AN EPIDEMIOLOGIC STUDY OF METHICILLIN-RESISTANT STAPHYLOCOCCI IN COMPANION ANIMALS

by

CHRISTINA L STILL

(Under the Direction of Susan Sanchez)

ABSTRACT

Increases in antimicrobial resistance among Staphylococcus genus is of growing concern in both human and veterinary medicine, principally because of the recent emergence of community-acquired methicillin-resistant *S. aureus* (MRSA). Veterinary medicine has seen a parallel increase in the number of cases of methicillin-resistant Staphylococci in companion animals. This is the most complete epidemiologic study comparing the genetic relatedness and characterization of human and companion animal MRSA isolates in the same study. It demonstrated that both companion animals and people may be infected by the same strains of MRSA and that a zoonotic potential exists. However, our study also revealed that some MRSA strains may be better suited to cause disease in animal hosts. To our knowledge, this is also the first report of these SCC*mec* type VII and its variant elements in *S. aureus*, *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans*, and *S. schleiferi* subsp. *schleiferi* isolated in the United States.

INDEX WORDS: *Staphylococcous* spp., MRSA, MRSP, companion animals, SCC*mec* VII, zoonosis

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DEDICATION

I dedicate this thesis to my husband Carter and my parents Doug and Patti Smith, who have always shown me tremendous love and support throughout my life. Thank you for always being there for me.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Phylogeny of the Genus Staphylococcus

The genus *Staphylococcus* belongs to the gram-positive bacterial family *Staphylococcaceae.* This family also includes the lesser known genera *Gemelia, Jeotgalicoccus, Macrococcus,* and *Salinococcus* and its close phylogenetic relatives include the families *Bacillaceae* and *Listeriaceae* (Todar 2008).

The Scottish surgeon Alexander Ogston is credited with first identifying *Staphylococcus aureus* in 1880 from the infected tissues of humans. He had postulated that abscess formation was caused by a microorganism. When viewing pus samples under a microscope, he observed clusters or bunches of cocci. He named the organism *Staphylococcus aureus*, which comes from the Greek: the word "staphyle," means "bunch of grapes," and cocci is derived from "kokkos," or berry, indicating the organism's spherical shape. "Aureus," meaning golden, was chosen for the species name because most *S. aureus* isolates produce golden-colored colonies on blood agar plates.

Staphylococci members can be distinguished as gram-positive, spherical cells approximately one micron in diameter, which form clusters and pairs. The organisms in this genus are nonmotile, nonsporeforming, facultative anaerobes. Staphylococci are also distinguishable by their catalase activity and lack of oxidase activity. Over forty species of staphylococci have been identified and are divided into two main groups due to their coagulase activity: into coagulase negative and coagulase positive (Euzéby 2008). Coagulase is the ability

to convert fibrinogen to fibrin, causing blood to clot. There are many more coagulase-negative species and this group is best represented and recognized for the member *Staphylococcus epidermidis.* The coagulase-positive Staphylococci include the following eight species: *S. aureus, S. intermedius, S. pseudintermedius, S. delphini* Group A, *S. delphini* Group B, *S. schleiferi* subspecies *coagulans*, *S. hyicus, S. lutrae* (Sasaki, Kikuchi et al. 2007).

The most medically-important coagulase positive species to humans is *S. aureus*. Nearly all strains of *S. aureus* are coagulase positive and β-hemolytic. *S. aureus* are hardy bacteria, which can grow at a wide temperature range of 15 to 45°C and at salt concentrations as high as fifteen percent (Todar 2008). This adaptability to their environment allows *S. aureus* to easily colonize the mammalian nasal passages, skin, oral cavity and gastrointestinal tract.

The *Staphylococcus intermedius* Group includes four closely-related species: *S. intermedius, S. pseudintermedius, S. delphini* Group A and *S. delphini* Group B, which are medically important in a variety of animal species (Sasaki, Kikuchi et al. 2007). Once all thought to be the single species *S. intermedius* advancements in molecular phylogenetic analysis have classified them as four distinct species. Phenotypically, there are no obvious differences in the biochemical reactions between *S. pseudintermedius, S. delphini* Group A and *S. delphini* Group B. These three species differ phenotypically from *S. intermedius,* however, by not producing arginine dihydrogenase and by producing acid from both β-gentiobiose aerobically and D-mannitol anaerobically (Sasaki, Kikuchi et al. 2007). *S. delphini* Group A has been noted as prominent within not only the dolphin population, but also in domestic pigeons, horses, and mink (Sasaki, Kikuchi et al. 2007). This is in contrast to *S. delphini* Group B which tends to infect more horses and a few wild pigeons. *S. intermedius*, first discovered in 1976 was once thought to be the primary cause of canine pyoderma. Molecular evidence now indicates that it primarily

infects wild pigeons. The actual leading cause of canine pyoderma has recently been elucidated as *S. pseudintermedius*. This organism tends to infect dogs and only rarely cats and humans (Sasaki, Kikuchi et al. 2007).

S. schleiferi subspecies *coagulans* is another medically-important coagulase-positive *Staphylococcus* for animals. It has recently been found infecting dogs with otitis externa and pyoderma (Igimi, Takahashi et al. 1990; Bes, Guerin-Faublee et al. 2002). Phenotypically, this species is quite similar to *S. intermedius* but can be differentiated on the basis of its acetoin production and acid production from trehalose (Yamashita, Shimizu et al. 2005).

Staphylococcus aureus Epidemiology and Disease

S. aureus is an often found as a part of the normal microflora, colonizing the skin and mucous membranes of a variety of mammalian species and birds. Approximately twenty percent of the human population carries *S. aureus* asymptomatically (Kluytmans, van Belkum et al. 1997). Some people are persistent carriers, being colonized by the organism nearly all of their life. Others are transient carriers, only being colonized by the organism briefly and temporarily in their life. A final group of people are categorized as non-carriers who are never colonized. *S. aureus* typically is known for only causing opportunistic infections. *S. aureus* is only considered pathogenic when a breach in the host's skin or mucous membrane has occurred. Individuals colonized by *S. aureus* do, however, have an increased risk for developing disease should their immune system be compromised by diabetes, AIDS, intravenous drug use, and surgery (Holden 2008). *S. aureus* can be transmitted, particularly from infected or heavily-colonized individuals, by direct person-to-person contact, fomites (inanimate objects such as door knobs and linens), and environmental sources (Holden 2008).

S. aureus has been considered a major cause of human disease, particularly in the hospital setting, for many years. It is a leading cause of pneumonia, surgical wound infections, and nosocomial blood infections. The main forms of disease are found in humans: superficial, invasive, and toxin mediated. Superficial infections involve skin and soft tissue infections such as pimples, impetigo, boils, carbuncles, scalded skin syndrome and abscesses. The invasive diseases include pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), and septicemia. Toxin-mediated diseases most often include TSS and food poisoning.

S. aureus has also been increasingly recognized as an important disease in many animal species. Most reports of these diseases focus on the avian, bovine, canine, equine, feline, and porcine species. *S. aureus* infection in animals was first reported in mastitis, inflammation of the parenchyma of the mammary gland (the breast in primates and the udder in other mammals) in sheep and cattle in the 1880s. Other diseases caused by *S. aureus* in animal infections include skin and soft tissue infections, botryomycosis (a chronic granulomatous bacterial skin infection), arthritis, pyoderma, urinary tract infections, bumble foot and discospondylitis (an infection of the invertebral disc or associated vertebrae) (Jonson 1993).

Staphylococcus aureus Virulence Factors

S. aureus is an important pathogen because it can cause disease by several different mechanisms. These include its ability to effectively colonize the host, invade to cause disease, produce a variety of toxins, and its antibiotic resistance. Colonization of the host usually begins in the anterior nares and subsequently disperses across the skin. *S. aureus* typically colonizes the mammalian nasal passages, skin, oral cavity, and gastrointestinal tract. *S. aureus* can colonize both humans and animals without causing disease. However, when the host's immune system is breached, *S. aureus* possesses a variety of virulence factors that aid in its invasion of the host and

ability to cause disease. The *Staphylococcus* genome consists of an approximately 2800kb circular chromosome with prophages, plasmids and transposons (Lowy 1998). Genes important for virulence can be found on the chromosome or on extrachromosomal elements, which are capable of being horizontally transferred between bacteria. Some of these important virulence factors include the bacterial cell wall, the capsule, surface proteins, toxins, and enzymes.

The cell wall, composed primarily of peptidoglycan and lipoteichoic acids, may contain endotoxin-like activity, which stimulates the release of cytokines by macrophages, activates complement, and aids in platelet aggregation (Lowy 1998). Most staphylococci produce a capsule, composed of antiphagocytic polysaccharides, which help to protect the bacteria during invasive infections (Lowy 1998). *S. aureus* produces a wide array of surface proteins, many of which play an important role in attachment and subsequent colonization of the host by the bacterium. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are a group of such surface proteins (Lowy 1998). The gene *spa* encodes Protein A, another of these surface proteins, which has antiphagocytic properties based on its ability to bind the F_C portion of immunoglobulins (Lowy 1998). The polymorphic X region of *spa* consists of a variable number of 24-base-pair repeats. The diversity in this region seems to arise from deletion and duplication of the repetitive units as well as by point mutations. Sequencing of this region is a rapid and consistent inter-laboratory method for investigation of potential outbreak situations and genetic relatedness of isolates.

Staphylococci can produce numerous toxins that act by many different mechanisms. Cytotoxins cause pore formation and induce proinflammatory responses from host cells, which may contribute to sepsis (Lowy 1998). Pyrogenic-toxin superantigens (PTSAgs) function as superantigens by binding to the major histocompatibility complex (MHC) II (Lowy 1998). This

causes widespread T cell activation, proliferation and cytokine release resulting in a "cytokine storm" and multi-organ-system disease. This group of toxins includes the staphylococcal enterotoxins (SEs) SEA-SEE (MJ Betley 1990), SEG-SER (Jarraud, Mougel et al. 2002; Omoe, Hu et al. 2003), and SEU (Letertre, Perelle et al. 2003), as well as toxic shock syndrome toxin (TSST-1) (Bergdoll, Crass et al. 1981). The SEs are responsible for staphylococcal food poisoning, however it is not yet known how these enterotoxins affect all host species. Toxic shock syndrome is a rare, but potentially fatal disease. It usually begins with a rash and symptoms progress to a high fever, decreased blood pressure, malaise and confusion. If not halted rapidly, this disease can progress to stupor, coma, and multi-organ failure in the patient. *S. aureus* also produce the exfoliative toxins *eta* and *etb,* which cause skin erythema and separation, as observed in staphylococcal scalded-skin syndrome. Panton-Valentine leukocidin (PVL) is a two-component leukocytolytic toxin, which is associated with necrotic skin and mucosa lesions. The two components form a ring with a central pore in host cells, causing the cell to leak and this acts as a superantigen to the body, broadly activating the host's immune system (Deresinski 2005).

Staphylococci also produce a number of enzymes, such as protease, lipase, and hyaluronidase that serve a role in virulence by destroying host tissue. Protease destroys proteins, lipase destroys fats, and hyaluronidase destroys connective tissue. Additionally, in coagulase-positive staphylococci, coagulase reacts with prothrombin in the blood, causing clotting by converting fibrinogen to fibrin (Lowy 1998). The fibrin coats the surface of the bacterium and may aid in the resistance of staphylococci to phagocytosis during invasive infections.

As the use of antimicrobials in recent years has increased, these bacteria have acquired resistance mechanisms to virtually all classes of available antibiotics (Holden 2008). Resistance

of particularly *S. aureus* to so many classes of antibiotics has led to increased treatment costs, longer hospital stays, and ineffectual treatment options for many patients and physicians.

The Emergence Methicillin-Resistant Staphylococcus aureus (MRSA)

The beta-lactam antibiotic penicillin was first discovered by Alexander Fleming in 1928 and gained significance in the Second World War. Currently, approximately 90% of human and animal *S. aureus* isolates are resistant to penicillin (Lloyd 1996; Cuevas O 2004). With the rise of penicillin-resistant *S. aureus* strains, production of other beta-lactams such as ampicillin, methicillin, oxacillin, and cephalosporins increased during the 1950s and 1960s. Since that time, numerous other classes of antibiotics have been marketed and utilized in the battle against *S. aureus*, but the bacteria are continually evolving, adapting, and acquiring resistance to new "miracle" drugs as they circulate through the population. Because the survival of bacteria critically depends upon their ability to adapt to ever-changing environments and to either avoid or overcome the effects of antimicrobials, the evolutionary response time for adapting and becoming resistant to antimicrobials is extremely short, often occurring in less than 3 years (Medeiros 1997). This rapid response time is likely due to the fact that most commercially available antibiotics are developed from naturally-produced chemicals by microbes (Barlow 2009). The occurrence of natural antimicrobials indicates that there was already selective pressure on microbes to develop resistance mechanisms before the development and widespread use of antimicrobials and their derivatives for medicine (Barlow 2009). This has allowed through horizontal gene transfer the rapid ability of microbes such as *S. aureus* to become resistant to a host of different antimicrobials.

There are four primary mechanisms by which bacteria exhibit resistance to antibiotics. These include drug inactivation or modification, the alteration of a target site, the alteration of a

metabolic pathway, and reduced drug accumulation (often through the use of efflux pumps). *S. aureus* uses all of these mechanisms to exhibit resistance to a variety of antibiotics (Holden 2008).

Penicillin binding proteins (PBPs) are enzymes in the bacterium that catalyze the transglycosylation and transpeptidation reactions responsible for the formation of the glycosidic and peptide bonds of peptidoglycan in the cell wall (Holden 2008). Beta-lactam antibiotics inhibit the transpeptidation reaction by acting as substrate homologues that bind almost irreversibly to the transpeptidase active site of PBPs (Holden 2008). This results in a loss of integrity for the cell wall. As such, penicillin was initially quite effective at treating *S. aureus* infections, which until that time had a 90% mortality rate. However, by the 1950s resistance to the antibiotic was noted (Beigelman PM 1950). Penicillin-resistant *S. aureus* carries the gene *blaZ*, which encodes for the expression of beta-lactamase enzymes. Beta-lactamases hydrolyze and thus inactivate beta-lactam antibiotics (Holden 2008).

In 1959, methicillin was introduced commercially for the treatment of *S. aureus* infections in humans. Within two years of its introduction, the first methicillin-resistant *S. aureus* (MRSA) isolate was reported (Eriksen 1961). The mechanism for resistance by *S. aureus* to methicillin and subsequently to beta-lactam antibiotics is through the alteration of the antibiotic's target site, PBP2. These MRSA strains have an altered PBP called PBP2a (also referred to as PBP2'), which has a decreased binding affinity for beta-lactams, resulting in clinical resistance (Holden 2008). The expression of PBP2a is encoded by *mec*A. It is located on a genetic element called the staphylococcal cassette chromosome (SCC*mec*), which is thought to be transferable among staphylococci.

The SCC*mec* is a mobile genetic element that integrates into the staphylococcal chromosome in an open reading frame (ORF) of unknown function, designated *orfX* (Ito, Katayama et al. 1999). SCC*mec* is neither a transposon nor a bacteriophage in its entirety, but does contain segments of each within the cassette, often in the functional form of antibiotic-resistance genes such as with the transposon Tn554 and the plasmid pUB110 (Ito, Katayama et al. 1999). The *mec*A gene is adjacent to a set of regulatory genes, *mec*I and *mec*R1, which together form the *mec*A gene complex (Hiramatsu, Asada et al. 1992). Also carried on SCC*mec* are the cassette chromosome recombinase (*ccr*) genes responsible for the movement of SCC*mec* (Ito, Katayama et al. 2001). SCC*mec* type is characterized by the *mec*A gene complex and *ccr* genes that the cassette carries. To date, seven different SCC*mec* types and several variants have been described worldwide (Ito, Katayama et al. 2001; Okuma, Iwakawa et al. 2002; Ito, Ma et al. 2004; Shore, Rossney et al. 2005; Oliveira, Milheirico et al. 2006; Higuchi, Takano et al. 2008). These cassettes vary in the combination of *mec*A complex and *ccr* genes, in addition to their size (varying from approximately 21kbp to 55kbp), and accompanying genes, such as those playing a role in antibiotic resistance. In the United States, SCC*mec* types I-IV and several of their variants have frequently been observed. Recently, reports of SCC*mec* V have also been noted in MRSA in pigs and pig farmers in the United States (Smith, Male et al. 2008). The *mec*A gene complex is widely distributed among many coagulase-positive and coagulase-negative staphylococcal species (Archer, Niemeyer et al. 1994), which has led to the speculation that SCC*mec* is likely transmissible among different staphylococcal species (Archer, Thanassi et al. 1996). However, the progenitor of *mec*A has yet to be determined and the mechanism for the import of SCC*mec* into the chromosome of the bacterial host remains to be elucidated.

The close relationship of MRSA strains to multiple methicillin-sensitive *S. aureus* (MSSA) strains suggests that there have been multiple origins of MRSA. It has been estimated that SCC*mec* has entered MSSA strains twenty times in total, indicating that the horizontal transfer of SCC*mec* to MSSA is very infrequent (Robinson and Enright 2003). Therefore, increases seen in MRSA are likely due primarily to a large clonal expansion of these few MRSA strains.

The Epidemiology of MRSA

Since the emergence of MRSA infections, the disease has primarily been considered strictly nosocomial (hospital-acquired) in nature. These hospital-acquired MRSA (HA-MRSA) infections occur in patients who have been hospitalized for greater than 72 hours prior to the staphylococcal infection. The high degree of selective pressure generated from a hospital environment where antibiotic use is extensive and there exists a large population of people within close proximity of one another who are immunocompromised by virtue of drug therapies, surgeries, and severe illnesses likely led to these infections. Poor infection control practices within the hospital as well as the resiliency of *S. aureus* in the environment allowed for the relatively-rapid spread of the bacterial strains through a number of hospitals across the globe in outbreak situations during the 1960s and 1970s (Boyce 1994). Generally these outbreaks remained limited to the hospital and often to only a specific ward within the hospital (Boyce 1994). The MRSA strains involved in these early nosocomial infections were typically characterized as SCC*mec* types I-III, which are the largest of the seven SCC*mec* cassettes because they tend (with the exception of SCC*mec* I) to encode for more antibiotic resistance genes than do the other types (Grundmann, Aires-de-Sousa et al. 2006). These additional genes

provide a selective advantage for these MRSA strains in the hospital environment where the use of antibiotics is much higher.

In 1989, community-acquired MRSA (CA-MRSA) emerged in Australia and has since gained significant interest globally as an important re-emergent pathogen in both the traditional hospital environment, but also in the community at large (Udo, Pearman et al. 1993). MRSA infections in people without prior hospitalization, surgery or antibiotic treatment within the past 12 months are considered community-acquired. These CA-MRSA strains show a marked genetic diversity, as evidenced by the number of genetic and phenotypic variants, which has never been seen in hospital-acquired (HA-MRSA) strains (Daum, Ito et al. 2002; Robinson and Enright 2003). The CA-MRSA strains are typically characterized as containing the SCC*mec* IV, V or VII, with a small population in Southeast Asia that harbors SCC*mec* VII (Grundmann, Aires-de-Sousa et al. 2006; Higuchi, Takano et al. 2008). These SCC*mec* cassettes are smaller, containing only the antibiotic-resistance gene *mec*A, and are thus considered more mobile and transferable among staphylococci (Grundmann, Aires-de-Sousa et al. 2006). Conventionally, CA-MRSA is considered more susceptible to non-beta-lactam antibiotics than are HA-MRSA strains. However, recent studies reveal that this is not always the case and CA-MRSA is becoming increasing resistant to antibiotics such as tetracyclines and clindamycin (Szumowski, Cohen et al. 2007). CA-MRSA isolates are often associated with the virulence factor Panton-Valentine leukocidin (PVL) (Dufour, Gillet et al. 2002). CA-MRSA has been isolated from children and adults with skin and soft tissue infections, bacteremia, diabetes, septic arthritis, necrotizing fasciitis, and necrotizing pneumonia. It has been reported in particularly-high number among groups with high-intensity physical contact, such as indigenous populations, homeless people, men who have sex with men, jailed inmates, military recruits, children in daycare, and athletes

(Grundmann, Aires-de-Sousa et al. 2006). Hospital outbreaks of traditionally CA-MRSA strains have also been reported, blurring the boundary of what truly defines a HA- versus CA- MRSA strain (Fridkin, Hill et al. 2002).

Asymptomatic carriage of methicillin-resistant *S. aureus* (MRSA), by segments of the population, once unheard of, has now been reported at low rates (less than 2%) by multiple studies across the globe since the emergence of CA-MRSA (Harbarth, Francois et al. 2005; Vlack, Cox et al. 2006; Lo, Lin et al. 2007; Gorwitz, Kruszon-Moran et al. 2008). Coinciding with this increase in asymptomatic carriage of MRSA and MRSA infections among humans is an increase in the number of methicillin-resistant staphylococcal species isolated from companion animals such as dogs, horses, and cats (Pak, Han et al. 1999; Tomlin, Pead et al. 1999; Duquette and Nuttall 2004; Weese 2005). In the United States in people approximately 80% of community-onset skin and soft-tissue infections caused by *S. aureus* are methicillin-resistant (Moran, Krishnadasan et al. 2006).

Due to the potential spill-over of antibiotic resistance between human and animals populations, which so often share the same treatment options, many have speculated that SCC*mec* may be easily transferred between human and animal staphylococcal species because of the frequency and extent of close, direct contact between humans and pets (Duquette and Nuttall 2004; Weese 2005; Malik, Coombs et al. 2006). There have been several reports of colonized pets serving as reservoirs of infection for humans and animals in close contact, as well as a few reports of animal-to-human and human-to-animal transmission of MRSA(Loeffler, Boag et al. 2005; O'Mahony, Abbott et al. 2005; Sasaki, Kikuchi et al. 2007). However, most of what is known of MRSA epidemiology among companion animal species (cats, dogs, domestic birds, and horses) is primarily limited to the screening healthy of animals for determining MRSA

carriage rates within specific geographic regions, which yields very low rates for these animal species. Much still needs to be done in this area of research, especially to gain better insight into how the relationship between humans and companion animals affects the epidemiology of MRSA and other methicillin-resistant staphylococcal infections and carriage at large.

Animals and Staphylococcus spp.

A number of pathogenic staphylococcal species cause disease in a variety hosts and can cause several infections of great economic importance. The primary pathogenic staphylococci in animals include *S. aureus, S. intermedius* Group, *S. schleiferi* subsp. *coagulans, S. epidermidis, S. hyicus,* and *S. scuiri* (Holden 2008).

S. aureus colonizes and infects a wide variety of animals, including mammals, reptiles and birds. It is known to cause mastitis in ruminants (cows, sheep and goats), as well as in rabbits. Additionally, *S. aureus* has been implicated in skin and urinary tract infections of the horse, and "Bumble foot" (foot abscesses), chondronecrosis (necrosis of the laryngeal cartilaginous framework) and septic arthritis in poultry (Holden 2008). The most economically important of these infections are the intra-mammary infections of lactating ruminants, which often leads to chronic mastitis. The infections are quite difficult to treat with antibiotic therapy, so culling of animals leading to substantial production losses is often used for control of the infection and to stem the spread of disease (Holden 2008). *S. aureus* is also an important bacterial pathogens of broiler chickens (Holden 2008).

Studies have demonstrated that although some *S. aureus* strains are capable of infecting different host species, there does appear to some host-adaptation, particularly in bovines and ovines (van Leeuwen, Melles et al. 2005). These animals tend to be infected by *S. aureus* strains which are not observed in other animal hosts. However, cross-infection has been noted

previously between humans and domestic animals, leading to the notion that these animals may be reservoirs for the transmission of *S. aureus* to humans (Simoons-Smit, Savelkoul et al. 2000). Studies have also shown that *S. aureus* isolates in humans and animals often share some of the same virulence determinants, such as genes for superantigens, exfoliative toxins, enterotoxins, and MSCRAMMS (Hazariwala, Sanders et al. 2002; Holden 2008). However, there are several virulence genes located on staphylococcal pathogenicity islands which demonstrate some host dependence, as in bovine MRSA specific pathogenicity islands (Fitzgerald, Monday et al. 2001).

The *Staphylococcus intermedius* Group includes *S. intermedius, S. pseudintermedius, S. delphini* Group A and *S. delphini* Group B, which are medically important in a variety of animal species (Sasaki, Kikuchi et al. 2007). *S. delphini* has been implicated in pyoderma cases in horses, mink, cows, dolphins and pigeons. *S. intermedius* primarily infects wild pigeons and *S. pseudintermedius* is a commensal and the leading causes of pyoderma in dogs. This organism tends to infect dogs and only rarely cats and humans (Sasaki, Kikuchi et al. 2007). Of the *S. intermedius* Group, *S. pseudintermedius* is the best studied. It produces a wide array of virulence factors, such as coagulase, proteases, thermonuclease, toxins, and MSCRAMMS with structural and functional similarity to those found in *S. aureus. S. pseudintermedius* has also been shown capable of forming biofilms (Futagawa-Saito, Sugiyama et al. 2004).

S. schleiferi subspecies *coagulans*, as mentioned previously, is another medically important coagulase-positive *Staphylococcus* for animals. It has recently been found infecting dogs causing otitis externa and pyoderma (Igimi, Takahashi et al. 1990; Bes, Guerin-Faublee et al. 2002).

The coagulase-negative staphylococci of interest for their ability to cause disease in animals include *S. epidermidis, S. hyicus,* and *S. scuiri*. *S. epidermidis* is an opportunistic

pathogens in animals and humans with the ability to persist and multiply in a variety of environments. It has been increasingly reported in recent years in intra-mammary infections of ruminants, though these infections are typically milder and acute compared with those caused by *S. aureus* (Holden 2008). *S. hyicus* is associated with "Greasy Pig Disease" (exudative epidemitis, a dermatitis which oozes greasy fluid), bovine mastitis, and with dermatitis in horses and chickens (Holden 2008). Complications arise due to the production of exfoliative toxins which are involved in the pathogenesis of this disease (Holden 2008). *S. scuiri* can be isolated from a variety of animals in addition to humans, but are usually non-pathogenic to animals. A recent report, however, has identified this organism in a case of acute exudative epidermitis in piglets (Chen, Wang et al. 2007).

All of the staphylococcal species described above are capable of colonizing and/or infecting both humans and animals. Many of these infections can be zoonotic, passing from animals to humans and vice versa. This is particularly common in veterinary practices, farming and domestic situations in which humans live with companion animals such as dogs, cats and domestic birds. For this reason, it is important that we continue to gain a better understanding of the pathogenesis of these diseases and the transmission potential between host species.

The Epidemiology of Methicillin-Resistant Staphylococci in Animals

MRSA was first isolated from the milk of a cow and has since been identified in wide variety of species including dogs, cats, horses, pigs and chickens (Holden 2008). As we have witnessed an increase in asymptomatic carriage of MRSA and MRSA infections among humans, there has been a parallel increase in the number of methicillin-resistant staphylococcal species isolated from companion animals such as dogs, horses, and cats (Pak, Han et al. 1999; Tomlin, Pead et al. 1999; Duquette and Nuttall 2004; Weese 2005). There is the potential of spill-over of

antibiotic resistance between human and animal staphylococcal populations, which often share the same treatment options. Because these host populations often share the same selection pressure, it is likely that through horizontal gene transfer, resistance to antibiotics may be passed between staphylococcal species quickly and easily. This has led to the speculation that SCC*mec* may be easily transferred between human and animal staphylococcal species because of the frequency and extent of close, direct contact between humans and pets (Duquette and Nuttall 2004; Weese 2005; Malik, Coombs et al. 2006). There have been several reports of colonized pets serving as reservoirs of infection for humans and animals in close contact, as well as a few reports of animal-to-human and human-to-animal transmission of MRSA (Loeffler, Boag et al. 2005; O'Mahony, Abbott et al. 2005; Sasaki, Kikuchi et al. 2007). Most of what is known in MRSA epidemiology among companion animal species (cats, dogs, domestic birds, and horses) is primarily limited to the screening healthy of animals for determining MRSA carriage rates within specific geographic regions, which yields very low rates for these animal species (ranging from 0% to 1% Actual infections rates of MRSA, however, appear higher (Baptiste, Williams et al. 2005). Of particular economic importance is the increase of MRSA infections observed in food animals such as ruminants and pigs. These infections are often more difficult to treat and lead to mass culling of the animals to clear the infection, resulting in production losses (Holden 2008). Research still needs to be done to gain better insight into how the relationship between humans and companion animals affects the epidemiology of MRSA and other methicillin-resistant staphylococcal infections and carriage at large.

Methicillin-resistant *S. pseudintermedius* (MRSP), though of less economic importance than MRSA in animals, is still a concern. Reports have shown infections rates to as high as 16% in the United States (Jones, Kania et al. 2007). One study recently demonstrated that the MRSP

strains found infecting dogs can also be carried transiently by their owners, which raised the question of interspecies transmission and the occurrence of resistance-gene transfer between different staphylococcal species (Guardabassi, Schwarz et al. 2004). This could have a tremendous impact on humans should it be shown that MRSP carried transiently in humans can spread methicillin-resistance to organisms such as *S. aureus* and *S. epidermidis* which are more often carried asymptomatically. This would have the potential of increasing that person's risk of a methicillin-resistant staph infection.

In summary, these infections have zoonotic potential and because it is suspected that SCC*mec* can be transferred horizontally, it is important for every species (humans and animals alike) that we continue to gain a better understanding of the epidemiology of these diseases and their transmission potential between hosts.

One Health

A recent study by Jones et al. reports that we have witnessed the emergence of 335 infectious diseases between 1940 and 2004 (Jones, Patel et al. 2008). These diseases include newly-evolved pathogenic strains (as occurs with drug resistance), pathogens which have recently entered human populations (such as severe acute respiratory syndrome – SARS), and pathogens which have likely been present in humans for many years, but which have recently increased in incidence (for example, Lyme's disease). The majority (54.3%) of these diseases are bacterial or rickettsial, followed by viruses and prions, protozoa, fungi, and helminthes. The incidence after controlling for reporting changes for diseases was shown to have increased since 1940, reaching a maximum in the 1980s, likely due to the emergence of new diseases associated with the human immunodeficiency virus (HIV). The majority of the emerging infectious diseases (60.3%) were noted as zoonotic pathogens (pathogens having a non-human animal source). This

included diseases with a wildlife origin and those from domestic animals. The study suggests that zoonotic emerging infectious diseases represent an increasing threat to global health. These findings highlight the importance of furthering our understanding about the epidemiology of zoonotic diseases.

The One Health Initiative is a movement focused on merging the concepts of both human and veterinary medicine ("One Health Initiative"). The mission statement for this movement is as follows: "Recognizing that human and animal health and mental health (via the human-animal bond phenomenon) are inextricably linked, One Health seeks to promote, improve, and defend the health and well-being of all species by enhancing cooperation and collaboration between physicians, veterinarians, and other scientific health professionals and by promoting strengths in leadership and management to achieve these goals." The mission is to be achieved through joint educational efforts between human medical, veterinary medical schools, and schools of public health; joint communication efforts in journals, at conferences, and via allied health networks; joint efforts in clinical care through the assessment, treatment and prevention of cross-species disease transmission; joint cross-species disease surveillance and control efforts in public health; joint efforts in better understanding of cross-species disease transmission through comparative medicine research; joint efforts in the development and evaluation of new diagnostic methods, medicines and vaccines for the prevention and control of diseases across species and; and joint efforts to inform and educate political leaders and the public sector through accurate media publications. In time, this joint initiative will lead to advancing health care in the 21st century by accelerating biomedical research discoveries, enhancing public health efficiency, increasing the scientific knowledge base, and improving overall medical education and clinical care. This could

have a profound impact of society, giving the means to better protect and even save millions of lives.

The time for thinking that human and animals infections are distinctly separate is past. With the recent emergence of zoonotic diseases such as Nipah virus and SARS from wildlife sources, human medicine can no longer afford to ignore the potentially devastating effects of remaining unaware of disease outbreaks and developments in animals (1999; Berger, Drosten et al. 2004). The case for drug-resistant staphylococci is no different. A number of reports have shown that a variety of host species ranging from domestic dogs and horses to food sources such as pigs to zoo animals like elephants are capable of being infected by MRSA and spreading their infection to humans, or vice versa (Duquette and Nuttall 2004; van Duijkeren, Wolfhagen et al. 2004; Weese, Archambault et al. 2005; Smith, Male et al. 2008; 2009). Since the start of the 21st century, MRSA has been increasingly considered a zoonotic disease and the key to elucidating the past, present and future trends in MRSA disease progression and its epidemiology lie in the collaborative efforts of human and veterinary medical research.

Research Goal and Hypothesis

Studying both human and animal epidemiology of MRSA is becoming increasingly important to gain a better understanding of the transmissions occurring between host species and their epidemiological consequences. The long-term goal of our research is to identify mechanisms of spread of antibiotic resistance genes in zoonotic pathogens. Our objective is to determine if there is a link between the increase in MRSA in the human population and in pets. Our overall hypothesis is that the increase in CA-MRSA in people is driving an increase in MRSA in animals through reverse zoonosis. MRSA in pets in turn may make its way back through direct contact to ultimately cause disease in humans. Therefore, our short-term goal is to test the hypothesis that

the increased MRSA and methicillin-resistant staphylococci in pets is due to transmission of isolates from people to their pets. Our intent here is to further characterize MRSA and methicillin-resistant staphylococci isolates cultured in our State Veterinary Diagnostic Laboratory, and to determine if and how they are related to MRSA in human in Georgia and the United States.

CHAPTER 2

EPIDEMIOLOGY OF CLINICAL ISOLATES OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS IN COMPANION ANIMALS AND HUMANS FROM THE SAME GEOGRAPHIC REGION[1]

[1]Still, C., Dorea, F., Wheeler, B., Foppe, S., Patel, S., Jensen, M., and S. Sanchez. To be submitted to *Emerging Infectious Diseases*.

Abstract

The link between domestic animal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) and human MRSA infection remains unclear. We hypothesized if pets are a reservoir of infection for humans, then the population of MRSA strains found infecting companion animals will reflect the MRSA strains infecting the human population within the same geographic region. SCC*mec* typing, pulsed-field gel electrophoresis (PFGE), *spa* tying, antimicrobial resistance patterns, and toxin genotyping were used to characterize MRSA strains from humans and animals. We found that most human isolates were identical to the common circulating strain USA300, while animal isolates were more diverse with respect to SCC*mec* types, PFGE patterns, and *spa* types. MRSA strains in horses were distinctly different from those of humans or other pets. This study suggests that despite reports of sporadic zoonotic MRSA cases, zoonosis accounts only for a small portion of the epidemiologic relationship between human and companion animal infectious MRSA strains.

Introduction

Staphylococcus aureus are gram-positive cocci that exist as part of the normal skin microflora in approximately 25% of the human population, only rarely causing opportunistic infections (Kluytmans, van Belkum et al. 1997). *S. aureus* can cause minor skin and soft tissue infections (SSTI) , such as impetigo and abscesses, and life-threatening diseases such as necrotizing pneumonia, septicemia, toxic shock syndrome and necrotizing fasciitis (Lowy 1998). These organisms can be easily spread through direct contact or through a variety of fomites (Romano, Lu et al. 2006). Companion animals are hosts to several staphylococcal species where *S. pseudointermedius* is the predominant coagulase-positive species. *S. aureus* is rarely among them and when present is often causing a SSTI or more serious conditions such as septicemia and pneumonia (Weese 2005).

Two years after the introduction of methicillin in human medicine, the first human isolate of methicillin-resistant *S. aureus* (MRSA) was reported in 1961 and through the 1980's remained a nosocomial infection. These hospital-acquired (HA-MRSA) infections resulted in limited outbreaks within a hospital or specific ward (Jevons 1961; Boyce 1994). The impact of MRSA has increased since 1989 when the first community-acquired MRSA (CA-MRSA) was described (Udo, Pearman et al. 1993). CA-MRSA strains show marked genetic diversity and an increased virulence, which was never seen in HA-MRSA infections (Daum, Ito et al. 2002). Asymptomatic carriage of MRSA by large segments of the population, once unheard of, has now been reported in multiple studies across the globe (Vlack, Cox et al. 2006; Lo, Lin et al. 2007; Gorwitz, Kruszon-Moran et al. 2008). This also coincides with an increase in the number of MRSA isolated from symptomatic companion animals such as dogs, horses, and cats (Duquette and Nuttall 2004; Weese 2005).

Although there is considerable information concerning the epidemiology and characterization of MRSA infections in human medicine ($MRSA^{H}$), there is much less known about infections in companion animals. Most studies were primarily limited to screening healthy animals to determine MRSA carriage rates within specific geographic regions (Malik, Coombs et al. 2006; Hanselman, Kruth et al. 2008). Many have speculated that MRSA strains with SCC*mec* may be easily transferred between human and animal staphylococcal species because of the frequency and extent of close, direct contact between owners and pets (Duquette and Nuttall 2004; Weese 2005; Malik, Coombs et al. 2006). There are several reports of colonized pets seemingly serving as reservoirs of MRSA infection for people and animals in close contact (van Duijkeren, Wolfhagen et al. 2004; Weese, Archambault et al. 2005; Strommenger, Kehrenberg et al. 2006).

In this study, we hypothesized that if it is true that pets are serving as a potential reservoir of infection for humans and other companion animals, then the population of MRSA strains found in companion animals will reflect the MRSA strains observed in the human population within the same geographic region. SCC*mec* typing, pulsed-field gel electrophoresis (PFGE), *spa* tying, antimicrobial resistance patterns, and toxin genotyping were used to characterize the MRSA strains collected from people and animals used in this study.

Materials and Methods

Bacterial Strains

The Veterinary Diagnostic Laboratory at the College of Veterinary Medicine at UGA identified 4823 coagulase-positive *Staphylococcal* isolates from June 2001 through December 2007 (Figure 1) from specimens submitted by clinicians from sick animals. 117 isolates were

phenotypically MRSA and 75 of these non-nosocomial isolates were stored and tested (Tables 1-2). Additionally, 71 MRSA^{H} isolates were randomly obtained from two local hospitals from 1999 and 2007. In total, 146 isolates were studied. Control strains used: NRS70, NRS384, NRS100, NRS123, NRS166, NRS227, NRS382, NRS383, NRS385, and NCTC8325.

Biochemical tests and API strips (bioMérieux, Marcy l'Etoile, France) were used for phenotypic identification of *S.aureus* isolates. Species confirmation was determined *nuc*A PCR (Brakstad, Aasbakk et al. 1992).

Antimicrobial sensitivity

Antimicrobial sensitivity was determined in accordance with Clinical and Laboratory Standards Institute (CLSI) for agar diffusion tests (CLSI 2006; CLSI 2007). Amikacin, clindamycin, enrofloxacin, erythromycin, gentamycin, orbifloxacin, oxacillin, tetracycline, and trimethoprim/sulfamethoxazole were tested. Weekly quality control used *Staphylococcus aureus* ATCC25923. Presence of *mecA* was confirmed in oxacillin resistant isolates by PCR (Higuchi, Takano et al. 2008).

SCCmec typing

DNA was extracted using the Microbial DNA Kit (MoBio Laboratories, Inc. Carlsbad, CA) following manufacturer's instructions. SCC*mec* type was determined by PCR using previously described primers and conditions (Zhang, McClure et al. 2005; Milheirico, Oliveira et al. 2007; Higuchi, Takano et al. 2008). Only SCC*mec*^{IV} isolates were subtyped (Milheirico, Oliveira et al. 2007; Higuchi, Takano et al. 2008). SCC*mec*^{IV} isolates that could not be subtyped were grouped as SCC*mec*$^{IV\text{-}nonA}$, with SCC*mec*$^{IVb\text{-}IVh}$.

Toxin gene profiling by PCR assays

Isolates were characterized by PCR-toxin testing. The following toxin genes were studied: *sea, seb, sec, sed*, *see* (Hazariwala, Sanders et al. 2002), *seg, seh, sei sem, sen, seo* (Jarraud, Mougel et al. 2002), *sek, sel, sep, seq* (Diep, Carleton et al. 2006), *sej* (Rosec and Gigaud 2002), *eta, etb, tsst-1* (Becker, Haverkamper et al. 2001), and *pvl* (Lina, Piemont et al. 1999).

Genotyping

Pulsed-field gel electrophoresis using restriction enzyme *Sma*I was performed and analyzed as previously described (de Lencastre, Couto et al. 1994; Tenover, Arbeit et al. 1995). PFGE patterns were analyzed by Bionumerics (version 4.6, Applied Maths, Kortrijk, Belgium), using the Dice coefficient to analyze the similarity of banding patterns, and the unweighted pair group method using arithmetic averages (UPGMA) for cluster analysis. A similarity cut-off of 70%, corresponding to a difference of ≤6 bands, was used to define a PFGE type. A group of isolates, pairs of which differed by no more than 6 bands, was considered a cluster. Clusters were numbered one through twenty, with cluster 1 containing the most number of isolates, followed by sequential decreases.

The X region of the *spa* gene was amplified using by PCR (Moodley, Stegger et al. 2006). DNA sequences were obtained with an ABI Prism™ 3730*xl* DNA sequencer (Seqwight Inc., Houston, TX). *spa* repeat types were identified with custom software (*spa*Typer) developed by us. Briefly, *spa*Typer is based on a position-specific scoring matrix (PSSM, (Henikoff and Henikoff 1994)) motif identification approach that searches input sequence for subsequences that are highly similar to previously-identified *spa* repeat units. *spa*Typer correctly identifies all currently known repeat types and repeat units, as provided at

http://spaserver2.ridom.de/spatypes.shtml and http://spaserver2.ridom.de/repeats.shtml, as well as random concatenations of known repeats. *spa*Typer is freely-available at http://fortinbras.us/spaTyper.

Statistics

Presence of specific genotypes or phenotypes was compared by species or SCC*mec* type using a test of independence, with Intercooled Stata® 9.2 for Windows (Stata Corp LP). P-values reported are with respect to the Fisher's exact test (when possible), or the Chi-squared test.

Results

Bacterial Strains

117 of 726 (16.1%) *Staphylococcus aureus* clinical isolates from companion animals (birds, cats, dogs and horses) were phenotypically MRSA. Trends show an increase in the number of companion animal MRSA infections from 2000-2006, while the total number of *Staphylococcal* infections in general and *S. aureus* infections in particular remained relatively stable (Figure 1). 146 (75 animal and 71 human) isolates were further characterized for this study (Tables 1-2). Only humans ($MRSA^{H}$), equines ($MRSA^{E}$) and canines ($MRSA^{C}$) were included in this study. $MRSA^{H}$ isolates from 1999 were derived solely from inpatients (HA-MRSA) compared with 74.1% of $MRSA^{H}$ isolates derived from outpatients presenting to the emergency department (CA-MRSA) in 2007.

SCCmec Typing

145 (99%) samples (70 human, 75 animal) were successfully typed for SCC*mec* (Table 1, Figures 2a-2c). Distribution of SCC*mec* types is not homogeneous among the three host species ($p<0.001$). The odds of MRSA being SCCmec^{II} instead of SCCmec^{IV} are 11 times higher for

canines than equines ($p<0.001$) and 3.6 times higher than for humans ($p=0.001$). MRSA^{E} and MRSA^{H} are equally likely to be SCC*mec*^{II} ($p=0.071$).

SCC*mec*^{IV} was identified in all host species studied. Humans show higher odds of being SCC*mec*^{IVa} subtype when compared to canines and equines [$p=0.001$ and $p<0.001$]. Canines and equines show similar odds of presenting either subtype ($p=0.052$). Additionally, two MRSA^{H} identified as SCC*mec*^{VII} are not included in this statistical analysis (Still et al., manuscript in preparation submitted to AAC).

Genotyping

140 (95.9%) isolates were analyzed by PFGE and could be grouped into 20 distinct clusters (Figure 3, Table 1). 85% of all isolates are represented in three clusters (1-3). Cluster 1 is the largest in this study and includes only isolates containing the SCC*mec*^{II} element. Cluster 2 is the second largest and includes 46 isolates containing SCC*mec*^{IVa} and 1 SCC*mec*^{IVg} MRSA^{E}. The entire cluster encompasses most SCC*mec*^{IVa} isolates. Cluster 3 contains only non-human isolates (9 equine, 1 canine) carrying SCCmec$^{IV\text{-}nonA}$. The 27 SCC*mec*$^{IV\text{-}nonA}$ isolates are in 13 different clusters and appear to segregate by clustering more on the basis of host species than SCC*mec* type. SCC*mec*^{IVa} isolates belong to two clusters, while SCC*mec*^{II} isolates belong to four clusters. The two human SCC*mec*^{VII} isolates belong to clusters 10 and 14, which include no other isolates and are genetically distinct from each other (Still et al., manuscript submitted to AAC).

spa typing distinguished 24 *spa* types, including two previously-unreported types for two MRSA^{H}: 14171 and 16338 (Table 1). *Spa* type t002 was most common, found in 64 of 145 tested isolates (44.1%). *Spa* type t008 was the next most frequent *spa* type, found in 33 (22.8%) isolates. Of the 67 SCC*mec*^{II} isolates, 8 *spa* types were identified, with t002 most common in 58 (86.6%) of these isolates. Of 49 SCC*mec*^{IVa} isolates, 12 *spa* types were identified. *Spa* type t008

was most common in 32 (65.3%) isolates. Dogs are predominantly SCC*mec*^{II} (68%) and as such *spa* type t002 (60%) is most common in this host species. Of 18 MRSA^{E}, 3 *spa* types were identified in the 13 SCC*mec*$^{IV\text{-}nonA}$ isolates, with t064 most frequent. Of the 71 MRSA^{H}, 14 *spa* types were identified. 23/26 SCC*mec*^{II} were t002 and 39 SCC*mec*^{IVa} isolates were all t008.

Toxin Gene Profiling (Table 2.3)

SCC*mec*^{II} isolates tend to have genes for *sed, seg, sei, sej, sem, sen,* and *seo* while SCC*mec*^{IVa} isolates tend to carry genes for *sek, seq,* and *pvl.* SCC*mec*$^{IV\text{-}nonA}$ isolates have a more-varied superantigen complement. Equines show agreement between their predominant SCC*mec*$^{IV\text{-}nonA}$ and the toxin genes *sea, sek,* and *seq*. The presence of *sea* distinguishes SCC*mec*^{IVa} from SCC*mec*$^{IV\text{-}nonA}$ isolates. The lack of *sek* in SCC*mec*^{II} distinguished it from all SCC*mec*^{IV} isolates. Interesting to note is the complete lack of the exfoliative toxins (*eta, etb*) and low prevalence of toxic shock syndrome toxin (TSST1) in all *mec* types, which are usually associated with increased strain virulence. Notably, TSST1 is not present in any SCC*mec*^{IVa} isolates and Panton-Valentine leukocidin (PVL) is present in nearly all SCC*mec*^{IVa} and a few SCC*mec*$^{IV\text{-}nonA}$ isolates.

Antimicrobial Sensitivity

SCC*mec*^{II} isolates were resistant to 63% of tested antibiotics, while SCC*mec*^{IVa} isolates were resistant to 53% and SCC*mec*$^{IV\text{-}nonA}$ isolates were resistant to 67.8%. SCC*mec*$^{IV\text{-}nonA}$ isolates are characterized by high frequencies of resistance to gentamycin, tetracycline, and trimethoprim/sulfamethoxazole, while resistance to clindamycin and the fluroquinolones are shared by most SCC*mec* types. Table 4 shows that the antibiotic resistance trends in the host species follows the same trends seen in the host species' most common SCC*mec* type.

Discussion

Although MRSA in the USA has been described extensively in the human population and some in the animal population, this is the most complete epidemiologic study comparing the genetic relatedness and characterization of human and companion animal MRSA isolates in the same study. Based on this study, we believe that there are specific cases of MRSA zoonosis, it only accounts for a small portion of the epidemiologic relationship between human and companion animal infectious MRSA strains.

The samples obtained for this study represent clinical non-nosocomial derived MRSA from symptomatic animals. The samples collected during 1999 and 2007 from humans reflect the national and statewide trend of predominant HA-MRSA to a majority of CA-MRSA infections, which appear to give an appropriate representation of what is happening in MRSA epidemiology in both humans (Klevens, Morrison et al. 2007). MRSA infections in animals in Georgia increased significantly from 11% in 2001 to 24% in 2006 ($p < 0.05$), in agreement with previous reports in the USA MRSA numbers decreased in 2007 although not significantly ($p > 0.05$) probably reflecting the overall decrease in *S. aureus* isolations as well as the lack of $MRSA^E$ that year. Horse owners and veterinarian's awareness of MRSA and introduction of new precautions and procedures may be responsible or a reduced number of equine samples submitted (Weese 2005; Weese, Archambault et al. 2005).

The parallel increase of MRSA in humans and animals raised the question of whether this increase in animal infections due to MRSA is due to a spill-over of CA-MRSA from humans. To answer this question, we employed the use of SCC*mec* typing, genotyping by PFGE and *spa* analysis, toxin genotyping and determination of antimicrobial resistance patterns.

MRSA strains are characterized by a large heterologous mobile genetic element – the staphylococcal cassette chromosome (SCC*mec*) (Ito, Katayama et al. 1999; Katayama, Ito et al. 2000). Currently, seven different SCC*mec* types have been identified worldwide (Higuchi, Takano et al. 2008). In the USA, SCC*mec* I-V and several variants have been observed (Smith, Male et al. 2008). In Georgia human MRSA strains causing clinical disease demonstrated a change from all HA-MRSA carrying SCC*mec*^{II} in 1999 to primarily CA-MRSA strains with SCC*mec*^{IVa} in 2007, likely due to an increase in community clinical presentations and patients seeking treatment at the emergency room (Prevention 2006). However, clinical MRSA^{C} and MRSA^{E} isolates maintain the same proportion of SCC*mec* types during 2001-2007, remaining primarily SCC*mec*^{II} and SCC*mec*$^{IV\text{-}nonA}$ respectively (Figures 2a-2b). Previous studies have shown that SCC*mec* $^{IV\text{-}nonA}$ isolates have only a prevalence of 4.2% (Weese, Archambault et al. 2005; Prevention 2006). The 14% of MRSA^{C} and 11% of MRSA^{E} that are SCCmec^{IVa} suggests that a small subset of animals share MRSA strains containing the same SCC*mec* type as people in the community. It is of interest to note the trend in the human isolates from only HA-MRSA to CA-MRSA, this trend it is not reflected in the animal populations study where the same mec type is predominant over the entire study period. The lack of change in SCC*mec* type among companion animal hosts could be explained by an increased incidence of animal-to-animal transmission instead of human-to-animal or vice versa. Furthermore, it seems that the virulence of MRSA strains in these animal hosts is the same regardless of SCC*mec* type.

PFGE analysis and *spa* typing (Figure 3) demonstrate the clonality of MRSA strains carrying a specific SCC*mec* type. With very few exceptions SCC*mec* types cluster together. Branching within clusters often further separates the isolates by host species. This is most notable in the SCC*mec*^{II} and SCC*mec*$^{IV\text{-}nonA}$ clusters where many branches completely lack any

MRSA^{H}. This raises the question of whether some MRSA strains are better adapted to certain host species. While all host species studied here are capable of being infected by MRSA having any SCC*mec* element, they are infected and sickened by different SCC*mec* types in different proportions. This seems to indicate that strains carrying particular SCC*mec* types may preferentially infect particular host species. Another possibility may be that strains such as USA300 (SCC*mec*^{IVa}), which are quite common and virulent in human SSTIs, are not as virulent in animals such as dogs and horses. Contrarily, USA100 (SCC*mec*^{II}) is the strain most commonly carried asymptomatically in humans in the United States (Tenover, McAllister et al. 2008), but dogs in our study were predominantly clinically sick by this strain type (Figure 3). The separation of host species by branching in PFGE clusters also supports the notion that there may be an increased incidence of human-to-human and animal-to-animal transmission instead of human-to-animal or vice versa. Due to differences in host, virulence or environmental factors, different MRSA strains may be better adapted to infection in one host species over another. Furthermore, it is notable that *S. aureus* is not the primary cause of staphylococcal disease in either dogs or horses (Busscher, van Duijkeren et al. 2006; Morris, Rook et al. 2006). This suggests that maybe only a subset of MRSA strains are successful pathogens in different animal hosts.

Additionally, we examined toxin gene distribution and antimicrobial resistance patterns in human and animal isolates. Our results show that SCC*mec* is associated with the presence of certain suites of toxin genes and antimicrobial resistance and because there are specific *mec* types prevalent in the host species, superantigens are also associated with host species (Table 3-4). Together with PFGE and *spa* typing, this study of toxins and antimicrobial resistance reveal that regardless of the host in which a SCC*mec* types resides in, the MRSA isolates containing the

same SCC*mec* type share the same characteristics. Some enterotxins form clusters that do not demonstrate horizontal transfer; these toxins remain restricted to the genomic background in *S. aureus*, as with SCC*mec*^{II} isolates tending to have the genes for *sed, seg, sei, sej, sem, sen,* and *seo* (Table 3) (Diep, Carleton et al. 2006). This is in agreement with the idea of a SCC*mec*^{II} element inserted into a SA background at some time in history and followed by large clonal expansion leading to the predominance of this MRSA strain (Deurenberg RH 2006; Diep, Carleton et al. 2006). The toxins ETA (chromosomally-encoded), ETB (plasmid-encoded), and TSST1 (encoded on a pathogenicity island) are typically associated with increased strain virulence, however, were either completely absent or of very low prevalence in all isolates studied here. Prophage-encoded SEA distinguishes the SCC*mec*$^{IV\text{-}nonA}$ MRSA^{E}. Even with the presence of multiple SEs, no suite of SEs or any SE individually appears to affect the host of the MRSA isolate. Again, demonstrating that both companion animals and people may be infected by the same strains of MRSA and that there does exist a zoonotic potential, although different MRSA strains may vary in their ability to cause disease in different animal hosts. Furthermore, the resistance patterns observed by species and SCC*mec* type are relatively conserved among the limited number of MRSA strains carrying these elements, and with further studies in MRSA susceptibilities in different host species these trends may have potential use in predicting treatment efficacy. For example, SCC*mec*$^{IV\text{-}nonA}$ isolates are characterized by high frequencies of resistance to gentamycin, tetracycline, and trimethoprim/sulfamethoxazole in all host species. However, because this SCC*mec* type is rarer in humans but frequent in horses, the question of interaction between an MRSA patient and a horse may indicate which antibiotic treatment would be most efficacious in treating infection.

In conclusion, the overall increase in the prevalence of MRSA-infected animals over this study period demonstrates the need of continued research in this field. We have seen a shift in the prevalence of SCC*mec* types in humans from SCC*mec* II to IV, but not in companion animals; SCCmec^{II} remains more prevalent in dogs, while SCCmec$^{IV\text{-}nonA}$ remains more prevalent in horses. PFGE, *spa* typing, toxin genotyping and antimicrobial resistance patterns indicate that regardless of the host, the MRSA isolates with the same SCC*mec* type share similar characteristics. This implies that most MRSA infections are caused by only a few MRSA background strains, which now predominate due to a large clonal expansion, confirming previous studies (Deurenberg, RH 2006). This study demonstrates that both companion animals and people may be infected by the same strains of MRSA; thus the zoonotic potential exists. Our study also reveals that only some MRSA strains may be better suited to cause disease in animal hosts. This may be combined with the fact that infections caused by CA-MRSA, due to their more pathogenic nature are more likely to be treated, therefore transmission of these strains to pets may be less likely. Studying both human and animal epidemiology of MRSA is important to gain a better understanding of the transmissions occurring between host species and their epidemiological consequences. Future research should focus on determining differences in gene expression between MRSA isolates originating from different host species, as well as a direct comparison between MRSA-infected animals and their owners.

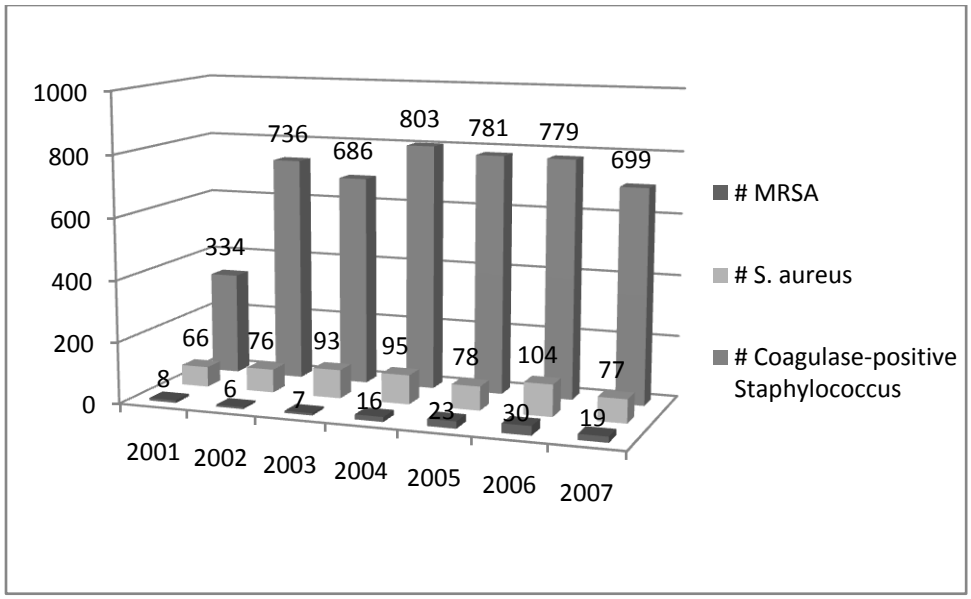


Figure 2.1 *Staphylococcus spp.* trends detected from laboratory-based surveillance clinical animal samples by the Veterinary Diagnostics Laboratory at the University of Georgia during the period of June 2001 through December 2007. 4823 coagulase-positive *Staphylococcal* isolates (726 *S. aureus*, 3929 *S. intermedius* Group, and 168 *S. schlieferi*) were collected from specimens submitted by clinicians from sick animals (alpaca, avian, bovine, camelid, canine, caprine, chicken, deer, duck, equine, feline, guinea pig, monkey, mouse, ovine, porcine, rabbit, and rat). Numbers indicate the actual number of positive cultures identified with coagulase-positive staphylococcus, *S. aureus*, and MRSA.

Table 2.1 Summary of 146 Isolates used for study

Sample	Host Species	Specimen Site	Date	SCC*mec* Type	PFGE Cluster	*spa* type
AVDL-1679	avian	skin	8/8/2001	II	1	t002
AVDL-55574	avian	Aspirate	5/10/2007	IV	9	NT
AVDL-18001	avian	culture	11/2/2007	IVb	16	t2922
AVDL-43041	canine	skin biopsy	4/20/2000	II	1	t002
AVDL-31679	canine	Ear swab	1/30/2001	IVd	7	t064
AVDL-23358	canine	drain fluid	1/2/2002	II	1	t002
AVDL-55574	canine	prostrate &urine	8/23/2002	IVc	9	t002
AVDL-24942	canine	chest tube	1/13/2003	II	1	t002
AVDL-57057	canine	Wound	6/13/2003	II	1	t002
AVDL-23700B	canine	Wound	12/4/2003	II	1	t002
AVDL-33546	canine	drain	2/19/2004	II	1	t002
AVDL-58835	canine	BAL	8/16/2004	II	1	t002
AVDL-1051	canine	pustule	8/17/2004	IVa	2	t008
AVDL-24575A	canine	Joint swab	11/18/2004	II	1	t002
AVDL-47964	canine	drain	4/4/2005	II	1	t062
AVDL-55002	canine	draining tract	4/29/2005	II	1	t002
AVDL-32822	canine	ND	5/31/2005	II	20	t211
AVDL-36579	canine	tissue	7/20/2005	II	1	t002
AVDL-29934	canine	wound	7/20/2005	IVd	19	t451
AVDL-47387-2	canine	urine	7/21/2005	II	1	t045
AVDL-51661-3	canine	drain	7/21/2005	II	1	t062
AVDL-1648	canine	abscess	7/21/2005	IVa	2	t008
AVDL-10202	canine	joint	8/30/2005	II	1	t002
AVDL-20332	canine	wound	11/2/2005	IVc	17	t002
AVDL-8958	canine	artery aspirate	11/14/2005	II	1	t045
AVDL-52275	canine	blood	4/12/2006	IVh	4	t1774
AVDL-52905-2	canine	bone	4/19/2006	IVg	4	t1774
AVDL-52490	canine	bone	4/20/2006	II	1	t002
AVDL-51236	canine	wound	4/21/2006	IVg	4	t1774
AVDL-53881	canine	incision	4/24/2006	II	1	t003
AVDL-589	canine	urine	7/11/2006	IVg	4	t1774
AVDL-9151	canine	wound	8/29/2006	IVa	2	t008
AVDL-17113	canine	wound	10/13/2006	IVa	12	t3016
AVDL-22565	canine	ND	11/16/2006	II	1	t002
AVDL-44094	canine	Joint fluid	3/21/2007	II	1	t002
AVDL-51587	canine	wound	4/26/2007	II	1	t002
AVDL-52673	canine	wound	5/1/2007	II	1	t002
AVDL-7275	canine	abscess	8/23/2007	IVa	2	t008
AVDL-8742	canine	Nasal swab	9/4/2007	IV	3	t064
AVDL-10291-2	canine	culture	9/12/2007	II	1	t002
AVDL-10506-1	canine	wound	9/13/2007	II	1	t002
AVDL-10764-2	canine	wound	9/14/2007	II	1	t002
AVDL-12013	canine	wound	9/24/2007	II	1	t002
AVDL-12014	canine	fresh tissue	9/24/2007	II	1	t002

AVDL-13446	canine	wound	10/3/2007	II	1	t002
AVDL-16053	canine	Tissue	10/19/2007	II	1	t002
AVDL-17422	canine	Joint fluid	10/30/2007	II	1	t002
AVDL-17423	canine	Joint fluid	10/30/2007	II	1	t002
AVDL-18098	canine	Aspirate	11/12/2007	II	1	t002
AVDL-20287	canine	wound	11/19/2007	IVa	2	t008
AVDL-20733	canine	wound	11/20/2007	II	1	t002
AVDL-20884	canine	Joint swab	11/21/2007	IVa	2	t126
AVDL-20971-1	canine	Nasal swab	11/26/2007	II	1	t002
AVDL-49420	equine	ND	ND	IVh	5	t064
AVDL-26824	equine	ND	ND	IVd	15	t451
AVDL-31222	equine	eye	1/31/2002	IV	3	t451
AVDL-4426	equine	incision	7/29/2002	IVd	6	t064
AVDL-19315-1	equine	Conjuctival swab	10/19/2004	IVg	2	t008
AVDL-35861	equine	cornea	2/7/2005	II	1	t002
AVDL-45407-1	equine	Trans Tracheal wash	3/15/2005	II	1	t002
AVDL-6409	equine	Fluid	8/17/2005	IVd	3	t064
AVDL-29623-1	equine	cornea	1/9/2006	IVa	2	t121
AVDL-38129	equine	Nasal swab	2/16/2006	IVd	3	t064
AVDL-37055-1	equine	Aspirate	2/16/2006	IVd	3	t064
AVDL-38155	equine	ND	2/16/2006	IVd	3	t064
AVDL-51878	equine	abscess	4/14/2006	IVd	3	t064
AVDL-49196-1	equine	Gutural punch	4/18/2006	IVd	3	t064
AVDL-4191	equine	cornea	8/2/2006	II	1	t002
AVDL-24066	equine	Tissue	11/28/2006	IVd	3	t451
AVDL-26805	equine	cornea	12/14/2006	IVa	2	t008
AVDL-12235	equine	draining tract	9/25/2007	IVg	3	t064
AVDL-41962A	feline	Urine swab	3/30/2004	II	1	t002
AVDL-50267A	feline	Nasal swab	5/11/2004	II	1	t002
AVDL-4401	feline	ND	8/2/2007	IVa	2	t008
AVDL-20969	feline	wound	11/26/2007	II	1	t002
AR-7	human	ND	1999	II	1	t002
AR-21	human	ND	1999	II	1	t002
2	human	ND	1999	II	1	t002
5	human	ND	1999	II	1	t002
6	human	ND	1999	II	1	t002
9138-8723	human	ND	1999	II	1	t002
9117-0645	human	ND	1999	II	1	t002
9131-6008	human	ND	1999	II	1	t002
9131-6321	human	ND	1999	II	13	t018
9105-5005	human	ND	1999	II	1	t002
9112-7166	human	ND	1999	II	1	t002
9128-4933	human	ND	1999	II	1	t002
9144-1754	human	ND	1999	II	1	t002
14173	human	Leg	10/8/2007	IVa	2	t008
14137	human	Axilla	10/8/2007	IVa	2	t681
14171	human	Scalp	10/8/2007	IVa	2	*
14192	human	Peripheral	10/8/2007	II	1	t002

14309	human	Shoulder	10/8/2007	IVa	2	t008
14292	human	Leg	10/8/2007	IVa	2	t211
14254	human	Urine	10/8/2007	II	1	t450
14285	human	Axilla	10/8/2007	IVa	2	t008
14301	human	Hip	10/8/2007	II	1	t002
14359	human	Peripheral	10/8/2007	II	1	t002
14367	human	Buttock	10/8/2007	IVa	2	t008
14107	human	Hand	10/8/2007	IVa	2	t068
14020	human	Groin	10/8/2007	II	1	t002
14423	human	Leg	10/8/2007	IVa	2	t008
14814	human	Arm	10/15/2007	IVa	2	t008
14837	human	Buttock	10/15/2007	IVa	2	t008
14803	human	Elbow	10/15/2007	IVa	2	t681
14745	human	Ear	10/15/2007	II	1	t002
10001	human	Endotracheal	10/22/2007	II	1	t002
10002	human	Hip	10/22/2007	IVa	2	t2168
10003	human	Urine	10/22/2007	II	1	t010
10004	human	Sputum	10/22/2007	II	1	t002
10005	human	Abdomen	10/22/2007	IVa	2	t002
10006	human	Ear	10/22/2007	NT	NT	t002
10007	human	Hip	10/22/2007	IVc	17	t002
10008	human	Hip	10/22/2007	II	1	t002
10009	human	Arm	10/22/2007	IVa	2	t008
15435	human	Face	10/29/2007	IVa	2	t008
15437	human	Hip	10/29/2007	IVa	2	t068
15513	human	Other	10/29/2007	II	1	t002
15327	human	Leg	10/29/2007	IVa	2	t008
15328	human	Sacrum	10/29/2007	IVa	2	t008
15315	human	Foot	10/29/2007	IVa	2	t211
15379	human	Nose	10/29/2007	IVa	2	t008
15596	human	Groin	11/5/2007	IVa	2	t008
15604	human	Face	11/5/2007	IVa	2	t024
15609	human	Buttock	11/5/2007	IVc	18	t002
15639	human	Thigh	11/5/2007	IVa	2	t008
15808	human	Perirectal	11/5/2007	IVa	2	t681
16188	human	Thigh	11/12/2007	IVa	2	t008
16234	human	Knee	11/12/2007	IVa	2	t008
16067	human	Left Cheek	11/12/2007	IVa	2	t024
15897	human	Buttock	11/12/2007	IVa	2	t008
16043	human	Urine	11/12/2007	II	1	t002
16115	human	Ear	11/12/2007	IVa	2	t008
16375	human	Foot	11/15/2007	VII^	10	t458
16259	human	Groin	11/19/2007	IVa	2	t008
16286	human	Urine Catheter	11/19/2007	II	1	t002
16302	human	Abdomen	11/19/2007	IVc	9	t2168
16353	human	Finger	11/19/2007	IVa	2	t008
16338	human	Buttock	11/19/2007	IVa	2	*
16347	human	Finger	11/19/2007	IVa	2	t008

16394	human	Abdomen	11/19/2007	IVa	2	t681
16469	human	Lab Draw	11/19/2007	IVa	2	t008
16489	human	Buttock	11/19/2007	IVa	2	t008
16483	human	Breast	11/19/2007	VII^	14	t017
16532	human	Leg	11/19/2007	IVa	2	t008
16576	human	Buttock	11/19/2007	IVa	2	t008

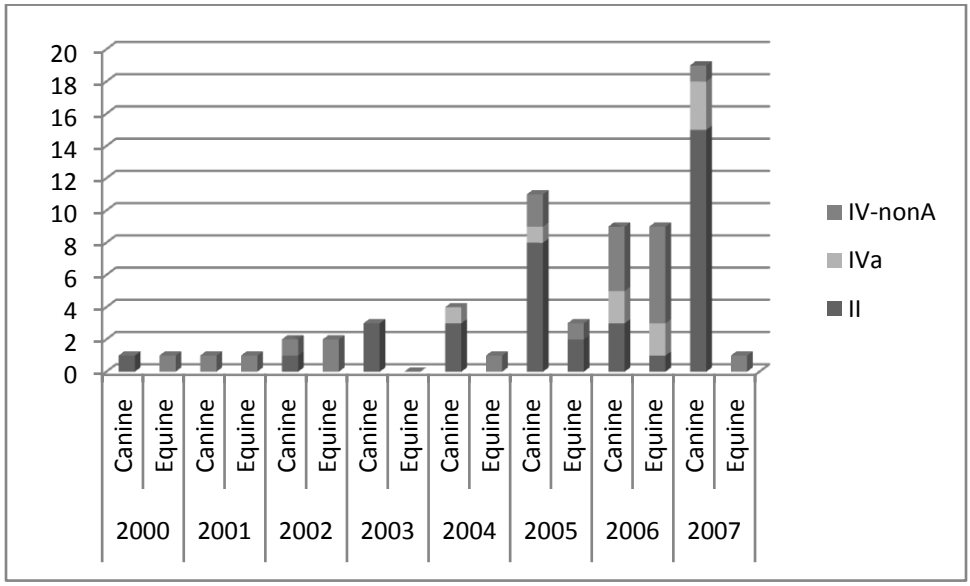
Laboratory-based surveillance in the Veterinary Diagnostics Laboratory at the University of Georgia identified and stored (in 15% glycerol stock at -80°C) 75 MRSA isolates from companion animals. To determine the most common MRSA strains circulating in the human population within the local community, we also obtained 13 human MRSA samples with Institutional Review Board (IRB) approval from two local hospitals in 1999 and 58 additional human MRSA isolates were obtained between October and December 2007. In total, 146 isolates (75 MRSA isolates from companion animals, 13 human MRSA isolates from 1999 and 58 human MRSA isolates from 2007) were used for this study. ND is not determined, UT is untypeable, * indicated a novel *spa* type, and ^ indicates Still et al., unpublished data.

Table 2.2 Summary of epidemiologic data collected on the isolates used in this study

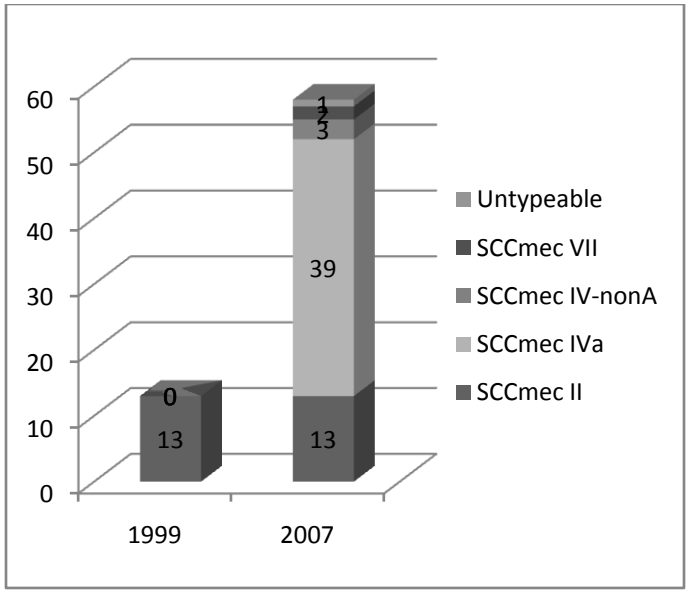
Animal Isolates (75 isolates)		**Human Isolates** (71 isolates)	
Host Species	Total # (%)	Date Collected	Total # (%)
Avian	3 (4.0)	1999	13 (18.3)
Canine	50 (66.7)	2007	58 (81.7)
Equine	18 (24.0)	Location	
Feline	4 (5.3)	North Georgia	71 (100)
Date Collected			
2000	2 (2.7)	Infection Origins (out of 58)	
2001	3 (4.0)	Inpatient	15 (25.9)
2002	4 (5.3)	Outpatient	43 (74.1)
2003	3 (4.0)	Specimen Site (out of 58)	
2004	7 (9.3)	SSTI	47 (81.0)
2005	14 (18.7)	Wound	0 (0)
2006	18 (24.0)	Urine	4 (6.9)
2007	24 (32)	Blood	1 (1.7)
Location (out of 74)		Other	6 (10.3)
Metro-Atlanta	34 (45.9)		
North Georgia	31 (41.9)		
South Georgia	3 (4.1)		
Out of State	6 (8.1)		
Specimen Site (out of 74)			
SSTI	46 (62.2)		
Wound	16 (21.6)		
Urine	4 (5.4)		
Blood	1 (1.4)		
Other	7 (9.5)		

Epidemiologic patient data was collected by clinicians and disclosed in a manner that did not compromise the patient's identity and personal information. SSTI indicates a skin and soft tissue infection, while a sample included in the "other" specimen site category is any infection from any part of the body that cannot be classified as a SSTI, wound, urine, or blood infection. Human isolates were collected from local hospitals and categorized on the basis of inpatient or outpatient. Inpatients developed the MRSA infection while in the hospital and were typically from long-term wards and intensive care units (ICU). Outpatients were those presenting with an MRSA infection to the emergency department.

2.2A


20
18
16
14
12
10
8
6
4
2
0
Canine
Equine
2000
2001
2002
2003
2004
2005
2006
2007
IV-nonA
IVa
II

2.2B

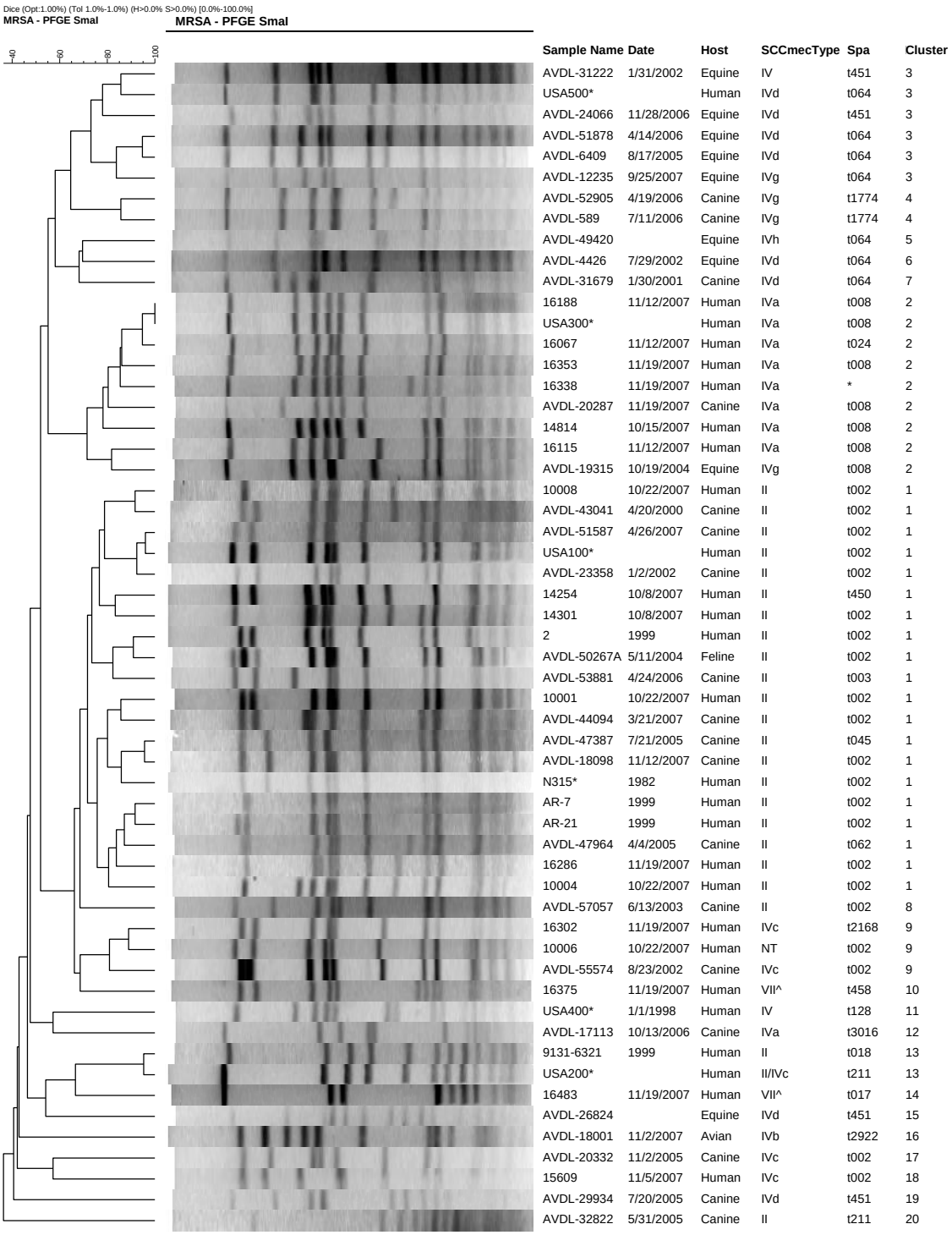

60
50
40
30
20
10
0
1
3
39
0
13
13
1999
2007
Untypeable
SCCmec VII
SCCmec IV-nonA
SCCmec IVa
SCCmec II

2.2C

Species	SCC*mec* type		Total	SCC*mec* subtype		Total
	II	IV		IVa	IV-nonA	
Canine	33	15	48	7	7	14
Equine	3	15	18	2	12	14
Human	26	42	68	39	3	42
Total	62	72	134	48	22	70
P-value		**<0.001**			**<0.001**	

Figure 2: SCC*mec* Typing

These figures examine the SCC*mec* types detected by PCR assay in the 146 MRSA isolates used for this study. **2A** examines the changes in numbers of MRSA infections in companion animals and the SCC*mec* type associated with those isolates throughout the study time period. **2B** compares the two sets of data collected on MRSA in human isolates from a local hospital, sampled once in 1999 and again in 2007. In **2C** the samples were tested for independence between host species and SCC*mec* type, using the Chi-square test. A p value < 0.05 was considered significant and indicative that the distribution of SCC*mec* types is not homogeneous along the three species.



Sample Name	Date	Host	SCCmecType	Spa	Cluster
AVDL-31222	1/31/2002	Equine	IV	t451	3
USA500*		Human	IVd	t064	3
AVDL-24066	11/28/2006	Equine	IVd	t451	3
AVDL-51878	4/14/2006	Equine	IVd	t064	3
AVDL-6409	8/17/2005	Equine	IVd	t064	3
AVDL-12235	9/25/2007	Equine	IVg	t064	3
AVDL-52905	4/19/2006	Canine	IVg	t1774	4
AVDL-589	7/11/2006	Canine	IVg	t1774	4
AVDL-49420		Equine	IVh	t064	5
AVDL-4426	7/29/2002	Equine	IVd	t064	6
AVDL-31679	1/30/2001	Canine	IVd	t064	7
16188	11/12/2007	Human	IVa	t008	2
USA300*		Human	IVa	t008	2
16067	11/12/2007	Human	IVa	t024	2
16353	11/19/2007	Human	IVa	t008	2
16338	11/19/2007	Human	IVa	*	2
AVDL-20287	11/19/2007	Canine	IVa	t008	2
14814	10/15/2007	Human	IVa	t008	2
16115	11/12/2007	Human	IVa	t008	2
AVDL-19315	10/19/2004	Equine	IVg	t008	2
10008	10/22/2007	Human	II	t002	1
AVDL-43041	4/20/2000	Canine	II	t002	1
AVDL-51587	4/26/2007	Canine	II	t002	1
USA100*		Human	II	t002	1
AVDL-23358	1/2/2002	Canine	II	t002	1
14254	10/8/2007	Human	II	t450	1
14301	10/8/2007	Human	II	t002	1
2	1999	Human	II	t002	1
AVDL-50267A	5/11/2004	Feline	II	t002	1
AVDL-53881	4/24/2006	Canine	II	t003	1
10001	10/22/2007	Human	II	t002	1
AVDL-44094	3/21/2007	Canine	II	t002	1
AVDL-47387	7/21/2005	Canine	II	t045	1
AVDL-18098	11/12/2007	Canine	II	t002	1
N315*	1982	Human	II	t002	1
AR-7	1999	Human	II	t002	1
AR-21	1999	Human	II	t002	1
AVDL-47964	4/4/2005	Canine	II	t062	1
16286	11/19/2007	Human	II	t002	1
10004	10/22/2007	Human	II	t002	1
AVDL-57057	6/13/2003	Canine	II	t002	8
16302	11/19/2007	Human	IVc	t2168	9
10006	10/22/2007	Human	NT	t002	9
AVDL-55574	8/23/2002	Canine	IVc	t002	9
16375	11/19/2007	Human	VII^	t458	10
USA400*	1/1/1998	Human	IV	t128	11
AVDL-17113	10/13/2006	Canine	IVa	t3016	12
9131-6321	1999	Human	II	t018	13
USA200*		Human	II/IVc	t211	13
16483	11/19/2007	Human	VII^	t017	14
AVDL-26824		Equine	IVd	t451	15
AVDL-18001	11/2/2007	Avian	IVb	t2922	16
AVDL-20332	11/2/2005	Canine	IVc	t002	17
15609	11/5/2007	Human	IVc	t002	18
AVDL-29934	7/20/2005	Canine	IVd	t451	19
AVDL-32822	5/31/2005	Canine	II	t211	20

Figure 2.3 Typing results obtained by PFGE with *Sma*I

Pulsed-field gel electrophoresis of MRSA isolates following chromosomal DNA digestion with restriction enzyme *Sma*I was performed and analyzed as previously described (de Lencastre, Couto et al. 1994; Tenover, Arbeit et al. 1995). The tree diagram includes six common control strains carrying SCC*mec* II and SCC*mec* IV elements (denoted by * beside strain name), along with 51 representative isolates (one isolate for every PFGE type identified). PFGE patterns were also analyzed by Bionumerics (version 4.6, Applied Maths, Kortrijk, Belgium) by using the Dice coefficient to analyze the similarity of banding patterns, and the unweighted pair group method using arithmetic averages (UPGMA) for cluster analysis. An optimization and 1.00 and position tolerance of 1.00 were used for cluster analysis. A similarity cut-off of 70% which corresponded to a difference of ≤6 bands was used to define a PFGE type. The PFGE type was considered different if seven or more band differences were observed (Tenover, Arbeit et al. 1995). A PFGE type, differing by no more than 6 bands was considered a cluster. Clusters were numbered one through twenty, with cluster 1 containing the most number of isolates, followed by sequential decreases. Human controls for frequently-identified strains in the United States are included for comparison purposes (denoted by * beside strain name). In SCC*mec* typing, ^ denotes unpublished data by Still et al. An * in *spa* typing indicated a novel *spa* type.

Table 2.3: Toxin genotyping by PCR assay

	II (n=66)	**IVa** (n=49)	**IV-nonA** (n=23)	**Canine** (n=49)	**Human** (n=71)	**Equine** (n=18)
sea	1.5[a]	0[a]	59.3	9.8[a]	1[a]	61.1
seb*	1.5	0	3.7	3.9	0	0
sec*	0	2	7.4	2	2	0
sed	88.1	0[b]	7.4[b]	60.8	23[b]	22.2[b]
see†	0	0	0	0	0	0
seg	94	4.1	20.6	74.5	29[c]	16.7[c]
seh†	0	0	0	0	0	0
sei	95.5	2	22.2	68.6	30[d]	16.7[d]
sej	86.6	12.2[e]	18.5[e]	66.7	29[e]	22.2[e]
sek	0	81.6	51.9	9.8	37[f]	72.2[f]
sel*	0	2	7.4	2	2	0
sem	95.5	4.1	25.9	72.5	29	16.7
sen	61.2	2	25.9	54.9	19[g]	0[g]
seo	98.5	4.1	25.9	74.5	31[h]	16.7[h]
sep	15[j]	0[i]	3.7[i,j]	5.9[*]	9[*]	0[*]
seq	11.9	87.8	55.6	13.7	46[k]	83.3[k]
eta†	0	0	0	0	0	0
etb†	0	0	0	0	0	0
tsst1*	7	0	11.1	7.8	3	11.1
pvl	0[l]	93.9	3.7[l]	7.8[l]	38	16.7[l]

Toxin genotyping by PCR assay as compared among both SCC*mec* types and host species. Numbers indicate percentages of toxin gene-positive isolates in that particular subset of isolates. The samples were tested for independence among host species or SCC*mec* type, and the presence of the toxin gene. Homogeneity along the three categories is denoted "*". A Chi-square of less than 0.05 was considered significant, and pair wise comparisons where then performed. In each row, pairs with the same letter superscript are not significantly different from each other (Fisher's exact p-value at a 95% confidence level adjusted for multiple comparisons). When isolates were all negative for the toxin a homogeneity test is not applicable (indicated in the Table by "†"). A category that is significantly higher than the others in the comparison is filled gray. When no category has a superscript, all are considered significantly different.

Table 2.4: Antibiotic Resistance Profiles

	II	IVa	IV-nonA	Canine	Human	Equine
AMK*	62.1	49	47.8	46.9	57.7	61.1
CLIN	83.3[a]	18.4	56.5[a]	61.2[*]	54.9[*]	50[*]
ENRO	93.9	69.4[b]	39.1[b]	79.6[b]	83.1[b]	33.3
ERY	100[c]	91.8[c,d]	78.3[d]	93.9[*]	94.4[*]	83.3[*]
GNT	3[e]	4.1[e]	65.2	8.2[e]	5.6[e]	72.2
OBX	95.4[f]	81.3[f,g]	54.5[g]	83.7[f]	90.1[f]	46.7
TET	1.5[h]	10.2[h]	65.2	8.2[h]	9.9[h]	72.2
SXT	9.1[i]	4.1[i]	78.3	16.3[i]	9.9[i]	66.7

Antimicrobial susceptibility was determined by using the Kirby-Bauer disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) for agar diffusion tests (CLSI 2006; CLSI 2007). The following antibiotics were tested: amikacin (AMK), clindamycin (CLIN), enrofloxacin (ENRO), erythromycin (ERY), gentamycin (GNT), orbifloxacin (OBX), oxacillin (OXA), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). Resistance profiles were compared between both SCC*mec* type and host species. Numbers indicate percentages of resistant isolates in that subset. The samples were tested for independence between host species or SCC*mec* type, and the presence of the toxin gene. Homogeneity along the three categories is denoted by "*". A Chi-square of less than 0.05 was considered significant, and pair wise comparisons where then performed. In each row, pairs with the same letter superscript are not significantly different from each other (Fisher's exact p-value at a 95% confidence level adjusted for multiple comparisons). When isolates were all negative for the toxin a homogeneity test is not applicable (indicated in the Table by "†"). A category that is significantly higher than the others in the comparison is filled gray.

CHAPTER 3

IDENTIFICATION OF SCC*mec* TYPE VII AND TYPE VII_{GA} VARIANT CASSETTES IN METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS AND STAPHYLOCOCCUS PSEUDINTERMEDIUS IN THE UNITED STATES[1]

[1]Still, C., Wheeler, B., Jensen, M., and S. Sanchez.
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Abstract

Two methicillin-resistant *Staphylococcus aureus* (MRSA) isolates from human wound samples and 46 clinical samples of methicillin-resistant staphylococci from dogs carrying the staphylococcal cassette chromosome (SCC) *mec* type VII and variants SCC*mec* VII_{GA} and SCC*mec* VII_{GA2} were identified in the United States. Both human MRSA isolates and 21 *Staphylococcus pseudintermedius* isolates were identified as carrying SCC*mec* VII. The variant SCC*mec* VII_{GA} was found in 17 isolates (one feline *S. pseudintermedius,* one canine *S. schleiferi* subsp. *coagulans*, and 15 canine *S. pseudintermedius*) and an additional variant, SCC*mec* VII_{GA2} was identified in 8 isolates (one canine *S. schleiferi* subsp. *schleiferi* and 7 canine *S. pseudintermedius*). These isolates were further characterized to their genetic relatedness by *spa* sequencing, MLST and PFGE. In addition, the antimicrobial susceptibilities and toxin gene profiles were also studied. To our knowledge, this is the first report of these SCC*mec* type VII and its variant elements in several *Staphylococcus* species isolated in the United States.

Introduction

Since the emergence of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in Australia in 1989, CA-MRSA has gained significant interest globally as an important re-emergent pathogen not only in hospital environments, but also in communities (Udo, Pearman et al. 1993). These CA-MRSA strains show a marked diversity, as evidenced by the number of genetic and phenotypic variants, which has never been seen in hospital-acquired (HA-MRSA) infections (Daum, Ito et al. 2002). Clinical symptoms and syndromes caused by CA-MRSA isolates range from skin and soft tissue infections to necrotizing pneumonia, severe sepsis, and necrotizing fasciitis. Asymptomatic carriage of methicillin resistant *S. aureus* (MRSA), by segments of the population, once unheard of, has now been reported at low rates (less than 2%) by multiple studies across the globe since the emergence of CA-MRSA (Harbarth, Francois et al. 2005; Vlack, Cox et al. 2006; Lo, Lin et al. 2007; Gorwitz, Kruszon-Moran et al. 2008). Coinciding with this increase in asymptomatic carriage of MRSA among humans is an increase in the number of methicillin-resistant staphylococcal species isolated from companion animals such as canines, equines, and felines (Pak, Han et al. 1999; Tomlin, Pead et al. 1999; Duquette and Nuttall 2004; Weese 2005). In the United States, while approximately 80% of community-onset skin and soft-tissue infections caused by *S. aureus* are methicillin-resistant (Moran, Krishnadasan et al. 2006), the prevalence of methicillin-resistant *S. pseudintermedius* (MRSP) infections in canines have been reported at levels as high as 16% in clinical samples (Jones, Kania et al. 2007).

MRSA strains are characterized by a large heterologous mobile genetic element that they carry – the staphylococcal cassette chromosome (SCC*mec*) - which contains the *mec*A gene responsible for conferring methicillin resistance to the bacterium and the *ccr* complex

responsible for gene transfer (Ito, Katayama et al. 1999; Katayama, Ito et al. 2000). While the SCC*mec* element has been studied mostly in MRSA isolates, it is also present in other staphylococcal species such as *S. haemolyticus, S. intermedius*, and *S. pseudintermedius* (Katayama, Ito et al. 2001; Sasaki, Kikuchi et al. 2007). Many have speculated that SCC*mec* may be easily transferred between human and animal staphylococcal species (Duquette and Nuttall 2004; Weese 2005; Malik, Coombs et al. 2006). Additionally, there have been several reports of colonized pets serving as reservoirs of infection for humans and animals in close contact, as well as a few reports of animal-to-human and human-to-animal transmission of MRSA (van Duijkeren, Wolfhagen et al. 2004; Loeffler, Boag et al. 2005; O'Mahony, Abbott et al. 2005; Weese, Archambault et al. 2005; Strommenger, Kehrenberg et al. 2006; Sasaki, Kikuchi et al. 2007). Reports have shown that in small animal MRSA infections, isolates correspond well to recognized, common human nosocomial isolates most often containing SCC*mec* II (Rich and Roberts 2004). MRSA colonization and infection in equines, however, seem to be dominated by one particular clone containing the SCC*mec* IVd, which is uncommon in humans other than veterinary personnel in contact with these animals (Weese 2005). To date, seven different SCC*mec* types have been identified worldwide (Ito, Katayama et al. 2001; Okuma, Iwakawa et al. 2002; Ito, Ma et al. 2004; Boyle-Vavra, Ereshefsky et al. 2005; Oliveira, Milheirico et al. 2006; Higuchi, Takano et al. 2008). In the United States, SCC*mec* types I-IV and several of their variants have frequently been observed, with SCC*mec* II common in nosocomial infections and SCC*mec* IVa in CA-MRSA. Recently, reports of SCC*mec* V have also been noted in MRSA in pigs and pig farmers (Smith, Male et al. 2008) and in *S. pseudintermedius* in dogs (Moodley, Stegger et al. 2008) within the United States. In light of these findings, we sought to compare the population of SCC*mec* types in methicillin-resistant *Staphylococci* between infected humans and

companion animals from the same geographic region. In the course of this study, we identified the SCC*mec* type VII element in canine *S. pseudintermedius* isolates and two human CA-MRSA isolates collected in Georgia, in addition to the variants SCC*mec* VII_{GA} and SCC*mec* VII_{GA2} in canine isolates of *S. pseudintermedius*. Only these isolates will be further discussed. The remainder of this larger epidemiologic study will be published elsewhere. (Still et al., manuscript in progress).

Materials and Methods

Bacterial Strains

Laboratory-based surveillance in the Veterinary Diagnostics Laboratory at the University of Georgia identified 4823 coagulase-positive *Staphylococcal* isolates (726 *S. aureus,* 3929 *S. intermedius* Group, and 168 *S. schlieferi*) during the period of June 2001 through May 2008 from specimens submitted by clinicians from sick animals (avian, bovine, camelid, canine, caprine, chicken, deer, duck, equine, feline, guinea pig, monkey, mouse, ovine, porcine, rabbit, and rat). 117 isolates were phenotypically methicillin-resistant *S. aureus* (MRSA) and a total of 76 were stored and further tested. 77 phenotypically methicillin-resistant non-aureus *Staphylococci* were also stored and used in this study. With the purpose of determining the most frequently encountered MRSA strains causing infection in the human population within our community, thirteen human MRSA samples were identified and obtained with Institutional Review Board (IRB) approval from two local hospitals in 1999. Fifty-eight more human MRSA isolates were obtained with IRB approval from a local hospital between October and December 2007. All these isolates came from a larger epidemiologic study which will published elsewhere (Still et al., manuscript in progress). Only SCC*mec* VII-containing isolates will be further discussed in this manuscript. Control strains for PCR assays were obtained from the Network on

Antimicrobial Resistance in *Staphylococcus aureus*: NRS70 (N315), NRS384 (USA300), NRS100 (COL), NRS123 (USA400, MW2), NRS166, NRS227, NRS 382 (USA100), NRS383 (USA200), NRS385 (USA500), and NCTC8325. Biochemical tests, PCR to detect the *nuc*A gene of both *S. aureus* and the *S. intermedius* Group and partial gene 16S and *sod*A sequencing were performed using previously described primers to confirm species designation (Brakstad, Aasbakk et al. 1992; PJ Quinn 1994; Poyart, Quesne et al. 2001; Becker, von Eiff et al. 2005; Sasaki, Kikuchi et al. 2007). DNA sequences were obtained with an ABI Prism™ 3730*xl* DNA sequencer (Seqwight Inc., Houston, TX).

Antimicrobial sensitivity

Antimicrobial sensitivity was determined by using the Kirby-Bauer disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) for agar diffusion tests (NCCLS 2002; CLSI 2006; CLSI 2007). The following antibiotics were tested for all gram-positive bacteria submitted to the Veterinary Diagnostics Laboratory at the University of Georgia: amikacin (AMK), ampicillin (AMP), cefoxitin (FOX), ceftiofur (XNL), cephalothin (CEPH), chloramphenicol (CHLR), amoxicillin-clavulanic acid (CLAV), clindamycin (CLIN), enrofloxacin (ENRO), erythromycin (ERY), gentamycin (GNT), orbifloxacin (OBX), oxacillin (OXA), penicillin (PEN), tetracycline (TET), vancomycin (VAN), trimethoprim/sulfamethoxazole (SXT), levofloxacin (LEV). All disks were supplied by REMEL, except orbifloxacin, supplied by Becton, Dickinson and Company, and quality control was carried out weekly using *Staphylococcus aureus* ATCC 25923. Presence of the *mec*A gene was confirmed by PCR detection (Hedin and Lofdahl 1993).

SCCmec typing

SCC*mec* typing by PCR-based assays were used in combination to identify the SCC*mec* type of all isolates (Zhang, McClure et al. 2005; Milheirico, Oliveira et al. 2007; Higuchi, Takano et al. 2008). The *mec* TypeV primers described by Zhang et al., were used for sequencing the *mec* V specific locus, hypothetical protein V011, of selected samples (Zhang, McClure et al. 2005). Additionally, using the ccrC primers described by Milheirico et al.(Milheirico, Oliveira et al. 2007), and the ccrC2 and ccrC8 primers described by Higuchi et al., several selected isolates were sequenced for these specific PCR-amplified loci (Higuchi, Takano et al. 2008). The primer sets listed in Table 3.1 were designed for the complete sequencing of the ccrC2 and ccrC8 genes using the sequences from GenBank Accession AB353125 as a reference. Sequence analysis was performed using Sequencher^{TM} 4.8 software and contigs were created (Gene Codes Corp., Ann Arbor, MI). Sequences for genes *ccr*C2 and *ccr*C8 were deposited as a group in GenBank (Accession numbers FJ931046 - FJ931053, consecutively).

All PCR assays were performed from a 1:20 dilution of the extracted DNA to water. An aliquot of 1µL of this DNA suspension was added to 9µL of a PCR mixture containing 3.0mM $MgCl_2$ (Idaho Technology, Inc., Salt Lake City, UT), 0.2mM of each deoxynucleotidetriphosphate (dATP, dUTP, dGTP, dCTP) (Roche, Basel, Switzerland), 0.1mM of the forward and reverse primers, and 0.5 units of *Taq* polymerase (Roche, Basel, Switzerland). The amplification was performed in the RapidCycler (Idaho Technology, Inc. Salt Lake City, UT) beginning with an initial denaturation step at 94°C, followed by 30 cycles at previously described annealing temperatures (Zhang, McClure et al. 2005; Milheirico, Oliveira et al. 2007; Higuchi, Takano et al. 2008) dependent upon the primer set, ending with an extension step at 72°C for 45 seconds. Amplified PCR products were visualized and saved using the BioRad Gel

Doc™ 2000, digital camera and the Quantity One software after electrophoresis on a 2% agarose gel containing 10mg/mL ethidium bromide solution (Bio-Rad Laboratories, Hercules, CA). The real-time PCR assay for *mec* complex C2 (C2a, C2b) (Higuchi, Takano et al. 2008) was performed with a SmartCycler II (Cepheid, Sunnyvale, CA), using the following cycling conditions: an initial single cycle for 2 minutes at 95°C, followed by 50 cycles of three-temperature cycling consisting of 15 seconds at 95°C, 60 seconds at 50°C, and 30 seconds at 72°C.

Genotyping

Pulsed-field gel electrophoresis using restriction enzyme *Sma*I was performed and analyzed as previously described (de Lencastre, Couto et al. 1994; Tenover, Arbeit et al. 1995). We used the following definitions of relatedness: identical isolates contained no band differences; epidemiologically closely related isolates differed in one to three bands; isolates possibly related epidemiologically differed in four to six bands. Isolates differing by more than six bands to the most common pattern were considered unrelated (Tenover, Arbeit et al. 1995). PFGE patterns for *S. pseudintermedius* were also analyzed by Bionumerics (version 4.6, Applied Maths, Kortrijk, Belgium), using the Dice coefficient to analyze the similarity of banding patterns, and the unweighted pair group method using arithmetic averages (UPGMA) for cluster analysis. An optimization and 1.00 and position tolerance of 1.00 were used for cluster analysis. A similarity cut-off of 80% corresponded to a difference of ≤6 bands and was used to define a PFGE type. A PFGE type, differing by no more than 6 bands was considered a cluster.

The "X" region of the *spa* gene in *S. aureus* isolates AGA-375 and AGA-483 was amplified using by PCR with previously described primers spa 1113f and spa 1514r (Moodley, Stegger et al. 2006). DNA sequences were obtained with an ABI Prism™ 3730*xl* DNA

sequencer (Seqwight Inc., Houston, TX). *Spa* repeat types were identified with custom software developed by us and described below. For MRSA AGA-375 and AGA-483 multilocus sequence typing (MLST) was also performed as previously described (Enright, Day et al. 2000). MLST types were identified using tools at http://saureus.mlst.net. Associations between *spa* types and MLST were made using the Ridom SpaServer (http://spaserver2.ridom.de/index.shtml).

Spa Typer

*spa*Typer is a freely-available web tool for finding and identifying spa repeats in uploaded sequences. A full description and validation of the method will appear elsewhere. Briefly, *spa*Typer is based on a position-specific scoring matrix (PSSM, (Henikoff and Henikoff 1994)) motif identification approach that searches input sequence for subsequences that are highly similar to previously-identified *spa* repeat units. The search proceeds along the sequence in a "sliding window"; the input sequence is divided into overlapping subsequences, and each subsequence is scored using the PSSM. When the score of the subsequence exceeds a cutoff, it is provisionally tagged as a *spa* repeat unit. Adjacent high-scoring subsequences are identified as *spa* repeats. The underlying PSSM was created using an alignment of known *spa* repeat units. The number of columns in the PSSM is 24, corresponding to 24bp, the most common repeat unit length. Because repeat unit length can differ from this, each subsequence is aligned to the PSSM using the Needleman-Wunsch algorithm (Needleman and Wunsch 1970), using the PSSM score to score the alignment. Once the sliding window analysis is complete, the entire repeat is identified as a concatenation of high-scoring repeats. The longest possible such concatenation is returned and checked against a list of known repeat types. *spa*Typer correctly identifies all currently known repeat types and repeat units, as provided at

http://spaserver2.ridom.de/spatypes.shtml and http://spaserver2.ridom.de/repeats.shtml, as well as random concatenations of known repeats.

*spa*Typer is available at http://fortinbras.us/spaTyper.

Toxin genotyping by PCR assays

Distribution of virulence genes in *S. aureus* isolates was determined by PCR. Toxin genes tested by referenced primer sets include the following: *sea, seb, sec, sed*, and *see* (Hazariwala, Sanders et al. 2002); *seg, seh, sei sem, sen,* and *seo* (Jarraud, Mougel et al. 2002); *sek, sel, sep, seq* (Diep, Carleton et al. 2006); *sej* (Rosec and Gigaud 2002); *eta, etb,* and *tsst-1 (Becker, Haverkamper et al. 2001)*; and *pvl* (Lina, Piemont et al. 1999). Previously listed NARSA strains and some in-house isolates were used as positive controls in each PCR. All PCR assays were performed from a 1:20 dilution of the extracted DNA to water. An aliquot of 1µL of this DNA suspension was added to 9µL of a PCR mixture containing 3.0mM $MgCl_2$ (Idaho Technology, Inc., Salt Lake City, UT), 0.2mM of each deoxynucleotidetriphosphate (dATP, dUTP, dGTP, dCTP) (Roche, Basel, Switzerland), 0.1mM of the forward and reverse primers, and 0.5 units of *Taq* polymerase (Roche, Basel, Switzerland). The amplification was performed in the RapidCycler (Idaho Technology, Inc. Salt Lake City, UT) beginning with an initial denaturation step at 94°C, followed by 30 cycles at previously described annealing temperatures (Lina, Piemont et al. 1999; Becker, Haverkamper et al. 2001; Hazariwala, Sanders et al. 2002; J.P. Rosec 2002; Jarraud, Mougel et al. 2002; Diep, Carleton et al. 2006) dependent upon the primer set, ending with an extension step at 72°C for 45 seconds. Amplified PCR products were visualized and saved using the BioRad Gel Doc™ 2000, digital camera and the Quantity One software after electrophoresis on a 2% agarose gel containing 10mg/mL ethidium bromide solution (Bio-Rad Laboratories, Hercules, CA).

Results

Bacterial Strains

76 phenotypically methicillin-resistant *S. aureus* (MRSA) isolates and an additional 77 phenotypically methicillin-resistant non-aureus staphylococci were used in this study. Species identity was confirmed by *nuc*A PCR (Brakstad, Aasbakk et al. 1992). Human MRSA isolate AGA-483 was PCR negative for *nucA*. However, biochemical tests run on API strips confirmed its species identity with 97% certainty as *S. aureus*. Additionally, we found from the partial gene sequence of the 16S rDNA that MRSA AGA-483 had 99% identity to *S. aureus* (Sasaki, Kikuchi et al. 2007).

Within the non-aureus staphylococci, we identified 5 coagulase-negative staphylococci (CNS) and 72 coagulase-positive staphylococci isolates. Partial 16S gene sequencing identified the CNS isolate AVDL-15372 as *S. schleiferi* subsp. *schleiferi*. The remaining four CNS isolates were irrelevant to this study and thus not identified further with regards to species. Partial gene sequencing of *sod*A from coagulase-positive staphylococci identified 21 isolates as *Staphylococcus schleiferi* subsp. *coagulans*, 48 isolates as *Staphylococcus pseudintermedius*. The remaining 3 isolates could not be confirmed beyond identification to the *Staphylococcus intermedius* Group. Biochemical tests for these isolates were positive for arginine dihydrolase activity, an enzyme seen in *S. pseudintermedius* and *S. delphini*, but absent in *S. intermedius* (Sasaki, Kikuchi et al. 2007). Given also that the isolates were collected from canine sources, these results indicate the isolate likely to be *S. pseudintermedius* (Sasaki, Kikuchi et al. 2007).

SCCmec Typing and Sequencing

All isolates contained the *mec*A gene. SCC*mec* typing using the primers by Zhang et al. identified two MRSA isolates (Table 3.2) and 46 non-aureus staphylococci (one *S. schleiferi*

subsp. *schleiferi*, one *S. schleiferi* subsp. *coagulans*, 42 *S. pseudintermedius* and two *S. intermedius* Group isolates) with the SCC*mec* V element (Table 3.3) (Zhang, McClure et al. 2005). Only these 48 isolates will be further discussed in this manuscript. A larger epidemiologic study will published elsewhere (Still et al., manuscript in progress.

Sequences generated using these primers showed 100% identity between the sequenced region, hypothetical protein V011 of the human MRSA isolates AGA-375 and AGA-483 and previously reported SCC*mec* type V strain JCSC3624 (Wis) (Ito, Ma et al. 2004). 99% identity was seen in this region between the canine non-aureus isolates AVDL-32616, AVDL-7923, AVDL-13449 and Wis. Sequence identities were as reported by NCBI BLAST searches. In an updated multiplex by Milheiriço, the primers for the *ccr*C complex were used to confirm the isolates as SCC*mec* type V (Milheirico, Oliveira et al. 2007), however the primers for the mecV J1 region were PCR-negative. These discordant results occurred for all isolates and were unexpected.

Recent sequencing by several groups have revealed that a SCC*mec* V variant in Taiwan (SCC*mec* V_T) showed high similarity to SCC*mec* type V strain JCSC3624 (Wis). SCC*mec* V_T sequencing distinguished two new ccr genes, *ccr*C2 and *ccr*C8, not found in the conventional *ccr*C-containing SCC*mec* V (Boyle-Vavra, Ereshefsky et al. 2005; Higuchi, Takano et al. 2008; Takano, Higuchi et al. 2008). SCC*mec* V_T was consequently renamed SCC*mec* VII (Higuchi, Takano et al. 2008). Because of the close relatedness of SCC*mec* VII to SCC*mec* V leading to the possibility of misidentification and the unusual PCR results found in our laboratory, we performed the multiplex described by Higuchi et al. and sequenced the resulting amplicons (Higuchi, Takano et al. 2008). MRSA isolates AGA-375 and AGA-483 and 21 canine non-aureus isolates were PCR positive for both *ccr*C2 and *ccr*C8, indicating the presence of SCC*mec*

VII. 17 canine non-aureus isolates were PCR positive for only *ccr*C2 and not *ccr*C8, an observation that has not been described previously. As such it is distinct from SCC*mec* VII and is referred to as the variant SCC*mec* VII_{GA1} element below. The remaining 8 isolates that were previously indicated to be SCC*mec* V, were PCR negative for both *ccr*C2 and *ccr*C8.

To further study the differences in the SCC*mec* VII variant, overlapping primer sets were designed for PCR assaying and sequencing the *ccr*C2 and *ccr*C8 genes (Table 3.1), which are distinguishing features in the SCC*mec* VII element (Higuchi, Takano et al. 2008). All 48 isolates (two MRSA and 46 non-aureus staphylococci) that tested PCR positive for SCC*mec* V by Zhang et al. and the *ccr*C primers from Milheiriço et al., were PCR positive for the three *ccr*C2 and the three *ccr*C8 primers designed by us. Isolates PCR-negative for *ccr*C2 and *ccr*C8 using the primers published by Higuchi et al. (Higuchi, Takano et al. 2008), but PCR-positive for the genes using our developed primer sets are referred to as the variant SCC*mec* VII_{GA2} element below. Additionally, all 48 isolates (23 SCC*mec* VII, 17 SCC*mec* VII_{GA1}, and 8 SCC*mec* VII_{GA2}) regardless of variation within the *ccr*C2 and *ccr*C8 genes were confirmed as containing the *mec* complex C2b, unique to SCC*mec* VII, by the real-time PCR assay as described by Higuchi et al (Higuchi, Takano et al. 2008).

Human MRSA SCC*mec* VII isolates AGA-375 and AGA483, *S. pseudintermedius* SCC*mec* VII_{GA1} isolate AVDL-32616 and *S. schleiferi* subsp. *schleiferi* SCC*mec* VII_{GA2} isolate AVDL-15372 were selected for *ccr*C2 and *ccr*C8 sequencing (Figure 1A-B, GenBank Accessions FJ931046 – FJ931053). AGA-375 had a total of 25 (1.5%) base pair differences from the published 1680bp *ccr*C2 gene, resulting in 154 amino acid changes (including 28 premature stop codons) and 14 (0.8%) base pair differences from the 1677bp published *ccr*C8 gene, resulting in 4 amino acid changes. AGA-483 contained no differenced from the published

*ccr*C2 and only 1 (.06%) from *ccr*C8, resulting in 1 amino acid change. AVDL-32616 had 42 (2.5%) base pair differences from *ccr*C2 and 77 (4.6%) base pair differences from *ccr*C8, resulting in 11 and 27 amino acid changes, respectively. AVDL-15372 was composed of 23 (1.4%) base pair differences from *ccr*C2 and 57 (3.4%) base pair differences from *ccr*C8, resulting in 5 amino acid changes and a deletion causing a frameshift, respectively. Sequence variation within the annealing region of the Higuchi et al. ccrC2 and ccrC8 primers resulted in negative PCR assays for 8 of our isolates and 17 others were only shown to contain ccrC2 by this method (Figure 3.1). Taken together, these results confirm the presence of SCC*mec* VII and variant SCC*mec* VII elements in *S. aureus, S. pseudintermedius, S. schleiferi* subsp. *coagulans*, and *S. schleiferi* subsp. *schleiferi* in the United States.

Genotyping

Are the SCC*mec* VII cassettes present in epidemiologically important and successful MRSA strains? PFGE, *spa* sequencing, and MLST were performed to answer this question for our *S. aureus* isolates, to identify the ancestral genetic background of our type VII strains. PFGE (Figure 3.2) revealed that the *Sma*I restriction pattern of our two MRSA isolates did not match any of the most common *S. aureus* strains currently circulating in the United States. Furthermore, the isolates were evidently unrelated to one another. *spa* sequencing was carried out only for the two MRSA strains. AGA-483 contained an 11-unit repeat with *spa* type repeat profile W1:G1:K1:K1:A1:K1:A1:O1:M1:Q1:Q1, Ridom type t017. This type has been previously associated with the multilocus sequence type (MLST) type ST30. AGA-375 contained a single repeat unit with the Kreiswirth designation T1, Ridom spa type t458. This *spa* type has not previously been associated with an MLST type. MLST was performed for the *S.*

aureus isolates, which confirmed that MRSA AGA-375 was indeed ST30. MRSA AGA-483 was determined to be ST5 (Table 3.2).

Are the SCC*mec* VII cassettes present in epidemiologically related and successful MRSP strains? PFGE analysis of *Sma*I restricted *S. pseudintermedius* revealed two major PFGE groups differing from each other by ten bands or more (Figure 3.3). The larger and more clonal group, Cluster 1, contains 40 (85%) of the isolates. 39 of these isolates contain the SCC*mec* VII and one non-typeable SCC*mec* element. The second group contains Clusters 2-6, which encompass 7 (15%) of the isolates. 1 isolates is SCC*mec* IVg, 2 are SCC*mec* VII, and four contain non-typeable SCC*mec* elements.

Toxin gene profiling

We examined the virulence gene complement for different SCC*mec* types among the MRSA isolates (Table 3.2). AGA-375 was PCR-positive for the toxins SEG, SEI, SEM, SEN, SEO, SEQ, while AGA-483 was PCR-positive for the toxins SEC, SEG, SEK, SEL, SEN, SEO, SEP, SEQ. Neither of the human MRSA SCC*mec* VII isolates contained the gene encoding for Panton-Valentine leukocidin (PVL) expression, which has been previously shown to be associated with community-acquired MRSA strains.

Antimicrobial sensitivity

The two SCC*mec* type VII MRSA isolates demonstrated significant antimicrobial resistance to a variety of the tested antibiotics (Table 3.2). Notably, the two isolates were resistant to tetracycline, ceftiofur (a third-generation cephalosporin), and clindamycin (a lincosamide). Additionally, 43 (92%) of the MRSP isolates exhibited antimicrobial resistance to at least five of the eight drug classes tested (Figure 3.4).

Discussion

In this study, we identified the SCC*mec* type VII element in 21 canine *S. pseudintermedius* isolates and two human CA-MRSA isolates collected in Georgia. Additionally, the variant SCC*mec* VII_{GA} was found in 17 isolates (one feline *S. pseudintermedius,* one canine *S. schleiferi* subsp. *coagulans*, and 15 canine *S. pseudintermedius*) and the variant SCC*mec* VII_{GA2} was identified in 8 isolates (one canine *S. schleiferi* subsp. *schleiferi* and 7 canine *S. pseudintermedius*) (Tables 2 and 3). It is important to note here the inability of these older PCR assays to distinguish some SCC*mec* VII variant cassettes. Due to variation within the primer attachment sites along the DNA in SCC*mec* VII_{GA} and SCC*mec* VII_{GA2} these variant cassettes from non-aureus staphylococci containing the genes for *ccr*C2 and *ccr*C8 may be missed if the real-time PCR assay for the *mec* complex C2b is not also tested (Figure 1).

We wanted to determine if the SCC*mec* VII cassettes found in our study are present in epidemiologically important and successful MRSA and MRSP strains. PFGE, *spa* typing, and MLST were performed to this end for our MRSA isolates. PFGE and *spa* typing indicated that that our isolates did not match most common currently circulating MRSA strains in the United States and were evidently unrelated to one another. *S. aureus* MLST types ST5 and ST30 have been observed previously in CA-MRSA isolates, suggesting that our isolates share a common ancestry with the most prevalent MRSA strains in the United States (Robinson and Enright 2004). It could be argued that the collection of two SCC*mec* VII isolates from human patients at the same hospital within 4 days of each other may be suspect. Without patient history we can make no conclusions about whether these patients were related or had close contact with one another, in addition to any relevant recent travels, such as to Taiwan, where these MRSA lineages have been found previously to harbor SCC*mec* VII. However, given that the two MRSA

strains are genetically distinct, it is unlikely that the two patients exchanged the strains harboring SCC*mec* VII.

PFGE analysis of our MRSP isolates revealed that these pathogenic isolates seem to belong to a very clonal group, which carries the SCC*mec* VII or one of two variants that have not been described previously in the United States in humans or animals. This suggests that the SCC*mec* VII element entered into a few different MRSP backgrounds at some time in history and then a large clonal expansion led to the predominance of the MRSP strain seen in Cluster 1 (Figure 3).

The distribution of SCC*mec* VII among different staphylococcal species recovered worldwide is not yet known and deserves further study. The primary reports of SCC*mec* VII include only MRSA isolates from Taiwan (Boyle-Vavra, Ereshefsky et al. 2005; Higuchi, Takano et al. 2008; Takano, Higuchi et al. 2008). The actual distribution of this element could be much more widespread, however. The presence of SCC*mec* VII in four different staphylococcal species (*S. aureus*, *S. pseudintermedius*, *S. schleiferi* subsp. *coagulans*, and coagulase-negative *Staphylococcus spp.*) suggests the likelihood of horizontal gene transfer between different species of staphylococci, which be passed between both human and animal hosts. We cannot infer which direction this gene transfer has occurred between either staphylococcal species or host species, but transfer has occurred and will likely continue to do so.

We examined the virulence gene complement for SCC*mec* VII MRSA isolates (Table 2) since this has not been examined previously. Neither of the human MRSA SCC*mec* VII isolates contained the gene encoding for Panton-Valentine leukocidin (PVL) expression, which has been previously shown to be associated with CA-MRSA and SCC*mec* VII (Boyle-Vavra, Ereshefsky et al. 2005). A broad spectrum of toxin genes was observed in a novel pattern. Neither isolate's

set of virulence genes is concordant with that of a prototypical SCC*mec* type II or type IVa strain (Diep, Carleton et al. 2006). Analysis of further type VII isolates may reveal a stable consensus pattern.

As the use of antimicrobials in recent years has increased, *S. aureus* has acquired resistance mechanisms to virtually all classes of available antibiotics. Antimicrobial sensitivity tests for the SCC*mec* VII MRSA isolates confirm an alarming trend observed recently that CA-MRSA infections are now much more likely to be resistant to non-beta lactam antibiotics, such as tetracyclines and clindamycin (Szumowski, Cohen et al. 2007). The resistance to both erythromycin and clindamycin, multi-drug resistance, and susceptibility to trimethoprim/sulfamethoxazole (SXT) observed in our MRSA isolates are consistent with previous reports on SCC*mec* VII-containing isolates (Boyle-Vavra, Ereshefsky et al. 2005). However, the human MRSA AGA-375 differs in that it is resistant to fluoroquinolones. Susceptibility testing for MRSP isolates containing SCC*mec* VII and its variants indicate that serious clinical infections in dogs are increasingly caused by multi drug-resistant staphylococci, with 43 (92%) of the MRSP isolates exhibiting antimicrobial resistance to at least five of the eight drug classes tested (Figure 4). These isolates show an increased incidence of multi-drug resistance and resistance to SXT than previously reported (Schwarz, Kadlec et al. 2008; Norstrom, Sunde et al. 2009). No differences in susceptibility were discerned between companion animal isolates containing SCC*mec* VII or either of its variants.

Based upon previous SCC*mec* typing in the United States we did not expect to find any SCC*mec* VII isolates during our study, predicting that the SCC*mec* types seen in animals would simply reflect types that are prevalent in human staphylococcal infections. It is important to note that SCC*mec* VII has been circulating in Georgia for several years now. Specific SCC*mec* VII

surveillance is needed to determine whether its prevalence is on the rise elsewhere in the country. As new clones arise, it is important for surveillance to keep pace, to rapidly distinguish changes in circulating MRSA and MRSP clones so that major epidemiological changes, such as clonal expansion, accompanied and perhaps assisted by virulence and antimicrobial susceptibility changes, may be monitored and used for public health and clinical guidance (Klugman 2002). We believe this study also raises the question of whether and how interactions between human and their companion animals may drive the development of new, clinically important CA-MRSA strains. Direct contact is necessary for the spread of MRSA. The relationship that owners and pets share allow for frequent exchange of commensal bacteria and their genes. Therefore, it would be of public health interest to not only to survey circulating MRSA clones, but also MRSP clones from companion animals. Studying both human and animal models of this bacterial infection will become more important in the coming years to gain a better understanding of the transmissions occurring between host species and their epidemiological consequences, and how we can better understand the dynamics of genetically different MRSA clones in the community.

Table 3.1: Primer sets used for sequencing *ccr*C2 and *ccr*C8 of SCC*mec* VII

Primer	Nucleotide Sequence	Amplicon Size (bp)	Reference
ccrC2 F	5'-ATAAGTTAAAAGCACGACTCA-3'	257	(Higuchi, Takano et al. 2008)
ccrC2 R	5'-TTCAATCCTATTTTTCTTTGTG-3'		
ccrC8 F	5'-GCATGGGTACTCAATCCA-3'	562	(Higuchi, Takano et al. 2008)
ccrC8 R	5'-GGTTGTAATGGCTTTGAGG-3'		
ccrC2a F	5'-AAGGAGCGGTATGATATGAA-3'	635	This study
ccrC2a R	5'-AGAATTGTCGTGATTGCTTT-3'		
ccrC2b F	5'-AGCTCATATTGTACGGGAAA-3'	608	This study
ccrC2b R	5'-CTTCTATCACACTTGACGCA-3'		
ccrC2c F	5'-ATTATTCAATCGCTCAGGAA-3'	692	This study
ccrC2c R	5'-AAACTCCTCCACTTTTGTCA-3'		
ccrC8a F	5'-GAGCGGTATGATATGAAGGG-3'	373	This study
ccrC8a R	5'-CTCCAGAAGCATTGGATGTA-3'		
ccrC8b F	5'-CTCGTTACAATGTTTGGGTT-3'	787	This study
ccrC8b R	5'-CTTCTATCACATTTGACGCA-3'		
ccrC8c F	5'-GAAGTCGTCGAGCGTGTAAT-3'	642	This study
ccrC8c R	5'-CATTTCGCTCACTTTTGTCA-3'		

Table 3.2: Characterization of MRSA isolates carrying SCC*mec* VII

Strain Code	Host	Specimen	Date of Collection	Pathogen	ccr	SCC *mec* Type	*spa* Type	Sequence Type (ST)	Antibiotic Resistance Profile[d]	Virulence Genotype
AGA-375	Human	Foot	11/15/2007	*S. aureus*	ccrC2 and ccrC8	VII	t458	ST5	FOX, CHLR, CLIN, ENRO, ERY, OBX, TET	SEG, SEI, SEM, SEN, SEO, SEQ
AGA-483	Human	Breast	11/19/2007	*S. aureus*	ccrC2 and ccrC8	VII	t017	ST30	FOX, CHLR, CLIN, ERY, OBX, TET	SEC, SEG, SEK, SEL, SEN, SEO, SEP, SEQ
N315[1]	-	-	-	*S. aureus*	ccrA2 and ccrB2	II	NT*	ST5[b]	AMK, FOX, CEPH, CHLR, CLIN, ENRO, ERY, SXT	SEC, SEG, SEI, SEL, SEM, SEN, SEO, SEP, TSST-1
USA300[2]	-	-	-	*S. aureus*	ccrA2 and ccrB2	IVa	t008[a]	ST8[c]	FOX, CEPH, ENRO, ERY, TET	SEK, SEQ, PVL

[1] N315 was acquired from NARSA (isolate NRS70).

[2] USA300 was acquired from NARSA (isolate NRS384).

[a] data from Ridom SpaServer (http://spaserver2.ridom.de/index.shtml)

[b] see Okuma et al. (Okuma, Iwakawa et al. 2002)

[c] data from NARSA

[d] The following antibiotics were tested: amikacin (AMK), cefoxitin (FOX), cephalothin (CEPH), chloramphenicol (CHLR), clavanulate (CLAV), clindamycin (CLIN), enrofloxacin (ENRO), erythromycin (ERY), gentamycin (GNT), orbifloxacin (OBX), oxacillin (OXA), tetracycline (TET), vancomycin (VAN), trimethoprim/sulfa (SXT), levofloxacin (LEV).

* not tested

Table 3.3: Characterization of Methicillin-Resistant *Staphylococcus* spp. Isolates Carrying SCC*mec* VII in Companion Animals

Strain Code	Species Identification	Host Species	Specimen	Date	SCC*mec* Type	PFGE Cluster	Resistance Pattern[e]
A1-15372*	SSsS^				VII_{GA2}	NT[c]	
A4-07923	S. pseudointermedius	Canine	Misc. swab	8/25/2003	VII_{GA2}	2	CLIN/ENRO/ERY/ TET/SXT
A5-017749	S. pseudointermedius	Canine	Misc. swab	10/11/2004	VII_{GA}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A5-027889	S. pseudointermedius	Canine	Wound	12/13/2004	VII	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A5-33311	S. pseudointermedius	Canine	Misc. swab	1/18/2005	VII	UT[d]	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A5-14921	S. pseudointermedius	Canine	Misc. swab	9/27/2004	VII	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A5-18568	S. pseudointermedius	Canine	Ear	10/14/2004	VII_{GA2}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A5-13449	S. pseudointermedius	Canine	Misc. swab	9/20/2004	VII_{GA2}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A3-57743	S. pseudointermedius	Canine	Skin	6/18/2003	VII	1	AMK/CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A7-56240	S. pseudointermedius	Canine	Misc. swab	5/15/2007	VII_{GA}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A7-56988	S. pseudointermedius	Canine	Misc. swab	5/18/2007	VII	1	CLIN/ENRO/ERY/OBX/TET/SXT
A7-16775	S. pseudointermedius	Canine	Joint swab	10/6/2006	VII_{GA}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A7-56032	S. pseudointermedius	Canine	Ear	5/14/2007	VII	1	CLIN/ENRO/ERY/OBX/TET/SXT
A6-5905-3*	S. pseudointermedius				VII_{GA}	1	CLIN/ENRO/ERY/TET/SXT
A6-22233	S. pseudointermedius	Canine	Skin	11/8/2005	VII	1	CLIN/ENRO/ERY/OBX/TET/SXT
A6-54690-2	S. pseudointermedius	Canine	Wound	4/24/2006	VII_{GA}	NT[c]	CLIN/ENRO/ERY/OBX/ TET/SXT
A7-56031	S. pseudointermedius	Canine	Ear	5/14/2007	VII	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A7-56493	S. pseudointermedius	Canine	Ear	5/16/2007	VII	1	AMK /CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-03331	SIG[a]	Canine	Abscess	7/25/2007	VII_{GA}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-01519	S. pseudointermedius	Canine	Ear	7/13/2007	VII	1	CLIN/ENRO/ERY/OBX/ TET/SXT
A8-2891L	S. pseudointermedius	Canine	Joint fluid	7/24/2007	VII	1	CLIN/ENRO/ERY/GNT/OBX/ TET/SXT
A8-2891S	S. pseudointermedius	Canine	Joint fluid	7/24/2007	VII	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-04689	SIG[a]	Canine	Muscle	8/3/2007	VII_{GA2}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-5077R	S. pseudointermedius	Canine	Ear	8/7/2007	VII	1	CLIN/ENRO/ERY/GNT/OBX/ TET/SXT

A8-14157	S. pseudointermedius	Canine	Pustule	10/9/2007	VII_{GA}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-09381	S. pseudointermedius	Canine	Tissue	9/7/2007	VII_{GA2}	1	GNT
A8-50776*	S. pseudointermedius				VII_{GA}	NT[c]	
A8-19030	S. pseudointermedius	Canine	Wound	11/9/07	VII_{GA}	NT[c]	CLIN/ENRO/ERY/OBX/SXT
A8-32639	S. pseudointermedius	Canine	Ear	2/14/2008	VII	1	CLIN/ENRO/ERY/OBX/ TET/SXT
A8-27924	S. pseudointermedius	Canine	Transtracheal wash	1/17/2008	VII	1	CLIN/ENRO/ERY/OBX/TET/SXT
A8-11195	S. pseudointermedius	Canine	Ear	9/18/2007	VII	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-22374	S. pseudointermedius	Canine	Ear	12/5/2007	VII_{GA}	1	CLIN/ENRO/ERY/OBX/TET/SXT
A8-24008	S. pseudointermedius	Canine	Draining tract	12/17/2007	VII_{GA}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-27634	S. pseudointermedius	Canine	Wound	1/16/2008	VII	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-10561	SSsC[b]	Canine	Ear	9/14/07	VII_{GA}	NT[c]	Only β-lactams
A8-22507	S. pseudointermedius	Canine	Misc. swab	12/6/2007	VII_{GA}	1	CLIN/ENRO/ERY/GNT/OBX/SXT
A8-21234	S. pseudointermedius	Feline	Wound	11/27/2007	VII_{GA}	1	ENRO/ERY/OBX/TET/SXT
A8-22004	S. pseudointermedius	Canine	Skin	12/3/2007	VII_{GA2}	1	CLIN/ENRO/ERY/OBX/TET/SXT
A8-9329-1	S. pseudointermedius	Canine	Ear	9/7/2007	VII_{GA}	1	CLIN/ENRO/ERY/OBX/TET/SXT
A8-41479-1	S. pseudointermedius	Canine	Ear	4/1/2008	VII	1	CLIN/ENRO/ERY/OBX/TET/SXT
A8-45700	S. pseudointermedius	Canine	Pustule	4/23/2008	VII	1	CLIN/ENRO/ERY/OBX/TET/SXT
A8-45699-1	S. pseudointermedius	Canine	Ear	4/23/2008	VII_{GA}	1	CLIN/ENRO/ERY/OBX/TET/SXT
A8-42808	S. pseudointermedius	Canine	Skin	4/8/2008	VII	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A8-39869	S. pseudointermedius	Canine	Skin	3/24/2008	VII	1	CLIN/ENRO/ERY/OBX/ TET/SXT
A8-34576	S. pseudointermedius	Canine	Skin	2/26/2008	VII_{GA2}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT
A6-32616	S. pseudointermedius	Canine	Catheter tip	1/17/06	VII_{GA}	1	CLIN/ENRO/ERY/GNT/OBX/TET/SXT

Table 3 lists all SCC*mec* VII non-aureus isolates collected from companion animals during the study period June 2001 through April 2008.

*indicates incomplete records

^ SSsS (*Staphylococcus schleiferi* subsp. Schleiferi)

[a] SIG(*Staphylococcus intermedius* Group)

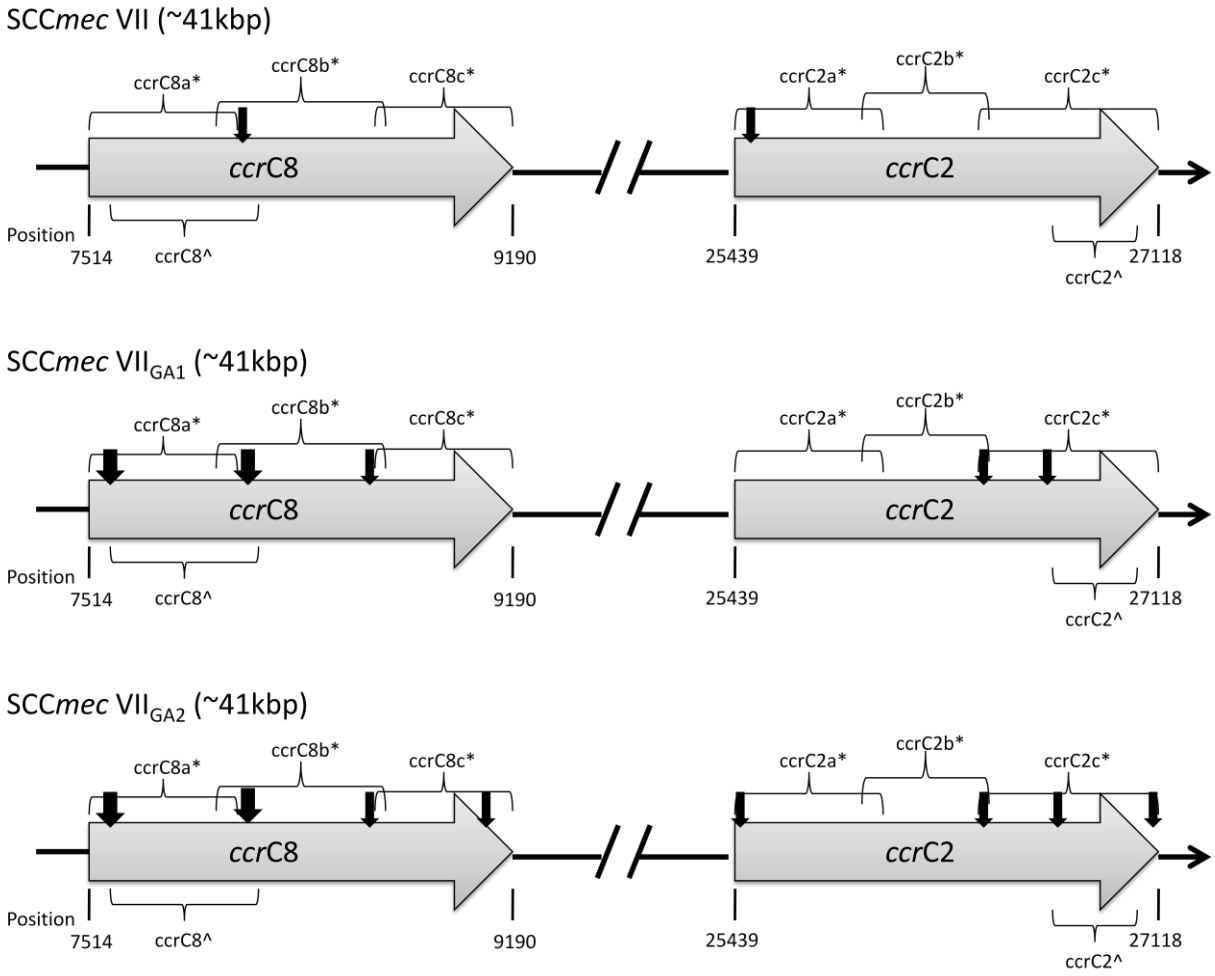
[b] SSsC (*Staphylococcus schleiferi* subsp. Coagulans)

[c] NT (not tested)

[d] UT (untypeable)

[e] The following antibiotics were tested: amikacin (AMK), clindamycin (CLIN), enrofloxacin (ENRO), erythromycin (ERY), gentamycin (GNT), orbifloxacin (OBX), oxacillin (OXA), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT).

A.


SCCmec VII (~41kbp)
ccrC8a*
ccrC8b*
ccrC8c*
ccrC2a*
ccrC2b*
ccrC2c*
ccrC8
ccrC2
Position
7514
ccrC8^
9190
25439
27118
ccrC2^
SCCmec VII$_{GA1}$ (~41kbp)
ccrC8a*
ccrC8b*
ccrC8c*
ccrC2a*
ccrC2b*
ccrC2c*
ccrC8
ccrC2
Position
7514
ccrC8^
9190
25439
27118
ccrC2^
SCCmec VII$_{GA2}$ (~41kbp)
ccrC8a*
ccrC8b*
ccrC8c*
ccrC2a*
ccrC2b*
ccrC2c*
ccrC8
ccrC2
Position
7514
ccrC8^
9190
25439
27118
ccrC2^

B.

```
                 40                                                                105   1087
ccrC2^           TCTGAGCAGTCAGAACATGGTTATTCTGAAAAGGAGCAGGAACAACTACTCATCAAAGAAGTTATG    GGAAGTTCT
16483-ccrC2      TCTGAGCAGTCAGAACATGGTTATTCTGAAAAGGAGCAGGAACAACTACTCATCAAAGAAGTTATG    GGAAGTTCT
16375-ccrC2      TCAGAGCAATCGGAGCATGGGTACTCAATCCATGAGCAAGAACAAGTACTCATCAAAGAGGTTGTG    GGAAGTTCT
15372-ccrC2      TCAGAGCAGTCGGAGCATGGGTACTCAATCCATGAGCAGGAACAAGTACTCATCAAAGAAGTTGTG    GGAAGTTCT
32616-ccrC2      TCTGAGCAGTCAGAACATGGTTATTCTGAAAAGGAGCAGGAACAACTACTCATCAAAGAAGTTATG    GGGAGTTCT


ccrC2^           GCCTGTCATAGTAATGCGATTAATGCTGAAGTCGTCGAACGCGTAATCAATGTTCATTTGAATCGTATTCTTTCACAA
16483-ccrC2      GCCTGTCATAGTAATGCGATTAATGCTGAAGTCGTCGAACGCGTAATCAATGTTCATTTGAATCGTATTCTTTCACAA
16375-ccrC2      GCCTGTCATAGTAATGCGATTAATGCTGAAGTCGTCGAACGCGTAATCAATGTTCATTTGAATCGTATTCTTTCACAA
15372-ccrC2      GCCTGTCATAGTAATGCGATTAATGCTGAAGTCGTCGAACGCGTAATCAATGTTCATTTGAATCGTATTCTTTCACAA
326165-ccrC2     GCATGTCACAGTAATTCCATCAATGCTGAAGTCGTCGAGCGTGTAATTAATGTTCATTTGAATCGTATTCTGTCTCAA


                           1184 1245
ccrC2^           CCTAATGTTATT    ATAAA-ATATGATATTGATAGTCTAGAAAAACAAAAAGCAAAAGTTAAAACACAACAAGAACG
16483-ccrC2      CCTAATGTTATT    ATAAA-ATATGATATTGATAGTCTAGAAAAACAAAAAGCAAAAGTTAAAACACAACAAGAACG
16375-ccrC2      CCTAATGTTATT    ATAAATATATGATATTGATAGTCTAGAAAAACAAAAAGCAAAAGTTAAAACACAACAAGAACG
15372-ccrC2      CCTAATGTTATT    ATAAA-ATATGATATTGATAGTCTAGAAAAACAAAAAGCAAAAGTTAAAACACAACAAGAACG
32616-ccrC2      CCAGATATTATC    ATTAA-ATATGACATTGATAGTTTAGAAAAACAAAAAGCTAAGCTTAAAACACAACAAGAACG


                                               1337
ccrC2^           ATTATTGGAATTGTTCTTAGATGATGAAAT
16483-ccrC2      ATTATTGGAATTGTTCTTAGATGATGAAAT
16375-ccrC2      ATTATTGGAATTGTTCTTAGATGATGAAAT
15372-ccrC2      ATTATTGGAATTGTTCTTAGATGATCAGAT
32616-ccrC2      ATTGTTAGAATTGTTCTTAGATGATCAGAT
```

C.

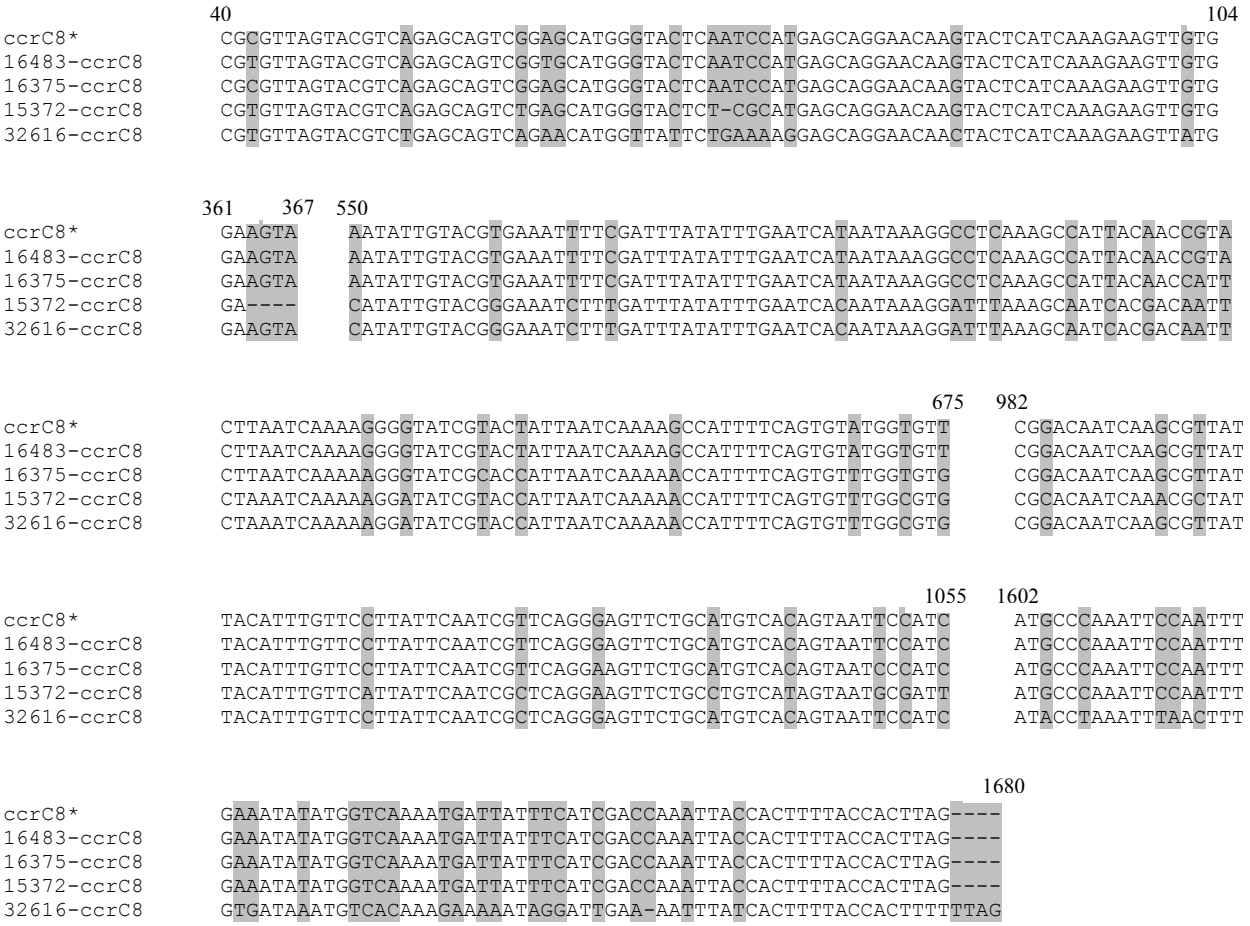


Figure 3.1: Schematic Diagrams and Sequencing results of *ccr*C2 and *ccr*C8 from isolates containing SCC*mec* VII, SCC*mec* VII_{GA} and SCC*mec* VII_{GA2}

Figure **1A** shows a schematic diagram of the genes *ccr*C2 and *ccr*C8 from MRSA isolates SCC*mec* VII, SCC*mec* VII_{GA}, and SCC*mec* VII_{GA2}. The position markings indicate the base pair positions at which the *ccr*C2 and *ccr*C8 genes were previously determined located in SCC*mec* VII by sequencing (Boyle-Vavra, Ereshefsky et al. 2005; Higuchi, Takano et al. 2008; Takano, Higuchi et al. 2008). Brackets situated above and below each of the genes indicated regions that were PCR-amplified and sequenced using primers described in this study (*) and primers

described by Higuchi et al. (^). Amplified DNA was sequenced from human MRSA SCC*mec* VII isolates AGA-375 (GenBank Accessions FJ931046 and FJ931050) and AGA-483 (GenBank Accessions FJ931047 and FJ931051), *S. pseudintermedius* SCC*mec* VII_{GA1} isolate AVDL-32616 (GenBank Accessions FJ931048 and FJ931052) and *S. schleiferi* subsp. Schleiferi SCC*mec* VII_{GA2} isolate AVDL-15372 (GenBank Accessions FJ931049 and FJ931053). Downward pointing arrows along the two genes indicate sequenced regions with higher variability in nucleotide presence. The thickness of these arrows is indicative of the size of the variable region. Figure **1B** shows in frame DNA sequences of regions with the highest variability within the *ccr*C2 and *ccr*C8 (Figure **1C)** genes for human MRSA SCC*mec* VII isolates AGA-375 and AGA-483, *S. pseudintermedius* SCC*mec* VII_{GA1} isolate AVDL-32616 and *S. schleiferi* subsp. Schleiferi SCC*mec* VII_{GA2} isolate AVDL-15372. Numbers indicate nucleotide position as compared with previously sequenced *ccr*C2 (GenBank Accession AB353125), dashes (-) indicate deletions of nucleotides, and grey-highlighted regions indicate nucleotide differences between isolates.

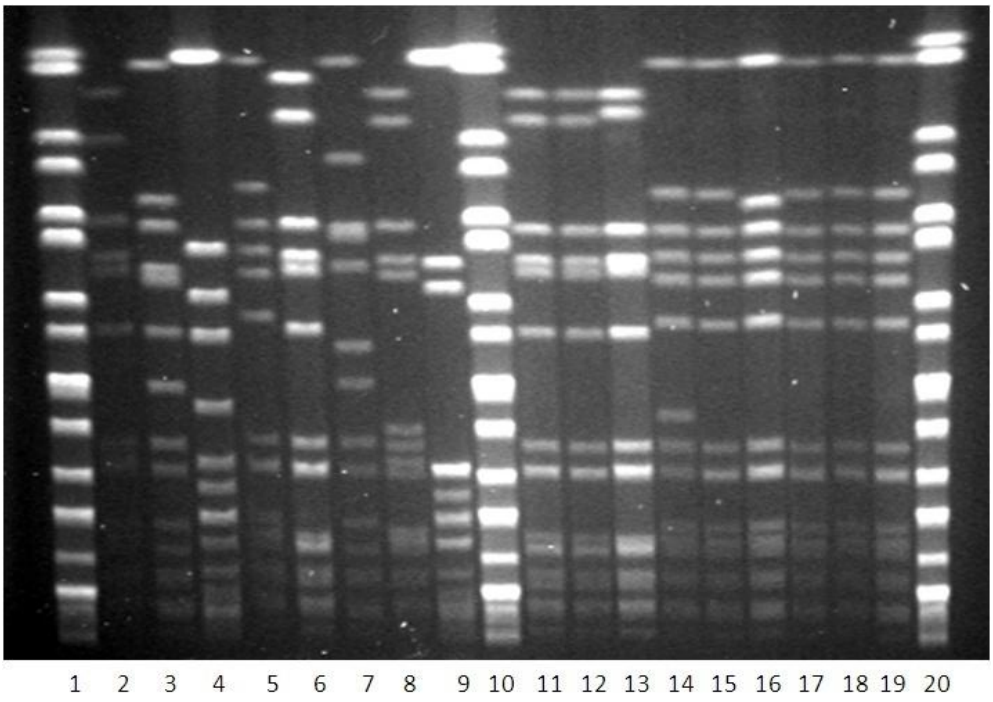


Figure 3.2: PFGE of MRSA isolates containing SCC*mec* VII

PFGE of *Sma*I restriction fragments among MRSA representative clones in the United States compared with SCC*mec* VII isolates. Lanes 1, 10, and 20 are a Salmonella Standard, ATCC BAA-664 digested by *Xba*I, used for comparison. Lanes 2-7 are control strains from NARSA. Lane 2: N315, Lane 3: NCTC8325, Lane 4: USA200, Lane 5: USA300, Lane 6: USA400, Lane 7: USA500. Lanes 8 and 9 contain our MRSA isolates of interest. Lane 8 is AGA-375. Lane 9 is AGA-483. Lanes 11-13 are prototypical MRSA strains collected in our laboratory containing the SCC*mec* II element. Lanes 14-19 are prototypical CA-MRSA strains collected in our laboratory containing the SCC*mec* IVa element.

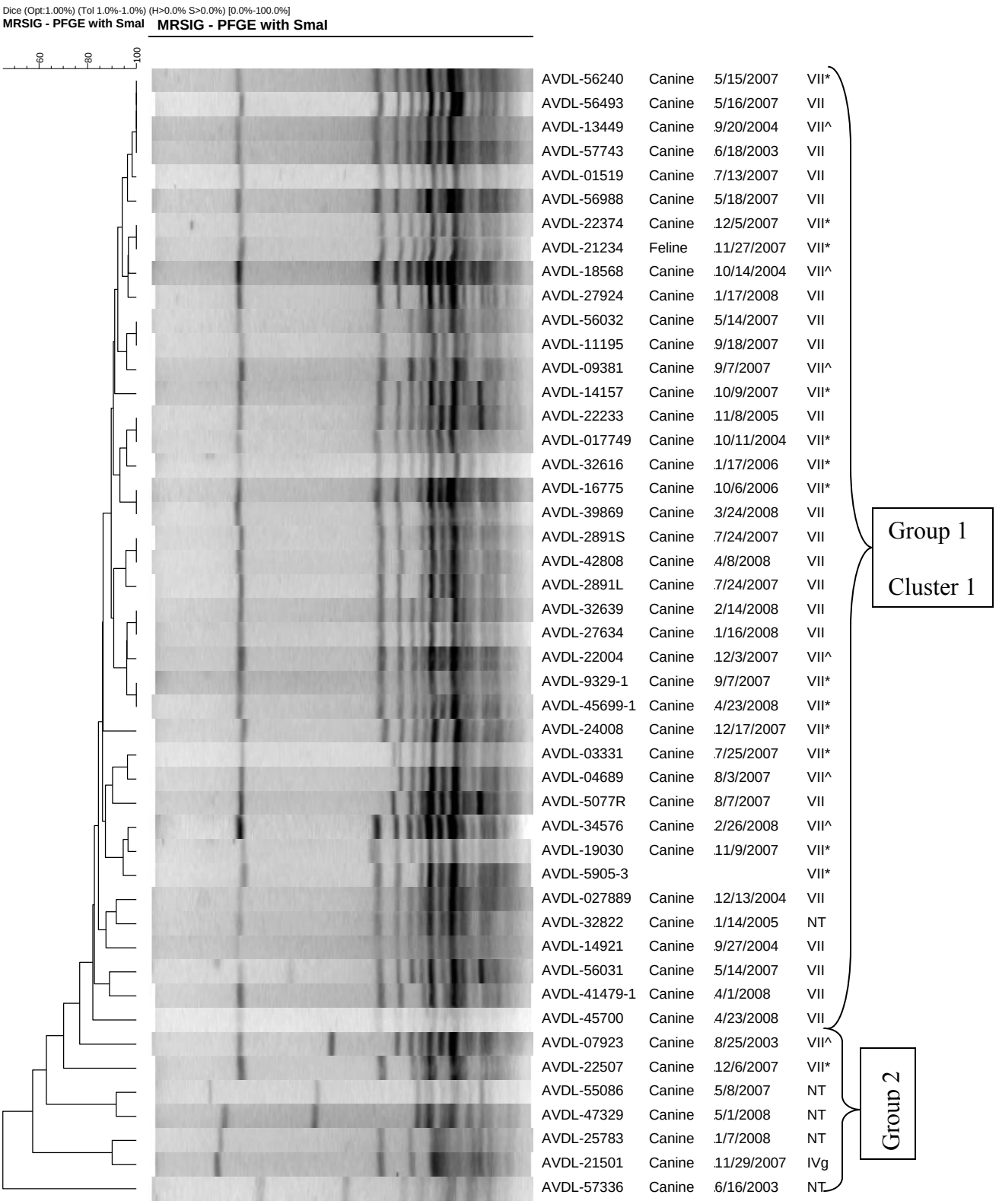

Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
MRSIG - PFGE with SmaI
MRSIG - PFGE with SmaI
60
80
100
AVDL-56240 Canine 5/15/2007 VII*
AVDL-56493 Canine 5/16/2007 VII
AVDL-13449 Canine 9/20/2004 VII^
AVDL-57743 Canine 6/18/2003 VII
AVDL-01519 Canine 7/13/2007 VII
AVDL-56988 Canine 5/18/2007 VII
AVDL-22374 Canine 12/5/2007 VII*
AVDL-21234 Feline 11/27/2007 VII*
AVDL-18568 Canine 10/14/2004 VII^
AVDL-27924 Canine 1/17/2008 VII
AVDL-56032 Canine 5/14/2007 VII
AVDL-11195 Canine 9/18/2007 VII
AVDL-09381 Canine 9/7/2007 VII^
AVDL-14157 Canine 10/9/2007 VII*
AVDL-22233 Canine 11/8/2005 VII
AVDL-017749 Canine 10/11/2004 VII*
AVDL-32616 Canine 1/17/2006 VII*
AVDL-16775 Canine 10/6/2006 VII*
AVDL-39869 Canine 3/24/2008 VII
AVDL-2891S Canine 7/24/2007 VII
AVDL-42808 Canine 4/8/2008 VII
AVDL-2891L Canine 7/24/2007 VII
AVDL-32639 Canine 2/14/2008 VII
AVDL-27634 Canine 1/16/2008 VII
AVDL-22004 Canine 12/3/2007 VII^
AVDL-9329-1 Canine 9/7/2007 VII*
AVDL-45699-1 Canine 4/23/2008 VII*
AVDL-24008 Canine 12/17/2007 VII*
AVDL-03331 Canine 7/25/2007 VII*
AVDL-04689 Canine 8/3/2007 VII^
AVDL-5077R Canine 8/7/2007 VII
AVDL-34576 Canine 2/26/2008 VII^
AVDL-19030 Canine 11/9/2007 VII*
AVDL-5905-3 VII*
AVDL-027889 Canine 12/13/2004 VII
AVDL-32822 Canine 1/14/2005 NT
AVDL-14921 Canine 9/27/2004 VII
AVDL-56031 Canine 5/14/2007 VII
AVDL-41479-1 Canine 4/1/2008 VII
AVDL-45700 Canine 4/23/2008 VII
AVDL-07923 Canine 8/25/2003 VII^
AVDL-22507 Canine 12/6/2007 VII*
AVDL-55086 Canine 5/8/2007 NT
AVDL-47329 Canine 5/1/2008 NT
AVDL-25783 Canine 1/7/2008 NT
AVDL-21501 Canine 11/29/2007 IVg
AVDL-57336 Canine 6/16/2003 NT
Group 1
Cluster 1
Group 2

Figure 3.3: PFGE of MRSP isolates containing SCC*mec* VII, VII_{GA1} and VII_{GA2}

Pulsed-field gel electrophoresis (PFGE) using restriction enzyme *Sma*I was performed and analyzed as previously described (de Lencastre, Couto et al. 1994; Tenover, Arbeit et al. 1995). The tree diagram includes 47 *S. pseudintermedius* isolates. PFGE patterns were also analyzed by Bionumerics (version 4.6, Applied Maths, Kortrijk, Belgium) by using the Dice coefficient to analyze the similarity of banding patterns, and the unweighted pair group method using arithmetic averages (UPGMA) for cluster analysis. An optimization and 1.00 and position tolerance of 1.00 were used for cluster analysis. A similarity cut-off of 80% which corresponded to a difference of ≤6 bands was used to define a PFGE type. The PFGE type was considered different if seven or more band differences were observed (Tenover, Arbeit et al. 1995). A PFGE type, differing by no more than 6 bands was considered a cluster. Clusters were numbered one through six, with cluster 1 containing the most number of isolates. * indicates isolates with SCC*mec* VII_{GA} and ^ indicates isolates with SCC*mec* VII_{GA2}.

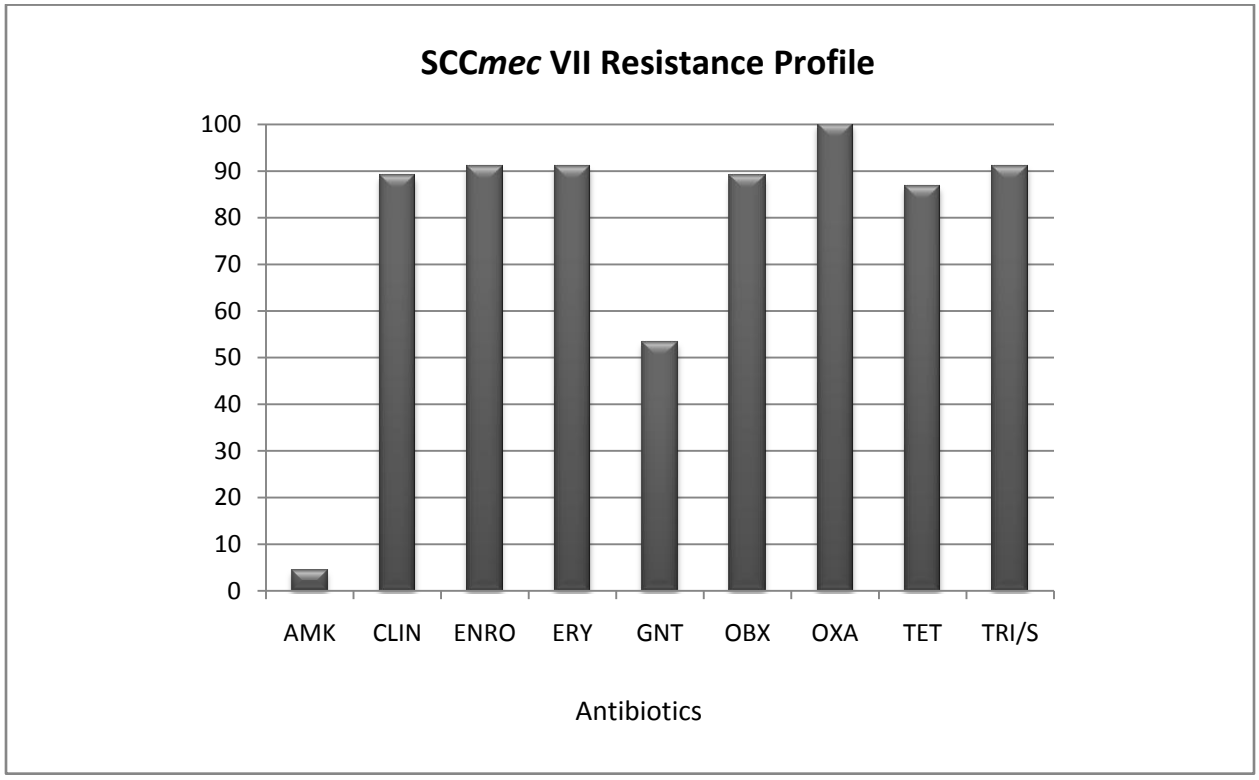


Figure 3.4: *Staphylococcus pseudintermedius* SCC*mec* VII, VII_{GA}, and VII_{GA2} Resistance Profile

The following antibiotics were tested: amikacin (AMK), clindamycin (CLIN), enrofloxacin (ENRO), erythromycin (ERY), gentamycin (GNT), orbifloxacin (OBX), oxacillin (OXA), tetracycline (TET), and trimethoprim/sulfamethoxazole (TRI/S).

CHAPTER 4

SUMMARY AND OVERALL CONCLUSIONS

MRSA Epidemiology in Companion Animals

Although MRSA in the United States has been described extensively in the human population and some in the animal population, this is the most extensive and complete epidemiologic study comparing the genetic relatedness and characterization of human and companion animal MRSA isolates in the same study. The clear increase in the prevalence of MRSA-infected animals observed during this study period demonstrates the need of continued epidemiological monitoring. In Georgia, we have seen a shift in the prevalence of clinical MRSA SCC*mec* types in people from SCC*mec* II to IV, but not animal MRSA; MRSA SCC*mec* II is more prevalent in dogs and that MRSA SCC*mec* IV-nonA is more prevalent in horses. Pulsed-field gel electrophoresis (PFGE), *spa* typing, toxin genotyping and antimicrobial resistance patterns indicated that the MRSA isolates containing the same SCC*mec* type share the same characteristics, whichever host a SCC*mec* types is in. Additionally, this study demonstrated that both companion animals and people may be infected by the same strains of MRSA and that the zoonotic potential is real. However, our study also revealed that some MRSA strains may be better suited to cause disease in animal hosts. Based on this study, we believe that though there have been previously reported specific cases of MRSA zoonosis, it only accounts for a small portion of the epidemiologic relationship between human and companion animal infectious MRSA strains.

Detection of SCCmec VII in Staphylococcus spp.

In this study, we identified the SCC*mec* type VII element in 21 canine *S. pseudintermedius* isolates and 2 human CA-MRSA isolates collected in Georgia. Additionally, the variant SCC*mec* VII_{GA} was found in 17 isolates (1 feline *S. pseudintermedius,* 1 canine *S. schleiferi* subsp. coagulans, and 15 canine *S. pseudintermedius*) and the variant SCC*mec* VII_{GA2} was identified in 8 isolates (one canine *S. schleiferi* subsp. schleiferi and 7 canine *S. pseudintermedius*) (Tables 3.2 and 3.3). Based upon previous SCC*mec* typing in the United States we did not expect to find any SCC*mec* VII isolates during our study, predicting that the SCC*mec* types seen in animals would simply reflect types that are prevalent in human staphylococcal infections. Importantly, SCC*mec* VII has been circulating in Georgia for several years now. Specific SCC*mec* VII surveillance is needed to determine whether its prevalence is on the rise elsewhere in the country. The presence of SCC*mec* VII in four different staphylococcal species (*S. aureus*, *S. pseudintermedius*, *S. schleiferi* subsp. coagulans, and coagulase-negative *Staphylococcus spp.*) suggested the likelihood of horizontal gene transfer between different species of staphylococci, which could be transferred between human and animal hosts.

Other Staphylococci in Animals

Our studies revealed that canines are primarily infected with *S. pseudintermedius*. PFGE analysis of our methicillin-resistant *S. pseudintermedius* (MRSP) isolates revealed that these pathogenic isolates seem to belong to a clonal group, which carries the SCC*mec* VII or one of two variants that have not been described previously in the United States in humans or animals. This suggests that the SCC*mec* VII element entered into a few different MRSP backgrounds at some time in history and then a large clonal expansion led to the predominance of the MRSP

strain seen in Cluster 1 (Figure 3). Susceptibility testing for MRSP isolates containing SCC*mec* VII and its variants indicated that these clinical infections in dogs are caused by multi drug-resistant staphylococci.

Future Directions

Studying both human and animal epidemiology of MRSA is becoming increasingly important to gaining a better understanding of the transmissions occurring between host species and their epidemiological consequences. Future research should focus on determining differences in gene expression between MRSA isolates originating from different host species, as well as a direct comparison between MRSA-infected animals and their owners. It would also be of public health interest to not only to survey circulating MRSA clones, but also MRSP clones from companion animals. Studying both human and animal models of this bacterial infection will become more important in the coming years to gain a better understanding of the transmissions occurring between host species and their epidemiological consequences, and how we can better understand the dynamics of genetically different MRSA clones in the community.

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