, rifampin, tetracycline, and co-trimoxasole (75, 126, 130, 265).

In human medicine, methicillin-resistant *S. aureus* (MRSA) has become notorious because it is one of the leading causes of nosocomial infections worldwide. Moreover, the emergence of MRSA as a cause of infection in the community in patients without a previous history of hospital contact, has contributed to an increased interest in this pathogen. In dogs, the presence of MRSA has been reported several times, (173, 198) and has been accompanied by an increase in the number of methicillin-resistance *S. pseudintermedius* (MRSP) and *S. schleiferi* subsp *coagulans* (MRSC), (156, 183, 195, 225). It is important to remember that there is a risk of potential zoonotic transfer when *Staphylococcus* species are colonizing canines especially in light of the reports of human patients infected with *S. pseudintermedius and S. schleiferi* subsp *coagulans* (98, 147, 257, 274). In another context, it is necessary to consider that clinical disease in dogs associated with a methicillin-resistance *Staphylococcus* species is a challenge for the veterinarian and is the cause of expensive and long treatments that owners assume.

The aim of this study was to analyze and display epidemiological information of *S. schleiferi* subsp *coagulans, S. pseudintermedius* and *S. aureus* isolated from canine clinical specimens. The presence of the genes *siet, lukI* and *lukF* which encode for important virulence factors was examined from the available canine clinical isolates.

Material and methods

Bacterial strains. A total of 577 coagulase positive *Staphylococcus* species were collected from canine clinical specimens processed by Auburn University, College of Veterinary Medicine, Bacteriology and Mycology Diagnostic Laboratory. Clinical samples were processed following established laboratory protocols. Culture specimens were inoculated on bovine blood agar (4%) and incubated at 37ºC for 24 – 48 hours. Isolates were selected based on colony characteristics: 1 to 3 mm of diameter, white, opaque, or golden color, in addition to hemolysis. Conventional biochemical assays including catalase (3% H_2O_2), tube coagulase test (BBL coagulase plasma; Becton Dickinson), acetoin production (1ml of 1.7% [MRVP medium]) and carbohydrate fermentation patterns (trehalose 1%; purple agar base: 3.7%, mannitol 1.2% and maltose 3.7%) were used for species identification. A restriction fragment polymorphism-PCR described by Bannoehr *et al* (11) was performed to confirm species identification. All the isolates were stored frozen at -80°C in 20% glycerol.

The period of collection (data and isolates) was variable. In the case of *S. aureus*, isolates and epidemiological data included in this study were collected from July 2000 to February 2011. *S. pseudintermedius* epidemiological data and antimicrobial susceptibility results were collected from July, 1998 to February 2011. For the analysis of genes related with virulence factors, *S. pseudintermedius* isolates from January 2009 to February 2011 were included. Epidemiological data and isolates of *S. schleiferi* subsp *coagulans* were collected from October 2006 to February 2011.

Epidemiological information, such as age, gender, source of infection, breed and previous antimicrobial therapy were collected with some limitations. Regarding the source of the specimen, isolates were separated in to the following groups: skin (biopsies, surgical wounds,

and abscesses), ear (ear swabs, surgical), bone/internal tissues (bone pieces, tissue biopsies, tissue from necropsies), joint fluid, urine, reproductive tract (vaginal or uterus swabs, lavages), upper respiratory (nasal, broncheoalveolar lavage) and blood culture.

Susceptibility testing and DNA isolation. With the aim of detecting patterns of resistance to methicillin, antimicrobial susceptibility testing to oxacillin (OXA) (1µg) and cefoxitin (FOX) (30µg) was determined by the disk diffusion method using Mueller Hinton salt agar (2%) according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (196). The antimicrobial susceptibility profiles of the isolates were determined by disk diffusion method using Mueller Hinton agar or broth dilution in accordance with CLSI guidelines. The antimicrobial agents tested were amikacin, amoxicillin/clavulanic acid, ampicillin, cefazolin, ceftiofur, cephalothin, chloramphenicol, clindamycin, enrofloxacin, erythromycin, gentamicin, marbofloxacin, orbifloxacin, penicillin, rifampin, tetracycline and trimethoprim-sulfamethoxazole. American Type Culture Collection (ATCC) quality control strains included *S. aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212.

For the extraction of bacterial DNA, two or three colonies were obtained from 18 – 48 hour cultures inoculated on to bovine blood agar (4%) or tryptic soy agar (4.1%). The individual colonies were picked and suspended in 100µl of RNAse-free distilled water. The bacterial suspension was boiled at 95°C for 7min and then centrifuged at 15,000 *g* x 1 min. Then the supernatant was collected. The DNA supernatant extracts were stored at -20°C until used for PCR.

Virulence genes detection. The presence of the *lukS*/F-PV genes, which encode for the PVL S/F bicomponent proteins, were determined by PCR. Primer nucleotide sequences are described

in Table 12. Briefly, PCR mixture contained, 25 µl of PCR master mix preparation (50units/ml *taq* DNA polymerase, 400µm [each] deoxynucleoside triphosphate and 3mM of $MgCl_2$ Promega® [M7502]). In addition, 0.08 µM of primers: Luk-PV-1 and Luk-PV-2 were added (157). Finally, 1-2 µl of DNA template and distilled RNase free water to give a 50µl total reaction volume was prepared for each isolate. Cycling reaction conditions were: 94°C for 4 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 4 min. PCR products (8 µl) were resolved in a 2% agarose gel in 0.5% Tris-borate-EDTA buffer. Electrophoresis was carried out at 4 V/cm for 1 hr and DNA visualized with ethidium bromide. Methicillin-resistant *S. aureus* reference strains from the ATCC were used as type-specific controls: BAA-1696 (PVL positive); BAA-1681 (PVL negative).

By means of PCR, primers NLukI-1 and NLukI-2 were employed to examine for the genes *lukS* and *lukF*, which encode the leukotoxin LUK-I described in *S. pseudintermedius*. The primers were designed using the Primer3 software (228) available in the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and based on the published sequence (GenBank accession number X79188) as the target site. Primers sequences are described in Table 12. The reaction mixture for LUK-I amplification contained 1µl of each primer (0.5 µM/µl) in 12.5 µl of PCR master mix preparation (25units/ml *taq* DNA polymerase, 200µm [each] deoxynucleoside triphosphate 1.5and 3mM of $MgCl_2$ Promega® [M7502]). In addition, 2.5 µl of DNA template and distilled RNase free water were added to give a 25µl total reaction volume. Cycling conditions were 94°C for 180 s; followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final extension 72°C for 300 s. PCR products (8 µl) were resolved in a 2% agarose gel in 0.5% Tris-borate-EDTA buffer. Electrophoresis was carried out at 4 V/cm for 1 hr and DNA visualized with 0.5 µg/ml ethidium

bromide stain. *Staphylococcus pseudintermedius* ATCC reference strain 49444 was used as control. To evaluate the specificity of the assay, size-specific PCR products obtained from the final PCR product were purified and DNA was isolated using a QUIAquick ® PCR extraction kit (Quiagen). The extracted DNA was submitted for automated nucleotide sequencing. Nucleotide sequencing results were analyzed using the NCBI database through BLAST 2.2.24., and GenBank accession number X79188.

To determine the presence of the *siet* gene in coagulase positive *Staphylococcus* species of canine origin, the primers and protocol of Lautz *et al.,* (2006) was followed. The primers that were used for the amplification of the *siet* gene are described in Table 13 (148). Briefly, the reaction mixture contained 1 µl each primer (10 pmol/µl) in 25 µl of PCR master mix preparation (50units/ml *taq* DNA polymerase, 400µm [each] deoxynucleoside triphosphate and 3mM of $MgCl_2$ Promega® [M7502]). In addition, 2 µl of DNA template and distilled RNase free water were added to give a 50µl total reaction volume. Cycling conditions were 94°C for 180 s; followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and a final extension 72°C for 300 s. PCR products (8 µl) were resolved in a 2% agarose gel in 0.5% Tris-borate-EDTA buffer and electrophoresis was carried out at 4 V/cm for 1 h and visualized with 0.5 µg/ml ethidium bromide. With the aim of testing the specificity of the assay, size-specific PCR products obtained from the final PCR product were purified and DNA was isolated using a QUIAquick ® PCR extraction kit (Quiagen) following the instructions of the manufacturer. The extracted DNA was submitted for automated nucleotide sequencing. Nucleotide sequencing results were analyzed using the NCBI database through BLAST 2.2.24., and GenBank accession number AB099710.

Statistical analysis. Statistical analysis was performed using statistical software (Statistical Analysis System; SAS version 9.2, SAS Institute, Cary, NC). Data were classified according to susceptibility or resistance to the antimicrobials being analyzed. Categorical data such as year, source, age, gender, breed and previous therapy were analyzed using the frequency procedure (PROC FREQ) and compared through the Chi-square test and Fisher exact tests. For all comparisons, values of $p < 0.05$ were considered significant. In addition, a Proc logistic was used to compare the frequency of occurrence of methicillin-resistant *Staphylococcus sp* associated with year of isolation and use of previous antimicrobial therapy.

Results

Staphylococcus species and epidemiological data. Five hundred seventy-seven (577) isolates of *Staphylococcus* were collected from canine clinical specimens processed for microbiological culture. After microbiological identification, RF-PCR and susceptibility testing; the microorganisms were classified by *Staphylococcus* species and grouped as methicillin-susceptible (MS) and methicillin-resistance (MR) as follow: *S. aureus*: 44 (13 MSSA, 31 MRSA); *S. pseudintermedius:* 459 (298 MSSP, 161 MRSP); and *Staphylococcus schleiferi* subsp *coagulans*: 74 (32 MRSC, 42 MSSC). The number of MR strains recovered represented 38.82% (224/577) of the total collected.

The frequency distribution of 516 staphylococcal isolates from canine clinical specimens in a four year period (2007 – 2010) included in this study are described in Table 13. Statistical analysis (Proc-logistic) was performed to investigate the increasing risk of finding methicillin resistant staphylococci in microbiology cultures from canine specimens in the most recent years.

A significant difference ($p = \leq 0.05$) between the frequency of *Staphylococcus* species isolated in 2007 with respect to 2009 and 2010 was found. Within the MRSP group, significant differences appeared when comparing the years 2007 to 2009 (p = 0.0001, OR 2.98, CI 1.71 – 5.20); 2007 – 2010 (p = 0.0001, OR 3.16, CI 1.82 – 5.49), and 2008 – 2010 (p = 0.0001, OR 2.98, CI 1.71 – 5.20). Similar results were found within the MRSC group. Within this group significant differences were found among years 2007 to 2009 (p = 0.004, OR 5.68, CI 1.59 – 20.32); years 2008 to 2009 (p = 0.02, OR 3.5, CI 1.11 – 11.0) and 2009 to 2010 (p = 0.01, OR 3.79, CI 1.21 – 11.8). Within the MSSC group, a significant difference appeared when comparing years 2008 and 2009 (p = 0.02, OR 2.95, CI 1.09 – 8.0) and among years 2009 to 2010 (p = 0.0037, OR 5.54, CI 1.64 – 18.7). No significant differences were found within the MRSA, MSSA and MSSI groups. Overall, these results indicate a higher risk of finding MRSP and MRSC during 2009 and 2010, when compared with 2007.

The source of the isolates was known in 572/577 cases. Table 14 provides an itemized description of the specimen sources for the isolates that were collected. As was expected, 52.6% (301/572) were collected from the skin; followed by ears, 19.2% (110/572); urine, 11.8% (68/572); bone/internal tissues, 8.3% (48/572); upper respiratory tract, 2.2% (13/572); reproductive tract 1.2% (7/572) and blood culture 1.2% (7/572); and 0.8% (5/572) were from a non-groupable source. *S. schleiferi* subsp *coagulans* is considered an opportunistic pathogen, which is associated with canine ear and skin infections. Therefore it is remarkable that *S. schleiferi* subsp *coagulans* was isolated from other body sites in this study. This microorganism was reported from bone/internal tissues (2 isolates), reproductive tract (1 isolate) and from urine (2 isolates).

A comparison of the three species, *S. aureus*, *S. pseudintermedius* and *S. schleiferi* subsp *coagulans* by host, gender and age is described in Table 15. In summary, 51% (279/547) and 48.9% (268/547) correspond to female and males, respectively. Table 15. When viewed by age, three groups were defined: young (<2 years of age), middle age (2 – 8 years of age) and old (>9 years old). Information about age was available in 532/577 cases; 7.7% (41/532) from animals <2 years of age; 59% (314/532) from animals 2 – 8 years old; and 33.2% (177/532) in >8 years old dogs.

Breed distribution is shown in Table 16 with breed information available on 535 of the dogs. A single group was created for breeds that had ≤4 isolates associated, and this group represented 15.7% (84/535) of the total. Mixed-breeds represented 9.53% (51/535) of the staphylococci found in canine clinical specimens. The most prevalent breeds associated with isolate collection were: 15.7% (84/535) Labrador Retrievers; 6.16% (33/535) Cocker Spaniels; 5.79% (31/535) Bulldog; 4.85% (26/535) German Shepherd Dogs; 4.67% (25/535) Boxer; 4.29% (23/535) Golden Retrievers; 3.17% (17/535) Doberman Pinscher; and 2.99% (16/535) Dachshund (refer to Table 16 to see the remaining variety of breeds).

The association between the use of antimicrobials and the finding of MR staphylococci has been reported in dogs. While there were some limitations in this study, the aim was to identify the influence of prior therapy with the presence of MR *Staphylococcus* in the canine. From the total of 577 isolates, information regarding previous antimicrobial use was available on 322 of the patients. Specific antimicrobial drugs or classes of drugs that were identified in the patient histories included: trimethoprim/sulfamethoxazole, chloramphenicol, mupirocin, rifamycin, metronidazole, the β-lactams, fluoroquinolones, tetracyclines, lincosamides, aminoglycosides, and antifungals. Isolates were categorized as collected from dogs that did not

receive any antimicrobial drug, dogs that receive specifically just one antimicrobial drug or class and dogs that received more than one class of antimicrobial. Within the group that received therapy, a significant difference ($p \leq 0.05$) indicated that isolates from dogs with a history of antimicrobial therapy, represented by 63.22% (98/155) had a higher risk of being MR positive, than dogs without antimicrobial therapy. However, when classes of antimicrobials were analyzed there was not any significant difference associated with *S. aureus* (MRSA). The significant difference observed within the group that received therapy was attributed to MRSC ($p = 0.01$, OR infinity) and MRSP ($p = 0.0005$, OR 2.84, CI 1.57 – 5.15).

***In vitro* susceptibility testing.** The result of the antimicrobial susceptibility testing was taken retrospectively. Therefore, different drug panels were used for testing some of the isolates. It is important to mention that in accordance with the CLSI guidelines, all methicillin resistant *Staphylococcus* isolates should also be considered resistant to all other β-lactams. For this reason diagnostic laboratories report MR isolates as resistant to all β-lactams irregardless of the *in vitro* findings. In this study, the phenotypic results of the *in-vitro* antimicrobial susceptibility testing are presented, meaning that any change of susceptibility results were performed based on methicillin resistance. In Table 17, the results are presented as the number resistant out of the total recovered in that category, Amikacin: 7.85% (33/420); amoxicillin/clavulanic acid 41.64% (182/437); ampicillin79.72% (350/439); cefazolin 38.91% (165/424); Ceftiofur 42.1% (177/420); Cephalothin 39.5% (113/286); Chloramphenicol 1.84% (8/434); Clindamycin 47.9 (212/442); Enrofloxacin 45.3% (189/417); Erythromycin 48.6 (215/442); Gentamicin 9.1% (41/446); Marbofloxacin 41.0% (176/429); Orbifloxacin 41.2% (103/250); Penicillin 82.6% (366/443); Rifampin 2.3% (9/391); Tetracycline 41.2% (113/274); and

Trimethoprim/sulfamethoxazole 37.1% (164/442). Refer to Table 17 for the susceptibility results according to *Staphylococcus* species grouping.

Virulence genes. Through the use of PCR, the genes that encode PVL (*lukS*-PV and *lukF*-PV), LUK-I (*lukS*-I and *lukF*-I) and SIET were amplified and analyzed. Genes that encode PVL were not identified either in *S. pseudintermedius* or *S. schleiferi* subsp *coagulans.* Within the *S. aureus* group, the PVL PCR was done for the MRSA group with four testing positive, 12.9% (4/31). PCR results of the *lukS*-I and *lukF*-I, as well as *siet* genes are listed in Table 18. LUK-I genes were present in a total of 254 isolates and distributed as follows, 1 in MRSC, 3 in MRSA, 91 in MSSP and 159 in MRSP. The *siet* gene sequence was amplified in 5 MRSA, 27 MRSC, 8 MSSC, 118 MRSP and 90 MSSP.

Discussion

The recovery of 38.82% methicillin-resistant *Staphylococcus* species from canine clinical specimens is considered a significant number. These findings are consistent with current studies that have reported an increasing frequency of isolation of MR staphylococci in small animal medicine (12, 31, 58, 67, 156, 220, 222, 237, 295). In addition, when comparing the annual frequency within the study period (2007 – 2010), a significant difference between the frequency of *Staphylococcus* species isolated in 2006 and 2007 with respect to 2009 and 2010 was found (Table 13). Isolates of *S. pseudintermedius*, previously known as *Staphylococcus intermedius*, were commonly susceptible to β-lactam antibiotics (174, 211, 270). However, since 2004, several studies have reported the finding of MR in *S. pseudintermedius* as an emerging animal

health problem in veterinary medicine (122, 220, 290). During the period 2007 – 2010, the recovery of MR staphylococci consisted of 29.8% (154/516) MRSP, 5.81% (30/516) MRSC and 2.51% (13/516) MRSA. The results indicate that MRSC is also an important pathogen associated with MR in dogs. Studies that have investigated *S. schleiferi* subsp *coagulans* in dogs are less frequents. However, the widespread resistance of this organism to methicillin has been reported previously (31, 122, 183). The prevalence of MRSA is variable, from 0% in dogs from the community to 9% in hospitalized canines (295).

Regarding the epidemiological information collected in this study, it is important to consider that all cases were identified in diagnostic material derived from clinically diseased dogs. Therefore, two important limitations of this study are the lack of case definitions and the collection of information from the patient's medical records. When interpreting the use of previous antimicrobial therapies as a risk factor for the development of methicillin resistance in *Staphylococcus* species, it is important to consider that the isolates included in this study do not represent a population-based study, instead include isolates recovered from patients referred to the veterinary teaching hospital at Auburn University or from private practices. Thus, the history of prior antimicrobial therapy may be not accurate. The authors of this study made every attempt to collect accurate comprehensive data on all culture submissions.

As reported in a myriad of studies, most of the isolates were collected from skin/abscess, 52.6% (301/572); followed by ears, 19.2% (110/572). In the present study, 54.7% (23/42) of the *S. aureus* isolates were associated with invasive infections (bone, body cavities, joint fluid, urine, respiratory, blood, and reproductive tract). In contrast, 71.8% (324/451) of *S. pseudintermedius* and 91.8% (68/74) of *S. schleiferi* subsp *coagulans* were associated with skin/ear infections. This is consistent with a previous study in which MRSA infections in small companion animals

were more commonly associated with invasive infections and MRSP infections were more associated with superficial infections (183). *S. schleiferi* isolates have been mainly associated with skin and external ear canal infections (72, 163, 183). However, six of the *S. schleiferi* subspecies *coagulans* included in this study were associated with invasive infections (bone/cavities, urine, respiratory and blood). Recently, Cain *et al* (2011) also reported the isolation of *S. schleiferi* from other body sites, specifically from respiratory tract, urogenital tract and eyes (31).

The American Kennel Club has reported the ranking of the most common dogs breeds since 2001 (http://www.akc.org/reg/dogreg_stats.cfm), which are Labrador Retriever, German Shepherd Dog, Beagle, Golden Retriever, Yorkshire Terrier, Bulldog, Boxer, Poodle, Dachshund, Rottweiler, Shih Tzu, miniature Schnauzers, Doberman Pinschers, Chihuahuas and German Shorthaired Pointers. In our study, breed popularity constituted a bias. The isolates were collected from 72 dog breeds with one collective group composed of 46 breeds that were represented by ≤ 4 isolates/breed yet consisted of 15.7% (84/535) of the total number of isolates. The most common breeds associated with this study were: Labrador Retrievers (15.7%) (84/535); Cocker Spaniels 6.16% (33/535); Bulldog 5.79% (31/535); German Shepherd Dogs 4.85% (26/535); Boxer 4.67% (25/535); Golden Retrievers 4.29% (23/535); Doberman Pinscher 3.17% (17/535); and Dachshund 2.99% (16/535). Epidemiological information about canine staphylococcal disease and breed is scarce. Breed aspects found in this study are similar to the results reported by Cain *et al.*, in *S. schleiferi.* (31), in which the same breeds are associated with staphylococcal infections. Some of these breeds are associated with allergic skin disease, which may be a predisposing risk factor. However, other breeds such as Beauceron, Boston terrier,

Cairn terrier, Chinese Shar Pei, Dalmatian, among others, are frequently associated with atopic dermatitis, (95) and were not represented in this study.

In human medicine, one of the risk factors associated with the increased rates of MRSA is the previous use of antimicrobials, such as β-lactams, fluoroquinolones and macrolides (3, 39, 91). Fluoroquinolones have been correlated with the highest incidence of hospital-associated MRSA (3). In the present study, 71.8% (411/572) of the isolates were associated with dogs with skin and ear infections. In veterinary medicine, these pathologic conditions have a tendency to be chronic and would be associated with prolonged antimicrobial therapies with fluoroquinolones and β-lactams (67, 97). Of the 577 isolates recovered, 335 were from cases classified as unknown status when previous therapy was considered. Information of non-previous antimicrobial usage was collected in 33% (80/242) of the cases. Within the group that received therapy, a significant difference ($p \leq 0.05$) indicates that isolates from dogs with a history of antimicrobial therapy represented by 63.22% (98/155), have a higher risk of being MR positive, than from dogs without therapy. However, when group classes of antimicrobials were analyzed there was not a significant difference associated with *S. aureus* (MRSA). The significant difference observed within the group that received therapy was attributed to MRSC (p = 0.01, OR infinity) and MRSP (p = 0.0005, OR 2.84, CI 1.57 – 5.15). This raises the question whether the use of antimicrobials in dogs may be considered as a risk factor for the development of resistantce in *S. pseudintermedius* and *S. schleiferi* subsp *coagulans,* but not for *S. aureus.*

B-lactams were used in 81 dogs as a single therapy and in 23 additional dogs in combination with others antimicrobial classes. Of the patients receiving β-lactam therapy, 61.7% (50/81) of the isolates were MR while only 38.2 (31/81) were MS. In addition, it is noteworthy that a significant difference between MRSC and MSSC was found (p = 0.0001).

Fluoroquinolone usage was associated with 12 isolates. However, this was not found to be a risk factor for MR in *Staphylococcus* species from canine clinical specimens.

Faires *et al.,* (2010) reported that β-lactams and fluoroquinolones were considered as risk factors for a MRSA infection in dogs (67). Similar results have been published by other authors for MRSP (195, 237). Specifically in dogs with MRSP infections, the use of antimicrobials within the 30 days prior to the onset of the infection was considered a risk factor when compared with dogs with MSSP infections (271). In addition, significant association with MR in *S schleiferi* isolates was found with the administration of β-lactam antimicrobials, such as cephalexin and amoxicillin-clavulanic acid. However, consistent with the results of this study, previous fluoroquinolone treatment was not significantly associated with culture of methicillin-resistant strains (31).

Results of the susceptibility testing indicate that chloramphenicol and rifampin exhibited the highest frequency of susceptibility when *S. aureus, S. pseudintermedius* and *S. schleiferi* subsp *coagulans* isolates were tested, 1.8% (8/434) and 2.3% (9/391), respectively. Resistance to aminoglycosides was relatively low, 7.86% (33/420) for amikacin and 9.1% (41/446) for gentamicin, as compared with resistance to β-lactams, fluoroquinolones tetracycline and trimethoprim/ sulfamethoxazole. Similar results have been reported previously (156, 233). In the present study, resistance to multiple non-β lactam drugs was present in the three *Staphylococcus* species examined. In particular, MRSC isolates were 100% susceptible to trimethoprim/sulfamethoxazole, while only 10.3% (3/29) were resistant to clindamycin and 17.2% (5/29) to erythromycin which is in contrast to the high frequency of resistance to clindamycin, trimethoprim/sulfamethoxazole and erythromycin within the MRSP and MRSA groups. Cain *et al.* reported similar results when investigating *S. schleiferi* (31).

Panton valentine leukocidin (PVL) is a pore-forming toxin secreted by strains epidemiologically associated with outbreaks of community-associated methicillin-resistant *S. aureus* (CA-MRSA) and often causing lethal necrotizing pneumonia (144). PVL is encoded by lukF-PV and lukS-PV genes (145, 223, 243). There are contradictory studies about the role of the PVL in human pulmonary disease. Some studies suggest that PVL has a minimal toxicity to mouse polymorphonuclear leukocytes (PMNs) compared with high toxicity to humans PMNs. However, it has been demonstrated that PVL is not required for lethal pulmonary disease in humans (242). Regional geographical differences in the prevalence of PVL, particularly in MRSA USA300, have been reported (189). This suggests the importance of active monitoring of PVL prevalence among MRSA clones in human and veterinary medicine. In this study, the PVL gene was detected in 12.5% (4/32) of MRSA isolates and was not found in any of the *S. pseudintermedius* or *S. schleiferi* subspecies *coagulans* isolates. Lin *et al*, (2011) reported a similar result in 4 of 24 animal MRSA isolates (156). The authors indicated that the PVL positive isolate were the genotypes of CA-MRSA identified in humans (CC8, SCCmec Type IVa, *luk*SF-PV +, and spa type t008) suggesting that transmission from humans to those animal species had occurred (156). The first report of PVL in MRSA isolates from companion animals was published by Rankin *et al* in 2004 (223). It was reported that 11 of the 23 MRSA isolates that were tested were positive for the presence of PVL, of which 8 were of canine origin. PVL negative results in MRSA from animal origin have been reported in others studies (229, 279, 304).

S. pseudintermedius produces Luk-I, a leucotoxin that is very similar to Panton–Valentine leukocidin (PVL) from *S. aureus* (77, 78). In addition, this microorganism produces SIET which is considered an exfoliative toxin (78, 148). Recent studies have investigated the

prevalence of Luk-I and SIET in *S. pseudintermedius* and the prevalence of both genes was significantly high (77, 88, 232, 283). In this study, *luk*I genes were present in a total of 254 isolates and distributed as follows: 1 in MRSC, 3 in MRSA, 91 in MSSP and 159 in MRSP. It is noteworthy that the *siet* gene was amplified by PCR in 5 MRSA, 27 MRSC, 8 MSSC, 118 MRSP and 90 MSSP isolates. To date, there is no published data documenting the finding of *luk*I and *siet* genes in *S. aureus* and *S. schleiferi* subsp *coagulans.* The crucial epidemiological and clinical importance of these findings has yet to be investigated, but the authors of this study speculate about the possibility of a process of genetic exchange among the *Staphylococcus* species isolated from the canine host. Through conjugation and transduction, genetic elements can be mobilized between strains and species of bacteria and horizontal gene transfer of virulence genes is known to occur between species of bacteria (22).

Moore and Lindsay in 2001, investigated the genetic variation among human hospital isolates of methicillin-sensitive *S. aureus,* and the authors concluded that horizontal transfer of mobile genetic elements encoding virulence genes occurs frequently, probably in the hospital environment, and possibly *in vivo* (181). In addition, Sung *et al.*, (2008), investigated whether human and animal MRSA isolates exchange mobile genetic elements encoding virulence and resistance genes. The results indicated that there is some evidence that exchange of mobile genetic elements such as bacteriophage and pathogenicity islands between animal and human lineages is feasible (255). It is possible that horizontal gene transfer of *siet* and *lukI* could have occurred among *S. intermedius, S. aureus* and *S. schleiferi* subsp *coagulans.*

The findings of this study support the increasing isolation of multi-drug resistant *Staphylococcus* species from the canine. In addition, isolates collected from dogs with a history of antimicrobial therapy, particularly β-lactams, have a higher risk of exhibiting MR. For these

reasons, veterinary practitioners are encouraged to perform antimicrobial cultures together with antimicrobial susceptibility testing for the selection of the most appropriate drug therapy. Another important finding in this study is the discovery of the genes *siet* and *luk*I in *S. aureus* and *S. schleiferi* subsp *coagulans.* Collectively, these findings support the need for further epidemiological and clinical studies concerning virulence factors in *Staphylococcus* species of canine origin.

V. Penicillin-binding proteins and cefoxitin in *Staphylococcus pseudintermedius* and *Staphylococcus* schleiferi subspecies *coagulans*

Summary

In human and veterinary medicine staphylococcal species are considered important opportunistic pathogens involved in nosocomial and community acquired infections. In veterinary medicine, the increased number of methicillin-resistant (MR) staphylococci reported in recent years is considered to be an emergent problem (220, 295). Although *S. aureus* has been associated with numerous pathologic conditions in dogs, (223, 250, 295) the most common staphylococci species found in the canine is *Staphylococcus pseudintermedius.* In dogs, *S. pseudintermedius* causes severe skin and soft tissue infections, as well as opportunistic infections in other body sites. Another staphylococcal species associated with canine disease is *Staphylococcus schleiferi* subspecies *coagulans* which is also implicated in canine dermatitis and otitis externa (184). In methicillin-resistant (MR) *S. aureus* (MRSA) the most important mechanism associated with β-lactam resistance is the production of an altered form of penicillin-binding protein (PBP) called PBP2a which is encoded by the *mec*A gene (114, 118, 158, 187). The PBPs of *S. aureus* has been characterized and there are 4 native PBPs (83). To date, the pattern of PBPs in *S. pseudintermedius* and *S. schleiferi subsp coagulans* has not been characterized. Our previous studies as well as other investigations have demonstrated that when the 2008 Clinical and Laboratory Standard Institute (CLSI) guideline for oxacillin (OXA) and cefoxitin (FOX) break points are applied there is a failure to accurately identify MR in both MR

S. pseudintermedius (MRSP) and MR *S. schleiferi* subsp *coagulans* (MRSC) (16, 17, 241). Therefore, there may be different mechanisms or factors influencing antimicrobial resistance for these different groups of *Staphylococcus.* It was hypothesized that differences in the reaction of MRSP and MRSC to FOX, when compared with MRSA, may be based on variations in the PBPs affinity for FOX (16, 17, 241). Therefore, the aim of this investigation was to characterize the PBPs in *S. pseudintermedius* and *S. schleiferi subsp coagulans.* In addition, the relative binding affinity of PBPs for FOX in MRSP and MRSC isolated from canine clinical specimens was determined. To identify the pattern of PBPs in *S. pseudintermedius* and *S. schleiferi* subspecies *coagulans*, bacterial membranes or whole bacteria were incubated with Bocillin-FL (Boc-FL) and separated through sodium-dodecyl sulfate polyacrylamide gel (SDS-PAGE) (219, 306). Competitive binding assays were done to obtain PBPs binding affinity data for cefoxitin in MRSC and MRSP. A five-band PBP pattern (PBP1, PBP2, PBP3, PBP4 and PBP5) was observed in *S. pseudintermedius,* while no difference was observed when PBPs correspond to methicillin-resistant or methicillin susceptible strains. Regarding *Staphylococcus schleiferi* subsp *coagulans*, a pattern of four heavy-weight PBPs (PBP1, PBP2, PBP3 and PBP4) was detected when the ATCC-49545 (methicillin-susceptible) was examined. In contrast, three heavy weights bands were observed in a methicillin-resistant *S. schleiferi* subsp *coagulans.* Results of the competitive binding assay between FOX and Boc-FL demonstrated differences in the binding affinity of canine *Staphylococcus* sp and *S. aureus.*

Introduction

In human and veterinary medicine, staphylococcal species are considered important opportunistic pathogens involved in nosocomial and community acquired infections. The development of resistance to β-lactam drugs and to other antimicrobials agents in *Staphylococcus* species has become a major challenge to health care providers. In human medicine, *Staphylococcus aureus* is one of the major causes of health-care-associated infections worldwide (54, 253). It is considered one of the most common causes of nosocomial bloodstream infections, health-care-associated pneumonia, surgical-site infections, and infections occurring in intensive care units (14, 29, 249). Nosocomial infections caused by methicillin-resistant *S. aureus* (MRSA) have been designated as hospital-acquired MRSA (HA-MRSA). Risk factors for HA-MRSA infections that are unique to the hospital population are well established (160). In the 90's, multiple cases of MRSA as a cause of infection in the community outside the hospital population were reported. Those cases were defined as community-acquired MRSA (CA-MRSA) and included patients who had never been hospitalized and had no other known risk factors for MRSA infection (105, 235).

In veterinary medicine, cases of MRSA infections are commonly reported (36, 295). In dogs, *S. aureus* is less prevalent than *Staphylococcus pseudintermedius.* However, *S. aureus* has been associated with numerous conditions, such as soft tissue infections, chronic pyoderma, post-surgical wound infection, joint invasion, urinary tract infections or even death (223, 250, 295). Commonly, *S. pseudintermedius* is associated with pyoderma and otitis. In addition, *S. pseudintermedius* causes severe skin and soft tissue infections, as well as opportunistic infections in other body sites. Another staphylococcal species associated with canine disease is *Staphylococcus schleiferi* subspecies *coagulans,* which is also implicated in canine dermatitis

and otitis externa (184). Currently, methicillin-resistance in *S. pseudintermedius* (MRSP) and *S. schleiferi* subspecies *coagulans* (MRSC) is considered an emerging problem in veterinary medicine (295).

Since the introduction of penicillin, β-lactam antibiotics have played an important role in the treatment against staphylococcal infections in humans and animals. However, the growing prevalence of resistance against β-lactams and other drugs is a problem in the clinical resolution of infections. Two main mechanisms have been associated with the development of resistance against β-lactams. The first mechanism is the production of hydrolases, called β-lactamases, which are able to hydrolyze the β-lactam ring. Once the antimicrobial is hydrolyzed it becomes inactive (252). The second mechanism is the production of an altered form of penicillin-binding protein (PBP), called PBP2a. The PBP2a is encoded by the *mec*A gene which is carried by a mobile genetic element called staphylococcal cassette chromosomal *mec* (SCC*mec*) and it is the most important mechanism associated with methicillin resistance (114, 118, 158, 187). The *mecA* gene has been identified in MRSA from canine origin and has been reported.

MRSP and MRSC also carry the *mec*A gene. There is a lack of information regarding the mechanism of β-lactam resistance in methicillin-resistant *S. pseudintermedius* (MRSP) and methicillin-resistant *S. schleiferi* subsp *coagulans* (MRSC). Because the SCC*mec* has been identified many times in MRSC and MRSP, (156, 222, 295) it has been assumed that the same mechanism of resistance is shared among these *Staphylococcus* species of animal origin.

The main component of Gram - positive bacteria is the peptidoglycan, which is a meshwork of glycan (polysaccharide) chains interconnected by peptide cross-links in the cell wall (285). The cross-linking is assembled by membrane-bound enzymes known as penicillin-binding proteins (PBPs) (240). Penicillins bind to PBPs and acylate an active serine site. This

molecular interaction is enough to inhibit the biological function of the PBP which then leads to cell death of the bacteria (37). Most bacterial species possess multiple PBPs. Differences among these proteins are based on enzymatic function, molecular weight, and affinity for β–lactam antibiotics (301). PBPs have been grouped according to molecular weight as high-molecular weight or low-molecular weight. High molecular-weight PBPs are characterized as essential proteins for the cell wall synthesis, containing transpeptidase and transglycosilase activity. Low-molecular weight PBPs are carboxypeptidase enzymes which participate in regulatory functions but are not considered essential (8). Alterations of PBPs have been found to mediate high-level β-lactam resistance in staphylococci, enterococci, and pneumococci (169, 306). In *S. aureus,* four native types of PBPs have been recognized and differentiated based on molecular weights. The four PBPs have been designated as PBP1 (87 Kd), PBP2 (80Kd), PBP3 (75), PBP3 (70Kd), and PBP 4 (41 Kd) (81, 166). As mentioned previously, MRSA has an additional PBP, the PBP2a (78 Kd) (81, 102).

The PBP1 of *S. aureus* was considered essential for growth due to the fact that it was the primary peptidoglycan transpeptidase (15, 287). However, in a recent study, Pereira *et al.,* has suggested that the PBP1 is not a major contributor to the cross-linking of the peptidoglycan. Instead the essential function of PBP1 may be intimately integrated into the mechanics of cell division (213). The PBP2 is a bifunctional (transglycosilase and transpeptidase activity) protein. This protein plays an important role in the cross-linking and elongation of peptidoglycans that constitute the bacterial cell wall (169, 193, 216). PBP 3 is a septation-associated transpeptidase while PBP4 has been described as a carboxypeptidase and more recently as a β-lactamase (193). PBP2a has a low affinity for β-lactam drugs and can function as a transpeptidase. When bacteria are challenged by β-lactam antibiotics, the other staphylococcal PBPs transpeptidases are

acylated and inhibited but PBP2a retains its activity. The PBP2a functional activity is not inhibited by β-lactams. Therefore, the PBP2a in association with the transglycosilase domain of the native PBP2 cooperates in the biosynthesis of the peptidoglycan (216, 217). It has been suggested that PBP4 also cooperates with PBP2a in staphylococcal cell wall biosynthesis in the presence of β-lactams (152). PBPs have different affinities for β-lactam drugs. The PBPs 1, 2, and 3 are reported to have high affinities for most β-lactam antibiotics and that the binding of β-lactams by these PBPs is lethal for *S. aureus* (38). Although PBP4 may be important in normal cell wall synthesis, low molecular-weight PBP4 is not considered a critical target and may be dispensable (82, 175).

The PBPs in *Staphylococcus* species of veterinary origin, specifically *S. schleiferi* subspecies *coagulans* and *S. pseudintermedius* have not been characterized. The PBPs of *Staphylococcus intermedius, Staphylococcus hyicus, Staphylococcus simulans* and *Staphylococcus delphini* from animal origin were characterized by Canepari *et al.,* (1985) (34). In recent years, *Staphylococcus intermedius* was classified within a cluster comprising *S. intermedius, S. pseudintermedius* and *S. delphini.* These three species have been associated with diseases in different animal species. Historically, *S. intermedius* was considered to be a commensal in dogs. Currently, *S. intermedius* has been re-classified as *S. pseudintermedius* (10, 58).

Veterinary microbiology diagnostic laboratories typically utilize guidelines established by the Clinical and Laboratory Standard Institute (CLSI) for antimicrobial susceptibility testing and interpretation. The CLSI recommends the use of oxacillin (OXA) and cefoxitin (FOX) break points standardized for *S. aureus* isolates to interpret the *in vitro* susceptibility of *S. pseudintermedius* and other coagulase-positives staphylococci of animal origin (43). Our

studies as well as other investigations have demonstrated that when the 2008 CLSI guideline for OXA and FOX break points are applied, there is a failure to accurately identify MR in both MRSP and MRSC. In this study, it was hypothesized that the differences in the reaction of MRSP and MRSC to FOX, when compared with MRSA is based on variations in the PBPs affinity for FOX (16, 17, 241).

In human and veterinary medicine it is crucial to have available antimicrobials, specifically β-lactams, with a high affinity for PBPs. Alterations (mutations or additions) of PBPs are one of the most important factors associated with the expression of resistance against β-lactams in different bacterial species (301). Understanding the mechanisms of resistance to β-lactam antimicrobials in *Staphylococcus* is imperative to finding a means of treatment and for the improvement of diagnostic methods. With the aim of characterizing the PBPs in *S. pseudintermedius* and *S. schleiferi* subspecies *coagulans,* bacterial membranes or whole bacteria were incubated with Bocillin-FL (Boc-FL) and the PBPs were separated through sodium-dodecyl sulfate polyacrylamide gel (SDS-PAGE) (219, 306). Competitive binding assays were performed to obtain PBPs binding affinity data for cefoxitin and Boc-FL in MRSC and MRSP.

Materials and methods

Bacteria strains and growth conditions. *S. pseudintermedius* and *S. schleiferi* subsp *coagulans* from canine origin were included in this study. A detailed description of each *Staphylococcus* sp is given in Table 19. Conventional biochemical assays including catalase (3% H_2O_2), tube coagulase test (BBL coagulase plasmas; Becton Dickinson), acetoin production (1ml of 1.7% MRVP medium) and carbohydrate fermentation patterns (trehalose, 1%; purple agar base: 3.7%, mannitol 1.2% and maltose 3.7%) were used for specie identification. In addition, due to the inability of conventional microbiological methods to differentiate the *S. intermedius* group: *S. pseudintermedius, S. intermedius* and *S. delphini*, a restriction fragment length-PCR described by Bannoehr *et al.* 2009 was used to verify *S. pseudintermedius* identification (11). Reference strains from the American Type Culture Collection (ATCC) were also included as controls: *S. pseudintermedius*-49444, *S. schleiferi* subsp *coagulans*-49545, and *S. aureus*-43300/1576. Bacteria were grown aerobically at 35ºC overnight on 4% bovine blood agar or 4.1% tryptose agar. For each isolate, one colony was selected and inoculated into 5 ml of Brain Heart Infusion (BHI) media (Difco Laboratories, Detroit, Mich.) and incubated overnight at 37°C in an incubator shaker cabinet at 150 rpm.

Fluorescent penicillin derivative. Historically, the identification of PBPs was based on the observed electrophoretic pattern exhibited by proteins labeled with radioactive penicillin (169, 219, 306). However, in 1999, Zhao *et al.* introduced the use of Boc-FL (Molecular Probes, Inc., Eugene, Oreg.), which is a synthetic, commercially available, fluorescent penicillin. Boc-FL is used as a labeling reagent for PBPs and the detection is performed with a FluorImager or by UV light (79, 305).

Susceptibility against β-lactams testing. Antimicrobial susceptibility testing to OXA (1µg) and FOX (30µg) was determined by the disk diffusion method using Mueller Hinton salt agar (2%) according to the CLSI guidelines (43, 192).

***In vivo* labeling**. The labeling methods described by Pucci and Dougherty were used, with some modifications (219). A 200-fold dilution of the overnight culture was made in 40 ml of BHI and grown at 37°C in an incubator shaker at 150 rpm. Bacterial growth was monitored using a Nanodrop spectrophotometer at 600nm untill an optical density of 0.3 to 0.4 nm was achieved which represented approximately 2 x 10^8 cells per ml (≈ 5 hours). To concentrate bacteria, 1 ml of the bacterial suspension was centrifuged at 2000 x *g* for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 50 µl of lysis buffer (50 mM Tris-HCl, pH 7.0, 10 mM MgCl2, 2.5 µg of DNase/ml, and 2.5 µg of RNase/ml followed by addition of lysostaphin to a final concentration of 200µg/ml). In some cases, to avoid degradation of the fluorescent β-lactam antibiotic by β-lactamases, samples were exposed for 10 min to 100µg/ml clavulanic acid. To the cell lysates, 25 µl of Boc-FL was added to achieve a final concentrations of 0- 0.5- 1- 2.5- 5- 10 and 50 µM and samples were incubated at 37°C for 30 min. Next, 5 µl of lysostaphin was added to achieve a final concentration of 200 µg/ml. The mixture was incubated at 37°C for 15 min to allow cell lysis to occur which was indicated by clearing of the sample solution. In preparation for electrophoresis, Laemmli sample buffer (Cat. Number: 161-0737, Bio-Rad Laboratories, Hercules, Calif.) was added at 1:1 dilution, boiled at 100°C for 3 – 5 min and placed on ice for 5 min. Samples were loaded (30µl) on SDS-PAGE gel and subjected to electrophoresis, or stored at - 20ºC until characterized.

Membrane preparation and labeling. A 200-fold dilution of the overnight culture was made in 500 ml or 1 liter of BHI, grown at 37°C in an incubator shaker at 150 rpm. Bacterial growth was monitored as previously described. For each isolate, 50 ml samples were centrifuged at 2000 x *g* for 10 min at 4 °C and the pellets were washed twice with 10 mM sodium phosphate buffer, pH 7.0. The pellet was transported to a 1.5ml microcentrifuge tube (~ 300µl) and 300µl of lysis buffer was added (50 mM Tris-HCl, pH 7.0, 10 mM MgCl2, 2.5 µg of DNase/ml, 2.5 µg of RNase/ml, 100µg/ml lysostaphin and 100µg/ml lysozyme) and incubated for 30 min, at 37ºC. Following cell lysis (as indicated by clearing of the sample solution), samples were immediately placed on ice. To ensure that bacteria were non-viable, 0.1 glass beads (~ 300µl) were added to the tubes, and samples were homogenized for 3 min at high speed using a bullet blender® (speed setting 8, Cat. Number BBX24, Next Advance, Inc., New York, USA). Samples were placed on ice, transferred to a 15 ml conical centrifuge tube, and diluted with PBS to 8 – 10 ml total volume. To remove any residual cell debris and glass beads, samples were centrifuged at 2000 x *g* for 10 min at 4 °C. Following centrifugation, the supernatants were transferred to clean tubes for ultracentrifugation at 100,000 x *g* for 30 min at 4 °C. After centrifugation, the supernatant was discarded and the pellets were resuspended using 1 ml of PBS in a microcentrifuge tube. The protein concentrations of the membrane preparations were determined by using a Bradford protein assay kit (27) using bovine serum albumin as a standard (Bio-Rad Laboratories, Hercules, Calif.). Reaction mixtures (100 µl each) contained 75 µl of each membrane preparation (300 µg of protein) and 25 µl of Boc-FL to achieve final concentrations of 0- 0.5- 1- 2.5- 5- 10 and 50 µM, and incubated at 37°C for 30 min. Laemmli Sample Buffer (Cat. Number: 161-0737, Bio-Rad Laboratories, Hercules, Calif.) was added to each reaction

mixture 1:1 and boiled at 100°C for 3 – 5 min and placed on ice for 5 min. Samples were loaded in SDS-PAGE gel and subjected to electrophoresis, or stored at - 20ºC until needed.

Competitive binding assays. Whole-cell and membrane preparations were used for competitive binding assays. For each concentration of test compound, an aliquot of freshly grown cells (equivalent to 1 ml of a bacterial suspension with an *A*600 of 0.4nm) or 75 µl of each membrane preparation (300 µg of protein) were incubated with increasing concentrations of cefoxitin: 0, 0.5, 1, 2.5, 5, 10 and 50 µg/ml final concentration, for 30 min at 35°C. Following incubation, BFL was added to a concentration of 10 µM Boc-FL and another incubation period was done for 30 min at 35 ºC. For whole-cell preparations, 5 µl of lysostaphin was added to achieve a final concentration of 200µg/ml and incubated at 37°C for 15 min to allow cell lysis to occur. Whole-cell and membrane preparations were prepared for SDS-PAGE with the addition of 75 - 100 µL of Laemmli Sample Buffer (Cat. Number: 161-0737, Bio-Rad Laboratories, Hercules, Calif.) and the reaction was stopped by incubation at 100°C for 5 min. Samples were loaded onto SDS-polyacrylamide gels and electrophoresed.

SDS-polyacrylamide gels and electrophoresis. Proteins were separated by electrophoresis using an SDS-PAGE system. Electrophoreses was conducted using a 10X Tris/Glycine/SDS running buffer (Cat. No. 161-10732, Bio-Rad Laboratories, Hercules, Calif.) and 30 µl of each reaction mixture were loaded in a discontinuous SDS-PAGE gel (5% stacking and 12% separating gels, Bio-Rad Laboratories). Electrophoretic conditions were 64V for the stacking gel (≈ 1 h) and then at 100V until the sample buffer migrated out of the separating gel. Following electrophoresis, the gels were immediately rinsed with water. To visualize the labeled PBPs, the gels were directly scanned with a Typhoon^{TM} 9410 (GE Healthcare) using an excitation of 488

nm and emission of 530 nm. In some cases, fluorescence detection was using an ImageQuant LAS 4010 (GE Healthcare). The fluorescence of PBPs with the bound FOX and fluorescent bocillin were measured using the software ImageQuant V 5.0. (GE Healthcare) software. Apparent Kd values were calculated to indicate the concentration of antibiotic required for 50% of maximum binding.

Results

Penicillin-Binding Proteins Patterns. Boc-FL is a fluorescent β-lactam that covalently binds and labels penicillin binding proteins (PBPs) (305). The highest affinity binding was obtained at a concentration of 10 to 30 µM using Boc-FL. The pattern of the PBPs obtained for *S.* aureus was as expected from previous reports, figure 7. A distinctly different pattern of binding was obtained when Boc-FL was used to label PBPs in *staphylococci* isolated from canine clinical specimens, figure 7 and 8. The PBPs pattern of the clinical isolate RA-1, classified as MRSA by OXA and FOX, is observed on lanes A and B. On lanes C and D the PBPs pattern of SA-1, a MSSA, are observed. The band patterns of the PBPs observed for MRSA and MSSA corresponded with previous studies (81, 83, 224). In the clinical MSSA, identified as SA-1 three high-molecular weight and one low-molecular weight proteins were detected ranging from 70 Kd to 100 Kd. In contrast, the MRSA isolate (RA-1) had the same 3 PBPs with an additional high-molecular band corresponding to PBP2a. Note that PBP2 and 2a migrated closely together in lanes A and B, while PBP4 was very light and detected ranging from 35 Kd to 55 Kd, figure 7.

Clinical isolates of *S. pseudintermedius* were loaded on lanes E, F, G and H, figure 7. On lanes E and F a MRSP (identified as RI-10) can be observed and on lanes E and F a MSSI (SI-89) is observed, (Fig. 7). Strains of *S. pseudintermedius* produced PBP patterns represented by

five PBP bands. Four heavy-weight bands (PBP1, PBP2, PBP3, and PBP4) were detected ranging from 70 Kd to 100 Kd. In addition, one light-weight band (PBP5) were detected ranging from 35 Kd to 55 Kd. Using this technique there was a similar PBPs pattern among MR and MS strains of *S. pseudintermedius,* figure, 7. Although, the PBP1 and PBP2 of *S. pseudintermedius* migrate together in the gel picture, figure 7, in other gels with improved resolution these proteins were completely separated, as demonstrated in figures 8 and 9. Bands corresponding to fluorescently labeled molecular weights were not visible on figure 8. Therefore, figure 9 was included as a duplicate of figure 8 to demonstrate the fluorescently labeled molecular weights in the same gel.

To avoid degradation of β-lactams by β-lactamases, the use of clavulanic acid has been recommended in experiments that use members of this group of drugs or analogs (219). In this study, the addition of clavulanic acid did not improve resolution or increase binding efficiency, figure 7, lane A, C, E and G. The addition of clavulanic acid generated significant background that prevented the light-molecular weight PBPs to be detected. Therefore, it was determined that the addition of clavulanic acid provided no benefit.

The PBP patterns for the selected ATCC strains can be observed in Fig. 8 and 9. Lanes A, B and C correspond to *S. aureus* ATCC 1756, 43300 and 25923 respectively. *S. pseudintermedius*-49444 correspond to lane E and *S. schleiferi* subsp *coagulans*-49545 to lane G. The four heavy-weight PBPs corresponding to *S. pseudintermedius* can be observed in figure 8, lanes D and E. Note that the PBP2 and PBP3 of *S. pseudintermedius*-49444 (lane E) are more intense than the same PBPs in the clinical MRSP (lane D) and the lighter band (PBP5) was not detected in *S. pseudintermedius* in this figure. The ATCC 49545 strain, *S. schleiferi* subsp *coagulans,* in lane G exhibited four heavy-weight PBPs (PBP1, PBP2, PBP3 and PBP4). Lane F

is represented by a MRSC clinical isolate and three heavy-weight PBPs were detected. The PBPs corresponding to ATCC 49545 strain, *S. schleiferi* subsp *coagulans* and the clinical MRSC were detected ranging from 70 Kd to 100 Kd. The PBP1 of S. *schleiferi* subsp *coagulans* displayed more fluorescent intensity than the other isolates.

Competitive binding assays. Competitive binding assays were performed to evaluate the binding capacity of each staphylococcal PBP for FOX. Saturation was completed using increasing concentrations (0.5- 1- 2.5- 5- 10 -30 and 50 µg/ml) of FOX. Therefore, only those PBPs with no or low affinity for FOX would be available to bind Boc-FL during the second incubation. The concentration of the FOX needed to block 50% of the subsequent binding of Boc-FL to each PBP of interest (50% inhibitory concentration [IC_{50}]) was detected in images processed by the LAS4010. The results of competition assays for PBPs with FOX are shown in figures 10 - 15.

The results using ATCC *S. aureus*-1756 in a competition assay are demonstrated in Fig. 10. FOX had a high affinity for MRSA PBPs 1, and 3, to the extent that Boc-FL inhibition of binding occurred a very low FOX concentrations (IC_{50} = 0.5µg/ml). The FOX affinity with PBP2 and PBP2a was lower, IC_{50} = 2.5µg/ml and 5 µg/ml, respectively. The PBP4 was not detected in this gel. The PBPs competition assay using *S. pseudintermedius* ATCC 49444 showed a total lack of affinity for FOX by PBP3 (IC_{50} = >50µg/ml) while PBP4 had a IC_{50}= 10µg/ml. The PBP1 and PBP2 exhibited much lower IC_{50}, 0.5 and 1µg/ml, respectively (Fig. 11). In contrast, for MRSP (RI-26) it was demonstrated that FOX had a high affinity for PBPs 1 and 2 by completely inhibiting Boc-FL binding, figure 12. Binding affinity of FOX to PBP3 and PBP4 was reduced (IC_{50} = 10µg/ml) and (IC_{50} = 0.5µg/ml), respectively.

The competitive binding of FOX by *Staphylococcus schleiferi* subsp *coagulans* PBPs is displayed in figures 14 and 15. For MRSC (RC-7) it was demonstrated that FOX completely prevents the binding of Boc-FL, because of a high affinity to PBP1, PBP2 and PBP4, figure 14. However, there was no affinity of FOX to PBP3 (IC_{50} = >50µg/ml). Similar results were observed when the PBPs of MSSC (SC-23) and MSSP (SI-89) were characterized, figures 15 and 13, respectively. The affinity of FOX to PBP3 of both microorganisms was very low (IC_{50} = >50µg/ml) in each case. The use of LAS4010 for the competition assays resulted in images with a lack of detectable on band resolution.

Discussion

Infections associated with methicillin resistant staphylococci are considered an emerging problem in veterinary medicine. Antimicrobials, specifically β-lactams, with a high affinity for PBPs are crucial for the treatment of infections making the accurate and rapid identification of MR staphylococci critical. Understanding the mechanisms of resistance to β-lactam antimicrobials in *Staphylococcus* is imperative to finding a means of treatment.

Alterations (mutations or additions) of PBPs are one of the most important factors associated with the expression of resistance against β-lactams in different bacterial species (301). In *S. aureus,* PBPs have been well studied, but this is little known regarding *Staphylococcus* species of animal origin. In this study, the pattern of PBPs was characterized in *S. pseudintermedius* and *S. schleiferi* subsp *coagulans.* Both staphylococcal species are commensal flora in dogs. However, the two species are considered important opportunistic pathogens with the potential to cause zoonotic infections. In the current study, a five-band PBP pattern (PBP1,

PBP2, PBP3, PBP4 and PBP5) was observed in *S. pseudintermedius* while no differences were observed when PBPs from MRSP and MSSP were compared. *Staphylococcus pseudintermedius* is one of the members of the *S. intermedius* group. PBPs pattern in *S. intermedius* were investigated by Canepari *et al.* (34). Canepari *et al,.* reported that identical three-band PBP patterns were observed in the five *S. intermedius* strains examined (PBP1, 85 Kd; PBP2, 82 Kd; and PBP3, 79 Kd) (34). It may be reasonable to conclude that although *S. pseudintermedius* and *S. intermedius* share a large genetic similarity, the pattern of PBPs is different between both species. Variations could be attributed to the use of radiolabeled benzylpenicillin for the identification of PBPs in *S. intermedius,* which was used in the cited study. However, it has been demonstrated that the sensitivity of Boc-FL is similar to those of the methods using radioisotope compounds (79, 306).

With regard to *Staphylococcus schleiferi* subsp *coagulans* a pattern of four heavy-weight PBPs (PBP1, PBP2, PBP3 and PBP4) ranging from 75 Kd to 100 Kd were detected when the ATCC-49545 was examined. In contrast, three heavy weights bands also ranging from 75 Kd to 100 Kd were observed in a clinical MRSC. No published data was found to compare with the results of this study. PBP1 corresponding to both MRSC (RC-1) and MSSC (ATCC-49545) strains was very wide and it is possible that with changes in the electrophoretic conditions, this band could separate and allow two different bands to be detected.

Conventionally, the *in vitro* identification of resistance to the isoxazolyl class of penicillinase-stable penicillin in staphylococcal species has been performed by testing OXA using disk diffusion or microbroth dilution methods (16, 43). OXA resistance in *Staphylococcus* sp predicts *mecA*-mediated resistance in staphylococci (43, 192). The use of FOX as a surrogate for OXA was recommended by the CLSI in 2008 together with changes in the break points for

OXA interpretation. Results of our previous studies, as well as other reports (16, 17, 241) demonstrated that when the 2008, OXA and FOX break points were applied, there was a failure to accurately identify MR in both MRSP and MRSC. In fact, for the detection of MR, FOX had a sensitivity of 4.35% and 14.8% for *S. pseudintermedius* and *S. schleiferi* subspecies *coagulans*, respectively. The finding of the SCC*mecA* element in *S. pseudintermedius* and *S. schleiferi* subsp *coagulans* has been reported multiple times and it has been assumed that this same mechanism produces methicillin resistance in both species. It is important to note that the PBP2a is encoded by the *mecA* gene. The PBP2a is considered the most important factor in defining MR in staphylococci and is described as an additional PBP in *S. aureus.* It was hypothesized that the differences in the sensitivity to cefoxitin by MRSP and MRSC, when compared with MRSA, is based on variations of their PBPs affinity by cefoxitin.

Competitive binding assays performed in this study demonstrated that one of the PBPs (presumably PBP3) in both, *S. pseudintermedius* and *S. schleiferi* subsp *coagulans,* exhibited a lack of affinity with FOX (IC_{50} > 50µg/ml). Overall a high degree of affinity was found when the other PBPs were evaluated in both species. In comparison, when MRSA (ATCC-1756) was examined, the affinity of its PBP2a by FOX was IC_{50} = > 5 µg/ml and the affinity of PBP2 by was IC_{50} = 2.5 µg/ml.

MR strains of *S. schleiferi* subsp *coagulans* and *S. pseudintermedius* are considered MS when tested by FOX. However, when OXA was used to identify resistance against β-lactams the same *Staphylococcus* sp strains were classified as MR. In addition, a penicillin latex agglutination test for the detection of the PBP2a and amplification of the *mecA* gene by PCR was performed in previous studies to confirm MR in these strains. Consequently it was hypothesized

that the differences in the reaction of MRSP and MRSC to cefoxitin, when compared with MRSA is based on variations in the PBPs affinity for cefoxitin.

The results of this study provide significant evidences to support this hypothesis by documenting that the PBPs of a MRSA strain (*S. aureus*-1756) exhibited higher affinity to FOX (Fig. 10) when compared with the PBPs of *S. pseudintermedius* and *S. schleiferi* subsp *coagulans,* figures 11 – 15. Specifically, the lack of affinity with FOX observed in *S. pseudintermedius* and *S. schleiferi* subsp *coagulans* was dependent on one of the PBPs which have been identified in this study as PBP3. A preliminary conclusion could be that PBP3 in both species were largely insensitive to cefoxitin, whereas the other PBP's were affected at very low concentrations.

β-lactam antibiotics bind to only certain number of PBPs and the affinities of PBPs by β-lactams is variable (251). The *mecA* gene is carried by MRSA, but also has been found in MRSC and MRSP. Results of this study raise the question whether is the *mecA* gene and its product (the PBP2a) are involved in the mechanism of resistance in MRSC and MRSP. The results from the competitive binding assays show that one of the PBPs (PBP3) in *S. schleiferi* subsp *coagulans* and *S. pseudintermedius*¸ express a lack of affinity by FOX; however, it is not likely that this is equivalent to the PBP2a in MRSA, because it is also detected in MS strains of *S. schleiferi* subsp *coagulans* and *S. pseudintermedius,* figures 11, 13 and 15. More studies are recommended to investigate the affinity binding of *S. pseudintermedius* and *S. schleiferi* subsp *coagulans* with other β-lactams.

General conclusions

The research information presented in this dissertation constitutes a three-part investigation that was conducted to investigate coagulase positive *Staphylococcus* species in companion animal medicine. Bacteria included in this study were isolates collected from horse and canine clinical specimens received in the Auburn University, College of Veterinary Medicine, Bacteriology and Mycology Diagnostic Laboratory. The studies were based on the molecular epidemiology and genetic analysis of *Staphylococcus aureus*, *Staphylococcus pseudintermedius* and *Staphylococcus schleiferi* subspecies *coagulans.* Overall, the species were identified using traditional microbiological methods and molecular techniques, including an evaluation of the most sensitive diagnostic method for recognition of methicillin-resistance. Molecular epidemiology studies were based on typing methods and analysis of virulence factor genes. Additionally, because the mechanisms of antimicrobial resistance are of great interest in staphylococci, the proteins that contribute to resistance in *Staphylococcus* sp were characterized.

In the initial studies, sixty-eight methicillin resistant *S. aureus* collected from horses were analyzed. Isolates were collected mainly from skin lesions (54%), followed by bones and joint fluids (12%), respiratory tract (9%), internal body tissues and cavities (7%), blood (6%), guttural pouch (3%), eye (4%) and urinary/reproductive tract (4%). A high pattern of resistance was observed against Ampicillin, Amoxicillin/clavulanic acid, Cephalothin, Erythromycin, Gentamicin, Penicillin, Trimethoprim/Sulfa, Tetracycline and Ceftiofur. Observations of this study are supportive of the premise that adequate biosafety methods (ie, improvements in hand

hygiene by personnel with animal contact) in veterinary environments will reduce nosocomial outbreaks of methicillin-resistant *Staphylococcus aureus* (MRSA) in equine medicine.

Results of PFGE and SCC*mec* multiplex PCR indicated that the MRSA isolates were highly clonal which was characterized by a predominance of the USA500, type IV strains. These results were in accordance with previously published reports. A relevant outcome of this investigation was the finding of MRSA USA100, SCC*mec* type-II clone in 11% (7/63) of the tested isolates. This finding suggests that the presence of the USA100 SCC*mec* type II clone in horses could be related to reverse zoonosis as it is not typically common in the horse population.

In the second part of the investigation, 577 canine *Staphylococcus* sp and were analyzed. The microorganisms were classified by *Staphylococcus* specie and grouped as methicillin-susceptible (MS) and methicillin-resistant (MR) as follow: *S. aureus*: 44, *S. pseudintermedius:* 459 and *Staphylococcus schleiferi* subsp *coagulans*: 74. The total of MR microorganisms is represented by 38.82% (224/577) of the isolates, which is considered a significant number. The frequency distribution analysis of MR isolates in a period of four years (2007 – 2010) demonstrated a higher risk of finding MRSP and MRSC associated with the more recent years 2009 and 2010.

As reported in other studies most of the isolates were collected from skin/abscess 52.6% (301/572), followed by ears 19.2% (110/572). In the present study, 54.7% (23/42) of the *S. aureus* isolates were associated with invasive infections (bone, body cavities, joint fluid, urine, respiratory, blood and reproductive tract). In contrast, 71.8% (324/451) *S. pseudintermedius* and 91.8% (68/74) *S. schleiferi* subsp *coagulans* were associated with skin/ear infections.

Collection of epidemiological information from the database was limited. However, the analysis of previous therapy as a risk factor related with the isolation of MR strains determined

that within the group that received therapy a significant difference ($p \leq 0.05$) indicated that isolates from dogs with a history of antimicrobial therapy represented by 63.22% (98/155) have a higher risk of being MR than from dogs without antimicrobial therapy. No significance was found when classes of antimicrobial used were analyzed. Overall, results of the susceptibility testing indicate that chloramphenicol and rifampin exhibited the highest prevalence of susceptibility when *S. aureus, S. pseudintermedius* and *S. schleiferi* subsp *coagulans* isolates were tested, with 1.8% (8/434) and 2.3% (9/391) respectively.

Analysis of the SCC*mec* demonstrated that 66% of the MR *Staphylococcus* sp from dogs carried the SCC*mec* type-V, which corresponds to 83% MR *S. pseudintermedius* (MRSP), 16% MR *S. schleiferi* subsp *coagulans* and 1% MRSA isolates. These results are remarkable, not just because this is the first time the SCC*mec* type-V has been reported in MRSC but also because 67% (18/27) carried SCC*mec* type-V and a single MRSC isolate harbored the type-IV. The SCC*mec* type-II was the most frequent in MRSA strains which was represented by 10% (17/171) of the total of MR isolates, but 57% (17/29) of the MRSA group. The type-IV was carried by 13% (23/171) of the strains included in this study and it was represented by 44% (10/23) MRSA, 52% (12/23) MRSP and 4% (1/23) MRSC. It is possible to speculate about the influence of human contact on these findings. Community – acquired MRSA is mostly characterized by these two structural variants of the mec element, the SCC*mec* type-IV and type-V.

Statistical analysis (sensitivity and specificity) of the laboratory methods to identify MR in *Staphylococcus* sp revealed the overall sensitivity and specificity of OX for the detection of MR strains to be 83% and 93%, respectively. However, with the use of FOX the sensitivity was 20% and the specificity 100%. These findings confirm that cefoxitin is not appropriate for the detection of MR in canine staphylococci. The use of OXA according to the 2004 CLSI breaking

points criteria is recommended. In addition, the PBP2a latex agglutination test and the subsequent screening by PCR amplification of the *mecA* gene by PCR are good alternatives for the confirmation of resistance against β-lactams in canine staphylococci.

Using PCR, the genes that encode PVL (*lukS*-PV and *lukF*-PV), LUK-I (*lukS*-I and *lukF*-I) and SIET were amplified and analyzed. In this study *luk*I genes were present in a total of 254 isolates and distributed as follows: 1 in MRSC, 3 in MRSA, 91 in MSSP and 159 in MRSP. In addition, it is noteworthy that in regards to *siet* gene, the *siet* gene sequence was amplified in 5 MRSA; 27 MRSC; 8 MSSC; 118 MRSP and 90 MSSP. To date, there is no published data documenting the finding of *luk*I and *siet* genes in *S. aureus* and *S. schleiferi* subsp *coagulans.* PVL gene was detected in 12.5% (4/32) of MRSA isolates, and was not found in any of the *S. pseudintermedius* and *S. schleiferi* subspecies *coagulans.*

The increased findings of MR in *Staphylococcal* sp from animal origin, together with the finding of virulence factors of *S. pseudintermedius* in the other staphylococci evaluated in this investigation allows one to speculate about the possibility of genetic exchange and transfer among the *Staphylococcus* sp isolated from canine.

Finally, results of the analysis of penicillin-binding (PBPs) in *S. pseudintermedius* and *S. schleiferi* subsp *coagulans* indicated that the PBPs pattern in *S. pseudintermedius* was represented by five PBPs bands. Four heavy-weight bands (PBP1, PBP2, PBP3, and PBP4) and one light-weight band (PBP5). The technique employed in this study did not identify a different PBPs pattern among MR and MS strains of *S. pseudintermedius.* In *S. schleiferi* subsp *coagulans* four heavy-weight PBPs (PBP1, PBP2, PBP3 and PBP4) were identified in a methicillin susceptible strain, and three heavy-weight PBPs were detected in a MRSC isolate. This is the first report characterizing the PBP pattern of these important *Staphylococcus* sp.

Competitive binding assays results demonstrated that one of the PBPs (PBP3) in *S. schleiferi* subsp *coagulans* and *S. pseudintermedius* expressed a lack of affinity by FOX. However, it is likely that is not the equivalent to the PBP2a in MRSA, because is also detected in MS strains of *S. schleiferi* subsp *coagulans* and *S. pseudintermedius.* To better understand these results, more studies, including competition assays for OXA have been initiated and need to be completed.

Because of the 'predictable' antibiotic susceptibility spectrum of *Staphylococcus* sp in small animals, it is not considered a standard practice to culture every case of canine pyoderma and otitis externa. However, because the increasing number of methicillin-resistant *Staphylococcus* sp reported in dogs and cats, this practice should become more common. The results of this investigation do not represent a population-based study, and this is the most important bias. However, the conclusions are considered valuable and consonant with staphylococcal diseases in canine and horses. Finally, it is important to mention that the three staphylococcal species investigated in these studies have the potential of being zoonotic pathogens.

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Appendix I: Tables

Table 1 Most common pathogenic *Staphylococcus* species in animals

SPECIES	HOST	INFECTION TYPE
S. aureus	Ruminants	Mastitis Skin infections
	Horse	Mastitis
	Rabbit	"Bumble foot", septic arthritis
	Poultry	
	Pigs	Skin infections (clone ST398)
S. pseudintermedius	Dog	Commensal, pyoderma
S. delphini	Horse, mink, cow, dolphin, pigeon	Pyoderma
S. intermedius	Pigeon	
S. epidermidis	Ruminants	Mastitis
S. hyicus	Pig	Exudative dermatitis
S. sciuri	Pig	Greasy pig syndrome
S. simulans	Ruminants	Mastitis
S. schleiferi subsp *coagulans*	Dog	Otitis

Taken from **Fitzgerald, J. R., and J. R. Penadés.** 2008. Staphylococci of Animals p. 255 - 269. *In* J. A. Lindsay (ed.), Staphylococcus: molecular genetics. Caister Academic Press, with some modifications

Table 2 Primers used in this study for the typing of the SCC*mecA*

Primer name	Primer sequence (5′→3′)	Amplicon size (bp)	Reference
CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	495	**(199)**
CIF2 R2	ATTTACCACAAGGACTACCAGC		
ccrC F2	GTACTCGTTACAATGTTTGG	449	**(177)**
ccrC R2	ATAATGGCTTCATGCTTACC		
RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	**(199)**
RIF5 R13	ATGGAGATGAATTACAAGGG		
SCC*mec* V J1 F	TTCTCCATTCTTGTTCATCC	377	**(177)**
SCC*mec* V J1 R	AGAGACTACTGACTTAAGTGG		
dcs F2	CATCCTATGATAGCTTGGTC	342	**(199)**
dcs R1	CTAAATCATAGCCATGACCG		
ccrB2 F2	AGTTTCTCAGAATTCGAACG	311	**(177)**
ccrB2 R2	CCGATATAGAAWGGGTTAGC		
kdp F1	AATCATCTGCCATTGGTGATGC	284	**(199)**
kdp R1	CGAATGAAGTGAAAGAAAGTGG		
SCC*mec* III J1 F	CATTTGTGAAACACAGTACG	243	**(177)**
SCC*mec* III J1 R	GTTATTGAGACTCCTAAAGC		
mecI P2	ATCAAGACTTGCATTCAGGC	209	**(199)**
mecI P3	GCGGTTTCAATTCACTTGTC		
mecA P4	TCCAGATTACAACTTCACCAGG	162	**(199)**
mecA P7	CCACTTCATATCTTGTAACG		

Table 3 Primers used in the SCC*mec* IV multiplex PCR[a]

Primer name	Primer sequence (5′→3′)	Primer specificity	Amplicon size (bp)
ccrB2 F	CGAACGTAATAACATTGTCG	ccrB2	**203**
ccrB2 R	TTGGCWATTTTACGATAGCC		
J IVa F	ATAAGAGATCGAACAGAAGC	Type IVa	**278**
J IVa R	TGAAGAAATCATGCCTATCG		
J IVb F	TTGCTCATTTCAGTCTTACC	Types IVb and IVF	**336**
J IVb R	TTACTTCAGCTGCATTAAGC		
J IVc F	CCATTGCAAATTTCTCTTCC	Types IVc and IVE	**483**
J IVc R	ATAGATTCTACTGCAAGTCC		
J IVd F	TCTCGACTGTTTGCAATAGG	Type IVd	**575**
J IVd R	CAATCATCTAGTTGGATACG		
J IVg F	TGATAGTCAAAGTATGGTGG	Type IVg	**792**
J IVg R	GAATAATGCAAAGTGGAACG		
J IVh F	TTCCTCGTTTTTTCTGAACG	Type IVh	**663**
J IVh R	CAAACACTGATATTGTGTCG		

[a]From Milheiriço *et al.* 2007. Update to the multiplex PCR strategy for assignment of *mec* element types in *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 51:3374.

Table 4 Differentiation of coagulase positive *Staphylococcus* species

***Staphylococcus* sp**	**Hemolysins**	**Catalase**	**Coagulase**	**VP**	**Trehalose**	**Mannitol**	**Maltose**
S. aureus	+	+	+	+	+	+	+
S. pseudintermedius	+	+	+	-	+	(d)	(POS/NEG)
S. schleiferi subsp. ***c****oagulans*	(+)	+	+	+	-	d	-

+ : 90% or more strains are positive. - : 90% or more strains are negative, ( ): delay reactions. d: 11 – 89% of strains are positive. POS/NEG: 90% or more are weakly positive. Reference: Manual of Clinical Microbiology, 7th ed. P 268 -269

Table 5 Disk diffusion breakpoints for oxacillin and cefoxitin recommended by the Clinical and Laboratory Standard Institute in 2004 and 2008

Year	Disk Content	Criteria: Zone Diameter, nearest whole mm			Comments
		Susceptible	Intermedius	Resistant	
2004	1 µg oxacillin	≥ 13	11 – 12	≤ 10	*S. aureus*
		≥ 18	-	≤ 17	Other *Staphylococcus sp*
2008	1 µg oxacillin	≥ 13	11 – 12	≤ 10	*S. aureus*, coagulase + *Staphylococcus sp*
	30 µg cefoxitin	≥ 22	-	≤ 21	*S. aureus*

Table 6 Primers used for *Staphylococcus pseudintermedius* identification and SCC*mec* multiplex PCR

Primer name	Primer sequence	Amplicon size (bp)	Reference
PTA F1	AAAGACAAACTTTCAGGTAA	320	(11)
PTA R1	GCATAAACAAGCATTGTACCG		
CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	495	(199)
CIF2 R2	ATTTACCACAAGGACTACCAGC		
ccrC F2	GTACTCGTTACAATGTTTGG	449	(177)
ccrC R2	ATAATGGCTTCATGCTTACC		
RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	(199)
RIF5 R13	ATGGAGATGAATTACAAGGG		
SCC*mec* V J1 F	TTCTCCATTCTTGTTCATCC	377	(177)
SCC*mec* V J1 R	AGAGACTACTGACTTAAGTGG		
dcs F2	CATCCTATGATAGCTTGGTC	342	(199)
dcs R1	CTAAATCATAGCCATGACCG		
ccrB2 F2	AGTTTCTCAGAATTCGAACG	311	(177)
ccrB2 R2	CCGATATAGAAWGGGTTAGC		
kdp F1	AATCATCTGCCATTGGTGATGC	284	(199)
kdp R1	CGAATGAAGTGAAAGAAAGTGG		
SCC*mec* III J1 F	CATTTGTGAAACACAGTACG	243	(177)
SCC*mec* III J1 R	GTTATTGAGACTCCTAAAGC		
mecI P2	ATCAAGACTTGCATTCAGGC	209	(199)
mecI P3	GCGGTTTCAATTCACTTGTC		
mecA P4	TCCAGATTACAACTTCACCAGG	162	(199)
mecA P7	CCACTTCATATCTTGTAACG		

Table 7 Number and percentage (%) of *Staphylococcus* sp isolated from canine clinical specimens by source of specimen.

Staphylococcus sp group	*n*	Number and percentage (%) of *Staphylococcus sp* by source					
		Skin/ear	Ears	Bone / Tissues	Joint	Urine	Others
MRSA	34	12	3	11	5	1	2
MSSA	5	0	1	1	0	1	2
MRSC	26	12	14	0	0	0	0
MSSC	17	4	11	0	0	0	2
MRSS	112	71	17	12	2	8	2
MSSS	103	53	13	6	0	21	10
Totals	297	152 (51)	59 (20)	30 (10)	7(2)	31(10)	18(6)

MRSA: Methicillin-resistant *S. aureus.* MSSA: Methicillin-susceptible *S. aureus.*
MRSC: Methicillin-resistant *S. schleiferi* subsp *coagulans.*
MSSC: Methicillin-susceptible *S. schleiferi* subsp *coagulans.*
MRSP: Methicillin-resistant *S. pseudintermedius.* MSSP: Methicillin-susceptible *S pseudintermedius.*

Table 8 Number and percentage (%) of *Staphylococcus* species isolated from canine clinical specimens by gender and age of patient.

Staphylococcus sp	n	Gender		*Staphylococcus sp*	n	Age (years)			
		F	M			< 2	2-5	6-8	≥ 9
MSSA	4	2	2	MSSA	4	0	2	1	1
MRSA	33	13	20	MRSA	32	17	6	9	0
MSSC	17	7	10	MSSC	26	0	6	4	16
MRSC	26	21	5	MRSC	17	1	3	3	10
MSSS	102	54	48	MSSS	99	5	27	29	38
MRSS	112	53	59	MRSS	107	8	34	31	34
Total	294	150(51)	144(49)	Total	285	31(11)	78(27)	77(27)	99(35)

MRSA: Methicillin-resistant *S. aureus.* MSSA: Methicillin-susceptible *S. aureus.*
MRSC: Methicillin-resistant *S. schleiferi* subsp *coagulans.*
MSSC: Methicillin-susceptible *S. schleiferi* subsp *coagulans.*
MRSP: Methicillin-resistant *S. pseudintermedius.* MSSP: Methicillin-susceptible *S pseudintermedius.*

Table 9 Characterization of *Staphylococcus sp* according to oxacillin and cefoxitin susceptibility patterns, penicillin-binding protein latex agglutination test (PLAT) and detection of *mecA* gene by PCR, *n*=299

Staphylococcus sp	CLSI 2008					CLSI 2004			PLAT		*mecA*	
	Oxacillin			Cefoxitin		Oxacillin			+	-	+	-
	S	I	R	S	R	S	I	R				
S. aureus	10	1	29	14	26	10	1	29	35	5	29	11
S. schleiferi subsp *coagulans*	18	2	24	40	4	17	-	27	27	17	27	17
S. pseudintermedius	105	15	95	210	5	91	-	124	112	103	115	100
Totals	133	18	148	264	35	118	1	180	174	125	171	128

PLAT: Penicillin-binding protein latex agglutination test. S: Susceptible. I: Intermediate. R: Resistant. (*n*): Percentages

Table 10 Distribution of the SCC*mecA* type between Methicillin Resistance *Staphylococcus* species (n = 171) isolated from canine clinical specimens

SCC*mecA*	*Staphylococcus sp*			Totals	%
	MRSA	MRSC	MRSP		
NT	0	8	8	16	9.35
II	17	0	0	17	9.94
II-III	0	0	1	1	0.58
III	1	0	0	1	0.58
IV	10	1	12	23	13.45
V	1	18	94	113	66.08

NT: No-typable

Table 11 Sensitivity and specificity expressed as % of the oxacillin (OXA) and cefoxitin (FOX) by *Staphylococcus* species when used to detect methicillin-resistance.

		***Staphylococcus* sp**					
		MRSP		**MRSC**		**MRSA**	
Criteria[a]	**TEST**	**OXA[b]**	**FOX[c]**	**OXA**	**FOX**	**OXA[b]**	**FOX**
CLSI 2008	% Sensitivity	78.2	4.35	85.1	14.8	100	89.6
	% False -	9.5	95.6	7.4	85.1	0	10.3
	% Specificity	94	100	94.1	100	90.9	100
	% False +	5	0	5.8	0	0	0
CLSI 2004	% Sensitivity	> 100	NA	100	NA	100	NA
	% False -	0	NA	0	NA	0	NA
	% Specificity	91	NA	100	NA	90.9	NA
	% False +	9	NA	0	NA	9.09	NA

[a] Clinical and Laboratory and Standard Institute Criteria for the detection of methicillin-resistance in *Staphylococcus* sp

[b] 2008 and 2004 CLSI criteria for OXA include intermediate values, which affect the sensitivity and specificity final results

[c] FOX is not included in the CLSI 2004 criteria: NA

Note: Results of the PCR *mecA* detection were considered as gold standard test

Table 12 Nucleotide primer sequences used for the amplification of virulence factor genes: *luk-P*, *luk*I and *siet.*

Primer name	Primer sequence (5′→3′)	Amplicon size (bp)	Reference
Luk-PV-1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	(157, 170)
Luk-PV-2	GCATCAAGTGTATTGGATAGCAAAAGC		
NLukI-1	TGCTACTGCAACATCCGTGGCA	557	This study
NLukI-2	GTGCTTCAACGCCCCAGCCA		
Siet-1	ATGGAAAATTTAGCGGCATCTGG	359	(148)
Siet-2	CCATTACTTTTCGCTTGTTGTGC		

Table 13 Frequency distribution of *Staphylococcus* species isolated from canine clinical specimens positives for *Staphylococcus* infection by year

	Staphylococcus sp grouped based on methicillin-resistance						
	***S. aureus,* *n* = 24**		***S. coagulans,* *n* = 70**		***S. pseudintermedius,* *n* = 422**		
	R	S	R	S	R	S	Total
Years	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)	% (*n*)
2007	38.46 (5)	45.5[a] (5)	13.3[b] (4)	27.5 (11)	14.93[a,b] (23)	22.01 (59)	20.73[a] (107)
2008	15.38 (2)	9.09[a] (1)	20.0[a] (6)	20 (8)	14.93[c,d] (23)	24.25 (65)	20.34 (105)
2009	30.76 (4)	18.18 (2)	46.6[a,b,c] (14)	42.5[a] (17)	34.41[b,c] (53)	27.98 (75)	31.97[b] (165)
2010	15.38 (2)	27.27 (3)	20.0[c] (6)	10.0[a] (4)	35.71[a,d] (55)	25.47 (69)	26.93[a,b] (139)
Totals	13	11	30	40	154	268	516

Same letters ([a, b, c, d]) within the same column indicate significant differences ($p \leq 0.05$) when years are compared. Abbreviations: R: resistant, S: susceptible. (n) = number of isolates

Table 14 Number (%) of *Staphylococcus* sp grouped as methicillin-resistant and methicillin-susceptible isolates by body site sources

	Source									
Staphylococcus sp	Skin/abscess	Ears	Bone/Internal	Joint Fluids	Urine	Reproductive	Respiratory	Blood Culture	Others	Totals
MRSA	15 (50.00)	1 (3.33)	9 (30.00)	4 (13.3)	0	1 (3.33)	0	0	0	30
MSSA	2 (15.38)	1 (7.69)	3 (23.08)	1 (7.69)	3 (23.08)	0	1 (7.69)	1 (7.69)	1 (7.69)	13
MRSC	15 (46.88)	16 (50)	1 (3.13)	0	0	0	0	0	0	32
MSSC	12 (28.57)	25 (59.52)	1 (2.38)	0	2 (4.76)	1 (2.38)	0	1 (2.38)	0	42
MRSP	108 (67.08)	21 (13.04)	11 (6.83)	4 (2.48)	13 (8.07)	0	3 (1.86)	0	1 (0.62)	161
MSSP	149 (50.58)	46 (15.64)	23 (7.82)	4 (1.36)	50 (17)	5 (1.7)	9 (3.06)	5 (1.7)	3 (1.02)	294
Totals	301 (52.6)	110 (19.2)	48 (8.3)	13 (2.2)	68 (11.8)	7 (1.2)	13 (2.2)	7 (1.2)	5 (0.8)	572

MRSA: Methicillin-resistant *S. aureus.* MSSA: Methicillin-susceptible *S. aureus.*
MRSC: Methicillin-resistant *S. schleiferi* subsp *coagulans.*
MSSC: Methicillin-susceptible *S. schleiferi* subsp *coagulans.*
MRSP: Methicillin-resistant *S. pseudintermedius.* MSSP: Methicillin-susceptible *S pseudintermedius.*

Table 15 Number (%) of *Staphylococcus* species grouped as methicillin-resistant and methicillin-susceptible isolates by gender and age

Staphylococcus sp	n	Gender		*Staphylococcus sp*	n	Age (years)		
		F	M			< 2	2-8	≥ 9
MSSA	11	6 (54.5)	5 (45.4)	MSSA	11	2 (18.1)	4 (36.2)	5 (45.4)
MRSA	30	12 (40)	18 (60)	MRSA	29	5 (17.2)	19 (65.4)	5 (17.2)
MSSC	41	16 (39)	25 (60.9)	MSSC	39	2 (5.1)	20 (51.2)	17 (43.5)
MRSC	32	24 (75)	8 (25)	MRSC	32	1 (3.1)	14 (43.7)	17 (53.1)
MSSP	272	147 (54)	125 (45.9)	MSSP	268	21 (7.8)	164 (61.1)	83 (30.9)
MRSP	161	74 (45.9)	87 (54)	MRSS	153	10 (6.5)	93 (60.7)	50 (32.6)
Total	547	279 (51.0)	268 (48.9)	Total	532	41 (7.7)	314 (59.0)	177 (33.2)

MRSA: Methicillin-resistant *S. aureus.* MSSA: Methicillin-susceptible *S. aureus.*
MRSC: Methicillin-resistant *S. schleiferi* subsp *coagulans.*
MSSC: Methicillin-susceptible *S. schleiferi* subsp *coagulans.*
MRSP: Methicillin-resistant *S. pseudintermedius.* MSSP: Methicillin-susceptible *S pseudintermedius.*

Table 16 Distribution of *Staphylococcus* species isolated from canine clinical specimens by breed

Breed	***n***	**%**	**Breed**	***n***	**%**
American cocker spaniel	33	6.16	Great Dane	15	2.80
American Staffordshire terrier	9	1.68	Jack Russell	7	1.30
Bassett	5	0.93	Labrador retriever	84	15.7
Beagle	8	1.49	Mastiff	14	2.61
Border collie	5	0.93	Mixed	51	9.53
Boxer	25	4.67	Poodle	9	1.68
Bulldog	31	5.79	Pug	10	1.86
Chihuahua	6	1.12	Rottweiler	12	2.24
Chow Chow	6	1.12	Schnauzer	6	1.12
Dachshund	16	2.99	Shih Tzu	15	2.8
Doberman pinscher	17	3.17	West highland white terrier	10	1.86
German shepherd	26	4.85	Yorkshire terrier	8	1.49
Golden retriever	23	4.29	Others (<4 isolate(s)/breed)	84	15.7

Table 17 *In-vitro* susceptibility testing results *Staphylococci* sp isolated from canine clinical specimens

	Staphylococcus sp groups						
Tested Drugs	MRSA % n/total	MSSA % n/total	MRSC % n/total	MSSC % n/total	MRSP % n/total	MSSP % n/total	Total % n/total
AMK	0 0/12	0 0/7	18.5 5/27	0 0/33	10.13 15/148	12.3 13/193	7.85 33/420
AMC	95.8 23/24	0 0/7	89.6 26/29	6.06 2/33	82.1 124/151	3.62 7/193	41.64 182/437
AMP	100 24/24	28.5 2/7	90.6 29/32	12.1 4/33	99.3 149/150	73.5 142/193	79.7 350/439
CFZ	91.6 11/12	0 0/10	89.2 25/28	6.0 2/33	82.99 122/147	2.57 5/194	38.9 165/424
CFAE	100 28/28	0 0/6	100 21/21	8 2/25	82.9 122/147	3.92 4/193	42.1 177/420
CEF	100 28/28	8 0/8	90.9 20/22	0 0/26	73.8 65/88	0 0/114	42.8 113/264
CHL	0 0/23	0 0/8	3.7 1/27	2.94 1/34	2.7 4/148	1.03 2/194	1.8 8/434
CLI	60.7 17/28	25 2/8	10.3 3/29	5.88 2/34	85.3 128/150	31.08 60/193	47.9 212/442
EFX	67.8 19/28	30 3/10	44.8 13/2	17.6 6/34	78.1 118/151	15.6 30/192	42.5 189/444
ERY	89.2 25/28	0 0/10	17.2 5/29	5.7 2/35	84.3 124/147	30.5 59/193	48.6 215/442
GEN	17.8 5/28	0 0/10	17.2 5/29	0 0/35	11.9 18/151	6.73 13/193	9.1 41/446
MAR	71.4 10/14	20 2/10	50 14/28	17.64 6/34	77.3 116/150	14.5 28/193	38.8 167/430
ORB	72.7 8/11	14.2 1/7	40.9 9/21	16 4/25	79.2 65/82	15.3 16/104	42.0 108/257
PEN	100 28/28	60 6/10	89.65 26/28	11.7 4/34	99.3 149/150	79.2 153/193	80.0 349/436
RIF	25 3/12	0 0/9	0 0/26	0 0/33	3.57 5/140	0.5 1/171	8.9 35/393
TET	28.5 8/28	0 0/10	21 4/19	0 0/28	84.7 72/85	27.8 29/104	38.7 109/281
SXT	17.85 5/28	0 0/10	0 0/27	0 0/33	79.4 120/151	20.2 39/193	38.7 168/434

Results were collected from retrospective sample database

Table 18 Results of the PCR for the detection of the *luk*I and *siet* genes in canine *Staphylococcus* species

	LUK-I		SIET	
	+	-	+	-
MRSA	3	28	5	28
MSSA	0	11	0	11
MRSC	1	28	27	4
MSSC	0	40	8	10
MRSP	159	2	118	0
MSSP	91	3	90	5
Totals	254	112	248	58

MRSA: Methicillin-resistant *S. aureus.* MSSA: Methicillin-susceptible *S. aureus.*
MRSC: Methicillin-resistant *S. schleiferi* subsp *coagulans.*
MSSC: Methicillin-susceptible *S. schleiferi* subsp *coagulans.*
MRSP: Methicillin-resistant *S. pseudintermedius.* MSSP: Methicillin-susceptible *S pseudintermedius.*

Table 19 Characteristics of the *Staphylococcus* species used for penicillin-binding proteins assays

		Test used for classification of methicillin-resistant			
		Susceptibility		PCR	
Strain	**Source**	**FOX**	**OXA**	**SCC*mec***	**Classification**
RA-1	Unknown	6, R	0, R	II	MRSA
SA-1	Cerebrum spinal fluid	31, S	12, I	-	MSSA
RC-1	Skin	24, S	0, R	*mecA* positive, no typable	MRSC
RC-7	Skin	38, S	0, R	V	MRSC
SC-23	Skin	42, S	28, S	-	MSSC
RI-10	Skin	31, S	16, R	IV	MRSP
RI-26	Skin	25, S	0, R	V	MRSP
SI-89	Skin	41, S	31, S	-	MSSP
1756	ATCC	R	R	IV	MRSA
43300	ATCC	R	R	II	MRSA
25923	ATCC	S	S	-	MSSA
49444	ATCC	S	S	-	MSSP
49545	ATCC	S	S	-	MSSC

Susceptibility: FOX = cefoxitin, OX = oxacillin. Results are based on Clinical and Laboratory Standard Institute in mm. S: susceptible, R: resistant.

Appendix II: Figures

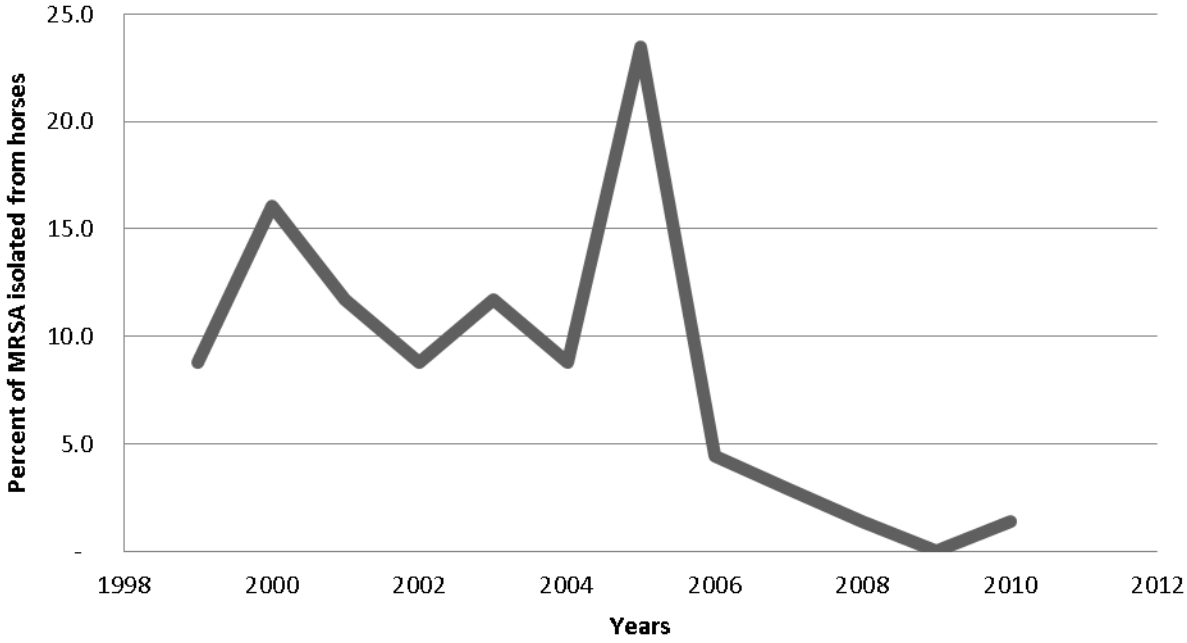


Figure 1 Percent of total MRSA isolated from Horses/Year

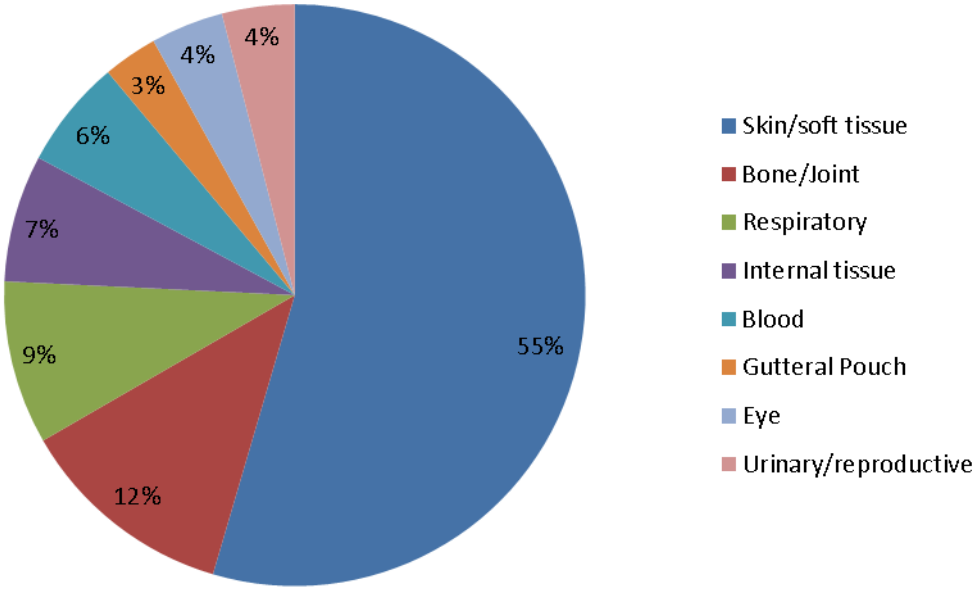


Figure 2 Source of MRSA isolates collected from Horses

Figure 3 Agarose gel electrophoresis of Multiplex PCR for typing of the SCC*mecA* gene. Lane 1: 100 bp – ladder. Lane 2: *S aureus* ATCC BAA-1756. Lane 3: MRSA BAA-42. Lane 4: *S. aureus* ATCC BAA-39. Lanes 5: canine clinical MRSA. Lane 6: canine clinical MRSP. Lane 7: canine clinical MRSC. Lane 8: *S. aureus* ATCC BAA-1688.

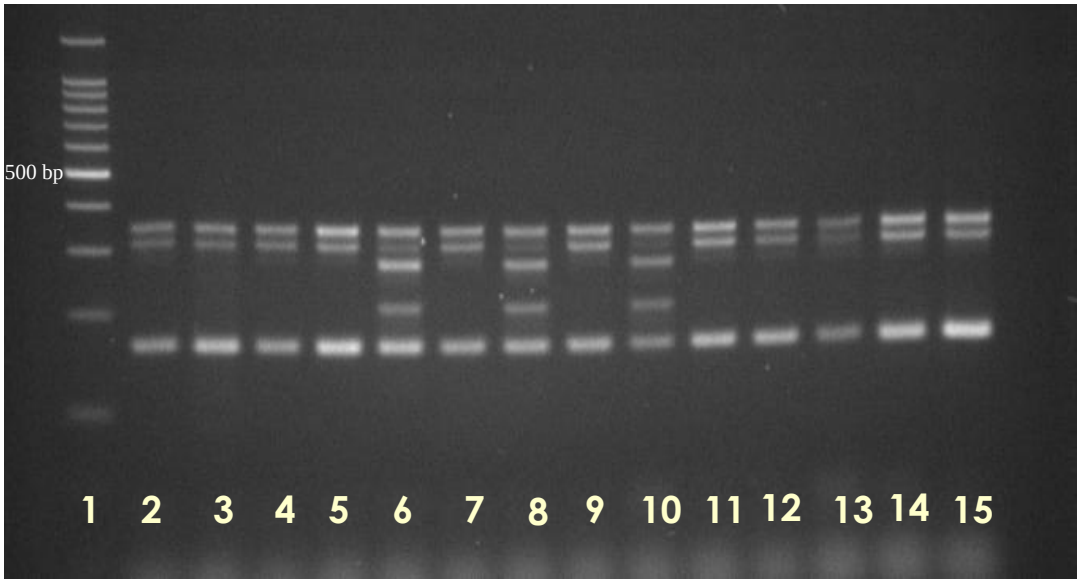


Figure 4 Agarose gel electrophoresis patterns of the Multiplex PCR products. Lane 1: DNA molecular size marker (1 Kb); lanes 2, 3, 4, 5, 7, 9, 11, 12, 13, 14 and 15, SCC*mec* gene type IV; lanes: 6, 8 and 10, SCC*mec* gene type II.

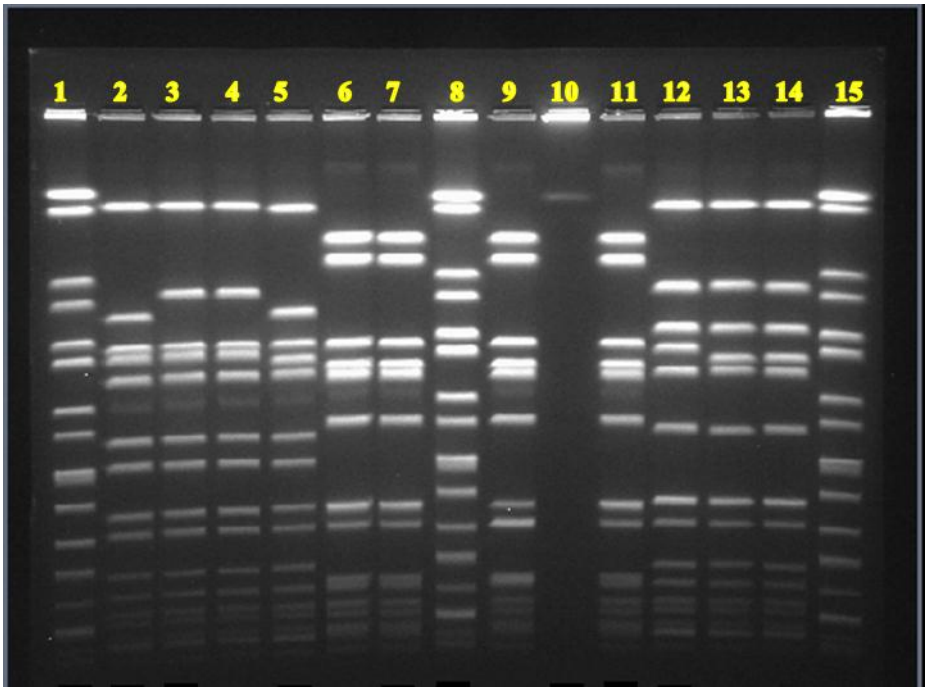
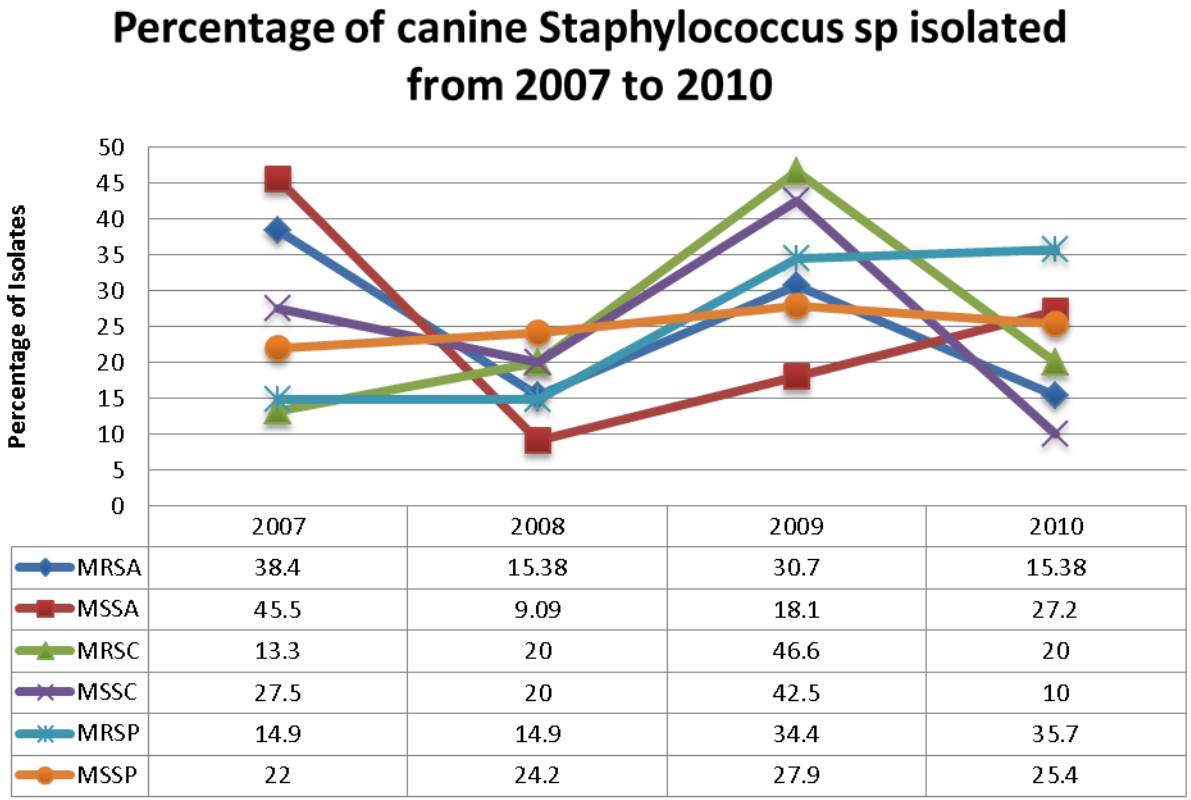


Figure 5 PFGE restriction patterns of isolates after SmaI digestion. Lanes 1, 8 and 15: *Salmonella* sp reference strain H9812. Lanes 2, 3, 4, 5: USA500; Lanes 6, 7, 9, 11: USA100; Lane 10: Non-cut by *SmaI*; 12, 13, and 14 non-classified as any USA clone.



	2007	2008	2009	2010
MRSA	38.4	15.38	30.7	15.38
MSSA	45.5	9.09	18.1	27.2
MRSC	13.3	20	46.6	20
MSSC	27.5	20	42.5	10
MRSP	14.9	14.9	34.4	35.7
MSSP	22	24.2	27.9	25.4

Figure 6 Frequency distribution of *Staphylococcus sp* isolated from canine clinical specimens positives for staphylococcal infection by year. *Staphylococcus* species were separated as Methicillin-Resistance (MR) and Methicillin-Susceptible (MS)

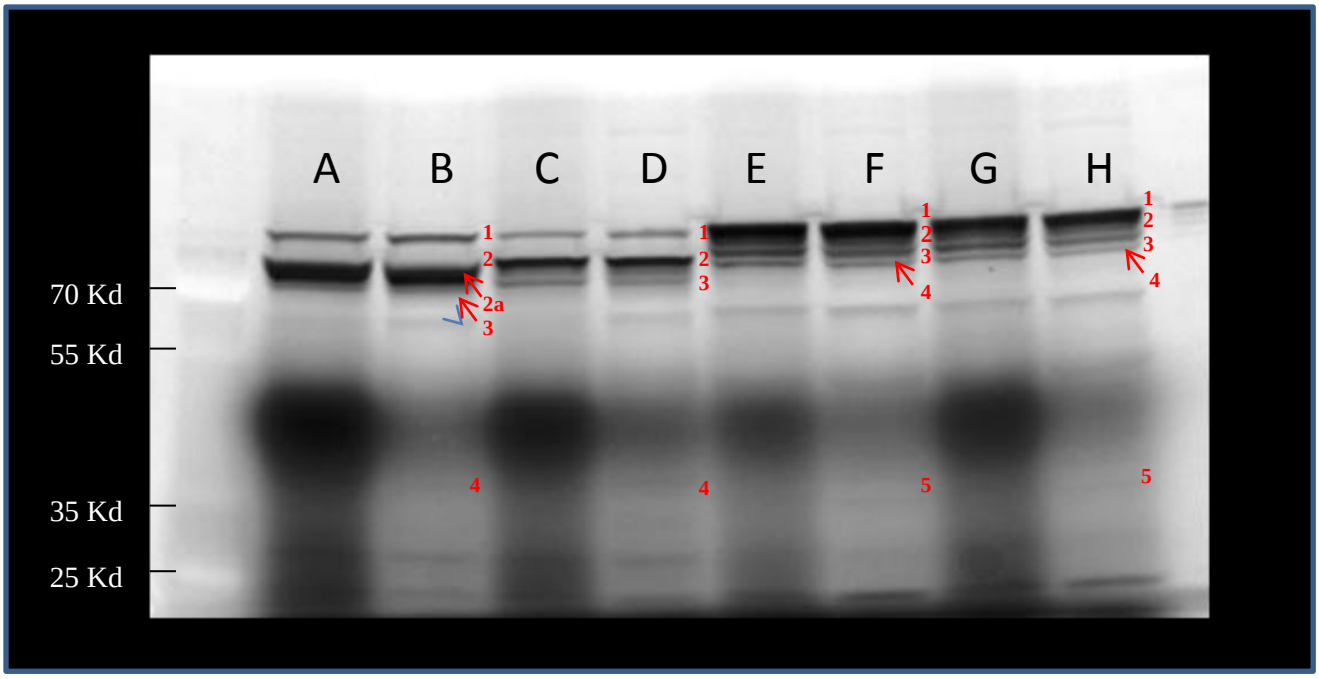


Figure 7 Binding of 10µM Bocillin FL to *Staphylococcus* sp. Lanes A and B: MRSA (RA-1). Designation of PBPs in red: PBP1, PBP 2, PBP2a, PBP3 and PBP4. Lanes C and D: MSSA (SA-1). Designation of PBPs in red: PBP1, PBP 2, PBP3 and PBP4 Lanes E and F: MRSP (RI-10). Lanes E and F: MSSI (SI-89). Designation of PBPs in red: PBP1, PBP 2, PBP3, PBP3, PBP4 and PBP5. Designation of PBPs in red: PBP1, PBP 2, PBP3, PBP4 and PBP5.

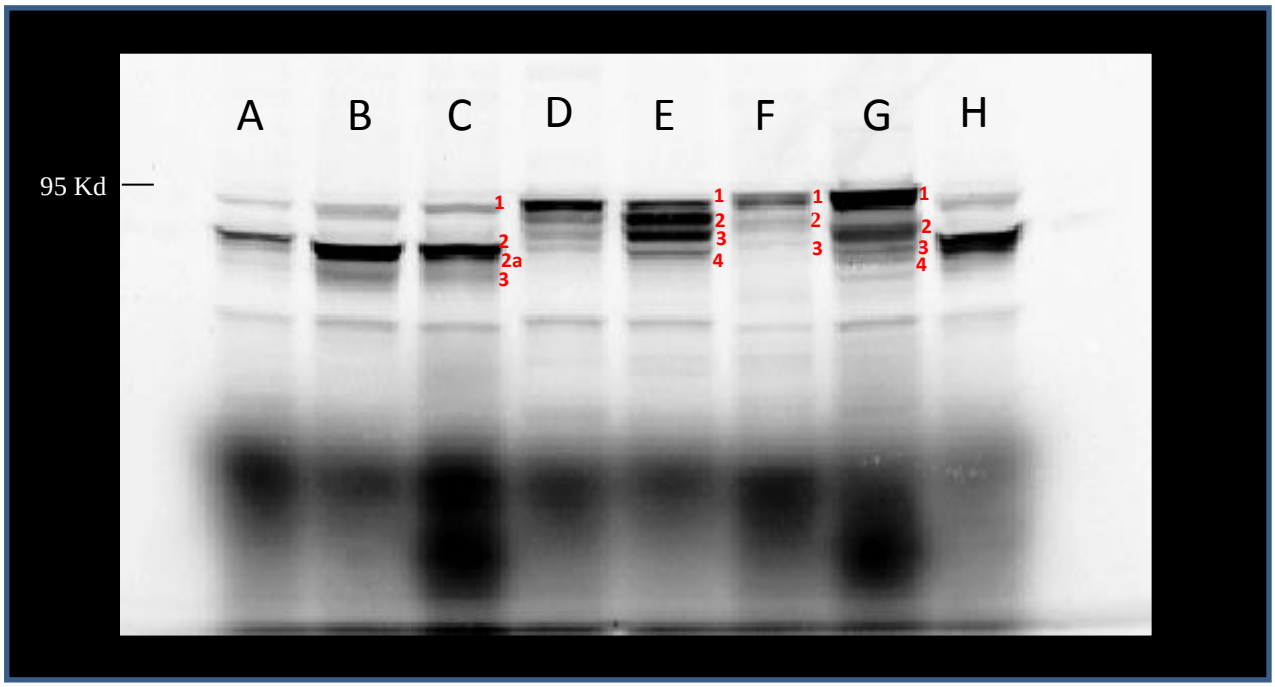


Figure 8 Binding of 10µM Bocillin FL to *Staphylococcus* sp. Lanes A: *S. aureus* ATCC 25923. Lane B: *S. aureus* ATCC 43300. Lane C and H: *S. aureus* ATCC 1756. Designation of PBPs in red: PBP1, PBP 2, PBP2a, PBP3 and PBP4. Lane D: MRSP (RI-10). Lane E: *S. pseudintermedius*-49444. Lane F: MRSC (RC-1). Lane G: *S. schleiferi* subsp *coagulans*-49545. Designation of PBPs in red: PBP1, PBP 2, PBP3 and PBP4

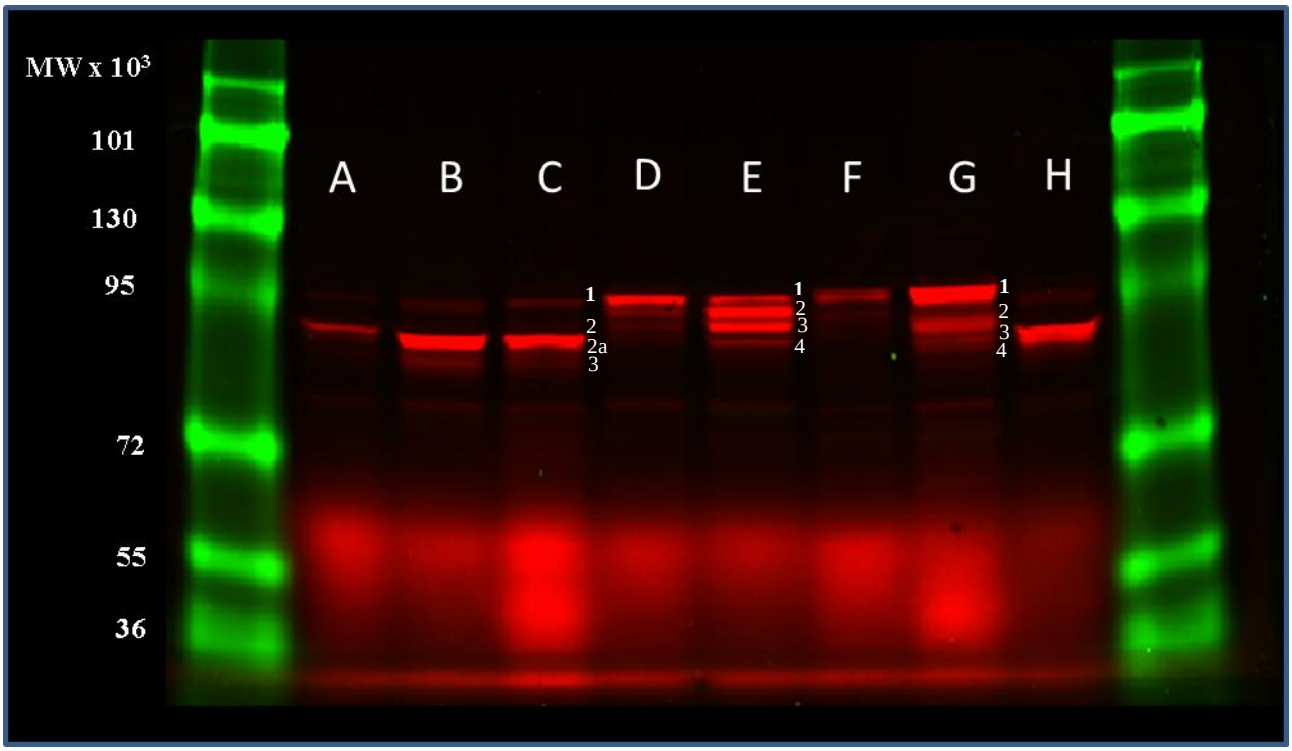


Figure 9 Binding of 10µM Bocillin FL to *Staphylococcus* sp Lanes A: *S. aureus* ATCC 25923. Lane B: *S. aureus* ATCC 43300. Lane C and H: *S. aureus* ATCC 1756. Designation of PBPs in red: PBP1, PBP 2, PBP2a, PBP3 and PBP4. Lane D: MRSP (RI-10). Lane E: *S. pseudintermedius*-49444. Lane F: MRSC (RC-1). Lane G: *S. schleiferi* subsp *coagulans*-49545. Designation of PBPs in red: PBP1, PBP 2, PBP3 and PBP4

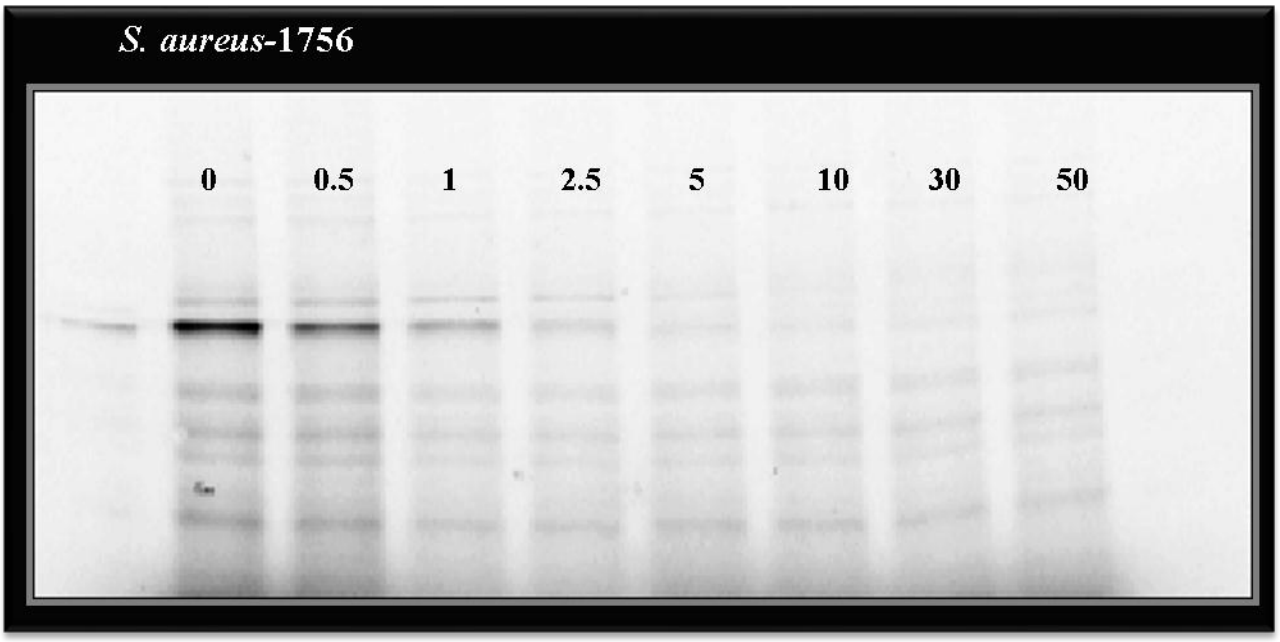


Figure 10 Competition assay for FOX binding to PBPs of *S. aureus*-1756, Bocillin FL added to a final concentration of 10μg/ml. The concentrations of FOX indicated: 0, 0.5, 1, 2.5, 5, 10, 30 and 50 μg/ml.

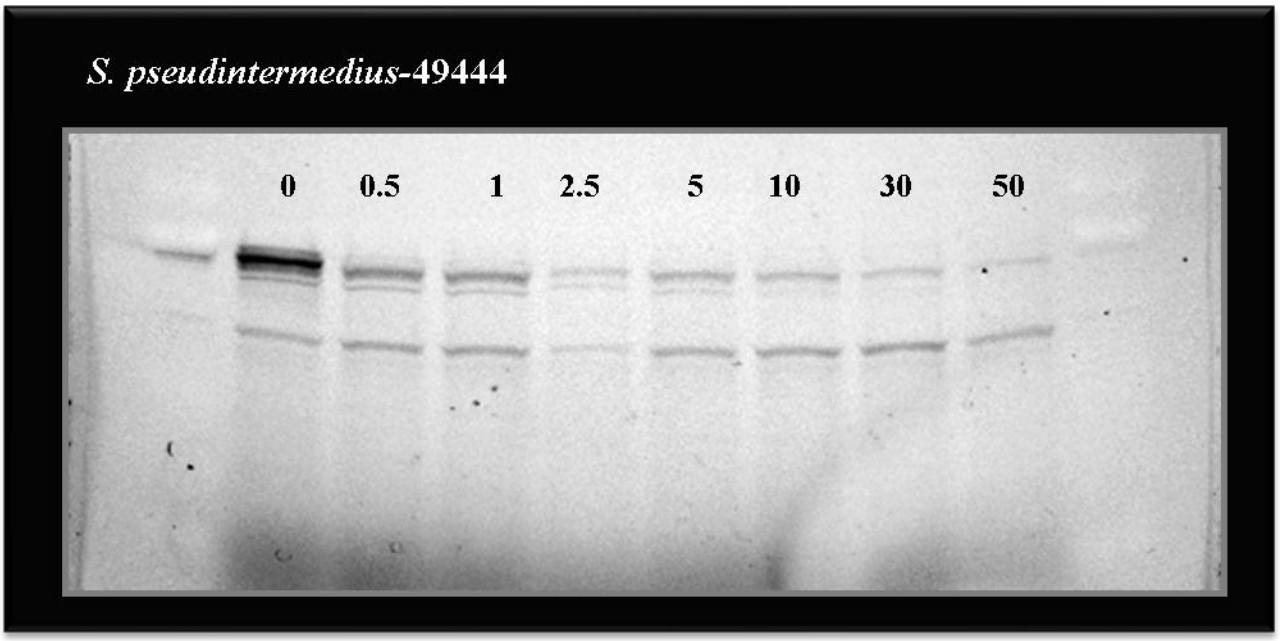


Figure 11 Competition assay for FOX binding to PBPs of *S. pseudintermedius*-49444, Bocillin FL added to a final concentration of 10μg/ml. The concentrations of FOX indicated: 0, 0.5, 1, 2.5, 5, 10, 30 and 50 μg/mL

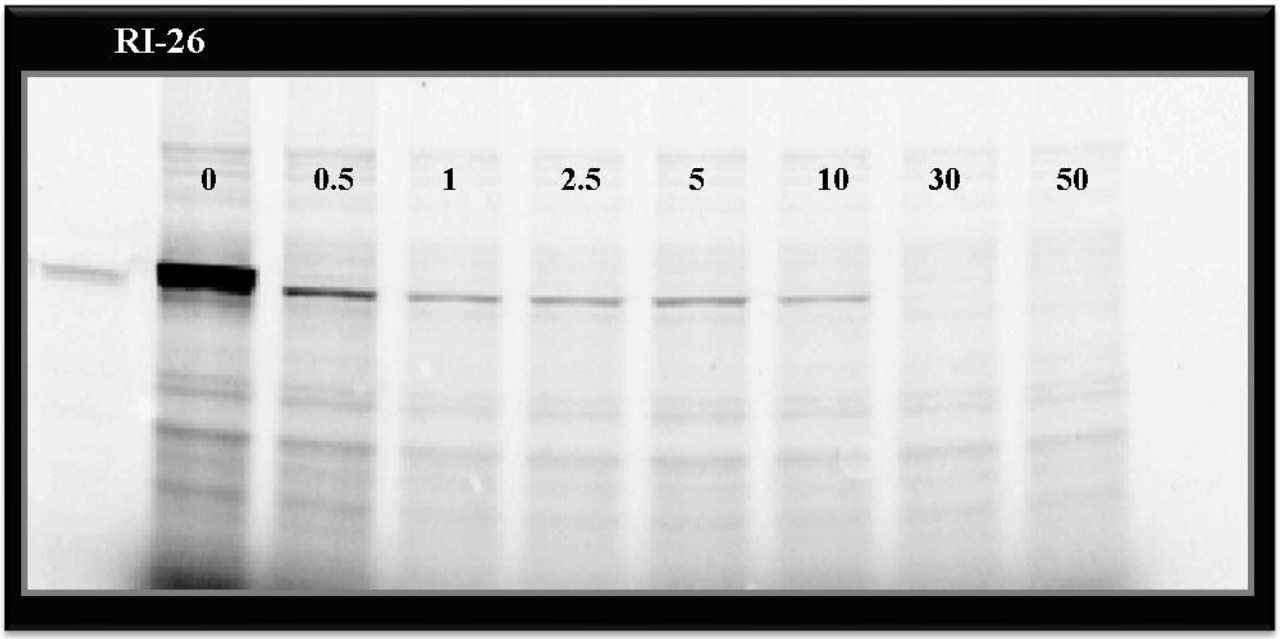


Figure 12 Competition assay for FOX binding to PBPs of RI-26, (clinical methicillin-resistant *S. pseudintermedius).* Bocillin FL added to a final concentration of 10μg/ml. The concentrations of FOX indicated: 0, 0.5, 1, 2.5, 5, 10, 30 and 50 μg/mL

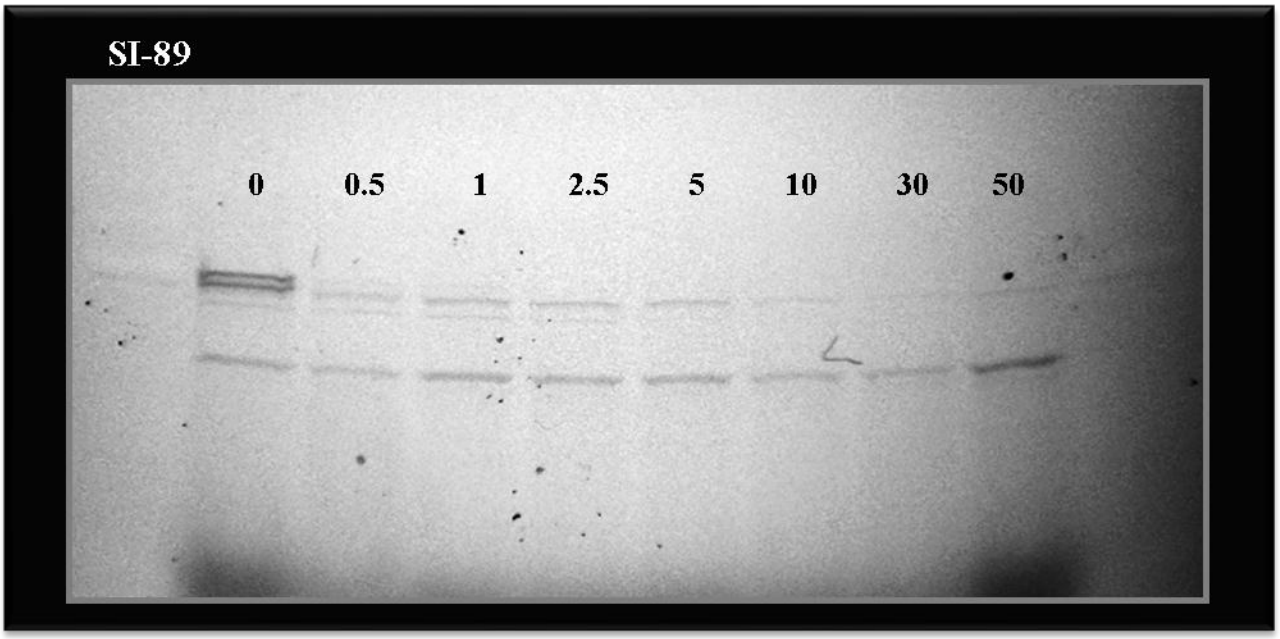


Figure 13 Competition assay for FOX binding to PBPs of SI-89, (clinical methicillin-susceptible *S. pseudintermedius*). Bocillin FL added to a final concentration of 10μg/ml. The concentrations of FOX indicated: 0, 0.5, 1, 2.5, 5, 10, 30 and 50 μg/mL

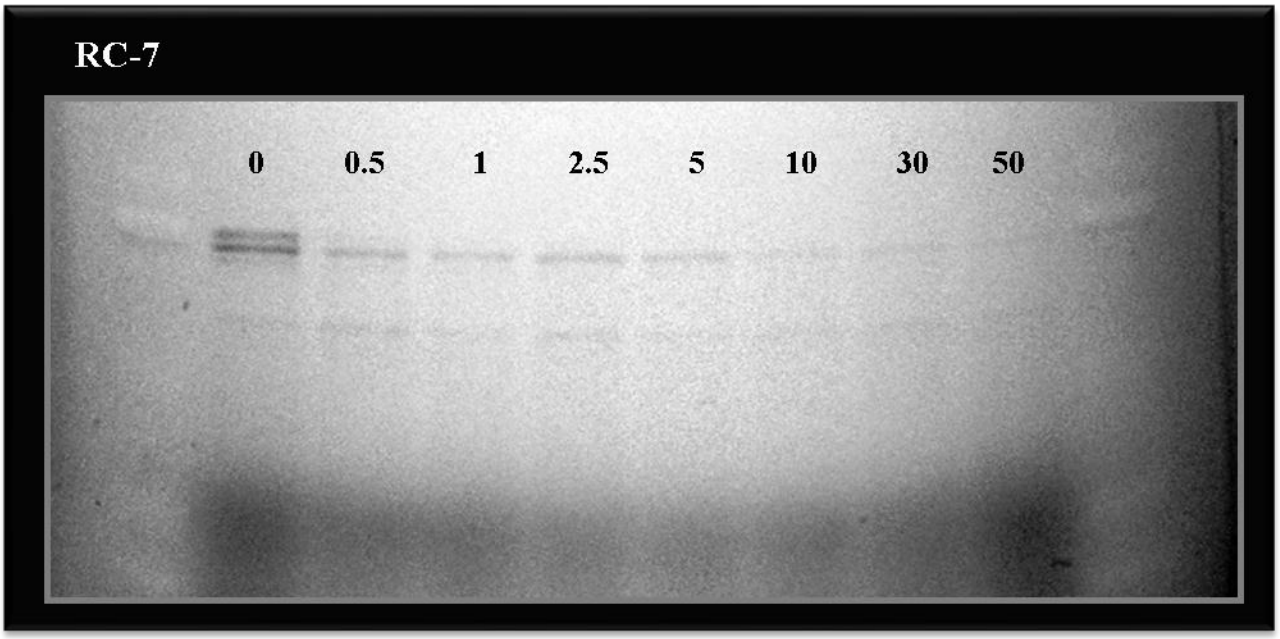


Figure 14 Competition assay for FOX binding to PBPs of RC-7, (clinical methicillin-resistant *S. schleiferi* subsp *coagulans*). Bocillin FL added to a final concentration of 10μg/ml. The concentrations of FOX indicated: 0, 0.5, 1, 2.5, 5, 10, 30 and 50 μg/mL

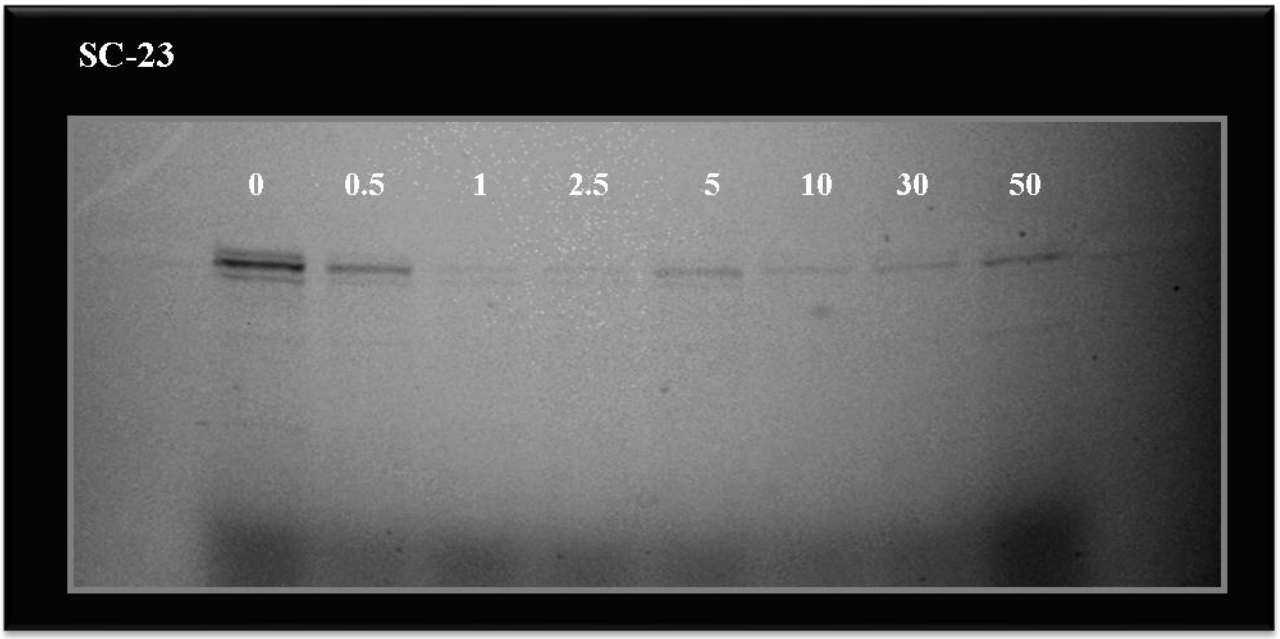


Figure 15 Competition assay for FOX binding to PBPs of SC-23, (clinical methicillin-susceptible *S. schleiferi* subsp *coagulans*). Bocillin FL added to a final concentration of 10µg/ml. The concentrations of FOX indicated: 0, 0.5, 1, 2.5, 5, 10, 30 and 50 µg/mL