

MICROBIOLOGICAL SURVEY FOR METHICILLIN-RESISTANT  
*STAPHYLOCOCCUS AUREUS* IN VETERINARY PATIENTS AND GENOTYPIC  
CHARACTERIZATION OF THE ISOLATES

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL  
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF  
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2010

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To my parents who taught me to follow my dreams and imbued me with an eternal curiosity and appreciation for life.

## ACKNOWLEDGMENTS

This work would not have been possible without assistance, guidance, and support from many. However, Dr. Maureen Long is due special thanks for her vision, support, and mentoring. Without her, none of this would ever have happened and I thank her for her constant belief in me. I also thank my other committee members, Dr. Judith Johnson and Dr. Charles Courtney for their valuable insight and help in the preparation of this manuscript and project. To my lab mate, Dr. Melissa Bourgeois without whom I would never have completed this work, I am especially thankful and forever indebted. Dr. Paul Gibbs has been a wonderful sounding board and friend and is due special thanks. I appreciate the help of Crystal Schuman and the members of the College of Veterinary Medicine's Microbiology Laboratory for their assistance in teaching me techniques and offering technical assistance in bacterial culturing. Dr. Paul Fiorella of the Florida Department of Health Bureau of Laboratories was instrumental in typing the strains. Amber Menendez, Shannon Roff, Carmen-Susan Glotfelty, and An Nguyen were all involved in various aspects of the project and their assistance is highly appreciated. I also thank the members of the EDART lab in which the majority of this work was carried out for their support and assistance. The staff members of the College of Veterinary Medicine's Veterinary Medical Center, All Cats HealthCare Clinic, and Equine Medical Center of Ocala were key in helping me obtain the samples used in this work and deserve my sincerest thanks. Lastly, I thank my family and friends for their constant support, love, and patience while undertaking this project - I know it was not always easy.

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## LIST OF ABBREVIATIONS

ACME	arginine catabolic mobile element
ATCC	American type culture collection
BA	blood agar
bp	base pair
CA-MRSA	community-associated methicillin-resistant <i>Staphylococcus aureus</i>
CC	clonal complex
CHIPS	chemotaxis inhibitory protein of staphylococci
CI	confidence interval
CNA	colistin and nalidixic acid
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
HA-MRSA	hospital-acquired methicillin-resistant <i>Staphylococcus aureus</i>
J	junkyard region of the SCC <i>mec</i>
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MRSI	methicillin-resistant <i>Staphylococcus intermedius</i>
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
OR	odds ratio
PBP/PBP2a/PBP2'	penicillin binding protein
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PFT	pulsed-field type
PMN	polymorphonuclear cell

PSM	phenol soluble modulin
PVL	Panton-Valentine leukocidin
SCC	staphylococcal chromosomal cassette
SE	staphylococcal enterotoxin
SP	sampling period
ST	strain type
TSST	toxic shock syndrome toxin

Abstract of Thesis Presented to the Graduate School  
of the University of Florida in Partial Fulfillment of the  
Requirements for the Degree of Master of Science

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August 2010

Chair: Maureen T. Long  
Major: Veterinary Medical Sciences

A convenience sampling was performed at 3 secondary and tertiary care veterinary facilities in Florida to determine the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) nasal colonization in the canine, feline, and equine pet populations. Nasal swabs were collected and processed following standard microbiological techniques. Full antibiograms were performed to confirm antibiotic resistance and pulsed-field gel electrophoretic (PFGE) characterization was used to determine the strain. Each MRSA isolate was matched to 2 methicillin-sensitive *S. aureus* (MSSA) and PCR was performed to identify virulence factors. Positive isolates were submitted for sequencing and the sequences were aligned. The overall prevalence was low as only 6 isolates were determined to be MRSA of 966 patients sampled; 4 of these were the community-acquired USA300 strain based on PFGE. The recovered MRSA isolates demonstrated multi-drug resistance. The 5 genes tested were present in all 4 USA300 and in 1 MSSA. Another MSSA had 4 of 5 genes. The nontypeable MRSA and the remaining MSSA only had 2 genes present. Though of low prevalence, 4 of the MRSA isolates obtained were USA300 clones and carried similar virulence factors to

the strains circulating in the human population. This finding is of particular concern given the potential threat this strain poses to people.

## CHAPTER 1 LITERATURE REVIEW

### **General Overview of *Staphylococcus***

*Staphylococcus* species are ubiquitous non-motile, non-spore-forming Gram positive cocci bacteria that produce catalase which grow principally as facultative anaerobes. Taxonomically, the *Staphylococcus* genus falls under the phylum Firmicutes, class Bacilli, Bacillales order, and Staphylococcaceae family. Members of this genus are characterized by being round, typically 1µm in diameter and can be found as single cells, pairs, or tetrads though they are prone to clumping into bunches (1, 2), giving rise to the cocci designation, a name derived from the Greek *kókkus* which means grain, seed, or berry (3). Phenotypically, the staphylococci are usually further classified according to the presence of the active coagulase enzymes which induces rabbit plasma to clot.

Staphylococci are found on the skin and on the mucous membranes of humans and animals; many strains are host specific and may or may not cause disease in the host. Commensal bacteria colonize the skin, filling an environmental niche and helping to protect the host from colonization by pathogenic bacteria by competing for nutrients and preventing the adherence of pathogenic bacteria (4, 5). They can also directly deter pathogenic bacteria by excreting toxic metabolites which make the local environment untenable to other bacteria (4). In humans, commensal staphylococci are generally coagulase-negative species with *S. epidermidis* and *S. hominis* being the most commonly identified.

Phenotypically, *S. aureus* itself is coagulase-positive and causes a double-zone of hemolysis on blood agar. Though coagulase-positive species are more frequently

pathogenic than coagulase-negative species, *S. aureus* can also be a normal commensal species of humans. A national study performed in 2000-2001 estimated the weighted *S. aureus* colonization prevalence to be 32.4% (95% CI, 30.7%-34.1%) or 89.4 million people (95% CI, 84.8-94.1 million people) in the USA with the highest prevalence seen in 6-11 year olds (6). A second prevalence study performed in 2003-2004 found that the rate of *S. aureus* colonization had decreased to a weighted prevalence of 28.6% (95% CI, 27.2%-30.0%), or 78.9 million people (95% CI, 75.0-82.9 million people) (7). Though these studies measured colonization at a single time point, some of those identified as colonized may only have been transiently populated with the organism. However, other studies have shown that colonization by *S. aureus* is a risk factor for developing subsequent opportunistic infection (8).

*Staphylococcus aureus* has evidence of antibiotic resistance. While investigating the rate of methicillin-resistant *S. aureus* (MRSA) nasal colonization in people in the USA, the authors of the study determined that the prevalence in 2003-2004 was 1.5% (95% CI 1.2%-1.8%), up from 0.8% (95% CI 0.5%-1.4%) in 2001-2002 (6, 7). As researchers have demonstrated that persons nasally colonized by MRSA have an increased risk of developing subsequent clinical conditions (8, 9) versus those colonized by methicillin-sensitive *S. aureus* (MSSA) or not colonized at all (10), the risk to the public cannot be discounted.

### **Pathogenicity of *Staphylococcus aureus***

#### **Bacterial Virulence Factors**

Several known and likely many still unknown features of *S. aureus* account for the tendency of this commensal to become an opportunistic infection of humans, causing as many as 50% of all nosocomial infections (11). Although sensitive to ultraviolet light

and drying, this bacterium can survive in organic debris and adapt. The bacteria's pathogenicity is determined by various virulence factors, the expression of which varies between various Staphylococci and can be chromosomally or plasmid encoded. The mobility of many of these elements allows rapid adaptation to a variety of host environments.

To avoid being ingested by the host's polymorphonuclear cells (PMN) during the immune response, some *S. aureus* strains produce capsular polysaccharides. These exopolysaccharides have been classified into 11 types though most pathogenic strains are of capsular serotype 5 or 8 (12, 13). The predominant serotype seen in strains demonstrating antibiotic resistance against the beta-lactamase class is serotype 5 (14). The presence of the capsule reduces the uptake of the bacteria by PMNs in the presence of opsonins (15).

Protein A is another cell wall and surface protein of *S. aureus* that binds the Fc region of the heavy chain of IgG. This protein causes coating of the bacterium with immunoglobulins in the incorrect orientation which interferes with opsonization preventing ingestion by the PMNs (15). Protein A is immunogenic and forms the basis of various rapid diagnostic tests in clinical laboratories testing for co-agglutination of other bacteria, such as *Streptococcus*.

The peptidoglycans and teichoic acids of the *S. aureus* cell wall serve a structural role providing rigidity but also contribute to virulence by instigating a chemotactic response by the host's PMNs and production of opsonic antibodies. However, about 60% of *S. aureus* strains secrete chemotaxis inhibitory protein of staphylococci (CHIPS) to inhibit PMNs chemotaxis and counter this innate defense system (15). Teichoic acids

help promote adherence of gram-positive bacteria to mucosal surfaces. Additional proteins that help the bacterium invade host-tissues such as adhesins, collagen-binding proteins, fibronectin-binding protein, and clumping factor are covalently bound to the peptidoglycan matrix of bacterium's cell wall, further increasing its virulence (1). These surface proteins help promote cell invasion (2).

In addition to structural proteins, *S. aureus* secretes many enzymes to assist in evasion of host immune responses or assume a niche in host cell tissue. Used also for diagnostic purposes, catalase production counters the free radicals formed by the myeloperoxidase system secreted by PMNs extracellularly or when it is phagocytized. This interaction creates local inflammation by inducing cytokines (16). Though clumping factor is bound to the peptidoglycan of the cell wall as noted before, it enables the bacteria to bind to fibrin and fibrinogen which then allows fibrinolysins to break down the fibrin and enable spread to surrounding tissues. Hyaluronidase functions similarly to fibrinolysins by hydrolyzing the matrix of nearby tissues and thereby allowing local bacterial spread. Coagulase also interacts with fibrinogen – following the interaction with prothrombin, it activates the conversion of fibrinogen to fibrin which then coats the bacteria and inhibits opsonization (1).

Another set of virulence factors seen in *S. aureus* is the hemolysins. Alpha-hemolysin, encoded by *hla*, has lethal effects on various cell types and can lyse erythrocytes of various species as well as human PMN cells. The toxin creates pores in the target cell, which disrupts the ion flow, causing osmotic swelling and rupture (17, 18). Diagnostically, this toxin creates the zone of hemolysis around colonies on sheep blood agar. Unlike alpha-hemolysin, beta-hemolysin is not dermonecrotic nor fatal to

animals when injected intravenously but appears to confer some selective advantage (18). Gamma-hemolysin and Panton-Valentine leukocidin (PVL) are two-component toxins, meaning that each is made up of two unassociated protein components that then assemble into one of six possible forms. Gamma-hemolysins are found in most strains of *S. aureus* and their virulence has not been fully characterized (18). In contrast, the *LukPV* operon is found only in some *S. aureus* strains, encoded on the prophage  $\Phi$ SA2usa. The operon encodes the two components, *lukS*-PV and *lukF*-PV of the Panton-Valentine leukocidin (PVL) (19, 20), an exotoxin implicated in causing PMN cell lysis via disruption of the osmotic gradient through pore-formation (1, 21). A large percentage of *S. aureus* strains also create delta-hemolysin, a toxin proposed to act as a surfactant, disrupting cell membranes allowing for cell lysis (18).

Generally associated with a particular strain of *S. aureus* which will be discussed in detail later, genome analysis of USA300 has identified a genetic island which contains a cluster of six genes known as the *arc* cluster. These genes allow for the conversion of L-arginine to carbon dioxide, ammonia, and ATP via arginine deiminase pathway (19). Two genes identified within the arginine catabolic mobile element (ACME) may enhance virulence: *arc* and *opp3*. The *arc* gene encodes for an arginine deiminase pathway which depletes L-arginine used in the production of nitric oxide, a molecule used in both innate and adaptive immune responses against bacteria. The *opp3* cluster encodes for an oligopeptide permease system, a transporter important to nutrient uptake, chemotaxis, quorum sensing, antimicrobial peptide resistance, and eukaryotic cell adhesion, among others (20). The two clusters serve as surrogate markers for ACME (19). The arginine deiminase pathway has been identified in the core genome of

*S. aureus* but it is not identical to the ACME found in USA300, where it is located downstream of the *SCCmec* in the *orfX* site (19). It is believed that the presence of redundant arginine catabolism pathways in this strain improve the strain's fitness since arginine has been proven critical for survival in anaerobic conditions (22). ACME has been shown to confer a bacterial advantage in a rabbit bacteremia model (23) though it did not cause dermonecrosis or increased severity of necrotizing pneumonia in a rat model (24).

More recently, bioactive peptides called phenol-soluble modulins (PSM) have been proposed as a principal virulence factor in *S. aureus*. Though still being investigated, *S. aureus* produces four shorter and two longer PSM-like peptides, alpha-PSM and beta-PSM respectively. It is proposed that these peptides are proinflammatory, thereby triggering an inflammatory response (25).

Certain strains of this species have also been shown to possess a variety of genes that allow it to produce exotoxins, which may persist and damage the host even if the bacteria are eliminated. The diseases associated with these exotoxins are primarily those associated with food-borne illness. Typically, *S. aureus* grows when refrigeration fails or processing requires growth-permissive temperatures as is the case in cheese-making (26). During bacterial growth, the enterotoxins are elaborated. If the foodstuff in which the bacteria are present is ingested, these enterotoxins can then cause inflammation of the lining of the stomach and intestinal tract. The result of this inflammation is gastroenteritis, generally manifesting itself with abdominal cramps, nausea, vomiting, and diarrhea (27). Although there are dissimilarities between these

proteins, the majority of the SEs have some shared amino acid sequences, particularly a cystine loop that is believed to play a role in causing emesis (26).

Foods that are commonly associated with staphylococcal foodborne illness include meat and meat products; poultry and egg products; milk and dairy products; salads such as egg, tuna, chicken, potato or macaroni; and several types of pastries, especially ones with cream fillings (28).

At least 20 different staphylococcal enterotoxins (SEs) have been identified. They are encoded on genes mostly located on mobile elements that allow the transfer of enterotoxin production ability between strains. However, some isolates carrying the *seb* enterotoxin have the SE gene integrated in the bacterium's chromosomal DNA while others have a mobile plasmid containing the gene (26). Once produced, these SEs are highly stable and resist heat and proteolytic degradation. The high heat processing used in sterilization will inactivate most SEs if these are present in low numbers. However, some of the SEs may retain function, depending on the foodstuff being treated and the environmental pH. *Staphylococcus aureus* enterotoxins can also be superantigens such as TSST which is associated with toxic shock syndrome (29). The superantigen capacity of these SEs is due to their ability to cause fever and non-specific T-cell activation. Rather than establishing an adaptive immune response, the enterotoxin T-cell stimulation results in a massive cascade of cytokines and inflammatory mediators which gives rise to the symptoms associated with foodborne illness. The clinical signs of abdominal cramps, vomiting, and diarrhea are thought to be associated with the direct effect of this cytokine release on the gastrointestinal epithelium. It may also stimulate the vagus nerve which may subsequently stimulate the central emetic center

(chemoreceptor trigger zone) and result in vomiting (26, 30). The cystine loop that is common to most of the SEs appears to play a role in stimulating the emetic center, as the SEs that lack the cystine loop based on mutant analyses have absent or reduced emetic capacity (26). Because enterotoxins activate T-cells and the subsequent cytokine cascade, symptoms of staphylococcal foodborne illness normally appear within 30 minutes to eight hours, depending on the infective dose of bacterial SEs consumed in the infected foodstuff. The disease, therefore, has an acute onset but symptoms are typically self-limiting and resolve spontaneously within 24 to 48 hours (26). Colonized food handlers, processors, and preparers could contaminate food and be the source of an outbreak. The presence of *S. aureus* in animals may also lead to the elaboration enterotoxins and start an outbreak in that fashion. Both populations can also have enterotoxin producing MRSA which would further complicate the issue.

Non-enterotoxin superantigens have been identified in staphylococci, which, unlike the enterotoxins, cause much greater systemic effects due to the body's more widespread response. Categorized as a pyrogenic superantigen toxin, toxic shock syndrome toxin-1 (TSST-1) shares three biologic characteristics with other superantigen in its class: pyrogenicity, superantigenicity, and the ability to enhance the lethal effects of minute amounts of endotoxin in rabbits up to 100,000 fold (1). The superantigen capacity of TSST-1 involves the nonspecific activation of T-cells and subsequent polyclonal T-cell proliferation and cytokine cascade. The resulting illness is a multiorgan dysfunction syndrome involving fever, hypotension, and a rash accompanied by a variety of other clinical symptoms dictated by the organ system involved, though

commonly gastrointestinal, renal, and hepatic signs are noted. Given the body's overwhelming immunologic response, TSST-1 can lead to death from multiorgan failure.

### **History of Antibiotic Resistance**

Medical advances in therapeutics have continuously sought to overcome the bacteria's incursions. Prior to the development of antibiotics, little could be done against this and other bacteria. But the addition of penicillin to the clinical formulary in 1940 revolutionized medical management of infectious bacterial diseases (31, 32). Penicillin and members of the beta-lactam antibiotic class are bacteriocidal as they target the bacterial enzymes involved in cell wall biosynthesis, known as the penicillin binding proteins (PBP), thereby preventing cross-linking of the peptidoglycans in the bacterial cell wall and inhibiting cell growth (1, 33). *S. aureus* soon adapted to the new environmental pressure with some strains developing resistance to the beta-lactam antibiotic within a year (34, 35). These resistant strains produced penicillinases (also known as beta-lactamases) that attacked the penicillin's four-membered beta-lactam ring structure by disrupting the amide bond of the ring and inactivating the antibiotic (33, 36). These plasmid-encoded penicillin-resistant strains soon became widespread and comprise the first wave of antibiotic resistance in the hospital setting, necessitating a new therapeutic option (36).

A new line of antibiotics, fortified against the beta-lactamases produced by the bacterium were developed to target these newly resistant strains. Methicillin, a beta-lactamase-resistant antibiotic was first used in 1959 and demonstrated efficacy in inhibiting cell wall synthesis by bacteria despite the presence of beta-lactamases. However reports of resistance to methicillin and members of its class surfaced by 1961, denoting the start of the second wave of antibiotic resistance (36, 37). These resistant

strains expressed an altered penicillin-binding protein designated PBP2a (or in some studies PBP2'), which is a transpeptidase that carries on cell wall synthesis despite the presence of beta-lactam antibiotics. Resistance is conferred upon the bacteria by the PBP2a protein's decreased affinity for binding the beta-lactam molecule (33, 38).

Though methicillin is no longer clinically used, *S. aureus* strains that exhibit resistance to the beta-lactamase antibiotic class are known as methicillin-resistant *S. aureus* (MRSA). To meet this classification, a MRSA strain must demonstrate a minimum inhibitory concentration (MIC) of oxacillin (a laboratory standard for the beta-lactamase class)  $\geq 4\mu\text{g/mL}$ . Alternatively, a zone of inhibition  $\leq 10\text{mm}$  around an oxacillin-impregnated disk would be considered resistant when performing a disk diffusion test (39).

Resistance to methicillin and the other beta-lactamases in *S. aureus* followed the acquisition of the *mecA* gene. The original source of the resistance gene is presumed to be a coagulase-negative staphylococci and studies have identified *S. sciuri* as the likely donor: a DNA probe for the MRSA *mecA* hybridized strongly to unrelated *S. sciuri* isolates. Interestingly, despite the presence of the gene, two-thirds of the *S. sciuri* isolates showed marginal if any resistance to methicillin (40).

The *mecA* gene is embedded in a chromosomal island known as the staphylococcal chromosomal cassette (*SCCmec*) that integrates into the *S. aureus* chromosome at the *orfX* site (19). Several classes of *SCCmec* have been identified and have been linked to the documented epidemic waves of resistance (36). Three major classes have been defined: class A includes the entire *mecA* regulon (*mecI-mecR1-mecA*) while classes B and C contain the regulon but the order is interrupted by

insertion sequences (41). The *SCCmec*'s ability to move between isolates as well as between species is due to the presence of recombinases encoded by the *ccr* gene complexes present on the cassette. The size of the *SCCmec* cassette is believed to impact its ability to transfer between isolates since the size depends on the number of additional resistance genes encoded in the junkyard (J) regions (36, 41). The presence of these additional resistance genes are used to classify the *SCCmec* subtype but also mean that a sensitive strain can become multidrug resistant if it obtains the entire cassette in a single horizontal gene transfer event (42). However, resistance to other antibiotics is not necessarily dependent upon acquisition of the SCC. Spontaneous mutations and positive selection have resulted in resistance to additional antibiotics and antibiotic classes such as the fluoroquinolones and linezolid (42). Methicillin resistance has also been demonstrated to occur via *mecA*-independent mechanisms, principally by overproduction of other PBPs or hyperproduction of beta-lactamases (33, 38, 43).

It stands to reason, therefore, that the presence of antibiotic resistance limits the clinician's ability to prescribe pharmacological agents that will curb the spread of the bacteria. Combine this with the presence of other virulence factors identified in *S. aureus*, and it is evident that the bacterium poses a threat to the wellbeing of people and animals.

## **Hospital-Acquired Versus Community-Associated MRSA**

### **Epidemiology of MRSA**

Following the first reports of resistance in 1961 (37), MRSA has been a constant hindrance and potential threat to the hospitalized patient. The bacterium has a predilection for catheter sites, in-dwelling devices, and surgical incisions; hospital-

acquired MRSA (HA-MRSA) has historically been a nosocomial infection of concern, but the risk has increased in recent years.

A study by Panlilio et al. found that nosocomial MRSA infections increased from 2.4% in 1975 to 29% in 1991. The number of beds in a facility impacted the rate of infection, with larger institutions demonstrating a faster climb in the number of resistant cases (44). In 2003, >60% of all isolates obtained from all adult patients in intensive care units were MRSA, which translates into a 3.1% annual increase from 1992 to 2003 (45).

A retrospective survey of the National Hospital Discharge Survey looking at *S. aureus*-related hospitalizations and deaths between 1999-2005 found that though annual admissions increased ~8% during those years, the number of *S. aureus* related hospitalizations increased 62% over the same time period, from 294,570 (95% CI 257,304-331,836) to 477,927 (95% CI 421,665-534,189). Strikingly, the estimated number of MRSA-related hospitalizations more than doubled in the same time period, from 127,036 (95% CI 112,356-141,716) to 278,203 (95% CI 252,788-303,619). The resulting overall rate of *S. aureus*-related diagnoses per 1,000 hospitalizations increased 50% from 9.17 to 13.79 while MRSA-related discharges per 1,000 hospitalizations more than doubled, from 3.95 to 8.02. In their study, the authors estimated that *S. aureus*-related deaths averaged ~10,800 (range 7,440-13,676) per year but that MRSA-related deaths averaged ~5,500 per year (range 3,890-7,372) (46). In another study, the authors used the Active Bacterial Core surveillance system to identify MRSA cases in a subset of the national population and extrapolated the findings to the national scale. They estimated that 94,360 invasive MRSA infections occurred

nationwide resulting in 18,650 deaths in 2005 (47). The estimated number of MRSA-related deaths was 3.3 times higher in the latter study as compared to the first.

However, the second study may have introduced bias into the sample by oversampling minorities and the elderly as both were found to have increased incidence and mortality (47).

In part, the increased rate of morbidity and mortality can be attributed to the increased antibiotic pressure these strains encountered with the advancement of therapeutics (43). These hospital-acquired strains developed a characteristic pattern of multi-drug resistance, because methicillin-resistance imparts resistance against the entire beta-lactam class, including the cephalosporins. Resistance to erythromycin, levofloxacin, and constitutive clindamycin resistance is also commonly found in these strains (48). Nosocomial transfer occurs among hospitalized patients, likely by means of fomite transfer via colonized staff or equipment.

Over the past 20 years, newer strains have been noted with increasing frequency. Initially labeled community-associated because patients diagnosed with these MRSA strains lacked traditional risk factors (49), these strains were thought to be escaped hospital strains that had circulated in the general population. Without antibiotic pressure, these strains had retained their resistance against penicillins but tended not to be multi-drug resistant. Community-associated MRSA patients tended to present to emergency rooms with skin and soft tissue complaints (50), commonly complaining of a spider bite. The characteristics that define these community-associated MRSA (CA-MRSA) and separate them from the traditional HA-MRSA are listed in Table 1.1

The emergence of MRSA in a population not previously considered at risk was, and continues to be, concerning. Though reports of isolations from indigenous populations in Australia cropped up in the early 1990s (36), the CA-MRSA strains first came to the forefront in the USA when the University of Chicago Children's Hospital reported on the increased prevalence of MRSA in patients with no previous predisposing risk factors. In their retrospective study, the authors looked at *S. aureus* isolates obtained from hospitalized children in 1988-1990 and then compared these to isolates from 1993-1995 and found that the number of hospitalizations from CA-MRSA rose and that the prevalence increased from 10 per 100,000 admissions to 259 per 100,000 admissions (51). However, concern was heightened when the CDC reported the death of four previously healthy children in the Midwest due to respiratory failure or secondary to multi-organ dysfunction caused by MRSA infection; none of the children had any risk factors for the development of traditional HA-MRSA (52).

Following the initial reports, CA-MRSA strains were reported in inmates (53), military recruits (54, 55), athletes (56), minority populations (47), men who have sex with men (57), and in poor urban adults (50). In a short amount of time, the frequency of outbreaks involving CA-MRSA increased dramatically. CA-MRSAs have become so prevalent, that though 59% of all skin and soft tissue infections presenting to 11 emergency departments nationwide were MRSA, 99% of these were community-associated strains (50). Given this rate of spread, it was not long before reports of CA-MRSA in the hospital setting followed, making the original definition for CA-MRSA strains debatable; in some cases the traditional HA-MRSA strains have been displaced

by CA-MRSA as the predominant nosocomial infection (58), making the naming nomenclature outdated (59, 60).

### **Molecular Epidemiology and Virulence of CA-MRSA**

Genetic analysis of MRSA strains has been carried out utilizing a variety of methods such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and *spa* typing (61) to better understand this spread. MLST has been used to study the evolution of the bacteria by performing sequence analysis on ~450bp internal fragments of seven housekeeping genes. Identical sequences are considered to be clones and are assigned to a sequence type (ST). If strains differ by less than three single nucleotide polymorphisms, they are grouped into clonal complexes (CC) (36, 48).

PFGE of MRSA strains, alongside epidemiological research, was used to create a national database of HA- and CA-MRSA strains due to the availability of technical knowledge and experience with the technique in laboratories nationwide. In creating the database, *Sma*I macrorestriction fragment analysis was used to identify several pulsed-field types (PFT). The HA-MRSA strains had PFTs USA100, 200, 500, 600, and 800. USA700 strains were both HA- and CA-MRSA while CA-MRSA strains were either USA300 or 400 (48). Additional PFT have been identified. The original CA-MRSA circulating in the Midwest, named MW2, was designated a USA400 strain. This strain has largely been displaced by the USA300 strain, the dominant CA-MRSA strain seen today. Using MLST nomenclature, USA300 is categorized as sequence type 8, clonal complex 8 (62).

Much of the recent scientific effort has been directed at USA300 strains, looking to understand the bacteria's remarkable spread and apparent increased virulence. A frequent cause of skin and soft tissue infections, a study by Johnson et al.

demonstrated ~4 fold increase in the incidence of primarily skin and soft tissue infections over the course of five years attributed to USA300 (63). USA300 has also been implicated as the cause of fulminant necrotizing pneumonias (21) and other invasive conditions such as osteomyelitis, bacteremia, and septic arthritis (64). Numerous studies have shown that the USA300 strain harbors a *SCCmecIV*. As noted previously, these cassettes vary in size depending on the presence of additional resistance genes and the *mecIV* is the smallest cassette (21-25kb) and does not harbor any additional virulence genes. In one study, CA-MRSA strains harboring the *SCCmecIV* grew faster than HA-MRSA carrying different *SCCmec* allotypes (65). The authors suggest that this may have allowed the CA-MRSA strains to outcompete other bacteria in the absence of antibiotic pressure and that this may offset the fitness cost that a larger *SCCmec* may impart on the HA-MRSA strains that carry them (65).

The rise of CA-MRSA infections, particularly those caused by the USA300 strain, has also been epidemiologically correlated with the rise in the number of strains containing PVL (48, 52, 56). However, conflicting results have been obtained evaluating the impact of PVL on USA300's virulence. A study by Lina et al. implicated the presence of PVL with *S. aureus* derived skin infections and community-acquired pneumonia, noting that hospital-acquired staphylococcal pneumonias were rarely associated with a necrotic process (21). Using a rabbit pneumonia, Diep et al. demonstrated that PVL-producing USA300 strains caused greater mortality and significantly more necrosis and disruption of the pulmonary architecture than a PVL negative strain. The researchers also demonstrated that PVL is produced in toxic amounts in the lung (66). In a different study, use of a rabbit bacteremia model demonstrated a modest but measurable effect

early in the disease process that could be attributed to the presence of PVL, which the authors surmised permitted the bacteria to seed the kidney early in the course of the disease (67). Another study determined that the presence of the PVL toxin in human *S. aureus* clinical samples was positively associated with skin and soft-tissue infections as well as bone and joint infections (OR, 2.5; 95% CI 1.2-5.2) (68).

This is in contrast to other studies which have investigated the contribution of PVL to disease processes and not found it to be the determining virulence factor in USA300-derived pneumonia (69) or in a mouse sepsis and abscess model study evaluating wild-type USA300 and LukS/F-PV-knockout. The latter study found similar levels of survival between mice inoculated with either strain as well as similar levels of PMN lysis upon exposure to the wild-type versus the knockout. In an evaluation of the strains' ability to cause abscesses and dermonecrosis, PVL negative strains produced a larger abscess than the PVL positive strains, refuting the claim that PVL is the factor conferring USA300 increased virulence (70).

A more recent study, however, validated both arguments by identifying PVL as a strong cytotoxic factor for human and rabbit neutrophils but not mouse or simian, demonstrating species-specific cytotoxicity. The group also found that no difference was detected between PVL-expressing and *pvl*-knockout *S. aureus*, including USA300 in their ability to induce neutrophil death when live bacteria were phagocytized. The authors proposed that the effect of PVL is masked in this model by cellular processes that delay the effect of PVL. Since production of PVL occurs mostly in the post-exponential phase of bacterial growth, it is reasonable to suspect that PVL may not be expressed in high concentration if the bacterium is immediately phagocytized (71). That

PVL's presence has been linked to USA300 strains causing skin and soft tissue infections is not refuted though its exact impact on pathogenicity, if any, is still being understood.

More recently, the ACME gene has been identified as a potential key determinant in the USA300 strain's pathogenicity. The SCC*mecIVa* has been demonstrated to be physically linked to the ACME (23, 72, 73), suggesting that antibiotic resistance and pathogenicity are interconnected. The strain's virulence was attenuated when ACME was eliminated but not when SCC*mec* was deleted in a rabbit bacteremia model (23).

As noted before, the presence of a redundant arginine deiminase operon in the USA300 strain is believed to confer a fitness advantage upon the bacteria allowing for improved survival in anaerobic conditions where arginine is the sole source of energy (22). Investigation of the effect of ACME in *Streptococcus pyogenes* has found that arginine deiminase inhibits proliferation of human mononuclear cells and improves the bacteria's ability to invade and survive intracellularly (74, 75). Because of the presence of the arginine deiminase pathway, the bacteria appear better equipped to survive in the face of the acidic conditions that exist on human skin.

Another group of researchers contends that ACME does not enhance virulence. Their study found that in a comparison of USA300-ACME wild-type and an ACME-deletion mutant in a rat model, the presence of the gene was not associated with an increase in mortality or lung pathology and no significant difference in expression of other virulence factors with the exception of alpha-hemolysin (24). More research is needed to further define the role of ACME in the pathogenesis of USA300.

Another potential source for the virulence noted in the CA-MRSA strains suggested by some researchers are the PSM. Wang et al. first described these peptides in *S. aureus* as they have previously been described *S. epidermidis*. The researchers detected PSM production *in vitro* at a higher concentration in CA-MRSA strains as compared to HA-MRSA strains. In testing alpha-PSM deletion mutants, the authors described reduced mortality and decreased levels of tumor necrosis factor-alpha peripherally in the affected mice as compared to mice infected with the wild-type. Monocyte infiltration and lysis were significantly increased when exposed to a wild-type CA-MRSA strain as compared to the deletion mutant while human neutrophils could be stimulated to respond and produce cytokines when similarly exposed (25).

Another study demonstrated PSM-mediated PMN cell lysis (both human and murine) when these were exposed to PSMalpha3 (the most potent peptide), though lysis occurred at high peptide concentrations. Based on this, the researchers concluded that the CA-MRSA strains would not be able to produce sufficiently high concentrations of PSM $\alpha$ 3 to create an appreciable amount of lysis. However, the researchers did find that the PVL-mediated lysis of human neutrophils was enhanced in the presence of the peptide (76). Correlating the findings of the previous study, Löffler et al. found that PSMs are active against many different species' neutrophils at high concentrations and that the cells are rapidly destroyed without a characteristic change in morphology (71). The PSMs appear to be under the control of the global regulator *agr* though how this might impact its virulence in CA-MRSA strains carrying the gene is not yet known (20).

It is evident that *S. aureus* has acquired a myriad of ways in which to prosper despite environmental pressures it encounters from our attempts to treat clinical

conditions caused by the bacteria. As the human medical field continues to attempt to curb the rising number of MRSA cases, potential spillover into the veterinary community is an area of heightened concern. Specifically, questions regarding the potential for reservoiring of MRSA in our pets and cross-transmission between humans and animals are focal topics. Additionally, as the virulence factors of the human strains are defined, efforts to characterize these virulence factors in the animal-derived strains should be performed.

### **MRSA in Animals**

Initial epidemiological studies into the presence of MRSA in pets have identified fairly low rates of infection or colonization. This is most likely due to the fact that *S. aureus* is a human-adapted strain so it would have to overcome species' specific immune responses every time the bacterium jumped host species. Notwithstanding, *S. aureus* does commonly cause pyodermas and other clinical diseases in animals.

The earliest report of veterinary MRSA isolation was in a mastitic dairy cow in 1972 (77). Since then, dogs, cats, horses, pigs, and poultry have been identified with the bacterium (78-81). MRSA has been more recently found in companion animals, with the first reports of a colonized dog arising in 1994 (82) and thereafter by the detection of MRSA in cats in 1998 (78). A study carrying out molecular characterization of dog and cat derived MRSA isolates concluded that these infections are rarely reported in pets. The 16 isolates obtained and tested were PVL negative, ST22, and *spa* type t032, with some strains carrying a type IV *mec* cassette, though subtyping was not successful (83). More recently, MRSA was identified in 2 dogs that demonstrated clinical signs at the time of sampling. Both isolates were obtained from the same veterinary clinic and harbored a SCC*mecIII* and were ST239 (84).

The low prevalence in the general population can be seen by comparing the results of MRSA isolations from a diagnostic laboratory and those obtained by screening a healthy population. A report by Rich and Roberts notes that only 1 in 255 healthy dogs was colonized during the course of one year as compared to 114 MRSA positive isolates received at the laboratory in the same time (85). A study sampling private practice populations and clinical cases at a teaching hospital found that dogs in the former were colonized 0.8% while MRSA was isolated in 7% of the latter cases (86). Though higher than other reported values, Jones et al. found that 23.5% of all *S. aureus* isolated from clinical samples submitted to their laboratory were MRSA (87).

These findings underscore the tendency in veterinary medicine to attempt empirical antibiotic therapy and then rely on culture and sensitivity when the infection does not resolve or respond. As in humans, the risk of developing clinical disease from MRSA is significantly impacted by the number of antibiotic courses received, the number of days the dog or cat is hospitalized, and having received surgical implants (88). In a case-control study evaluating comparing MRSA and MSSA cases in dogs, the receipt of antibiotics (OR 3.84, 95% CI 1.21-14.74,  $p=0.02$ ), beta-lactams (OR 3.58, 95% CI 1.04-14.79,  $p=0.04$ ), or fluoroquinolones (OR 4.61, 95% CI 1.08-27.37,  $p=0.02$ ) within 90 days of admission were significantly associated with the development of MRSA in canine patients (89), corroborating the previous study's findings. Fortunately, no significant difference was noted with regards to surgery or outcome.

Following an outbreak, sampling at a Canadian university's veterinary teaching hospital large animal clinic was instituted. Subsequently, 4% of horses in 2000 and 8% of horses in 2002 were found colonized with MRSA (90). In the study, 14% ( $n=17$ ) of the

staff at the veterinary hospital were found to be colonized with MRSA, which, combined with case histories and repeat sampling, lead the authors to conclude that 63% (n=17) of the equine cases were nosocomial transmissions. A subset of these isolates were typed and all demonstrated SCC*mecI*V but lacked PVL genes (90).

A surveillance program instituted at the Ontario Veterinary College following the initial outbreaks found that 5.3% of all equine patients were either colonized at admission or became colonized with MRSA during hospitalization over an almost 2 year period. The colonization rate of horses by CA-MRSA was 2.7% (91).

In an Irish study, healthy horses were colonized 1.6% of the time (86). Environmental swabbing by the Canadian group identified 9.6% of sites sampled were MRSA positive. The most common locations that cultured MRSA were stalls housing MRSA-positive horses (62%). In contrast, only 6.9% of stalls housing MRSA-negative horses were culture positive for MRSA (92).

In a broad study surveying *S. aureus* isolates submitted by diagnostic laboratories at seven veterinary teaching hospitals in the USA, 14% (n=9) of the isolates were found to be MRSA, though none of the PFGE patterns matched each other; they were not compared to existing human strains (80).

Recent attention has revolved around the high prevalence of colonization of MRSA in pigs and pig farmers, which has been evaluated primarily in the Netherlands. An initial study found that 23% (n=6) of pig farmers sampled were colonized, representing a >760x higher risk of colonization by swine farmers as compared to the rest of the Dutch population. The authors were also able to demonstrate transmission between species and found that *spa*-type t108 was the most prevalent MRSA, though all the recovered

strains were nontypeable using PFGE (81). In a larger subsequent colonization study, de Neeling et al. identified 39% (n=209) of the swine sampled were colonized with MRSA at the time of slaughter and that all the samples belonged to ST398 (93). Three *SCCmec* types were identified in the Dutch swine population sampled: type III (3%), type IV (39%), and type IV (57%). All of the isolates demonstrated resistance to tetracycline, which is a reflection on the high level of use of that antibiotic in pig husbandry (93). To evaluate the potential for widespread colonization of pig farmers on the North American continent, pigs and pig farmers in Ontario, Canada were swabbed and 20% (n=5) and 24.9% (n=71), respectively, were found to be colonized with MRSA, though the predominant strain was a HA-MRSA (94).

As the human-animal bond tightens and pets play increasingly integral roles in the home, the potential for cross-colonization and infection has raised concerns in both the human and veterinary medical fields. With this possible host-range expansion, the epidemiology of the disease could be further complicated if reservoiring occurs in pets as has been suggested by one group (95). An example of this complication was described by van Duikjeren et al. when they reported a recurring outbreak in a nursing home which was only resolved once the affected nurse was treated, in addition to her affected infant and colonized dog (96). Weese et al. reported several case studies of companion animal MRSA isolations that were identical to strains obtained from either attending veterinary technician staff or the animal's owner (97). Cases such as this underscore how detrimental it may be to both people and animals if MRSA gains a solid foothold in non-human species and develops an additional host predilection.

Fortunately, to date, there does not appear to be reports of USA300 having been detected.

It is the goal of this research to investigate the prevalence of MRSA in companion animals and to assess *Staphylococcus aureus* for evidence of shared virulence factors between the human and animal-derived strains. This is a needs study to further define MRSA in animals.

Table 1-1. CDC case definition for community-associated MRSA

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Diagnosis of MRSA within 48hrs of admission or as an outpatient

No previous history of MRSA infection or colonization

No history of hospitalization, admission to a long term care facility, dialysis, or surgery in the past year

No indwelling catheter or medical device that passes through the skin into the body

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CHAPTER 2  
MICROBIOLOGICAL SURVEY AND NASAL COLONIZATION RATE  
DETERMINATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* IN  
PATIENTS AT THREE SECONDARY/TERTIARY VETERINARY CARE FACILITIES IN  
FLORIDA

**Background**

Originally detected in humans only two years after methicillin was introduced to treat infections caused by penicillin-resistant staphylococci (37), methicillin-resistant *Staphylococcus aureus* (MRSA) is today considered an emerging disease in humans because of the increased numbers of isolations and clinical ramifications of reported infections (46). Historically, these infections were associated with hospitalized patients with in-dwelling catheters and devices, the elderly, and the immunocompromised and were thereby termed hospital-acquired (HA)-MRSA. These HA-MRSA infections have become much more frequent during the last two decades. Whereas in 1974 only 2% of the *S. aureus* infections in US intensive care patients were classified as MRSA, by 1992 this number had risen to 35.9% and was 64.4% by 2003 (45, 98). Resistance to penicillin in *S. aureus* is associated with beta-lactamases produced by resistant strains that attack the antibiotic's beta-lactam ring. Methicillin and similar penicillins of this class were fortified by the addition of an ortho-dimethoxyphenol group to the beta-lactam ring to counter these resistant strains. The next wave of resistance against these fortified antibiotics ensued (37), mediated via the *mecA* gene found on the staphylococcal chromosomal cassette *mec* (SCC*mec*). These mobile elements transfer horizontally between bacteria so sensitive strains become rapidly resistant. The traditional HA-MRSA carry SCC*mec* types I, II, or III (22), with the rise in type II and III SCC*mec* allotypes forming the basis for the third wave of resistance (36). Resistance to all antibiotics in the beta-lactam family as well as cephalosporins is phenotypically

designated by the detection of resistance to oxacillin on an antibiogram. Additional resistance to other antibiotic classes such as macrolides and fluoroquinolones is frequently noted with these infections, leaving practitioners limited treatment options (99). The fourth wave of antibiotic resistance involves the rise in vancomycin-intermediate *S. aureus* (VISA) strains (36).

A second set of MRSA strains have come to the forefront in the past two decades as community-associated strains (CA-MRSA) have been increasingly isolated from people lacking traditional risk factors such as contact with hospital settings (58). Often, these CA-MRSA show less antibiotic resistance than the hospital-acquired strains (48) and typically have SCC*mec* type IV or V (22). The USA300 strain, which carries a SCC*mec*IV cassette has been particularly concerning as it has developed increased virulence and causes various primary disease syndromes predominant now in the US. This trend towards the displacement of typical HA-MRSA by CA-MRSA strains, particularly by USA300, is resulting in infections of new patient cohorts previously not at risk for *S. aureus* infections. The syndromes themselves have higher morbidity and in some cases mortality (46, 47, 58, 60). Understanding of the epidemiology of the bacterium, its transmission, and reservoir patterns is still incomplete.

MRSA was first reported in the veterinary literature in a dairy cow in 1972 (77). The first companion animal reported to have MRSA was a dog in 1994 (82) and colonized cats were first reported in 1998 (78). There have been sporadic reports of MRSA infection in horses in surgical wounds with more recent reports of nasal colonization rates as high as 5.3% (91).

Whether or not animals serve as either the originating source of human infection or disseminate acquired *S. aureus* (from humans) has not been fully determined. Work performed in the Netherlands demonstrated contact with pigs or pig farming is a risk factor for developing MRSA infection, and the frequency of colonization by pig farmers was over 760 times higher than in the general Dutch population (81). In another study, there was a significant correlation on Canadian farms with the presence of MRSA positive pigs and MRSA positive nasal colonization in farm personnel. In these studies, the strains found in personnel were of swine origin (94). Concomitant carriage between cows and humans has also been demonstrated though the direction of transmission could not be shown (100). A study investigating colonization rates in horses and associated caretakers found similar subtypes circulating among the two populations with evidence of human-to-horse and horse-to-human transmission (90). In 2004, a case report described recurring MRSA colonization in a nursing home nurse which only resolved once her child and dog were also treated to eliminate the same strain (96).

Given that human-animal-human transmission can occur, the potential for zoonotic transmission of MRSA bears investigation. The use of molecular epidemiology to perform comprehensive characterization of MRSA should be carried out to truly identify if animals are an originating source of resistant *S. aureus* epidemics in humans. The purpose of the study reported here was to determine the degree of nasal colonization in companion animals at tertiary and secondary veterinary care facilities in Florida and to perform basic genetic subtyping of any MRSA isolates.

## Methods

### Animal Sampling

Sampling was performed at several secondary and tertiary animal treatment centers in north central Florida including the small (dog and cat) and large animal (equine) hospitals of a veterinary teaching hospital (facility 1 and facility 2, respectively); a primary and secondary care private care feline clinic (facility 3); and a primary, secondary and tertiary care private care equine facility (facility 4) during the second sampling period. Two sampling periods (SP) took place, the first during July-August (SP1) and the second during the following March-April (SP2) to reflect seasonal variation in Florida and seasonal reproductive differences in the horse. Detailed patient signalment was obtained, recording the specie, age, gender, breed, and neuter status of each animal sampled. Access to medical records at facilities 1 and 2 was used to determine the animal's originating geographic location from the owner's address. Admission date, hospital service visited, and length of stay were also obtained for these animals and placed in a database. When possible, hospitalized patients were swabbed within 24 hours of admission and again between 72 and 96 hours. Most of the cats sampled were swabbed once at the time of their outpatient visit given the large majority of these samples came from facility 3.

Samples were obtained by passing rayon-tipped culture swab against the mucosa of a single nostril and then placed in Stuart's media (BD BBL CultureSwab, Sparks, MD) and frozen at -20°C if not processed immediately. A subset of the swabs was frozen without any media during SP2 to determine storage options for future broader studies that may be performed in animals under different management conditions.

Animal sampling protocols were evaluated and approved by the University of Florida Institutional Animal Care and Use Committee. Additionally, owners of patients seen at facilities 1-3 signed an informed consent prior to having their pet sampled.

### **Microbiological Testing**

Briefly, samples were plated onto Columbia blood agar with 5% sheep blood (BA), CNA, and (during SP2) mannitol salt plates. Dry swabs were plated to BA and then inoculated into tryptic soy broth, which was incubated for enrichment and subsequently plated to BA, CNA, and mannitol salt plates. All plates and broths were incubated at 37°C with 5% CO<sub>2</sub> overnight. The plates were evaluated for round, raised white to yellow colonies with a zone of beta-hemolysis on the BA and CNA plates as well as growth on the mannitol salt plate. Suspect colonies meeting these criteria were reisolated onto BA after a Gram stain confirming gram positive status and a positive catalase test was obtained. A coagulase tube test (BD BBL Coagulase Plasma, Rabbit, Sparks, MD) was set up for these suspect colonies by inoculating a loop into rabbit plasma and allowing it to incubate for a total of 24 hours with an initial read at 4 hours. Isolates that coagulated were subjected to a latex agglutination test evaluating the presence of penicillin binding protein 2a (PBP2a) (Remel BACTi STAPH, Lenexa, KS). Any isolate that demonstrated latex agglutination was then inoculated into maltose, trehalose, and lactose broths (Hardy Diagnostics Purple Broth with Maltose, Trehalose, Lactose, Santa Maria, CA) and incubated overnight. Evidence of sugar fermentation by the bacterium was based on a purple to yellow color change, though evidence of partial fermentation was further evaluated. If all the criteria were met, the isolate was inoculated into tryptic soy broth to a 0.5 McFarland turbidity standard and subsequently plated onto Mueller Hinton agar with 4% NaCl and oxacillin (6ug/ml) (Hardy Diagnostic

MRSA screen plate, Santa Maria, CA) using the alternate swab procedure outlined by the manufacturer. Any growth after 24 hours of incubation was considered a methicillin resistant suspect strain. Following reisolation, the suspect strain was subjected to full antibiogram performed by minimum inhibitory concentration (MIC) using a standard microwell format.

### **MIC Analysis of MRSA and MSSA Isolates**

Each of the six MRSA isolates was matched to two MSSA controls that had been submitted for antibiograms and confirmed to be oxacillin sensitive. The MSSA controls were matched to the MRSA isolates by species (canine, feline, or equine) and represented the stored isolate that was obtained temporally closest to the case isolate at that facility. Each strain's antibiogram was evaluated to determine the prevalence of resistance to the penicillins, cephalosporins, fluoroquinolones, and macrolides. These were then combined to determine overall prevalence of resistance to the classes in the MRSA as compared to the MSSA group.

### **Pulsed Field Gel Electrophoresis**

Once an isolate was identified as being methicillin resistant *S. aureus* based on microbiological screening and MIC antibiogram (resistant to oxacillin at >2ug/ml), isolates were submitted to the Florida Department of Health Bureau of Laboratories for genotypic characterization using pulsed-field gel electrophoresis (PFGE) following the protocol developed by McDougal, et al.(48). Briefly, a colony was inoculated into broth and incubated while shaking. The turbidity was adjusted and the suspension was centrifuged. The pellet was resuspended and a lysostaphin solution was added in addition to agarose in buffer. The entire mixture was then dispensed into the wells of a

96 well plate and the plugs were allowed to solidify. The plugs were then placed in lysis buffer and incubated prior to washing several times.

The plugs were cut to the desired size, equilibrated in restriction buffer, and then subjected to *Sma*I restriction enzyme prior to further incubation. The plugs were loaded onto the comb tooth and placed in the casting platform to which the equilibrated agarose was added. Pulsed-field gel electrophoresis (PFGE) was carried out and the gel was stained and photographed. A dendrogram of the isolates was derived using the unweighted pair group method using arithmetic means and Dice coefficients to generate percent similarities. A similarity coefficient of 80% was used to define the pulsed-field type (PFT) clusters (48).

## **Results**

### **Microbiological Results**

A total of 966 patients were sampled from 3 domestic animal species: 263 patients were sampled in SP1 (27%) while 703 patients were sampled in SP2 (73%). Of these, 132 patients were swabbed again during their hospitalization with 62 and 70 isolates obtained during SP1 and SP2, respectively. No detectable differences were observed on *Staphylococcus* spp. isolations based on sample storage as well as use of the dry versus Stuart's media swabs (data not shown).

Dogs were most commonly sampled (n=536, 55%) followed by cats (n=257, 27%), and horses (n=173, 18%). General population demographics showed that 50% of the animals sampled were male and 80% were adult. The majority (n=585, 61%) of the patients sampled came from facility 1, while 22% of the samples were submitted by facility 3 (n=208). Additionally 17% (n=173) of the samples were from large animal services with facility 2 contributing 7% (n=72) and facility 4 contributing 10% (n=101).

Facilities 1 and 2 yielded 68% (n=657) of the total submissions while 32% (n=309) of the study population represented the two private secondary care facilities.

Six MRSA isolates were identified which represented 0.62% of the total animals sampled for this study population during the entire sampling period. Three cats, 2 dogs, and 1 horse grew a MRSA isolate. Based on these findings, MRSA colonization prevalence rates were 0.37%, 1.17%, and 0.58% respectively for dogs, cats, and horses. Only 1 patient was found to be colonized in 2006 (sampling prevalence 0.38% or 1 in 263 patients) while the remaining 5 isolates were all obtained in 2007 (sampling prevalence 0.71% or 5 in 703 patients). The results are summarized in Table 2.1.

### **Antibiotic Resistance**

All MRSA and most of the control MSSA isolates demonstrated beta-lactamase activity and were resistant to penicillin and ampicillin (Table 2.2). All 6 MRSA isolates were resistant to the remainder of the antibiotics in the penicillin class, while none of the MSSAs were multi-resistant to penicillins. One MRSA isolate was sensitive to imipenem. In addition to penicillin and cephalosprins, all of the MRSA isolates were resistant to macrolides (Table 2.2). Resistance against the fluoroquinolones class was noted but was variable among the MRSA isolates while no MSSA demonstrated resistance to this antibiotic class. No resistance was detected in any *S. aureus* against vancomycin, rifampin, synergid, or linezolid (Figure 2.2).

### **Pulsed-Field Gel Electrophoresis**

The isolates demonstrating growth on the MRSA Screen plate were subjected to PFGE. Four of the MRSA isolates recovered (2 dog, 2 cats, 66%) were consistent with USA300 strains (Figure 2.1). The pulsed-field type of 2 feline isolates and 1 of the canine isolates aligned with the USA300-0014 strain (19). The other canine isolate

aligned with the circulating USA300-aJCI strain (48). The remaining two isolates did not align with CA- or HA-MRSA reference strains.

### **Discussion**

This study sought to evaluate the rate of nasal colonization of a veterinary patient population presented for evaluation to secondary and tertiary care facilities in North-Central Florida. The study did identify MRSA in pets and helps to underscore the potential implications of MRSA colonized pets on human health. Low rates of colonization were detected in the 3 species sampled, an overall 0.58% prevalence rate. This deviates from a previous report by Weese et al. who identified 5.3% of the equine patients at a Canadian veterinary college hospital as colonized or who became colonized during hospitalization. Reasons for this discrepancy were not elucidated by this study but may be related to differences in geographic distribution and impacting environmental conditions, such as temperature and weather. The present study was not performed alongside an increase in surveillance due to MRSA detection during routine monitoring or due to a hospital outbreak, a condition which preceded the surveillance program reported above (90). This study was cross-sectional in nature, with only 13.7% of the patients being sampled a second time as opposed to the almost 2 years during which surveillance occurred in the Canadian study, with weekly sampling of hospitalized patients (91). An increase in MRSA isolations was noted between the two sampling periods but cannot be validated due to the uneven distribution of sample collection.

The results of antibiogram analysis of suspect strains demonstrated that resistance to the beta-lactam antibiotic class was widespread. All MRSA and most MSSA isolates demonstrated beta-lactamase activity against penicillin and ampicillin. Only the MRSA demonstrated resistance to all the antibiotics in the penicillin class with

the exception of case #3 which was sensitive to imipenem, an intravenous beta-lactam antibiotic. Given the resistance detected against the fortified beta-lactamases in this isolate, the clinical use of this antibiotic would have been questionable despite in vitro results since the presence of a PBP2a would impart resistance to the entire class. All of the MRSA isolates were also found to be resistant to the macrolides tested and to all the cephalosporins. Resistance against members of the fluoroquinolone class was more variable among the MRSA isolates. The results of the antibiogram analysis demonstrated multi-drug resistant attributes of the MRSA isolates obtained. Fortunately, no resistance was detected against vancomycin, rifampin, synercid, and linezolid. However, as these are the last line of defense in many resistant human cases, use in the pet population is to be undertaken with extreme caution so that further resistance in animal-derived isolates does not impact the human population.

The isolation of USA300 from two dogs and two cats is significant given the increasing concerns regarding this strain in people (58). The recovered strains demonstrated typical patterns of resistance associated with USA300, indicating that the strain has continued to propagate without great genomic diversification (19, 101) and by means of clonal expansion (102) similar to what has been previously reported in people. The multi-drug resistant pattern was seen in both USA300 and non-USA300 strains which raises concern that other strain types may be acquiring multi-drug resistance. The combination of PFGE and antibiograms were used to demonstrate similarity in the isolates obtained in this study. Employing whole genome analysis of each MRSA would determine if these animal-derived isolates are as closely associated to each other and

also to known human strains as they appear to be or if various single nucleotide polymorphisms exist that could substantially impact the strains' virulence (102).

Of note, a USA300 positive dog was resampled a month later at the time of its recheck admission and was found to no longer have MRSA but instead was colonized with a methicillin-resistant *S. intermedius* (MRSI). The antibiogram profile for the MRSI was similar to the MRSA's with additional full or partial resistance to various members of the fluoroquinolone family, full resistance to clindamycin, tetracycline, and trimethoprim-sulfa, and intermediate resistance to gentamicin, using the CLSI set points for *S. intermedius* (data not shown). Since colonization in humans increases the risk of developing a subsequent MRSA infection (9, 47), this finding raises the question as to the duration of MRSA colonization in domestic pets and their ability to reservoir the bacterium (95). Reports demonstrating repeated human infections that have been traced back to the household pet (91, 96) point to the need for protocols and control measures that help medical facilities address the issue of colonized pets. A goal in the management of these animals would be to prevent the acquisition of multi-drug resistant bacteria that could serve as sources of reinfection or may transmit virulence factors to normal animal bacterial flora.

As the human MRSA epidemic continues to grow and pets play a more central role in the lives of their owners, increasing reports pointing to transmission between people and pets are likely. Additional studies investigating owners and pets for colonization rates and assessing the genotypic similarities of resulting isolates are necessary to elucidate the epidemiology of zoonotic transmission and thereby provide important foundations for the management of this epidemic and prevention of further

spread in the pet population. Further evaluation of animal-derived MRSA isolate for the presence of virulence factors associated with human disease is also warranted.

Table 2-1. Number of species swabbed, number of samples processed, and resulting prevalence rates of MRSA in dog, cat, and horses patients of 3 secondary and tertiary care facilities in North-Central Florida

	No. Sampled per Species (Samples Processed per Species)			
	Dog	Cat	Horse	Total
Sampling period 1	107 (126)	84 (89)	72 (110)	263 (325)
Sampling period 2	429 (459)	173 (180)	101 (134)	703 (773)
Total	536 (585)	257 (269)	173 (244)	966 (1098)
Total MRSA	2	3	1	6
Prevalence	0.37%	1.17%	0.58%	0.62%

Table 2-2. Overall antibiotic class resistance between MRSA isolates and the MSSA control isolates obtained from dog, cat, and horse patients of 3 secondary and tertiary care facilities in North-Central Florida

Antibiotic Class	Percent of Resistant Isolates (No. isolates)	
	MRSA (n=6)	MSSA (n=11)
Penicillin*	97.2%	28.8%
Cephalosporin†	100%	1.6%
Fluoroquinolone‡	74.4%	0%
Macrolide§	100%	9.1%
Vancomycin	0%	0%

\*\* Penicillins tested include: amoxicillin/clavulanic acid, ampicillin sulbactam, ampicillin, penicillin, oxacillin, imipenem

† Cephalosporins tested include: cefazolin, cefepime, cefotaxime, ceftriaxone, cephalothin, ceftiofur (equine only), cefpodoxime (2007 only)

‡ Fluoroquinolones tested include: ciprofloxacin, enrofloxacin, gatifloxacin, levofloxacin, marbofloxacin, moxifloxacin, ofloxacin

§ Macrolides tested include: azithromycin, erythromycin

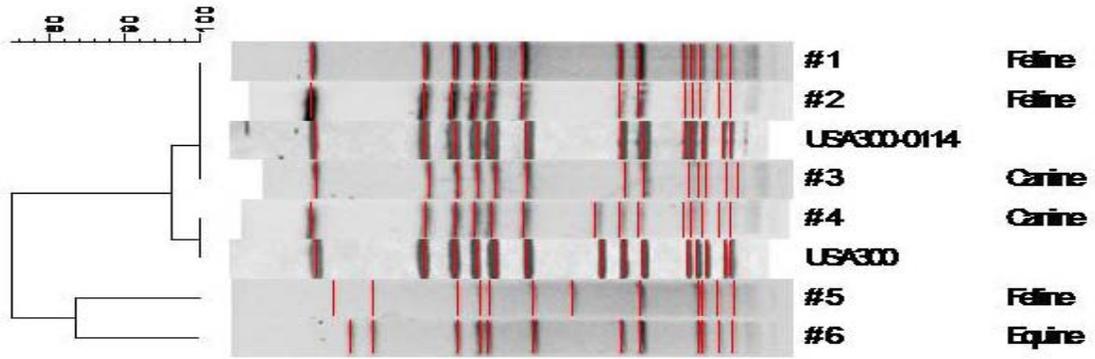


Figure 2-1. Dendrogram showing the relatedness of the 6 MRSA isolates obtained from dog, cat, and horse patients of 3 secondary and tertiary care facilities in North-Central Florida as compared to commonly circulating USA300 strains. Source species is listed. Scale bar indicates genetic relatedness.

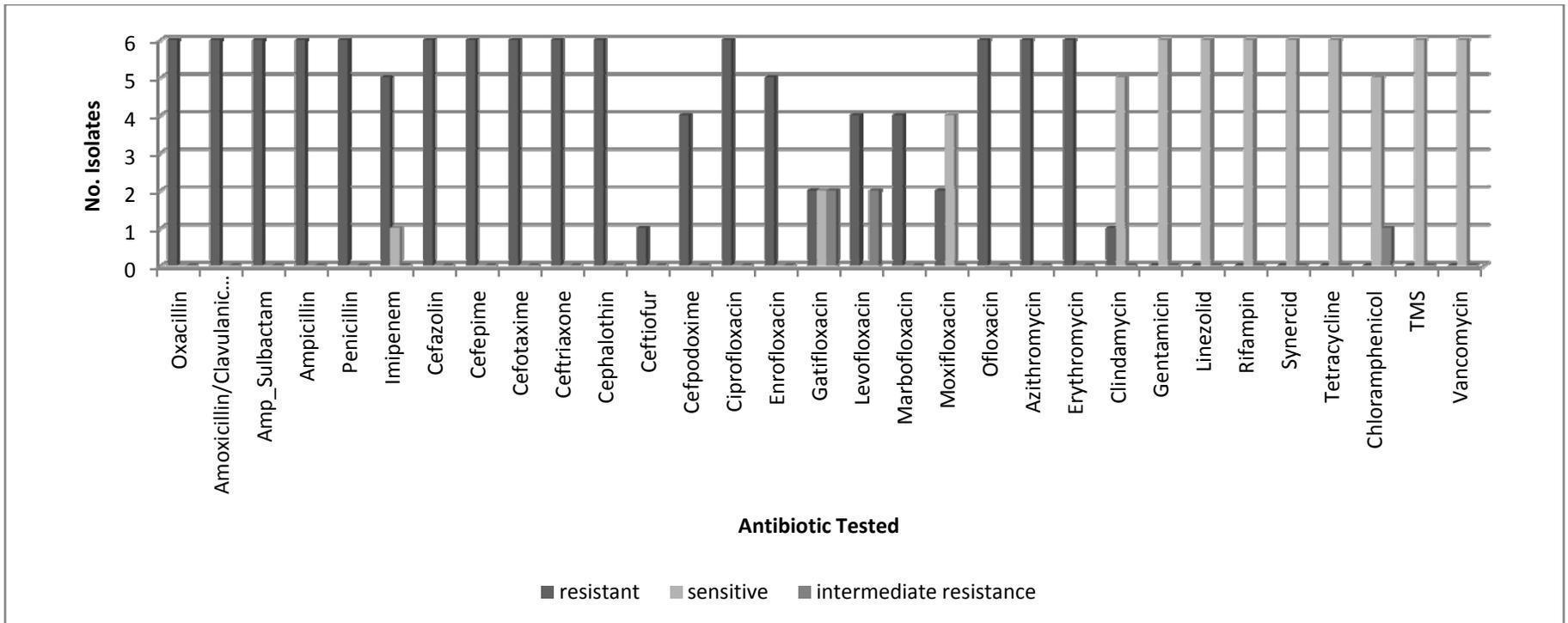


Figure 2-2. Antibiograms obtained from 6 MRSA isolates from dog, cat, and horse patients of 3 secondary and tertiary care facilities in North-Central Florida.

## CHAPTER 3 GENOTYPIC CHARACTERIZATION AND SEQUENCING OF THE MRSA ISOLATES

### **Background**

Known for its propensity to cause disease in humans, methicillin-resistance *S. aureus* (MRSA) is more recently being investigated for the role it may play in animal disease. Not a usual commensal of animals, *S. aureus* has been identified as a colonizer of healthy cats (78, 95), dogs (82, 95, 96), and horses (90, 95). In molecular comparisons of some animal derived MRSA strains to human isolates, many animal MRSA are not typeable, raising the idea that these strains are circulating primarily in the animal population (94, 103). Several studies have found these untypeable animal strains in outbreaks or colonization surveys of humans, suggesting that characterization of all strain has not yet occurred. Conversely, through molecular epidemiology, several clinical infections and nosocomial outbreaks in animals have been caused by MRSA strains primarily associated with human colonization and infection (97). Strains that have been successfully isolated from animals and typed according to human derived MRSA schemes, have been common hospital-acquired MRSA (HA-MRSA) strains such as USA100 (94, 97) though strains carrying *SCC<sub>meclV</sub>* have been obtained as well (95).

During the past two decades there has been a sharp increase in both HA- and community-associated MRSA (CA-MRSA) colonization as well as disease. Colonization is an important risk factor for subsequent primary disease (9) and complications after elective invasive procedures. Whether or not this bacterial evolution diverged from the initial hospital acquired to community-associated genotypes is not known but generally it is accepted that MRSA was initially confined to humans in hospital settings. This strain

was then disseminated into the community and colonized people readily. It continued to circulate and eventually made its way back to the hospital setting. Much effort has been spent in evaluating the genetic changes that MRSA has undergone in its transition from being a hospital-based infection to one detected routinely in non-healthcare associated people. Specifically, researchers have focused particular attention on understanding what virulence factors, in addition to antibiotic resistance, have allowed the CA-MRSA to successfully adapt to the community setting as well as in hospitals to which it has returned. The focus of understanding the full ecology and evolution of the bacterium has broadened to also investigate the role that animals have played in the process.

The first phase of this study determined the nasal colonization rate of dog, cat, and horse patients at three facilities in North-Central Florida. These animals were swabbed upon admission and the swabs were processed using standard microbiological technique. MRSA cases were confirmed with full antibiogram and then subjected to pulsed field gel electrophoresis (PFGE). A total of six isolates were obtained, four of which were typed USA300 and two which were untypeable. In addition, several beta-lactam resistant, but methicillin-sensitive, *S. aureus* were isolated allowing for molecular characterization of virulence factors. It is important to determine the genotypic background of these isolates to establish the genetic relatedness of *S. aureus* and its pathogenicity determinants from a comparative species standpoint. This will allow assessment of the role of animals and assessment of the value of these determinants as tools to perform complete epidemiologic characterization of epidemics. Animal-derived MRSA strains were evaluated for the presence of virulence factors including the staphylococcus chromosomal cassette *meclVa* (*meclVa*), Panton-Valentine leukocidin

(PVL) gene *lukPV*, and the arginine catabolic mobile element (ACME)-encoded *arcA* gene. Accessory gene regulator protein C (*agr*) was used as a marker protein for phenol soluble modulins in addition to directly detecting phenol soluble modulin  $\alpha 3$  (*psm*). These are virulence factors currently under investigation for their role in the pathogenesis of MRSA associated diseases in humans.

## **Materials and Methods**

### ***S. aureus* Isolates**

In a convenience sampling of 966 dogs, cats, and horses performed in North-Central Florida, six isolations of MRSA were made, as discussed in Chapter 2. The species and date of acquisition of MRSA case samples obtained from the colonization prevalence study were determined. Each of the 6 MRSA isolates was matched to two MSSA controls that had been submitted for antibiograms and confirmed to be oxacillin sensitive. The MSSA controls were matched to the MRSA isolates by species (canine, feline, or equine) and represented the stored isolate that was obtained temporally closest to the case isolate. Control isolates ranged in collection dates from 22 days before the MRSA in question to 21 days later as seen in Table 3.1. The average difference among date of collection of all controls as compared to the date of collection of the case isolates was -1.75 days.

### **Microbiological Techniques**

The 6 MRSA case isolates and the 12 control MSSA isolates obtained while performing the colonization prevalence study were recovered from stocks stored on nutrient agar slants or in glycerol stock aliquots at  $-80^{\circ}\text{C}$  and recultured in brain-heart infusion broth at  $37^{\circ}\text{F}$ . A CNA plate was also plated and incubated for 24 hours at  $37^{\circ}\text{F}$  in addition to a new nutrient agar slant for storage. The MRSA-USA300 ATCC #BAA-

1556 (positive control) isolate and the MSSA ATCC #29213 (negative control) isolate were also prepared in this fashion, having previously been rehydrated and stored at -80°C on nutrient agar slants. Turbidity of the broth was evaluated at 24 hours and colony morphology and evidence of beta-hemolysis on the CNA plate was used to confirm monoculture. Glycerol stocks were made from all samples and frozen at -80°C for future use.

### **DNA Isolation and PCR**

Isolation of DNA from each isolate was performed utilizing the QIAGEN DNeasy Blood & Tissue kit (Valencia, CA). Briefly, the bacteria were pelleted by centrifugation and resuspended in a lysozyme solution. The mixture was incubated to promote cell wall lysis prior to adding proteinase K and lysis buffer. The solution was again incubated then centrifuged after which the supernatant was pipetted off into a clean microcentrifuge tube for further use. This step was added to eliminate loss of product due to large proteins clogging up the spin column in future steps. The manufacturer's instructions were then followed as written. To this solution, ethanol (96–100%) was added and the entire mixture was then applied to the QIAamp Mini spin column and subjected to a two-step wash process. Lastly, DNA product was eluted with and the resulting DNA concentrations were determined using a spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE).

Polymerase chain reactions (PCR) for the five genes of interest were set up for each isolate (Table 3.2). Each 50  $\mu$ L reaction contained 50 ng of isolate template, 1  $\mu$ L of 0.5  $\mu$ M forward and reverse primer of the corresponding primer pair, 25  $\mu$ L of Sigma Ready-Mix Taq. The reactions were run on a thermocycler (Perkin Elmer GeneAmp DNA Systems 9600, Waltham, MA) using the following method: 3 minutes at 94°C

followed by 40 cycles of 1 minute at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C, then a final elongation phase of 7 minutes at 72°C before an indefinite hold at 4°C.

The reactions were loaded onto 1% agarose gels stained with ethidium bromide and exposed to current. Each gel had a 100 bp DNA ladder, the USA300 MRSA positive control, the MSSA negative control, and a water negative control loaded as well. The gel was transilluminated using a UV viewer and bands corresponding in size to the target band seen in the MRSA USA300 positive control were identified. PCR purification was performed on these positive reactions following the QIAGEN QIAquick PCR Purification Kit Protocol (Valencia, CA). Briefly, the sample was diluted 5:1 and applied to a QIAquick spin column and centrifuged to allow binding. A buffered wash was applied to the column before the final product was eluted off the spin column with DEPC. The concentration of the final product was determined using the spectrophotometer.

Purified products were loaded onto 1% agarose gels stained with ethidium bromide using a 100 bp DNA ladder and exposed to current. The resulting gels were again imaged on the transilluminator to verify product size. Sequencing was performed by the Sanger sequencing method (Interdisciplinary Center for Biotechnology Research, Gainesville, FL). The sequences were analyzed for similarity to known sequences (BLAST, NCBI, Bethesda, MD) and sequence alignment was performed on the sequence products obtained and compared to reference standards (ClustalW2, European Bioinformatics Institute, Hinxton, UK) employing a blosum matrix (104).

## Results

PCR analysis identified the presence of the 5 genes of interest in all 4 USA300 strains. One of the control MSSA strains, control #2A, also harbored all 5 genes of interest. A second control strain, control #4A was weakly positive for 2 genes, *meclVa* and *lukPV* in addition to being strongly positive for the presence of the *agr* and *psm* genes. All MRSA and MSSA isolates were strongly positive for the *agr* and *psm* genes. However these two genes were the only genes identified in the two non-typed MRSA. The results of the PCR analyses are summarized in Table 3.3.

Each sequence returned a match to the target area for *S. aureus*. Alignment of the sequences to each other demonstrated close homology between the isolates (data not shown).

The *meclVa* gene was identified in 6 isolates: 4 USA300 and 2 MSSA controls, though 1 was weakly positive. When these were subjected to alignment against known sequences, the isolates matched 6 sequences with 99-100% homology. The resulting phylogram of the 6 sequences aligned to the USA300 control can be seen in Figure 3.1.

The *lukPV* gene was identified in 5 isolates: 4 USA300 and 1 MSSA control. When these were subjected to alignment against known sequences, the isolates matched 109 sequences with 71-100% homology, though the majority of the matches were 99-100% (data not shown).

The *arcA* gene was identified in 6 isolates: 4 USA300 and 2 MSSA controls, though 1 was weakly so. When these were subjected to alignment against known sequences, the isolates matched 38 sequences with 70-100% homology with the majority of the matches in the mid 70s% range (data not shown).

The *agr* gene was identified in all 18 isolates, as all 6 MRSA and all 12 controls were positive. When these sequences were subjected to alignment, the isolates matched 100 sequences with 89-100% homology (data not shown).

Similarly, the *psm* gene was identified in all 18 isolates, as all 6 MRSA and all 12 controls were positive. When these sequences were subjected to alignment, the isolates matched 19 or 20 sequences with 82-100% homology (data not shown).

### **Discussion**

Virulence factors associated with USA300 invasive disease in people were detected in the animal-derived USA300 strains. All 4 of the USA300 strains had *meclVa*, PVL, and ACME as well as *agr* and PSM. That the two untypeable strains did not have a *meclVa* gene suggests that they have a different SCC or that their methicillin resistance is mediated by another fashion. It is not surprising that the SCC*meclVa* gene was identified in the 4 USA300 strains as this strain has been shown to carry a typeIV *mec* cassette. MSSA have also been found to have a USA300 PFT, so finding 2 strains that harbored this cassette is not unusual. Control #2A was sensitive to all antibiotics except penicillin and ampicillin suggesting the presence of beta-lactamases (data not shown). An antibiogram of control #4A demonstrated resistance to penicillin and ampicillin, but the strain was not oxacillin resistant. Control #4A also had evidence of resistance against members of the macrolide family (data not shown).

The presence of PVL has been linked epidemiologically to skin and soft tissue outbreaks caused by USA300 strains. The *lukPV* gene was identified in the 4 USA300 strains, supporting the apparent link between the 2 genes. A single MSSA, control #2A, harbored this gene. The *arcA* gene is a marker gene for the presence of the ACME gene cluster. The 4 USA300 strains appeared to harbor this gene cluster, in addition to

both control #2A and control #4A. Neither of the untypeable MRSA strains had evidence of either the PVL or ACME genes, further differentiating them from the USA300 strains.

Not surprisingly, the *agr* and *psm* genes were identified in all the isolates tested. PSMs have been described in all *S. aureus* so their presence in MSSA as well as MRSA strains was expected. What role, if any, these genes play in the virulence of the MRSA strains as compared to the MSSA strains has yet to be fully understood. The primers for *psm* gene were generated by selecting for closest match since detection was of interest given it is believed to incite an inflammatory response. Now that we have shown that all the strains harbor this sequence, extending the target length and sequencing the product will help to evaluate the true importance of this detection by determining if any significant differences exist between the resistant and sensitive strains. The identification of *agr* is similarly important since, again, all isolates proved to harbor this gene which is used as a marker for *psm*. However, the ACME gene cluster is believed to be under regulation by the *agr* gene as well so this may have additional implication as to the virulence of the USA300 strains and warrants further investigation.

The MSSA control #2A deserves further consideration and brings to light questions regarding bacterial fitness upon host-range expansion. Control #2A demonstrated the presence of the virulence factors detected in the USA300 MRSA strains but did not have oxacillin resistance on antibiogram. The bacterium was PBP2a positive on latex agglutination test but did not grow readily on the MRSA screen plate as discussed in Chapter 2. Based on the genetic analysis in addition to the previous microbiological analysis, this strain may actually be a MRSA with heterogenous

expression of methicillin resistance. Further classifying this strain's PFT and subjecting it to different growth parameters to evaluate for altered expression of methicillin resistance would help resolve the classification.

This study sought to evaluate animal-derived MRSA isolates against MSSA isolates for the presence of virulence factors noted in human strains. It also evaluated the virulence factors obtained against banked sequences to evaluate the level of relatedness and thereby confirm their presence. We have shown that animal-derived MRSA strains harbor many of the same virulence factors as those found in people. We have also described 2 MSSA strains which harbor all (control #2A) or most (control #4A) of these factors and suggest that one strain (control #2A) was likely a MRSA with heterogenous expression of methicillin resistance. Once the bacterium overcomes fitness costs associated with the host-range expansion, we may detect MRSA more frequently in animals but at present it appears that the bacteria colonizes animals at a very low rate.

Table 3-1. Date of collection for case (MRSA) and control (MSSA) isolates, the number of days difference between the collection dates, and the species of origin.

Isolate	Date Acquired	Control	Date Acquired	Variance in Days	Species
Case #1	4/3/07	Control #1A	3/30/07	-4	Feline
		Control #1B	3/12/07	-22	
Case #2	8/1/06	Control #2A	7/13/06	-19	Feline
		Control #2B	8/3/06	2	
Case #3	4/3/07	Control #3A	4/5/07	2	Canine
		Control #3B	3/19/07	-15	
Case #4	3/13/07	Control #4A	3/13/07	0	Canine
		Control #4B	3/13/07	0	
Case #5	3/21/07	Control #5A	3/21/07	0	Feline
		Control #5B	3/22/07	1	
Case #6	3/2/07	Control #6A	3/15/07	13	Equine
		Control #6B	3/23/07	21	

Table 3-2. Primer sequences used for PCR analysis

Gene Target	Forward	Reverse
<i>meclVa</i> *	TTTGAATGCCCTCCATGAATA AAAT	AGAAAAGATAGAAGTTCGAAAGA
<i>pvl</i> †	ATCATTAGGTAAAATGTCTGG ACATGATCCA	GCATCAAATGTATTGGATAGCAA AAGC
<i>arcA</i> ‡	GAGCCAGAAGTACGCGAG	CACGTAACCTTGCTAGAACGAG
<i>agr</i>	AGATGACATGCCTGGCCTAC	ACGCGAATGATAGGGTCATC
<i>psm</i>	GGGGGCCATTACATGGAATT	GCCATCGTTTTGTCCTGTA

\* Okuma K, Iwakawa K, Turnidge JD, Grubb WB, Bell JM, O'Brien FG, et al. Dissemination of New Methicillin-Resistant *Staphylococcus aureus* Clones in the Community. J Clin Microbiol. 2002 November 1, 2002;40(11):4289-94.

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‡ Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, et al. Complete Genome Sequence of USA300, an Epidemic Clone of Community-Acquired Methicillin-Resistant *Staphylococcus aureus*. The Lancet. 2006 March 4, 2006;367(9512):731-9.

Table 3-3. Results of PCR analysis for all five genes in every MRSA case and its corresponding two MSSA control isolates

Isolate	Gene				
	<i>meclVa</i>	<i>lukPV</i>	<i>arcA</i>	<i>agr</i>	<i>psm</i>
Case #1	+	+	+	+	+
Control #1A				+	+
Control #1B				+	+
Case #2	+	+	+	+	+
Control #2A	+	+	+	+	+
Control #2B				+	+
Case #3	+	+	+	+	+
Control #3A				+	+
Control #3B				+	+
Case #4	+	+	+	+	+
Control #4A	w+		w+	+	+
Control #4B				+	+
Case #5				+	+
Control #5A				+	+
Control #5B				+	+
Case #6				+	+
Control #6A				+	+
Control #6B				+	+
USA300 control	+	+	+	+	+
MSSA control				+	+

## CHAPTER 4 CONCLUSIONS

The research presented herein sought to identify and characterize MRSA isolates obtained from pets sampled at 3 veterinary hospitals in North-Central Florida. The dogs, cats, and horses receiving veterinary care at these secondary and tertiary care facilities have, by the very nature of their presence in the hospital, owners that are interested in providing for their pet's needs and care. In light of this, it would be safe to suggest that these dogs and cats likely participate as a member of the family in many, if not most cases. Though horses may be viewed in such a light by recreational riders, Florida has a large Thoroughbred industry which generally regards horses as a financial investment and these animals make up a large proportion of the equine caseload at the facilities sampled. However, horses are intensely managed so most are in direct contact with people daily. The intense management on one hand and the close contact with owners on the other provides a natural source of interaction that may allow for transmission of bacteria between people and these animals.

In light of the prevalence of MRSA in the human population, isolation of this bacterium in pets was anticipated. The overall prevalence of MRSA in our pet population was low, suggesting that it is not very common, but similar to findings by others in the veterinary literature (86) as well as in the human literature (6). There is also some evidence to suggest that animals are able to clear the infection, both in this work and in others (95), which supports the idea that the threat MRSA poses to veterinary patients is less than in people. The MRSA strains that were isolated did demonstrate multi-drug resistance, so early identification of these cases will help improve clinical outcomes by directing therapeutic choices. Submitting culture and

sensitivity of infected wounds, recurring abscesses, effusions and the like from symptomatic animals earlier in the course of disease would be clinically indicated in general, but especially in cases where the owner has known risk factors. Empirical antibiotic therapy is prevalent in the veterinary field and may work against clinicians in the long run as it will help propagate antibiotic resistance. However, obtaining culture status early on would allow the veterinary staff and owners to institute appropriate measures, such as barrier controls to prevent spread, if the culture identifies MRSA as the causative agent. Discouraging drastic measures such as euthanasia based on a MRSA culture should be highlighted as outcome may not be negatively impacted by the MRSA-positive status (89) and the risk to people can be mitigated through barrier controls such as gloves and masks.

The identification of 4 USA300 strains was not anticipated. These strains harbored known virulence factors seen in human-derived USA300 strains. It would be of value to know the colonization status as well as the occupation of the owners of these cases to help determine the original source of infection. Further studies investigating the colonization of the general population and their pets would be of value to helping fully determine the potential role that pets serve in reservoiring. The identification of *agr* and *psm* in all the MRSA and MSSA isolates tested requires further investigation to determine the true relevance of these findings. Performing full isolate sequencing or performing PCR with overlapping targets would help identify what lies to either side of the sequence obtained and allow assessment of its role in disease processes. The two MSSA isolates which had harbored 4 or 5 of the genes of interest also deserve further evaluation. Having these two isolates typed with PFGE would determine if they are truly

USA300 MSSAs or if they have an altered SCC*medI*Va. This would also help fully establish the identity of control #2A as a MSSA or, as we suggest, as a MRSA. Additionally, it would be interesting to evaluate these strains for virulence in a rabbit model or against human PMN cells and determine if they produce the degree of disease expected. Moving forward, typing of animal-derived MRSA strains should also be pursued more actively to determine strain source and further characterize the circulating strains. For diagnostic laboratories, the use of a bench-side latex agglutination test to identify PBP2a could be implemented to assist in identifying cases that demonstrate heterogenous resistance of methicillin.

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## BIOGRAPHICAL SKETCH

Katherine Lynn Maldonado attended the University of Florida where she received her Bachelor of Science in animal sciences with an emphasis in animal biology in 2002. She was admitted to the early admission program at the College of Veterinary Medicine and received her Doctor of Veterinary Medicine degree in 2006. Following a short time in mixed animal practice, she returned to the University of Florida to pursue further graduate studies. In December 2009 she received her Master in Public Health degree. While finishing her graduate studies, Dr. Maldonado worked as a full-time veterinary associate with Banfield, the Pet Hospital in small animal practice.