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Staphylococcus pseudintermedius: Population Genetics and Antimicrobial Resistance

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To the Graduate Council:

I am submitting herewith a thesis written by Ricardo Videla entitled "Staphylococcus pseudintermedius: Population Genetics and Antimicrobial Resistance." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

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Staphylococcus pseudintermedius: POPULATION GENETICS AND ANTIMICROBIAL RESISTANCE

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ABSTRACT

Staphylococcus pseudintermedius is a Gram-positive coagulase-negative coccus. It is a normal inhabitant of the skin of dogs. However, clinical disease can be observed in animals that are immunossuppressed or if the skin barrier is altered. This bacterium is recognized as the main cause of canine pyoderma and has also been associated with other conditions such as infection of the urinary tract, the ears, and surgical wounds.

Methicillin resistance and resistance to other antimicrobials regularly used by veterinarians is common among *S. pseudintermedius* which can complicate treatment. The first report of *mecA*, gene responsible for methicillin resistance, in *S. pseudintermedius* is from 1999. Since then, resistance to methicillin and to other antimicrobials has become increasingly more common, making this bacterium a possible reservoir for antimicrobial resistance genes. The reason for the increase in the presence of antimicrobial resistance among *S. pseudintermedius* is still not well understood.

This research focuses on characterization of *S. pseudintermedius* isolates from the United States in order to determine their genetic diversity, antimicrobial susceptibility profiles, and possible relationships among the two. A description of the genetically related populations that are present in the country may help in the understanding of the mechanisms of expansion of this microorganism. Also, the availability of more current information on the susceptibility to antimicrobials should help in the reestablishment of the consequences of misusage of antimicrobials and highlights the need for the development of novel treatment alternatives.

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CHAPTER I
LITERATURE REVIEW

Introduction

Staphylococcus pseudintermedius is a coagulase-positive *Staphylococcus*. It is a canine commensal and opportunistic pathogen, which is analogous to *S. aureus* in human beings. The bacterium is part of the normal flora of the skin of dogs and typically does not represent a clinical problem. However, if the skin barrier is broken (due to trauma, abrasions, surgery, etc,), or if the animal is immunosuppressed, the organism can become pathogenic. In fact, *S. pseudintermedius* is recognized as the main cause of skin infection in the dog and it is also associated with other clinical conditions such as infections of the ears, the urinary tract, and surgical sites [1].

In the past few years *S. pseudintermedius* has gained importance due to the increasing rate of resistance to methicillin and non-β-lactamic antibiotics [2]. This complicates treatment when disease is present and also represents a zoonotic risk since *S. pseudintermedius* may serve as a reservoir of antimicrobial resistance genes. Until now, no research studies have been able to demonstrate that *S. pseudintermedius* can successfully transfer genes responsible for antimicrobial resistance to other *Staphylococcus* species; however, there is clinical evidence to believe this is possible [3].

Even though *S. pseudintermedius* shows specificity for canines and is not usually isolated from people [4], there are reports of identification of this bacterium in human beings [5, 6] and other species such as cats, horses, a donkey, and a parrot [7-11].

Taxonomy

The word ”staphylococcus” comes from the Greek “staphule”, which means a bunch of grapes. It was first discovered in 1882 by Alex Ogston, in 1884, Rosenbach subdivided staphylococci based on the color on the culture media [12]; where *S. aureus* forms gold colonies, and *S. albus* white ones. Around 1950, Smith observed that in canine samples, not all strains were uniform [13]. In 1967, a report proposed a new strain called *S. aureus* var canis, which described those differences observed by Smith in the 50’s [14]. It wasn’t until 1976 that Hajek discovered a new species considered to be the staphylococcal normal flora as well as opportunistic pathogen of dogs, which he named *S. intermedius* [15-20].

For a long time, *S. intermedius* had been considered the agent causing skin and soft tissue infections in canines. However, the advance in technology and the development of new molecular techniques with more powerful discriminatory capabilities, allowed further distinction of 3 different species within *S. intermedius*: *S. intermedius*, *S. pseudintermedius*, and *S. delphini*. The latest was first isolated in 1988 from skin lesions of dolphins; *S. intermedius* has so far only been isolated from pigeons; and *S. pseudintermedius,* first described by Devriese in 2005, was recognized as the common cause of canine cutaneous infection [21]. The name “pseudintermedius” reflects the close genetic relatedness (99% similar) to *S. intermedius* and the inability of discriminating among the two when phenotypic tests are used [22].

The term *Staphylococcus intermedius* Group (SIG) is used to refer to the three previously mentioned isolates (*S. intermedius*, *S. pseudintermedius*, and *S. delphini)* as a group [23, 24]. Based on whole genome analysis, the average nucleotide identity (ANI) between these 3 species is 93.61% [25], very close to the threshold for species delineation (ANI 95%). Therefore, for differentiation of the species, DNA-DNA hybridization was used, and this determined that most canine isolates phenotypically identified as *S. intermedius*, were, in fact, *S. pseudintermedius* [23, 24]. Consequently, since the reclassification of the species, it has been proposed that all canine isolates belonging to the SIG should be considered as *S. pseudintermedius* unless proven otherwise by genetic typing methods [26]. One recent study showed that 100% (44/44) of the isolates that had been classified as *S. intermedius* based on phenotypic properties and PCR amplification of the *S. intermedius*-specific fragment of the 16S rRNA gene, were reclassified as *S. pseudintermedius* once more discriminatory methods were used [27].

Horizontal Gene Transfer

Most familiar eukaryotes have obligatory sexual reproduction, which means that the new organism will carry a combination of the genes present in the progenitors. However, bacteria reproduce by binary fusion, where the DNA of a mother cell is replicated and then divided to generate two daughter cells that are identical among each other and to the progenitor. Based on this, one could assume that microbial populations should be formed by clones of almost identical individuals [28]. However, in reality, bacterial populations are extremely diverse because their genomes are very dynamic.

Genetic information is frequently deleted or incorporated into the bacterial DNA by mutations or by transfer of genetic material from one organism to another through a process other than reproduction or vertical transmission. The later process is known as horizontal gene transfer (HGT). This genetic dynamism contributes to microbial diversification and speciation and has a strong ecological impact [29]. Point mutations will usually result in subtle refinement and alteration of the existing metabolic functions but HGT can immediately introduce novel traits typically associated with antibiotic resistance, pathogenicity, and bacterial metabolism [29]. Taking in consideration that bacterial genomes do not grow in size, the acquisition of foreign genes must be counter-balanced with the loss of native genes. Consequently, it is not always advantageous for bacteria to maintain the foreign genes. If the newly acquired genes confer a selective benefit for the recipient bacteria, they will be more likely to persist in the host chromosome [30] and be transferred to future generations by vertical transmission, otherwise they may be lost.

There are three major mechanisms that bacteria utilize to incorporate foreign DNA, and potentially acquire antimicrobial resistance: transformation, transduction and conjugation.

In transformation, the bacteria take up DNA from the environment[31], through this mechanism DNA can be transmitted between two organisms even if they are distantly related [29]. In transduction the DNA is transferred from one bacterium to another by bacteriophages [31] and both organisms must be recognized by the phage [32, 33]. An advantage of this process is that phage-encoded proteins can promote the integration of the transferred sequence into the recipient's chromosome protecting it from degradation by enzymes such as host restriction endonucleases [29]. Conjugation requires direct contact between bacteria [29]. The transmission

of DNA is mediated by a plasmid or through conjugative transposons. With this mechanism, genetic materials can be transferred between different types of cells. Conjugation is believed to be the most frequent method of antimicrobial resistance acquisition in bacterial populations [31].

Antibiotic Resistance

Definition and Significance

Bacteria originated almost 4 billion years ago and based on the genetic divergence of antibiotic biosynthetic gene clusters, antibiotics are at least hundreds of millions of years old. Bacteria, therefore, have been exposed to natural antibiotics for a very long period of time [34].

Antibiotics represent one of our most effective therapeutic defenses against infectious diseases. However, the continuous use of antibiotics is under enormous threat due to bacterial resistance [34]. The development of antibiotic resistance is a major issue that can compromise the treatment of infectious diseases as well as other advanced therapeutic procedures [35].

Antibiotic resistance in bacteria can occur from acquisition of foreign resistance genes (HGT), from a mutation of the genes, or from a combination of both [31]. Mutations are normally rare, but under stress their frequency is increased [36, 37]. This is known as "mutator state" which can be involved in the development of resistance *in vivo* during antimicrobial treatment [38]. Horizontal transfer of genes is a common event between microbes, and it has the capacity of introducing novel qualities such as antibiotic resistance [29].

The use of antibiotics causes selection of bacteria. The elimination of the susceptible organisms will favor the replication of the resistant isolates due to lack of competition with susceptible flora, facilitating the development of antibiotic resistant strains. A similar effect is seen when susceptible bacteria, for different reasons (incorrect dosing, poor penetration, etc), are exposed to sub-therapeutical concentrations of antimicrobial at the site of infection. A logical action to prevent the spread of antibiotic resistance genes would be to minimize antibiotic use [39]. However, in many instances, the lack of other therapeutically effective agents complicates their replacement.

Resistance to commonly used antimicrobials is frequently encountered within two main species of *Staphylococcus*: *S. aureus* and *S. pseudintermedius*. Resistance to penicillin was

reported in the 1940's, shortly after its introduction, among *S. aureus* strains collected from humans [40, 41]; and beta-lactamase production is now widely disseminated among *S. aureus* and *S. pseudintermedius* in the community [42]. Even though, resistance to antibiotics was not proven until 1940's, a recent report has provided the first direct molecular evidence for antibiotic resistance in ancient sediment samples [43].

In summary, we can say that many of the resistance genes have their evolutionary origin in the antibiotic-producing microbes, which have to protect themselves from the antibiotics they produce. The resistance genes may also originate from environmental organisms, especially soil microorganisms, which have been exposed to various antibiotics throughout their evolutionary history [39].

Antibiotic Resistance in *S. pseudintermedius*

Antibiotic resistance in staphylococci is of great concern due to a continuously increasing incidence of methicillin resistance among *S. pseudintermedius* and other members of the SIG group [44, 45]. Also, a high rate of multidrug resistance is observed among methicillin-resistant *S. pseudintermedius* (MRSP) strains.

Methicillin resistance in '*S. intermedius'* from a canine isolate was first reported in a study published in 1999 [44]. It is important to take in consideration that, since it was not uncommon to misclassify *S. intermedius* as *S. aureus* based on phenotypic tests, MRSP isolates could have been present long before 1999 and erroneously reported as methicillin-resistant *S. aureus* (MRSA). In recent years, an increasing number of MRSP isolates have been identified [45-50]. A study published in 2006 by Morris et al found that as many as 17% of the isolates studied were methicillin resistant [51].

Analogous to that seen in *S. aureus*, the overwhelming majority of resistance to beta-lactamase-resistant penicillins (methicillin being the prototype) in *S. pseudintermedius* isolates is due to the *mecA* gene, which encodes a supernumerary penicillin binding protein (PBP2a) with reduced affinity for beta-lactams [2, 44, 52]. Resident PBPs play important roles in the formation of the bacterial cell wall peptidoglycans [53]. These PBPs can be inactivated by the presence of beta-lactam antimicrobials, leading to abnormal cell wall synthesis and bacterial death. However, the poor affinity for beta-lactams associated with the carriage of the *mecA* gene [54], serves as a mechanism of protection for the bacteria, evading disruption of the peptidoglycan layer and

preventing bacterial death [53, 55]. A recent study from Youn et al [56] suggests the possibility of horizontal transmission of the *mecA* gene from *S. pseudintermedius* between different species. It has been proposed that the *mecA* gene now possessed by MRSA may have originally been present in coagulase-negative staphylococci and later transferred to *S. aureus* [57].

Staphylococcal Chromosome Cassette mec

In staphylococci, the *mecA* gene is located in mobile genetic elements, which are recognized as staphylococcal chromosome cassette mec (*SCCmec*) [58]. These mobile genetic elements are small pieces of DNA that are known to be carriers of virulence and resistance genes. In *S. aureus*, the most important mobile genetic elements are bacteriophages, pathogenicity islands, plasmids, transposons and SCC [59].

It is known that *SCCmec* can be transferred between different staphylococcal species *in vivo* [3], but the mechanisms responsible for *mecA* transfer is still poorly understood. Many studies suggest that *SCCmec* is transferred by HGT in different staphylococcal species [60, 61]. Structural *SCCmec* differences among *S. pseudintermedius* strains can be analyzed and used as a typing method, which is discussed with more detail in a different section of this manuscript.

Multidrug and Methicillin Resistance

Multidrug resistance is recognized as resistance to several antimicrobials, usually resistance to at least three antimicrobials of different classes. It is generally caused by the acquisition of different genes that code for resistance to a single drug, in different acquisition events. This accumulation of antibiotic resistance genes generally occurs on resistance plasmids, known as “R plasmids”, that are not only stably maintained, but that are also passed along between bacterial cells at a very high efficiency. Multidrug resistance can also occur by the increased expression of genes that code for what is known as multidrug efflux pumps. The efflux of drugs play a major role in the resistance to some specific drugs such as tetracyclines and fluoroquinolones [39]. The first multidrug efflux pump discovered in bacteria was the QacA and it was found in isolates from hospital-acquired infections from *S. aureus* [62].

Methicillin-resistant staphylococci are considered resistant to all beta-lactam antibiotics. As discussed above, methicillin resistance in *S. pseudintermedius* is based on the expression of the *mecA* gene. Different antimicrobial resistance genes have been identified in *S.*

pseudintermedius, most of which have also been detected in other staphylococcal species as well as in a few other Gram-positive bacteria [2].

The gold standards for determination of methicillin resistance in *S. pseudintermedius* are *mecA* PCR and PBP2a serology, but other phenotypic methods such as oxacillin and cefoxitin disk diffusion test can also be used [63, 64].

A large number of MRSP strains also show multidrug resistance [27]. In one study from South Korea, where 11 different species of *Staphylococcus* were recovered, *S. pseudintermedius* showed the highest rate of multidrug resistance. All multidrug-resistant *S. pseudintermedius* were resistant to antibiotics commonly used in the treatment of pyoderma, otitis and enterocolitis in dogs [65]. Multidrug resistance is frequent in *S. pseudintermedius* and includes resistance to: tetracycline; macrolides; lincosamides and streptogramins; aminoglycosides and aminocyclitols; fluoroquinolones; and methicillin [25].

The genome of a *S. pseudintermedius* methicillin-susceptible strain (ED99) revealed the presence of four transposons containing one or more antibiotic resistance genes, where two of those contained the *bla* operon, which is responsible for beta-lactamase mediated-resistance. The close similarity of transposons found in human-associated staphylococcal species and *S. pseudintermedius* suggests interspecies horizontal transfer of antibiotic resistance. It should be noted that the mentioned strain, ED99, is resistant to penicillin but susceptible to methicillin since it lacks the *mecA* gene.

The clinical importance of *S. pseudintermedius* is responsible for a high antibiotic selective pressure, which plays a role in the spread of mobile genetic elements encoding antibiotic resistance [25].

Methods for determination

The methods for determination of antimicrobial resistance can be classified as phenotypic methods or molecular methods.

Phenotypic Methods: different phenotypic methods such as disk diffusion, broth microdilution, and the gradient diffusion have been used to phenotypically analyze the antimicrobial resistance of *Staphylococcus* isolates [8, 9, 11, 52, 63, 66-70].

The *disk diffusion* test is the most common method used in veterinary medicine due to the large number of drugs that can be tested and its low cost. This test is based on the diffusion of an antimicrobial agent from a disk that is placed on an agar surface that has been previously seeded from a pure culture bacterial inoculum adjusted to contain aproximately 1-2X10^8 CFU/ml of a pure culture of the bacterium to test. Once the disk is placed on the agar, and after enough time for bacterial replication is allowed, there will be competition between the diffusion of the drug and the bacterial growth. At a certain point, the drug will be too dilute to inhibit the growth of the bacterium and a zone of inhibition will be formed. Thus, the larger the zone of inhibition, the smaller the concentration of the drug that is required to inhibit the pathogen [31].

The *Etest*, also known as the "concentration gradient strip", is a modification of the diffusion test, but in addition, it generates quantitative results. The antimicrobial diffuses from a plastic strip into an agar medium plated with the bacterium. The strip has a defined concentration of stabilized dried drug and an interpretative MIC scale. The dilution susceptibility test can be performed using agar dilution, broth macrodilution, or broth microdilution; of these, agar dilution is the gold standard [31].

Agar dilution and broth macrodilution are too complex for their routine use. On the other hand, the broth microdilution test is being used more frequently in veterinary laboratories. This test is done in microtiter plates, in V bottom wells with antimicrobials of known potency in progressive two fold dilutions; and several drugs can be tested against the selected isolate. This type of test is more expensive than disk diffusion test and has less flexibility [31].

Molecular (Genotypic) Methods: The presence of genes associated with antibiotic resistance can be promptly assessed by PCR. When conventional culture methods are used, results are typically not available until 48 hours later. However, in a clinical setting, a faster method would facilitate rapid implementation of proper antimicrobial therapy and reduction of the usage of broad-spectrum antibiotics for empirical treatment [71]. Heterogeneous phenotypic expression of the *mecA* gene has been described in *Staphylococcus*. This means that an isolate may carry the gene but does not express it, which would lead to misclassification as a methicillin susceptible isolate when phenotypic methods are used. Molecular detection of *mecA* using PCR and PBP2a detection by serological testing are considered the gold standard methods to detect methicillin resistance [72, 73]. Conventional or real time PCR can be used. The use of real time PCR can be

advantageous in certain circumstances because it is faster and it is a semi-quantitative method, meaning that it can, to a certain extent, quantify the amount of DNA present in a sample.

Typing of *Staphylococcus pseudintermedius*

Species Identification

The genus *Staphylococcus* holds 42 species and subspecies of Gram-positive, catalase-positive cocci [74]. Seven different species of coagulase-positive staphylococci have been identified: *S. aureus*, *S. intermedius*, *S. schleiferi* subsp. coagulans, *S. hyicus*, *S. lutrae*, *S. delphini*, and *S. pseudintermedius*. The correct identification of these bacteria is necessary in order to determine methicillin resistance because the MIC breakpoint of oxacillin (a more stable class representative that is used for in vitro detection of methicillin resistance) varies among different species.

Various molecular DNA-methods for the identification of the different *Staphylococcus* species have been developed, but these methods cannot usually be used to distinguish all species simultaneously. A common test used broadly with many different types of bacteria is the analysis of 16S rRNA gene sequences; however, this method gives results that do not correspond with polyphasic taxonomy of the SIG making it inappropriate for differentiation at the species level [75].

As discussed above, isolates previously identified as *S. intermedius* are differentiated into three different species. It is also known, that *S. pseudintermedius* cannot be clearly distinguished from the rest of the members of the SIG by phenotypic methods. Consequently, due to the lack of standardized and specific phenotypic tests, the routine presumptive identification of *S. pseudintermedius* is based on the fact that it is the only member of the SIG that has been isolated from dogs. Therefore, definitive identification of *S. pseudintermedius* relies on molecular methods [21].

Different molecular methods have been developed since the discovery of *S. pseudintermedius*. The first method described was based on *hsp60* and partial *sodA* gene sequences [24]. Later on, in 2009 a PCR-restriction fragment length polymorphism was developed by Bannoehr based on a single MboI restriction site in the *pta* gene of *S.*

pseudintermedius, which is absent in the other SIG members [76]. This results in the production of two characteristic restriction fragments in DNA from *S. pseudintermedius* isolates that will be observed as two separate bands on the agarose gel. In other related species such as *S. delphini*, *S. intermedius*, and *S. schleiferi* this restriction fragment is not present so no changes on the original PCR band are seen after exposure to the enzyme. In the case of *S. aureus*, only one MboI restriction site is present which results in the visualization of a single band on the agarose gel. One disadvantage of this method is that a small proportion of the *S. pseudintermedius* isolates (about 1%) can be misclassified due to heterogeneity of the Mbol restriction site [77]. Another technique that can be utilized for routine species identification of coagulase-positive staphylococci species of veterinary significance is a multiplex PCR targeting the thermonuclease *(nuc)* gene [78]. Proteomic mass spectrometry (MALDI-TOF MS or matrix assisted laser desorption ionization-time-of-flight mass spectrometry) is a rapid and cost effective technique currently being introduced into human and animal diagnostic laboratories [21]. One recent report indicated that this might be an accurate tool for *S. pseudintermedius* species identification [79].

Typing Methods

The importance of typing relies on the fact that different methods can be used to track sources, pathways of spread of infection, and to study the population genetics. An ideal typing technique should be simple, inexpensive, reproducible in different laboratories, highly discriminatory, and easily available [80].

In the case of staphylococci, accurate typing methods are necessary for the monitoring and reduction of its spread [27]. The typing methods that have been used for the typing of *S. pseudintermedius* are based on genetic variability among the isolates. The most commonly used are: pulsed-field gel electrophoresis (PFGE), multiple-locus variable-number tandem-repeat analysis (MLVA), *spa* typing, and multi-locus sequence typing (MLST) [81].

Phenotypic methods are usually easier to perform, easier to interpret, cheaper and easily available, but in general, they are less discriminatory, and usually classify isolates into broad groups. These broad groups are only good at the initial stages and for identification of known epidemic strains. On the other hand, DNA sequence-based methods, such as the ones that will be described, are more practical, able to detect evolutionary changes, and capable of discriminating epidemic strains from endemic ones [80].

Pulsed-Field Gel Electrophoresis

This test is considered one of the most discriminatory methods for bacterial typing, but up until now there is no standardized protocol for *S. pseudintermedius*, therefore an adapted protocol for *S. aureus* is used [21]. PFGE can compare large genomic DNA fragments after digestion with restriction enzymes [82]. The basis of this technique is that when comparing clonal strains of DNA, the lengths of DNA fragments after the exposure to the restriction enzyme should be the same. Consequently, once the DNA is digested, the fragments will be run through an agarose gel, in which the orientation of the electric field across the gel is changed periodically permitting large fragments to be separated and decreasing their overlapping [80]. If two isolates show identical band patterns, then these isolates will be considered to be from the same strain. On the other hand, if they show different patterns due to the action of the restriction enzymes at different sites giving different sizes of DNA fragments, the isolates will be considered from different strains [83]. These gel band patterns are analyzed by a statistical software that classifies the isolates based on a set percentage of similarity among each other [84]. Studies have shown that PFGE application on long term epidemiological studies is not as trustworthy because genetic changes, typically due to point mutations, occurring on the restriction sites will lead to loss of band pattern similarities among isolates that originated from a clonal population [80]. That is why the use of this technique is more useful when comparing isolates from a limited area in a short period of time within a close population [81, 85, 86], such as in an outbreak situation. Another disadvantage is that PFGE is difficult to reproduce due to variations in different factors such as the gel or the electric fields [82], and the fact that some isolates lack the restriction sites for the enzyme, and thus, cannot be evaluated with this technique. A relevant step is the selection of the restriction enzyme, since with this technique we should attempt to generate the simplest pattern with the least number of bands possible, making the interpretation of the data easier.

In summary the major difficulties associated with this technique are the technical demands, the cost of the material (reagents and machinery) as well as the time required to execute the test. The interpretation of the results is complex, but recently, guidelines for the interpretation of the bands have been published which facilitates the association of the results with the existing epidemiological data [80].

Multiple-Locus Variable-Number Tandem-Repeat Analysis

MLVA is a PCR method that analyzes the variation in the number of repeats in several genes. In 2003, a MVLA method was developed for *S. aureus* based on seven genes (*sspA*, *spa*, *sdrC*, *sdrD*, *sdrE*, *clfA* and *clfB*) [87]. In different MRSA studies, MLVA has been proven to be as discriminatory as PFGE [81, 88]. This technique is cheaper and does not need highly specialized training; therefore, it is thought that MLVA will soon replace PFGE [89-91]. In the case of *S. pseudintermedius* MLVA has not yet been developed.

Staphylococcal Protein A (spa) Typing

Spa typing was first developed for *S. aureus* in 1996 [92]. It is a single-locus PCR typing method based on tandem repeat sequence analysis of a highly polymorphic region of the *spa* gene. The relatedness between isolates is determined by statistical software [92]. This technique, based on sequence variation of region X of the *spa* locus [92], has progressively replaced PFGE in outbreak investigations for *S. aureus*, since it is more reproducible and takes less time [21]. A *spa* protocol for *S. pseudintermedius* has been developed, and it is generally used for rapid typing of MRSP. Its discriminatory power is comparable to PFGE and higher than MLST [93]. Among its disadvantages, *spa* typing is not an effective method when typing methicillin-susceptible strains, since more than 50% of them are not typeable due to failure of the current primers to detect the target region or due to production of multiple non-specific bands that complicates sequencing. Another disadvantage is that unusual homogeneous spikes in *spa* types might require other methods such as PFGE or MLVA for finer characterization due to possible mischaracterizations [55]; and it does not have the resolving power of PFGE sub typing [27]. So far, 53 spa types have been assigned.

Multi Locus Sequence Typing (MLST)

This technique analyzes sequence variation at slowly evolving genes with high discrimination. It compares DNA sequences of around 500 bp fragments within typically seven to eight housekeeping genes. An allelic profile is generated based on the combination of differences found at the different sites of variation for each gene, and then a sequence type (ST) is assigned for each isolate based on the combination of alleles for the different loci [21]. Those

isolates that show identical sequences at all loci are considered to be from the same clone, and therefore will have a unique ST [94]. The purpose of MLST is to identify the isolate, not to determine what the differences identified are responsible for. The genes used in MLST are chosen to provide a "population framework", which means that isolates exhibiting similar or identical genotypes are intimately related, and that they descended from a common and recent ancestor [95]. Due to the unmistakable character of DNA sequences, this method achieves data that can be highly reliable [96]. MLST is useful for detecting and studying major changes of the lineages between isolates. It is also functional for periodic typing and global epidemiology [97], and for studies of evolution and population genetics [98-102]. A web-base database for MLST is available (www.mlst.net) for comparison of results.

MLST can be expensive to execute due to the process of DNA sequencing. It is also labor intensive and time consuming since it involves various gene targets [80].

The use of MLST could be different depending on the strain being tested. In the case of *S. aureus* the MLST data does not give information regarding the virulence potential. On the other hand, in the case of *N. meningitidis* for example, the data provides relevant information regarding properties of the isolate. This means that in the case of *S. aureus*, changes in the accessory genome are the ones that cause changes in the virulence of the strain [95]. In the case of MRSA, MLST has been used in combination with PCR analysis of *SCCmec* for the definition of the clonal type of MRSA strains [80].

MLST for *S. aureus* uses 7 loci [99], out of these 7 loci, only *pta* is also used in the MLST method developed for SIG [23]. A 4-locus MLST [16S rRNA, heat shock protein (*cpn60*), elongation factor (*tuf*), phosphate acetyltransferase (*pta*), and the accessory gene regulatory (*agrD*)] based on a sequencing approach developed by Bannoehr et al has been used to study the distribution of MRSP clones [23]. In our laboratory, a more discriminatory-7 locus MLST for *S. pseudintermedius* was recently developed [103]. The new scheme included 3 loci of the previously used MLST (*tuf, cpn60*, and *pta*) and 4 newly selected loci [adenylosuccinate synthetase (*purA*), formate dehydrogenase (*fdh*), acetate kinase (*ack*), and sodium sulfate symporter (*sar*)]. This new MLST has detected multiple STs within the main North American MRSP clone (ST68), and it has revealed methicillin resistance in different genetic backgrounds. It also suggests slow evolution between the lineages that have methicillin resistance[21].

Staphylococcal Cassette Chromosome (SCCmec) Typing

This typing technique is based on the structural differences of *SCCmec*. This method can be used in epidemiological studies to distinguish among MRSP strains or to define an MRSP clone. In 1999 the first *SCCmec* was discovered; to date, eleven *SCCmec* types have been defined[104]. In 2009 the "International Working Group on Classification of SCC Elements" was created. The main purpose of this association is to establish guidelines for identification of *SCCmec* elements for epidemiological studies, determine specific requirements for the description of SCC elements, and have a uniform nomenclature system (IWG-SCC, 2009). Within the *SCCmec* typing, there are three different methods based on: a) restriction enzyme digestion, b) PCR or multiplex PCR (M-PCR), c) real time PCR (Q-PCR) [105].

As mention before, *S. aureus* obtains methicillin resistance through MGE *SCCmec* that contains the *mecA* and the *ccr* gene complexes [80]. Different types of *SCCmec* have been identified and each of them confers resistance to specific antibiotics [106, 107]. The variation between these *SCCmec* types, can be used to identify different MRSA strains [80]. For reliable typing, a combination of MLST and *SCCmec* typing is recommended for surveillance, international transmission studies, and studies of evolution of the different MRSA strains [108].

Staphylococcus pseudintermedius SCCmec elements had previously been classified as *SCCmec* III [24], but in 2008, a study from Descloux et al. reported that some *SCCmec* elements from *S. pseudintermedius* could not be classified using standard PCR methods previously developed [58]. In their study, they discovered two more *SCCmec* elements, which were named *SCCmec* II-III and *SCCmec* VII. In a recent study from South Korea *SCCmec* V was the most prevalent cassette type amongst MRSP [65].

Antibiogram typing

This is a phenotypic method based on the antibiotic resistance profile of the isolate being studied. Isolates that differ in their susceptibility profile will be considered as different strains. The main advantages of this technique are that it is easy to execute, it is inexpensive, and it is available in any microbiology laboratory. On the other hand, in most cases, this method should not be used as the only typing method since it does not have much discriminatory power. It is also important to be aware that there are other factors such as the local environment, antibiotic

pressure, acquisition or loss of genes through plasmids or other mechanisms, that could change the patterns observed [80].

Epidemiology

As previously mentioned, *S. pseudintermedius* is an opportunistic pathogen that is part of the normal flora of the dog and does not cause disease unless the host is immunosuppressed and/or has alteration of the skin barrier. Therefore, exposure between a sick and a healthy dog is typically not sufficient to produce clinical disease. Transmission of *S. pseudintermedius* can occur in several ways:

- Vertical or pseudo vertical transmission. The skin of puppies is normally colonized after birth, probably due to transmission from the bitch, and *S. pseudintermedius* can be detected as early as 1 day after birth [109].
- Horizontal transmission between dogs. Not many studies have looked at this type of transmission in dogs. However, in the case of an MRSP infection, healthy pets in contact are at a high risk of carrying the pathogen [110].
- Interspecies transmission. *Staphylococcus pseudintermedius* does not usually colonize humans, although transmission between pet and owner has been reported [21]. Human beings may become transient carriers if in close contact with an infected dog [5, 47, 110, 111].

The carriage rate of *S. pseudintermedius* was reported to ranges from 46 % to 92% [21]. This variation may be related to differences in sample collection and analysis among the different studies. Dogs with atopic dermatitis have been shown to have a higher carriage rate (87%) when compared with healthy dogs (37%) [19].

Epidemiologic research of the genetic relations between methicillin-resistant staphylococci is important because it helps to understand the spread of the bacteria as well as the relationship between human and animal infections [112]. Human beings frequently carry MRSA and other staphylococci in their anterior nares. Transient carriers of *S. aureus* can be as high as 60% of the people studied [113]. However, this varies depending on the occupation and chance of exposure. People involved in health care show twice the prevalence than the general

population [112]. In the case of veterinarians, around 20% were positive for MRSA in a study done in a teaching hospital in the UK [114]. Colonization with *S. aureus* does not mean infection, but it increases the possibility of MRSA infection up to 10 fold [112]. Transmission is easy, and can occur by direct contact or fomites. Colonization can be transient, persistent, or may not even occur [115]. In pets, colonization with MRSA or MRSP is also common; and as in human beings, being infected with a methicillin-resistant strain does not necessary imply the presence of a more virulent strain, but will certainly increase the rate of treatment failure when compared with a MSSP infection [112]. MRSP prevalence measured from cultures from pets has been reported to be as low as <5% and as high as 17%. Nevertheless, it is believed that the real prevalence may be much higher, since methicillin-resistant isolates can be missed by disk diffusion or broth macrodilution [44]. More recent data indicates that the prevalence in certain regions may be as high as 30% [63, 116].

It is still unclear if, once dogs become infected with MRSP, they became long-term carriers or not. In a study done in Sweden, 31 dogs previously diagnosed with MRSP were sampled for a period of 8 months or until two consecutive negative culture results were obtained [117]. In this study, isolates were compared by PFGE from each dog and the SmaI restriction profiles showed 85% or more similarity between isolates and all of them but two showed similar antibiograms. The results obtained from the study showed that 61% of dogs harbored MRSP for at least 8 months, but re-infection of dogs during the study could not be ruled out. In the same study, non-purulent wound samples had the highest frequency of MRSP isolations (up to 81%). This study indicates that dogs can be carriers of MRSP for months even if they don't show clinical signs, and that the presence of signs does not seem to influence the length of carriage. They were also able to show that longer treatment with antibiotics to which the bacteria were resistant prolongs the carriage of MRSP [117].

Based on what is known so far, the population structure of *S. pseudintermedius* seems to be very heterogeneous. The level of genetic diversity reported in different studies was dependent on the method used (due to the difference in discriminatory power) as well as the body sites sampled. However, all of the studies reported high levels of genetic diversity [21]. On the other hand, in the case of *S. aureus*, five major clonal complexes are recognized as the main human commensal and clinical isolates [118]. Since 2006 the emergence of MRSP has increased significantly due to spread of the main clonal populations [23, 68, 85, 86].

Another study done by Perreten et al [68] determined the phenotypic and genotypic resistance profiles of MRSP and examined their clonal distribution in Europe and North America. In this study they evaluated 103 canine samples from USA, Canada, and different countries in Europe. They identified two major clones, one in Europe (ST71) and another in North America (ST68). MRSP ST71 has also been detected in isolates from dogs from Canada, USA, and Hong Kong [119], which suggest a global spread of the clone. Up to the beginning of 2012, a total of 155 STs based on MLST 4 had been assigned by the curator of the database [21].

Studies on *S. pseudintermedius* characterization have been performed in several countries. In China, a large study done in Guangdgong province, recovered 144 *S. pseudintermedius* isolates from 785 sampled dogs and cats. Almost 50% of the isolates were classified as MRSP. In this study, 24 different STs were identified demonstrating that MRSP in South China has high genetic diversity [97]. In a study from South Korea, staphylococci was isolated in 55.2% (111/201) of the samples obtained from staff, hospitalized animals, and medical equipment. The most prevalent species was *S. pseudintermedius* (46.8%). Of importance, among the MRSP isolates, *SCCmec* V was the most prevalent. The highest detection rate and diversity were found in the staff and not in the animals or equipment, this is a relevant issue since it indicates that people could serve as reservoirs for the dissemination of staphylococci [65]. One study where 146 MRSP isolates from Germany, Netherlands, France, Italy, Austria and Luxembourg were analyzed, showed that ST71 was the main clone detected (145/146), with only one isolate pertaining to a different ST (ST5) [86]. Another study conducted in Spain [120] supported the findings that ST71 is the main MRSP lineage in Europe. On the other hand, a more heterogeneous clonal distribution was reported in Norway, where ST106 (8/23) was the main MRSP clone, followed by ST71 (4/23), ST28 and ST127 (2/23 each), and STs 10, 26, 69, 78, 100, 128 and 129 (1/23 each) [121].

MRSP: a Pet and Zoonotic Pathogen

Healthy dogs have *S. pseudintermedius* as part of their normal microflora of the skin, coat and mucocutaneous sites like the nose, mouth and anus [17, 122-124]. The incidence of colonization varies significantly among different studies, more than likely due to difference in

number and sites of sample collection. Pets such as dogs and cats are usually colonized with *S. pseudintermedius*. It has been reported that 87% of atopic dogs are colonized by *S. pseudintermedius*, in contrast to "only" 37% in healthy dogs [19]. On the other hand, carriage rates in cats is much lower than in dogs, which may imply that cats are not natural hosts of *S. pseudintermedius* [21].

Staphylococcus pseudintermedius is a nosocomial pathogen in veterinary settings, just like MRSA in human medicine [27]. Additionally, people working in animal hospitals have been shown to be carriers of MRSP [24, 47] and therefore could transfer MRSP to animals [27].

Human infections with MRSP have been previously described, however these are uncommon [125]. People can get infected with MRSP after direct contact with pets that are colonized or infected. Also, in one study, similar or non-distinguishable MRSP isolates were isolated from patients, contact animals, and the environment indicating transmission within the household [125]. Infection from dog bite wounds have been reported [21]. In certain cases, human infections with MRSP are difficult to treat and have an increased risk of mortality [110, 126]. Another relevant issue of MRSP infection in humans is that MRSP could provide genetic material by the transfer of *SCCmec* and convert MSSA into MRSA [127].

It is not known if dogs and human beings are either colonized persistently or transiently or if they are just contaminated with MRSP. However, MRSP is rarely isolated form human beings, and very rarely more than once, which suggests either sporadic contamination or rapid elimination if colonization occurs [125]. On the other hand, MRSP can be repeatedly and intermittently isolated from dogs. MRSP was isolated from one particular dog more than a year after the initial sampling, meaning that MRSP can persist in dogs for a long time [125].

In 2009, a study by Frank et al [5] studied the risk of colonization or gene transfer to owners of dogs from which MRSP had been isolated. The study was done in the USA with 25 dog-owner pairs, and the isolates were collected from lesions of infected dogs and the nasal cavity of the owners. Eighteen out of the 25 dogs studied had methicillin-resistant *Staphylococcus spp*, and out of those, 15 (83.3%) were MRSP. MRSP was only found in 2 people. Interestingly, they each had the same susceptibility pattern and *SCCmec* type as the isolates from their dogs. Another study where dogs with deep pyoderma and their owners were sampled, showed that an identical *S. intermedius* was isolated from dogs and their respective owners in 46% of the cases [66]. This is an important issue, since there is evidence to believe

that human beings can acquire an infection from their pet dogs and therefore *S. pseudintermedius* should be considered as a zoonotic pathogen [126, 128]. However, MRSP was no longer present in the owners involved in the first study after the dogs had been treated for a month. It appears that colonization of humans by MRSP is transient and not common. Thus, owners are not at great risk of zoonotic transfer of antimicrobial resistance genes from their dogs and prolonged infections in humans, when present, are believed to be associated with re-infection due to continuous exposure to an infected pet [5]; however, persistent infection should also be considered.

The proper diagnosis of MRSP is of importance not only for the proper treatment of infected animals but also for its zoonotic potential. There has been a raise in the number of human infections with bacteria that are resistant to different antimicrobial drugs, and a major concern is finding effective drugs to combat these diseases [112].

Clinical Relevance

Staphylococcus pseudintermedius is recognized as the main cause of canine pyoderma, which represents the most common dermatological pathology seen in dogs. It is also associated with infections in other body sites such as ears, urinary tract, surgical sites, wounds, mammary gland, and endocardium.

Treatment is generally required when infection is caused by MRSA or MRSP. Treatment for the infection can be topical therapy, combined or not with systemic antibiotics. For the topical treatment, usually lavage and debridement will be done if possible. Conventional treatment relies on antimicrobial ointments such as mupirocin. Unconventional therapy is based on natural products such as oak bark and honey [112].

For systemic antibiotic treatment, drugs have to be chosen based on the susceptibility of the isolate. It is also important to know if the antimicrobial will reach therapeutic concentrations at the site of infection. Irrespective of the culture and susceptibility results, MRSA and MRSP should not be treated with beta-lactams. It is also relevant to know that even if the isolate is susceptible to fluoroquinolones *in vitro*, rapid resistance can develop *in vivo*. Thus, fluoroquinolones are not recommended to treat MRSA [129].

Screening of healthy pets is not recommended, unless animals are exposed to infected human beings [112]. In pet animals, the ideal site for collection of a sample has not been determined. Some studies suggested the use of nasal and perineal swabs [112, 127, 130] but a more recent work revealed low yields when the nostrils were swabbed and discouraged their use for sampling [117]. Decolonization of MRSA in human beings is usually done with the application of an antibiotic ointment nasally and either oral medication or antiseptic baths. In the case of pet animals, application of the ointment would be difficult and decolonization recommendations have not been described.

Hand hygiene is an important precaution to avoid transmission of MRSA or MRSP between human beings and pets in the clinic. If the pet animal is suspected of MRSP, use of gloves and gowns by clinic staff is also recommended. It should be taken into consideration that staphylococci can survive in the environment for as long as months [131], depending on the conditions, therefore, routine disinfection is compulsory.

Immunity

The emergence of resistance to different antimicrobials brings the need for the development of new therapeutic methods to control Staphylococcal infections. Some of the proposed methods include: anti-virulence therapy, vaccines and quorum sensing molecules.

Staphylococcal sp are part of the natural flora of the skin. Different mechanisms help the staphylococcal sp to colonize the host's skin surface. In the case of *S. aureus*, different factors facilitate its adherence and interfere with immune responses. As in *S. aureus* a key factor in the development of anti-staphylococcal immunotherapy is highly dependent on the identification of the bacterial antigens expressed in vivo that will provide protection by the immune system. In the case of humoral immune responses, IgG has shown different results. In some cases, IgG responses to some staph antigens were protective, whereas in other did not show any protective role.

One study performed in Norway, examined the antimicrobial resistance patterns and biofilm-forming abilities of isolates [121]. Isolates of ST71 formed more biofilm than any other MRSP isolates belonging to other studied STs. This is important since previous studies suggest

that the ability to form biofilm helps in bacterial persistence and survival in the environment [132, 133]. They also showed that even though ST106 was the most frequent ST found in their isolate collection, this clone was less resistant to antimicrobial agents when compared to ST71.

Until now, most studies have been based on other staphylococcal species, mainly *S. aureus*. However, *S. pseudintermedius* is different from *S. aureus.* The fact that methicillin resistance was not recognized in *'S. intermedius'* until 50 years after its detection in *S. aureus* and that some of the *SCCmec* types found in MRSP were never described in MRSA, indicate that these two species have evolved separately. Consequently, they manifest important differences in relation to their ecology and epidemiology [21]. Therefore, cross development of diagnostic, therapeutic, or prophylactic strategies are not always recommended. Further characterization of *S. pseudintermedius* would provide resources to better understand and study the pathogenic properties associated with this bacteria.

Research Statement

The goal of this research is to study the population genetics of *S. pseudintermedius* in the United States. Isolates collected from throughout the country have been genetically characterized in order to identify the presence of different clonal groups and sequence types. The newly described MLST 7 typing method was applied to our isolate collection, which allowed further discrimination among groups previously considered as single sequence types. Recognition of the different STs and possible relationships with geographical distribution and antimicrobial resistance patterns can provide further information about the clones that are currently present and may help to better explain the mechanisms behind the rapid emergence of antimicrobial resistance among *S. pseudintermedius.* Furthermore, this epidemiological information can facilitate selection of representative isolates to further study their genetic and immunological properties for better understanding of the factors that influence the pathogenicity of the isolates in order to target new treatment alternatives. Also, eBurst analysis of the MLST data was used to determine which of the STs are expected to be the founders of the different clonal complexes and to identify new STs that could represent a future threat.

From a clinical standpoint, this work provides information about the antimicrobial susceptibility profile of *S. pseudintermedius* in the United States, which elucidates possible trends in the emergence of antimicrobial resistance. Being aware of the high rate of antimicrobial resistance emphasizes the importance of proper use of antimicrobials and stresses the need for development of novel therapeutical alternatives.

CHAPTER II

Molecular Characterization and Antimicrobial Susceptibility of Canine *Staphylococcus pseudintermedius* Isolates from the United States

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Abstract

Staphylococcus pseudintermedius, the primary cause of canine pyoderma, is therapeutically challenging due to a high, and increasing, rate of resistance to antibiotics.

The objectives of this study were: to investigate the genetic diversity within canine *S. pseudintermedius* isolates in the United States, to characterize their antimicrobial susceptibility profile, and to explore possible associations based on geographical origin, genetic relatedness, and antimicrobial susceptibility patterns. Samples from 221 dogs were obtained from veterinary diagnostic facilities located in different regions of the United States. Species identity of 194 isolates was confirmed by PCR-restriction fragment length polymorphism. Pulsed-field gel electrophoresis (PFGE) allowed differentiation of 22 clusters with one major clonal group. Eighty different sequence types were detected based on Multi-Locus Sequence Typing (MLST).

Conventional or real-time PCR was performed to detect presence of the *mecA* gene as an indicator of methicillin resistance, and the Kirby-Bauer disk diffusion method was used to test the susceptibility to 13 antimicrobials. A total of 24 different susceptibility profile groups were generated. An association between the largest clonal group and the most commonly encountered susceptibility pattern was found indicating the presence of one major clonal population. These findings represent a significant contribution for the better understanding of *S. pseudintermedius* genetic diversity in the United States and also reflect the degree of antimicrobial resistance at the time the study was performed. Our findings emphasize the need for the development of innovative therapeutics to address the increasing number of *S. pseudintermedius* isolates resistant to the most clinically useful antimicrobials that are available to veterinarians.

Introduction

Staphylococcus pseudintermedius, a coagulase-positive *Staphylococcus*, is a canine commensal and opportunistic pathogen [1]. It was first described in 2005 [22], before then it was classified as *S. intermedius*. This bacterium is part of the normal flora of the skin of dogs and typically does not represent a clinical problem [1]. However, when the skin barrier is altered, clinical disease can occur. *Staphylococcus pseudintermedius* is recognized as the leading cause of skin and post-operative infections in dogs and cats [134].

Methicillin resistance in *S. pseudintermedius,* same as in *S. aureus,* is mediated by the gene *mecA*, which encodes the penicillin-binding protein 2a (PBP2a). PBP2a has low affinity for beta-lactam antimicrobials, and therefore confers beta-lactam resistance to *Staphylococcus* [65]. The *mecA* gene is located on the staphylococcal cassette chromosome mec (*SSCmec*), a mobile genetic element that may be transferred between different species of staphylococci [3]. Methicillin-resistant *S. pseudintermedius* (MRSP) is usually resistant to various antimicrobial agents used in veterinary medicine, and it can act as a potential reservoir for different antimicrobial resistance genes [68].

The prevalence of MRSP has been increasing significantly over the past years. Based on previous reports, clonal spread of specific sequence types (STs) is the most likely factor responsible for the raising level of methicillin resistance in canine *S. pseudintermedius* [68, 85,

86, 134]. In 2009, Black et al reported that 37 out of 38 methicillin-resistant isolates submitted to one veterinary clinical laboratory in the USA belonged to the same clone, ST68. On the other hand, a larger genetic diversity was observed among methicillin-susceptible isolates. In 2010, Perreten et al found that two major and independent clones, ST71 and ST68, have disseminated in Europe and in North America respectively [68].

Our objective was to determine genetic diversity among *S. pseudintermedius in* the United States, to identify the most frequent STs, to characterize the antimicrobial susceptibility profile of the isolates, and to explore possible associations based on geographical origin, genetic relatedness, and antimicrobial susceptibility patterns. For that purpose we studied canine isolates collected from different regions of the country. Pulsed-field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) of 7 loci were used to investigate the genetic diversity. Also, eBurst analysis of the MLST data was performed to identify the predicted founders within the major clonal complexes.

Genetic characterization of the *S. pseudintermedius* population present in the United States is important to establish if there is expansion of a single or a few clonal groups or if there is presence of several, less frequent, clonal groups. This information can be useful to determine the direction of future research to find out what makes certain STs succeed over others and also to explore novel therapeutical targets. In addition, antimicrobial susceptibility characterization provide information on the current frequency of antimicrobial resistance for the most commonly antimicrobials used in veterinary medicine.

Materials and Methods

Isolate collection

Isolates were obtained as convenience samples from private, state, and university-associated veterinary diagnostic laboratories located in different regions of the United States. These were non-duplicate, recently acquired, canine clinical isolates. They were identified as *S. pseudintermedius*, or a member of the *S. intermedius* group, based on each originating laboratory's routine phenotypic tests. Samples were received over a 3-year period (2008 to 2010).

Regional diversity

To facilitate regional diversity the states were grouped as proposed by the Center for Disease Control based on geographical proximity in 10 regions. At least 9 isolates per region were collected. The distribution of states among each of the regions were as follow: region I (CT, ME, MA, NH, RI, VT), region II (NJ, NY, Puerto Rico, US Virgin Islands), region III (DE, DC, MD, PA, VA, WV), region IV (AL, FL, GA, KY, MS, NC, SC, TN), Region V (IL, IN, MI, MN, OH, WI), region VI (AR, LA, NM, OK, TX) Region VII (IA, KS, MO, NE), region VIII (CO, MT, ND, SD, UT, WY), region IX (AZ, CA, Guam, HI, NV), and region X (AK, ID, OR, WA).

DNA isolation*:*

Isolates were grown on blood agar plates overnight at 37 C° and bacteria derived from a single colony were suspended in 0.5 ml of TE buffer mixed with an equal volume of 0.5 μm glass bead. Cell disruption and DNA extraction were obtained through-pulsed vortexing. For PCR related to MLST, 3 to 4 bacterial colonies were suspended in 3 ml of sterile tryptic soy broth media, incubated at 37 C° overnight, and a commercially available kit (UltraClean® Microbial DNA isolation Kit, Mo Bio Laboratories, inc) was used for DNA extraction.

Isolate identification:

Identification of the isolates by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based on single MboI restriction site in the *pta* gene of *S. pseudintermedius* was performed as previously described [76]. PCR amplification of a 320-bp fragment of the *pta* gene was carried out in a 50 ul reaction volume containing the following: a 0.2 uM oligonucleotide primers (pta fwd 5'- AAAGACAAACTTTCAGGTAA -3', and pta rev 5'- GCATAAACAAGCATTGTACCG -3'), 0.2 mM deoxynucleoside triphosphates, 1.5 mM MgCl2, 0.5 U rTaq DNA polymerase, and 5 ul of DNA template in a 1X reaction buffer. Thermocycling conditions were as follows: 95 °C for 2 min, 30 cycles of 95 °C for 1 min, 53 °C for 1 minute, and 72 °C for 1 min, with a final incubation of 72 °C for 7 min. Samples containing 25 μl of the PCR mixtures were incubated with 5 U of MboI and 5 ul of 5X digestion buffer for 2 h at 37 °C, and the digestion products were resolved in 1.4% agarose gel by electrophoresis. The *pta* PCR product of 320 bp was amplified from all isolates. Restriction analysis products *S.*

pseudintermedius were recognized as two fragments of 213 bp respectively, since this species contains a single MboI site in the *pta* gene.

Antimicrobial Susceptibility

Antimicrobial susceptibility was evaluated for the 194 isolates recognized as S. pseudintermedius. Initially, PCR was performed to detect the *mecA* gene and subsequently standard reference susceptibility testing was performed.

PCR for *mecA* detection: After DNA extraction, detection of the *mecA* gene was performed by conventional PCR as previously described[135] using the following primers: mecA fwd 5'-CATATCGTGAGCAATGAACTGA -3'; mecA rev 5'- AGCAACCATCGTTACGGATT -3'. Isolates that tested negative using *mecA* conventional PCR were tested with a real time *mecA* PCR with previously described oligonucleotide primers and a *mecA* fluorescently labeled probe[136]. Isolates that tested negative to conventional and real-time PCR were considered to be *mecA*-negative.

Disk diffusion susceptibility test: The standard refernce disk diffusion method was performed and interpreted by recommended by the Clinical and Laboratory Standards Institute (CLSI) guidleines (cite). Prepared Mueller Hinton Agar Plates and antimicrobial disks were obtained commercially (BD Diagnostic Systems, Sparks, MD). The antimicrobials tested included: amoxicillin-clavulanic acid, cefoxitin, cephalothin, cefpodoxime, chloramphenicol, clindamycin, erythromycin, gentamicin, marbofloxacin, oxacillin, penicillin, tetracycline, and trimethropin-sulfa. Erythromycin and clindamycin disks were placed approximately 15 mm apart on the plates so that interpretation of inducible clindamycin resistance could be made for isolates that were resistant to erythromycin but otherwise susceptible to clindamycin. By convention, every isolate that was positive on the *mecA* PCR was considered resistant to all β-lactam antimicrobials regardless of the disk diffusion results. The following antimicrobials were tested using the breakpoints recommended by CLSI at the time of performing the study: amoxicillin-clavulanic acid (≥20 mm), penicillin (≥29 mm), cefpodoxime (≥21 mm), cefoxitin (≥25 mm), cephalothin (≥18 mm), oxacillin (≥18 mm), clindamycin (≥21 mm), erythromycin (≥23 mm), tetracycline (≥19 mm), gentamicin (≥16 mm), trimethropin-sulfa (≥16 mm), marbofloxacin (≥20 mm), and chloramphenicol (≥18 mm). Isolates that fell into the intermediate category were classified as resistant to facilitate grouping and analysis. Antimicrobial susceptibility groups were

generated for isolates with identical susceptibility patterns. Multidrug resistance was defined as resistance to 3 or more drugs from different antimicrobial classes.

Genetic relatedness

All the isolates confirmed to be *S. pseudintermedius* were typed based on PFGE, and MLST.

PFGE:

PFGE was performed using the protocol previously described with minor modifications [137]. A single colony from each catalogued isolate was grown aerobically on a blood agar plate for 24 h at 37 °C. From the plate, a cell suspension in TE buffer (2.8 ml, 10 mM Tris–HCl, 1 mM EDTA [pH 8]) was made to a reading of 0.55 (range 0.5 – 0.6) using the MicroScan turbidity meter (Dade Behring Inc., Deerfield, IL). Pre-incubation solution was made with 200 ul cell suspension, lysozyme (10 µl, 10 mg/ml) and lysostaphin (20 µl, 10 mg/ml). This solution was incubated for 45 minutes in a 55-degree water bath. After pre-incubation, plugs were formed in disposable ~100 µl molds by mixing equal amounts of the pre-incubated cell suspension with 1.2% SeaKem Gold agarose (FMC, Rockland, Maine). Formed plugs were incubated in 5 ml of cell lysis buffer (1 M Tris HCL, 0.5 M EDTA, 10% sarcosyl solution, sterile type 1 water) and 10 U proteinase K at 54 °C for 2 h in a shaking water bath. Plugs were washed twice with Type 1 water for 5 min and four times with TE buffer for 15 min each. All washes took place in the 54 °C shaking water bath. Plugs were cut in half and digested using 2 µl BSA, 20 µl of buffer four New England BioLabs (NEB, Ipswich, MA), 40 U SmaI (NEB) and 176 µl of Type 1 water per section. Digestion was achieved over a minimum of 4 h in a 25 °C shaking hybridization incubator. Restriction fragments were separated by PFGE using a CHEF Mapper (Bio-Rad Laboratories, Hercules, CA). Running conditions consisted of 6.0 V/cm, optimized for separation of 30 kb low molecular weight to 600 kb high molecular weight fragments, and 5 s initial switch time and 40 s final switch time for 18 h. Isolates unable to elicit a distinct band pattern with SmaI were restricted with ApaI using the same protocol. Gels were stained with ethidium bromide, destained in deionized water and the images were digitally captured using a GelDoc 2000 UV transilluminator and Quantity One software (Bio-Rad Laboratories, Hercules, CA). The Salmonella Braenderup H9812 global standard was used for gel normalization using

BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and banding patterns were compared in the normalized view using PulseNet E. coli scripts. PFGE DNA fingerprint types were assigned using the Tenover criteria (Tenover et al., 1995).

MLST:

Genetic diversity of *S. pseudintermedius* was also determined by MLST of seven genes as previously described [103]. PCR conditions were as follow: initial denaturation at 95 °C for 90 seconds, 35 cycles of annealing for 30 seconds at 52 °C, extension for 1 min at 72 °C, denaturation for 30 seconds at 94 °C, followed by annealing at 52°C for 30 seconds, and a final extension for 5 minutes at 72°C. Following the reaction, PCR products were resolved and visualized in electrophoresis gels containing 1.4% agarose and 0.5 μg/m ethidium bromide. PCR amplicons were treated with an enzyme that digests single-stranded DNA (ExoSap-IT, USB Corp., Cleveland, OH) and sequenced at the University of Tennessee Molecular Biology Resource Facility (Knoxville, TN). MLST sequences were analyzed using the commercial software Lasergene and compared with allele sequences previously described[103] in order to determine the allele number and ST. New numeric designations were assigned to alleles and ST that had not been previously described.

Data analysis: The contingency tables were constructed and analyzed using the FREQ procedure in SAS (SAS Institute Inc., Cary, NC, USA) to explore for associations among the following variables: PFGE groups vs. MLST results; geographical regions vs. MLST results, PFGE groups, and ASP groups; and ASP groups vs. PFGE groups and MLST results. The MLST data was analyzed with eBURST (http://eburst.mlst.net/).

Results

Isolate collection

A total of 221 non-duplicate canine isolates were collected within the study period. Twenty-seven isolates (12.2 %) were excluded from the study because they were considered to be from species other than *S. pseudintermedius* based on PCR-restriction fragment length

polymorphism. The remaining 194 samples were distributed as follows among the different regions of the country: I (11), II (11), III (10), IV (31), V (17), VI (29), VII (16), VIII (9), IX (34), X (26).

Methicillin Resistance

The 194 isolates studied were classified as methicillin susceptible or resistant based on *mecA* PCR. Forty-six (23.7 %) isolates were susceptible to methicillin, and 148 (76.2 %) were methicillin-resistant.

Antimicrobial susceptibility profile

The isolates were grouped based on shared antimicrobial susceptibility profile (ASP) to 13 antimicrobials. Twenty-two isolates had a unique susceptibility pattern so they could not be grouped, the rest of the isolates were distributed among 24 groups. Table 2.1 shows the susceptibility information for all the ASP groups that were encountered. The largest group (group 8) contained 67 isolates (34.53%) characterized by resistance to all tested antimicrobials except for chloramphenicol. The other main groups included: groups 5, 6, and 9 with 11 isolates (5.67%) each. Group 5 isolates were susceptible to all tested antimicrobials except for penicillin; group 6 isolates were susceptible to all tested antimicrobials; and group 9 isolates were resistant to all tested antimicrobials except for
chloramphenicol and tetracycline. Seven isolates classified as group 7 (3.6%) were resistant to all the tested antimicrobials.

Figure 2.1 shows the number of susceptible and resistant isolates to each of the antimicrobials tested among the methicillin-resistant isolate collection. Figure 2.2 shows the number of susceptible and resistant isolates to each of the antimicrobials tested among the methicillin-susceptible isolate collection.

Multidrug Resistance

Of the 194 isolates, 138 (71.1%) were resistant to at least 3 antimicrobials of different classes. For this purpose, contrary to what was done to generate the ASP groups, the resistant and intermediate categories were not merged. The percentage of multidrug resistance was 85.6%

(125/146) among the methicillin-resistant isolates, and 27.1% (13/48) among the methicillin-susceptible isolates.

Table 2.1. Susceptibility profiles of *Staphylococcus pseudintermedius* from dogs in the United States. .

ASP group	Amoxi/Clav	Cefoxitin	Cefpodoxime	Cephalothin	Oxacillin	Penicillin	Chloramph.	Clindamycin	Erythromicin	Gentamicin	Marbofloxacin	Tetracycline	Trimeth. Sulfa	% of isolates
1	S	S	S	S	S	R	S	R	R	R	S	R	R	1.03
2	S	S	S	S	S	R	S	S	S	S	S	R	R	1.55
3	S	S	S	S	S	R	S	S	S	S	S	R	S	2.06
4	S	S	S	S	S	R	S	S	S	S	S	S	R	3.61
5	S	S	S	S	S	R	S	S	S	S	S	S	S	5.67
6	S	S	S	S	S	S	S	S	S	S	S	S	S	5.67
7	R	R	R	R	R	R	R	R	R	R	R	R	R	3.09
8	R	R	R	R	R	R	S	R	R	R	R	R	R	34.54
9	R	R	R	R	R	R	S	R	R	R	R	S	R	5.67
10	R	R	R	R	R	R	S	R	R	R	S	R	R	1.55
11	R	R	R	R	R	R	S	R	R	R	S	R	S	1.03
12	R	R	R	R	R	R	S	R	R	S	R	R	R	1.55
13	R	R	R	R	R	R	S	R	R	S	R	S	R	1.03
14	R	R	R	R	R	R	S	R	R	S	S	R	R	1.55
15	R	R	R	R	R	R	S	R	R	S	S	R	S	2.06
16	R	R	R	R	R	R	S	R	R	S	S	S	S	2.57
17	R	R	R	R	R	R	S	S	S	R	R	R	R	2.06
18	R	R	R	R	R	R	S	S	S	R	S	R	R	1.55
19	R	R	R	R	R	R	S	S	S	R	S	S	R	1.03
20	R	R	R	R	R	R	S	S	S	S	R	S	R	1.55
21	R	R	R	R	R	R	S	S	S	S	S	R	R	3.09
22	R	R	R	R	R	R	S	S	S	S	S	R	S	1.03
23	R	R	R	R	R	R	S	S	S	S	S	S	R	1.55
24	R	R	R	R	R	R	S	S	S	S	S	S	S	2.06

- ASP-Antimicrobial susceptibility profile group

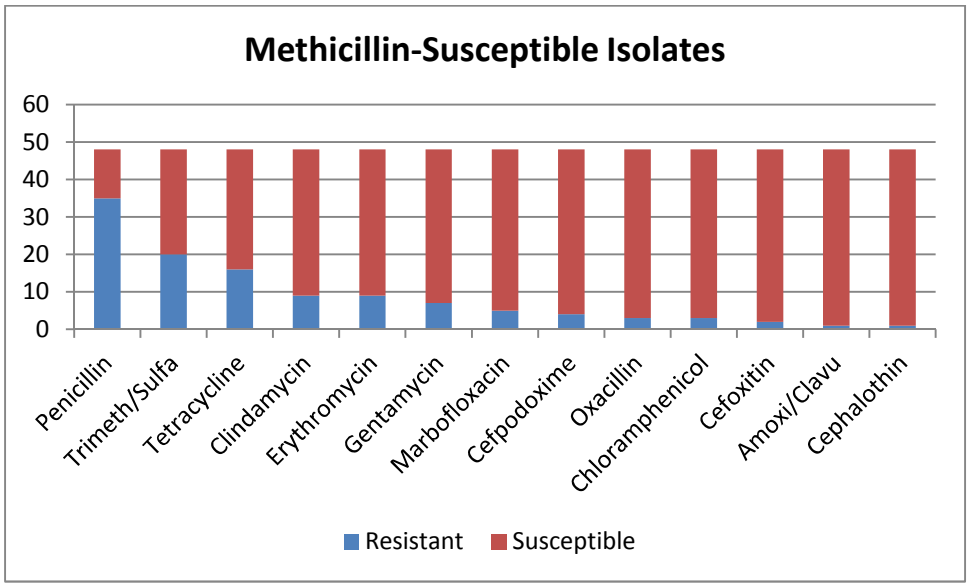

Figure 2.1. Antimicrobial resistance profile among the 48 *mecA* PCR-negative isolates.

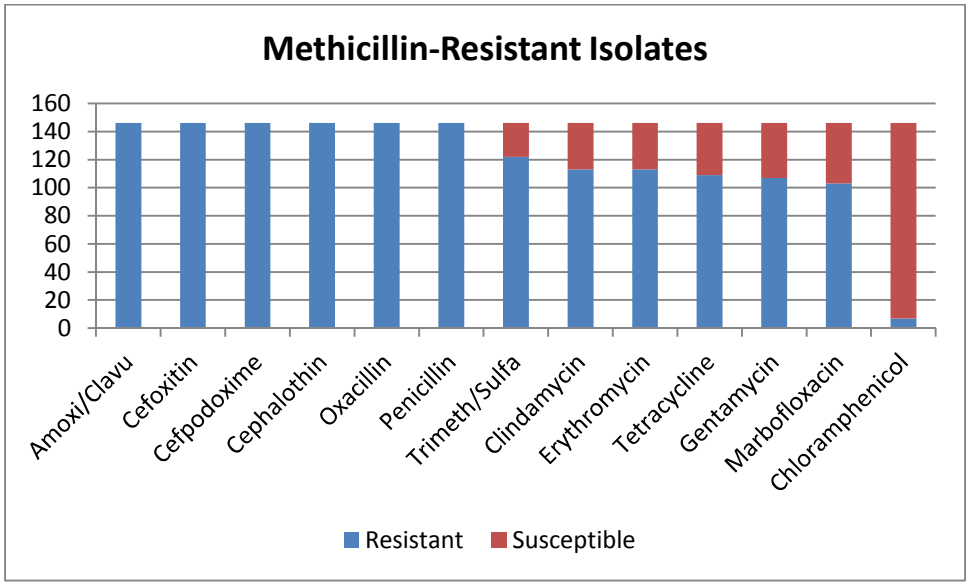

Figure 2.2. Antimicrobial resistance profile among the 146 *mecA* PCR-positive isolates.

Genetic Relatedness

PFGE: 15 isolates could not be evaluated by this technique. Of the remaining 179 isolates, 175 were classified in 22 groups and 4 isolates were unique and could not be grouped. The largest group (group "D") contained 81 isolates. The other main groups were group "L" (27 isolates), group "H" (9 isolates), and groups "C", "J", and "Q" (7 isolates each).

MLST: This method was completed for 177 isolates because some sequences had not been analyzed at the time this manuscript was written. Eighty different STs were recognized. Sixty-seven STs had not been described before. The most frequent ST was ST68 (53 isolates). Other important identified STs were ST71 (16 isolates), and ST84 (10 isolates). None of the other sequence types had more than 5 isolates. Among methicillin-resistant isolates with assigned ST (141 isolates), 47 different STs were detected, and the 36 methicillin-susceptible isolates with assigned ST were distributed among 33 different STs. The detailed allelic profile for each isolate is depicted in table 2.2.

Table 2.2. Allelic profile and ST of all the isolates studied. The letter "M" is utilized when the information is missing for that particular locus and ST.

NA #	*tuf*	*cpn60*	*pta*	*purA*	*fdh*	*sar*	*ack*	ST
1	1	10	4	1	1	1	1	68
2	1	10	4	1	1	5	1	130
3	2	10	1	5	2	5	1	152
4	2	10	1	5	2	1	2	84
6	2	10	1	5	2	2	2	153
7	1	10	1	1	1	1	1	124
8	2	10	1	5	2	2	2	153
9	1	10	4	1	1	1	1	68
10	1	10	4	1	1	2	1	128
11	2	7	11	5	2	2	2	64
12	2	7	11	5	2	2	2	64
13	1	10	4	1	1	1	1	68
14	1	10	4	1	1	2	1	128
15	2	10	1	5	2	1	2	84
16	1	9	2	1	1	2	3	71
17	1	10	4	1	1	2	1	128
18	1	9	2	1	1	1	3	123
19	1	7	1	13	1	1	1	17
24	1	10	4	1	1	1	1	68
26	2	3	1	1	2	1	3	136
27	1	10	4	1	1	1	1	68
28	2	29	1	8	1	1	6	168
29	2	13	1	7	2	2	2	161
30	1	7	1	23	6	1	1	107
31	2	7	1	4	3	1	1	56
32	2	13	1	7	2	1	1	178
33	2	7	1	18	5	1	2	138
35	1	10	4	1	1	1	1	68
36	2	10	4	1	1	1	3	154
37	1	10	4	1	1	1	1	68
38	2	8	1	4	4	2	1	142
39	1	10	4	1	1	1	1	68
40	1	10	4	1	1	1	1	68
41	1	10	4	1	1	1	1	68
42	1	10	4	1	1	1	1	68
43	2	10	1	5	2	1	2	84

Table 2.2. Continued.

NA #	*tuf*	*cpn60*	*pta*	*purA*	*fdh*	*sar*	*ack*	ST
44	1	10	4	1	1	1	1	68
45	2	10	1	5	2	1	2	84
46	1	10	4	1	1	1	1	68
47	2	7	1	4	3	1	1	56
48	1	10	4	1	1	1	1	68
49	2	10	1	5	2	1	2	84
50	1	10	4	1	1	1	1	68
53	1	9	2	1	1	2	3	71
54	1	10	4	1	1	1	1	68
55	1	9	2	1	1	2	3	71
63	2	13	1	7	3	1	3	162
64	1	10	4	1	1	1	1	68
65	2	13	25	1	1	2	4	163
66	2	24	24	7	1	1	7	165
67	1	2	8	1	2	1	2	11
68	1	10	4	1	1	1	1	68
69	1	10	4	1	1	1	1	68
71	1	10	1	1	2	1	2	125
72	2	8	23	8	2	1	3	144
73	2	8	2	14	4	2	6	143
74	2	2	1	7	1	2	8	133
75	2	26	1	14	2	1	3	166
76	2	2	1	13	3	2	3	134
77	2	2	1	13	3	2	3	134
78	1	27	1	25	2	1	1	132
79	1	13	1	M	3	1	1	M
80	2	9	25	M	3	1	3	M
81	2	13	1	M	2	1	2	M
82	2	8	1	4	1	1	3	172
83	1	7	8	M	2	1	7	M
84	2	9	2	M	2	5	1	M
85	2	9	1	3	2	1	1	145
86	5	2	1	M	2	1	4	M
87	1	8	1	M	2	1	4	M
88	2	2	1	M	1	2	4	M
89	1	10	4	M	1	1	1	M
90	1	2	1	M	8	2	1	M
91	1	9	2	M	1	2	3	M
92	1	10	4	1	1	1	1	68

Table 2.2. Continued.

NA #	*tuf*	*cpn60*	*pta*	*purA*	*fdh*	*sar*	*ack*	ST
95	2	13	1	M	2	1	4	M
101	1	10	4	1	1	1	1	68
102	1	10	4	M	1	1	1	M
103	1	10	4	1	1	1	1	68
104	2	7	2	10	2	2	2	139
105	2	9	1	4	1	1	6	146
106	1	3	8	M	1	2	13	M
107	2	11	1	1	2	1	2	155
108	1	2	8	4	2	1	3	103
109	1	2	1	1	2	1	10	101
110	2	13	25	24	2	5	4	175
111	1	2	1	1	2	1	10	101
112	2	2	2	4	1	1	3	135
113	2	13	22	4	1	1	6	177
114	2	13	22	4	1	1	6	177
115	2	13	25	1	2	1	2	164
116	2	9	1	5	2	1	2	147
117	1	8	1	23	2	1	4	109
118	1	7	1	1	2	1	7	104
119	1	10	1	1	1	1	1	124
120	1	10	4	1	1	1	1	68
121	2	28	2	10	3	4	3	167
123	1	8	22	4	2	1	9	120
124	1	10	4	1	1	1	1	68
125	2	8	1	4	1	2	1	141
126	1	9	2	1	1	2	3	71
127	1	10	4	1	1	1	2	127
128	1	2	1	4	2	1	3	3
130	2	7	11	5	2	2	2	64
131	2	11	1	5	2	1	2	156
132	2	7	1	5	2	2	2	137
133	1	10	4	1	1	1	1	68
135	1	9	2	1	1	2	3	71
136	1	7	1	13	1	1	1	17
137	1	10	4	1	1	1	1	68
138	5	10	1	5	2	1	2	179
139	1	9	2	1	1	2	3	71
140	2	7	1	5	2	2	2	137
141	1	7	1	M	1	1	1	M
142	1	10	1	1	2	1	2	125

Table 2.2. Continued

NA #	*tuf*	*cpn60*	*pta*	*purA*	*fdh*	*sar*	*ack*	ST
144	2	7	1	4	3	1	1	56
145	1	10	8	1	2	1	2	131
146	1	7	4	13	1	1	1	108
147	2	10	1	5	2	1	2	84
148	2	7	1	4	3	1	1	56
149	2	13	1	4	1	3	1	160
152	2	10	4	1	1	1	4	188
153	1	7	1	6	1	1	1	105
154	1	10	1	22	2	1	2	126
155	2	10	1	1	1	1	1	150
156	1	10	4	1	1	1	1	68
157	1	9	1	1	3	2	2	121
158	1	9	2	1	1	2	3	71
159	2	10	1	5	2	1	2	84
160	1	10	4	1	1	1	1	68
161	2	10	1	5	2	1	2	84
162	1	9	1	1	3	2	2	121
163	2	11	11	5	2	1	2	157
164	2	10	1	4	7	1	7	151
165	1	10	4	1	1	1	1	68
166	1	10	4	1	1	1	1	68
167	1	10	4	1	1	1	1	68
168	2	9	1	7	1	2	11	173
169	1	9	2	1	1	2	3	71
170	1	10	4	1	1	1	1	68
171	1	10	4	1	1	1	1	68
172	1	9	2	1	1	2	3	71
173	2	7	1	5	2	2	2	137
174	2	7	1	5	2	1	1	171
175	2	13	1	4	1	3	1	160
176	1	10	4	1	1	1	1	68
177	1	10	4	1	1	1	1	68
178	1	9	2	1	1	2	3	71
179	2	9	2	4	5	1	1	148
180	1	7	1	14	4	1	12	106
181	1	9	2	1	1	2	3	71
182	2	13	1	4	1	2	1	159
183	1	10	4	13	1	1	1	170
184	2	9	2	4	4	5	14	80
185	2	10	1	5	2	1	2	84

Table 2.2. Continued

NA #	*tuf*	*cpn60*	*pta*	*purA*	*fdh*	*sar*	*ack*	ST
186	1	10	4	1	1	1	1	68
187	2	7	1	5	2	2	2	137
188	2	7	1	5	2	2	2	137
189	2	13	1	4	1	2	1	159
190	1	10	4	1	1	1	1	68
191	1	9	2	1	1	2	3	71
192	1	10	4	1	1	1	1	68
193	2	7	11	5	2	2	2	64
194	1	10	4	1	1	1	1	68
195	2	10	1	5	2	1	2	84
197	1	9	2	1	1	1	2	169
198	2	7	11	5	2	2	2	64
199	1	9	2	1	1	2	3	71
201	1	10	4	1	1	1	1	68
202	2	13	1	4	1	2	1	159
203	1	10	4	1	1	1	1	68
204	1	10	4	1	1	1	1	68
205	1	10	4	1	1	1	1	68
206	2	19	2	21	2	1	3	176
207	1	10	4	1	1	1	1	68
208	1	10	4	1	1	1	1	68
209	1	10	4	1	1	1	1	68
210	1	10	4	1	1	1	1	68
211	1	10	4	1	1	1	1	68
212	1	10	4	1	1	1	1	68
213	2	10	1	1	2	1	2	174
214	1	10	4	1	1	1	1	68
215	2	7	11	5	2	1	2	140
216	1	10	4	1	1	1	1	68
217	2	7	1	7	3	1	6	181
218	2	24	1	13	3	2	2	182
220	1	9	2	1	1	2	3	71
221	1	9	2	1	1	2	3	71
222	2	10	1	1	1	1	1	150
223	1	7	1	13	1	1	1	17
224	M	M	M	M	M	M	M	M
225	1	10	4	1	1	1	1	68
226	1	9	2	1	1	2	3	71
232	1	10	4	1	1	1	1	68

Analysis of Associations

The classification of the isolates based on the results to *mecA* PCR, oxacillin disk diffusion, ASP, PFGE, MLST, and region of origin are illustrated in table 2.3.

PFGE and MLST: 49/50 (98%) of the ST68 isolates belonged to the main PFGE group (D), and 13/16 (81.2%) of the ST71 isolates belonged to the second largest PFGE group (L). The ST84 isolates were distributed among 8 different PFGE groups.

PFGE and ASP: 55/67 (82%) of the isolates classified as ASP group 8 belonged to the main PFGE group ("D"). 4/11 of the ASP group 6 belonged to PFGE group "L", and 9/11 isolates classified as ASP group 9 also belonged to PFGE group "L".

ST and ASP: 43/53 (84.9%) isolates classified as ST68 also belonged to ASP group 8, and 9/16 isolates classified as ST71 belonged to ASP group 9.

Region and PFGE: The two main PFGE groups ("D" and "L") contained isolates from all 10 regions.

Region and ST: ST68 isolates were obtained from 9 different regions and ST71 isolates were obtained from 8 different regions. Diagnostic facilities located in 4 different regions provided the 10 isolates classified as ST84.

Region and ASP: The ASP group 6 isolates were acquired from 3 different regions: region 4 (5/11), region 6 (5/11), and region 10 (1/11). Each of the other main ASP groups (8, 5, and 9) contained isolates provided by diagnostic facilities from at least 5 different regions.

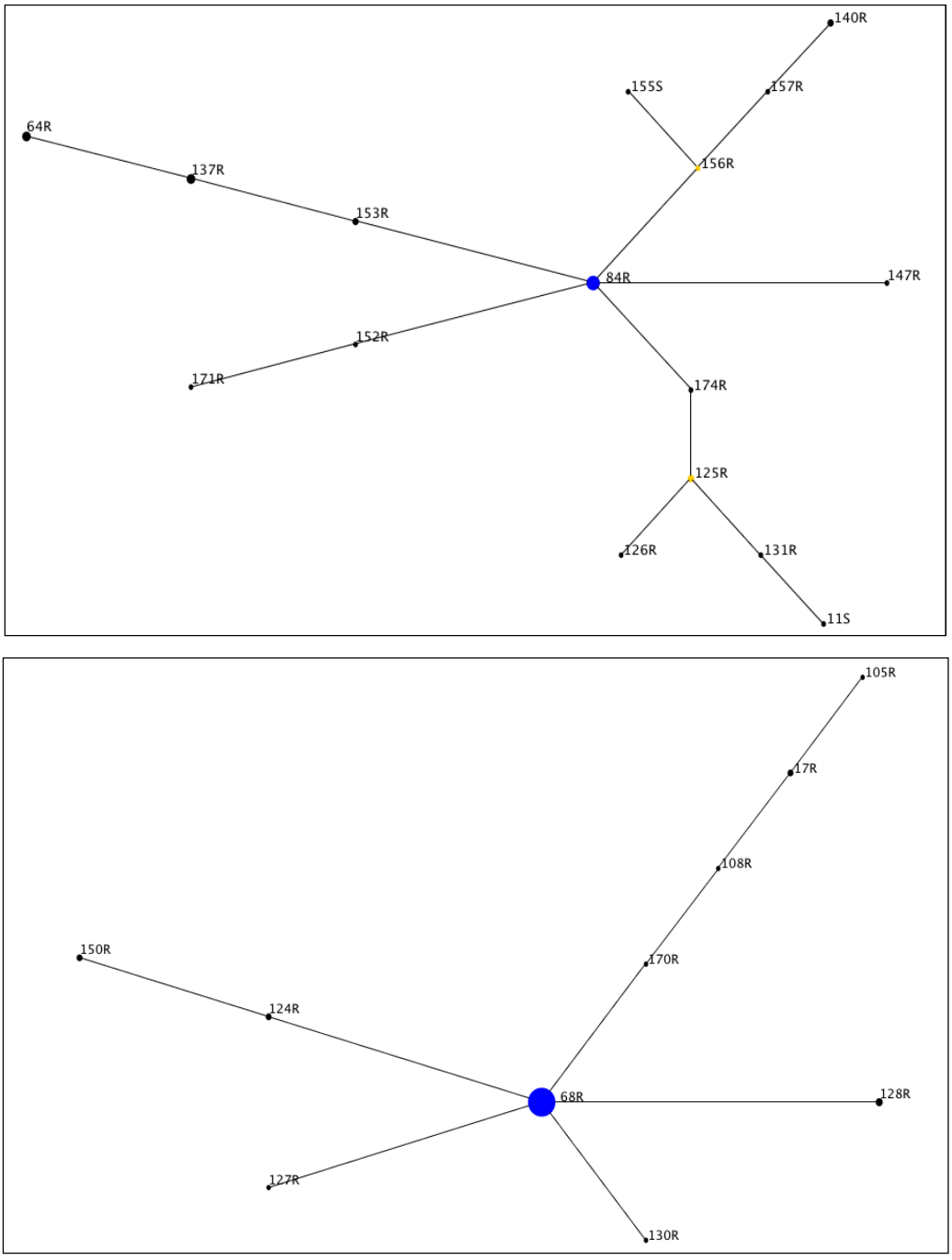

Figure 2.3. eBurst diagrams showing the two main clonal complexes and their predicted founders (ST84 and ST68). The numbers represent the sequence types. Methicillin-resistant isolates have an “R” suffix, and methicillin-susceptible isolates have a “S” letter.

Discussion

Since the reclassification of *S. pseudintermedius* in 2005 by Devriese et al [22, 97], several reports have been published where MRSP has been isolated in healthy and diseased dogs from different countries [44, 68, 85, 97]. MRSP is usually resistant to various antimicrobial agents used in veterinary medicine, and it has the potential to act as a reservoir for antimicrobial resistant genes [68, 97].

In our study, we collected *S. pseudintermedius* isolates from veterinary diagnostic facilities located in different regions of the United States and studied their genetic diversity, characterized their antimicrobial susceptibility profile, and explored possible associations among geographical origin, genetic relatedness and antimicrobial susceptibility patterns. Surprisingly, 12.2% of the samples received by the different diagnostic facilities were not considered to be *S. pseudintermedius* based on PCR-RLP and were eliminated from this study, indicating that the use of phenotypic methods to classify canine staphylococcal infections is not always adequate. However, it should be noted that these isolates were obtained from multiple veterinary diagnostic facilities and it is likely that a different level of accuracy may exist among different institutions. Proper identification at the species level is important to be able to determine precise antimicrobial susceptibility.

In recent years there has been an increase in the number of *S. pseudintermedius* isolates that are methicillin-resistant [46, 47, 50]. Methicillin resistance in staphylococci is mediated by PBP2a, which is encoded by *mecA* on *SCCmec*. The *mecA* gene sequences found in *S. pseudintermedius* are very similar to the ones found in MRSA [50]. As in MRSA, it is believed that expression of *mecA* in *S. pseudintermedius* has the same potential to confer a broad resistance to beta-lactam antibiotics. In this study, a *mecA* PCR was performed to determine methicillin resistance. Our results showed that 75.3% of the isolates in our collection were *mecA* positive. This is important for characterization of the isolates in our collection but it is not representative of the population due to the nature of the sample collection (convenience samples) and the fact that only clinical isolates were included in the study. Molecular detection of the *mecA* gene using PCR is considered the gold standard for making a definitive classification of methicillin resistance [112]. Nevertheless, it should be clarified that there could be heterogeneous expression of the *mecA* gene, meaning that some staphylococci may possess but

do not express the gene, causing a misclassification when using a phenotypic method [112]. In our collection, 6 of the *mecA* PCR positive isolates were susceptible to methicillin based on oxacillin disk diffusion. Also, in a study by Shore et al [138] two MRSA isolates were phenotypically identified as MRSA but lacked the *mecA* gene. Further characterization of one of those isolates indicated presence of the *SCCmec* element, implying that MRSA isolates that test negative for *mecA* could still be resistant to beta-lactams. Due to the similarities between MRSA and MRSP, this could also hold true for MRSP. In fact, 3 of the isolates studied here were classified as *mecA* PCR negative but methicillin-resistant based on the oxacillin disk diffusion test, indicating that, as previously reported, oxacillin resistance does not always indicate a *mecA*-mediated resistance [116]. An isolate could be PCR-*mecA* negative and methicillin resistant if there are mutations in genes that encode PBPs or molecules associated with methicillin resistance, if a highly divergent *mecA* gene is present, or if there are other mechanisms associated with resistance to beta-lactams, such as the production of beta-lactamases [104].

In the analysis of the antimicrobial susceptibility, a major ASP group (group 8) that contained 34.5% of all the isolates in our collection was found. Interestingly, 80% of the ST68 isolates belonged to this group characterized by resistance to all the antimicrobials tested except for chloramphenicol. This, added to the high rates of multidrug resistance among MRSP isolates (85.6%) and MSSP isolates (27.1%) reported here and in previous studies [25], reflects the importance of this bacteria in veterinary medicine. Additionally, the high prevalence of infection with a bacteria associated with such a high rate of multidrug resistance may denote a public health issue since the *SCCmec*, although not yet proven, may be transferred between staphylococcal species *in vivo* [3]. As shown in Figures 1, chloramphenicol is the only tested antimicrobial to which most methicillin-resistant isolates were susceptible in the United States. However, the use of this antimicrobial should be restricted to prevent expansion of resistant bacteria and prolong its efficacy.

MLST, a typing method based on the sequence variation of housekeeping genes, is a valuable method for understanding the molecular epidemiology of bacteria [139, 140]. The recently described MLST scheme that uses 7 loci for typing of *S. pseudintermedius* [103] was used in this study. MRSP had been linked to two major clonal lineages: ST71 in Europe, and ST68 in North America [68, 85]. Interestingly, in our results 80 different STs were identified suggesting a heterogeneous bacterial population. The level of heterogeneity was notably higher

among methicillin-susceptible isolates (33 STs among 36 isolates) in comparison to methicillin-resistant isolates (47 STs among 141 isolates), suggesting clonal expansion in the MRSP population (but not in the MSSP) and a broad range of clonal diversity within the MSSP population. The 3 main STs identified in this study were ST68 representing 28.2% of the isolates, ST71 representing 9% of the studied isolates, and ST84 representing 5.6% of the isolates. All the isolates distributed among these 3 main STs are MRSP. Emerging ST71 clones in USA warrant attention, as they are associated with multidrug resistance and could play an important role in MRSP epidemiology within the United States. This clone, is the most commonly identified MRSP clone spreading worldwide to date [103] and our data indicate that it is also emerging in the US. In the case of *S. aureus,* the population structure is very homogenous, the majority of the commensal and clinical isolates are distributed among five major clonal complexes [118].

PFGE is considered one of the most discriminatory methods for bacterial typing, but until now there is no standardized protocol for *S. pseudintermedius*; therefore, a previously described protocol for *S. aureus* with minor modifications was followed [137, 141]. In our study, 179 isolates were successfully studied with this technique. A correlation was found between PFGE group D (the main PFGE group) and ST68 (the most frequent ST), and between PFGE group L (the second largest PFGE group) and ST71 (the second most frequent ST). In the case of ST84, the third most frequent ST, we found that it was distributed among 8 different PFGE groups. A possible hypothesis to explain the lack of correlation between ST84 and a specific PFGE group could be that this ST may be more ancient and genetic changes had more time to occur.

eBurst analysis of the MLST data, used to study the bacterial population structure of our samples, predicted ST84 and ST68 as the founders of the two main clonal complexes.

We were unable to detect associations between geographical origin of the isolates and a specific ST, PFGE group, or ASP group. It should be noted that, even though the samples were obtained from diagnostic centers located in different regions of the country, they could have potentially originated in regions other than the region where the facilities are located. This, combined with the high transit of dogs across the country, could have compromised our chances of finding geographical associations.

To our knowledge, this is the largest and most comprehensive study done in the United States until now to better understand the molecular epidemiology of *S. pseudintermedius.* The

presence of *mecA* in such a genetically diverse population suggests independent acquisition of *mecA* by each lineage [23]. The data presented here show the presence of emerging MRSP lineages and clonal expansion of the more successful clones, with ST68 being the most frequent clone in the country. The data reported in this epidemiologic study support the need for development of novel therapies to combat disease caused by *S. pseudintermedius* and other multidrug resistant bacteria.

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APPENDIX

Table 2.3. List of the 194 isolates studied detailing results of *mecA* PCR, oxacillin disk diffusion, and group they belonged based on ASP, PFGE, geographical region, and ST. Unique isolates that could not be grouped are referenced as "single". The word "missing" is used when the information for that variable was not available.

Isolate NA#	*mecA* PCR	Oxacillin	ASP group	PFGE	ST	Region
1	+	R	8	D	68	VI
2	+	R	8	D	130	VI
3	+	R	10	L	152	VI
4	+	R	single	K	84	VI
6	+	R	15	D	153	VI
7	+	R	8	D	124	VI
8	+	R	16	L	153	VI
9	+	R	8	D	68	VI
10	+	R	8	D	128	VI
11	+	R	18	missing	64	VI
12	+	R	18	missing	64	VI
13	+	R	8	D	68	VI
14	+	R	8	D	128	VI
15	+	R	8	N	84	VI
16	+	R	9	L	71	IX
17	+	R	8	D	128	IX
18	+	R	9	L	123	IX
19	+	R	8	C	17	IX
24	+	R	8	D	68	IV
26	-	S	5	J	136	X
27	+	R	8	D	68	X
28	+	S	24	P	168	X
29	+	S	21	Q	161	X
30	+	S	24	Q	107	X
31	+	R	single	D	56	X
32	-	S	single	H	178	X
33	-	S	2	C	138	X
35	+	R	7	D	68	X
36	-	R	single	D	154	IV
37	+	R	single	D	68	IV
38	+	R	15	S	142	V

Table 2.3. Continued

Isolate NA#	*mecA* PCR	Oxacillin	ASP group	PFGE	ST	Region
39	+	R	8	D	68	V
40	+	R	8	D	68	V
41	+	R	8	D	68	V
42	+	R	8	D	68	V
43	+	R	21	R	84	V
44	+	R	8	D	68	V
45	+	R	24	L	84	V
46	+	R	8	D	68	V
47	+	R	20	F	missing	V
48	+	R	8	D	68	V
49	+	R	16	K	84	V
50	+	R	8	D	68	V
53	+	R	13	L	71	IX
54	+	R	8	D	68	IX
55	+	R	9	L	71	IX
63	-	R	single	single	162	IX
64	+	R	8	D	68	IX
65	-	S	single	D	163	IX
66	-	S	2	D	165	IX
67	-	S	3	G	11	IX
68	+	R	8	D	68	IX
69	+	R	8	D	68	IX
71	+	R	8	D	125	IX
72	-	S	3	Q	144	VI
73	-	S	6	A	143	VI
74	-	S	6	O	133	VI
75	-	S	4	Q	166	VI
76	-	S	6	L	134	VI
77	-	S	6	L	134	VI
78	-	S	3	C	132	VI
79	-	S	6	D	missing	VI
80	-	S	single	L	missing	VI
81	-	S	5	C	missing	VI
82	+	S	22	single	172	VI
83	-	S	4	N	missing	VI

Table 2.3. Continued.

Isolate NA#	*mecA* PCR	Oxacillin	ASP group	PFGE	ST	Region
84	-	R	single	L	missing	X
85	-	S	5	C	145	V
86	-	S	4	I	missing	V
87	-	S	4	D	missing	V
88	-	S	1	missing	missing	V
89	+	R	8	D	missing	IX
90	-	S	single	U	missing	IX
91	-	S	5	V	missing	IX
92	+	R	8	D	68	IX
95	-	S	2	D	missing	IX
101	+	R	8	D	68	IV
102	+	R	8	D	missing	IV
103	+	R	8	D	68	IV
104	+	R	22	N	139	IV
105	-	S	6	J	146	IV
106	-	S	single	G	missing	IV
107	-	S	1	L	155	IV
108	-	S	5	E	103	IV
109	-	S	6	L	102	IV
110	-	S	3	F	175	IV
111	-	S	6	L	101	IV
112	-	S	4	single	135	IV
113	-	S	6	H	177	IV
114	-	S	6	H	177	IV
115	-	S	5	M	164	IV
116	+	S	23	L	147	IV
117	-	S	5	B	109	IV
118	-	S	4	O	104	IV
119	+	R	8	D	124	IV
120	+	R	8	D	68	IV
121	-	S	5	H	167	IV
123	-	S	4	Q	120	IV
124	+	R	17	D	68	I
125	+	R	21	J	141	II
126	+	R	9	L	71	IV

Table 2.3. Continued

Isolate NA#	*mecA* PCR	Oxacillin	ASP group	PFGE	ST	Region
127	+	R	8	D	127	IV
128	+	R	7	missing	3	II
130	+	R	single	missing	64	IV
131	+	R	14	U	156	III
132	+	R	10	missing	137	IV
133	+	R	8	D	68	II
135	+	R	8	L	71	IX
136	+	R	8	J	17	IX
137	+	R	12	D	68	IX
138	+	R	15	R	179	IX
139	+	R	9	L	71	VI
140	+	R	18	missing	137	IX
141	+	R	8	H	missing	IX
142	+	R	single	D	125	IX
144	+	R	21	F	56	IX
145	+	R	11	D	131	IX
146	+	R	single	C	108	IX
147	+	R	16	T	84	VI
148	+	R	21	Q	56	VI
149	+	R	20	A	160	IX
152	+	R	8	D	180	IX
153	+	R	8	D	105	IX
154	+	R	11	D	126	IX
155	+	R	12	D	150	I
156	+	R	17	D	68	I
157	+	R	7	T	121	I
158	+	R	single	J	71	I
159	+	R	single	L	84	I
160	+	R	8	D	68	I
161	+	R	10	D	84	I
162	+	R	7	H	121	I
163	+	R	single	single	157	II
164	+	R	7	P	151	II
165	+	R	8	M	68	II
166	+	R	8	D	68	II

Table 2.3. Continued.

Isolate NA#	*mecA* PCR	Oxacillin	ASP group	PFGE	ST	Region
167	+	R	8	D	68	II
168	-	S	5	missing	173	II
169	+	R	9	L	71	II
170	+	R	8	D	68	II
171	+	R	8	D	68	III
172	+	R	7	D	71	III
173	+	R	19	missing	137	III
174	+	R	23	A	171	III
175	+	R	20	H	160	III
176	+	R	8	D	68	III
177	+	R	12	D	68	III
178	+	R	8	L	71	III
179	+	R	14	D	148	VII
180	-	S	6	C	106	X
181	+	R	9	L	71	VII
182	+	R	8	Q	159	VII
183	+	R	8	D	170	VII
184	+	R	13	B	80	VII
185	+	R	15	G	84	VII
186	+	R	8	D	68	VII
187	+	R	23	missing	137	VII
188	+	R	single	missing	137	VII
189	+	R	8	S	159	VII
190	+	R	8	D	68	VII
191	+	R	9	J	71	VII
192	+	R	8	D	68	VII
193	+	R	21	missing	64	VII
194	+	R	8	D	68	VII
195	+	R	16	H	84	VII
197	+	R	8	I	169	VIII
198	+	R	single	missing	64	VIII
199	+	R	9	L	71	VIII
201	+	R	8	E	68	VIII
202	+	R	16	D	159	X
203	+	R	8	D	68	X

Table 2.3. Continued.

Isolate NA#	*mecA* PCR	Oxacillin	ASP group	PFGE	ST	Region
204	+	R	8	D	68	X
205	+	R	8	D	68	X
206	+	S	24	V	176	X
207	+	R	17	D	68	X
208	+	R	8	D	68	X
209	+	R	8	D	68	X
210	+	R	8	D	68	X
211	+	R	17	D	68	X
212	+	R	8	D	68	X
213	+	R	14	K	174	X
214	+	R	9	D	68	X
215	+	R	19	missing	140	X
216	+	R	8	D	68	X
217	-	S	5	H	181	I
218	-	S	5	J	182	I
220	+	R	9	L	71	VIII
221	+	S	single	L	71	VIII
222	+	R	8	D	150	VIII
223	+	R	8	L	17	VIII
224	+	R	single	missing	missing	VIII
225	+	R	8	D	68	IV
226	+	R	7	L	71	IV
232	+	R	single	D	68	III

VITA

Ricardo Videla was born in January 4th 1980 in Buenos Aires, Argentina. He obtained a veterinary degree from the Universidad del Salvador in 2003. In 2005 he moved to the United States to pursue further professional development and in 2011 he became a diplomat of the American College of Veterinary Internal Medicine.