The clonal diversity and antimicrobial resistance of methicillin resistant *Staphylococcus pseudintermedius*.

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DEDICATION

I dedicate my work to my late grandmother MATAMELA ELINAH PHOPHI
1936-11-16 to 2020-02-21

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ABSTRACT

Staphylococcus pseudintermedius acts both as a commensal and an opportunistic pathogen in dogs. Methicillin-resistant *S. pseudintermedius* (MRSP) is a form of *S. pseudintermedius* that is inherently resistant to methicillin due to the presence of the *mecA* gene which encodes penicillin-binding protein 2a (PBP2a). The *mecA* gene is usually harbored on a mobile genetic element, SCC*mec*, that can be transferred among *staphylococcus* species through horizontal gene transfer. In addition to β-lactams, MRSP isolates can be resistant to aminoglycosides, tetracyclines, macrolides, and folate dehydrogenase inhibitors. In clinical settings, MRSP presents a challenge because of ineffective antibiotic treatment and the persistence of infections. Therefore, the development of alternative therapeutics for MRSP treatment is important.

The characterization of essential MRSP strains is an important step in the development of alternative therapeutics. MRSP strains in the United States were previously characterized using Multilocus Sequence Typing (MLST) and Based Upon Related Sequence Types (BURST) analysis. This study however lacked whole genome sequencing (WGS) to distinguish antimicrobial resistance and virulence genes between the MRSP strains. To characterize MRSP isolates from dogs in the United States, we used antimicrobial susceptibility, WGS, MLST, geoBURST, antimicrobial resistant gene identification, virulence gene characterization, and phylogenetic analysis. Using similar methods, we have also reported on the presence and molecular epidemiology of MRSP in South Africa. Additionally, we characterized *S. pseudintermedius* of human origin compared to canine origin. The initial results were the detection of new clonal lineages and antibiotic resistance of MRSP in the United States. Secondly, phylogenetic analysis, recombination, and positive selection of the MLST data showed that MRSP lineages currently circulating in the United States do not share a recent common ancestor with isolates reported 10 years ago. The antimicrobial resistance of *S. pseudintermedius* isolated from dogs has also increased over the past ten years. The molecular epidemiology of *S. pseudintermedius* among dog isolates from South Africa showed a high prevalence of MRSP and a non-clonal population structure. High antimicrobial susceptibility and a non-clonal population structure of *S. pseudintermedius* were observed among human isolates compared to canine isolates. Finally, the study suggests that the bla regulators are important in the control of oxacillin-resistant phenotype in *S. pseudintermedius*. These results have interesting epidemiological implications regarding the evolution and host adaptability of MRSP around the world, which may impact the treatment of MRSP infections and development of alternative therapeutics in the future.

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LIST OF ABBREVIATIONS

Aac (6')-Ie-aph (2')-Ia - acetyltransferase/phosphotransferase gene
ARIBA - Antimicrobial Resistance Identification by Assembly
Ack - acetate kinase
Ant (6)-Ia - adenyl nucleotidyl transferase gene
Aph (3')-III - phosphotransferase gene
blaZ - gene encoding B-lactamase
BLAST- Basic Local Alignment Search Tool
BURST- Based Upon Related Sequence Types
catpC221 - chloramphenicol acetyltransferase genes
CARD - Comprehensive Antibiotic Resistance Database
CC - clonal complexes, determined by MLST
CLSI - Clinical and Laboratory Standards Institute
CoNS - coagulase-negative staphylococci
Cpn60 - chaperonin protein
DLV - double locus variant
ermB - macrolide resistant gene
fdh - formate dehydrogenase
HGT - horizontal gene transfer
icaA - intracellular adhesion gene
luk - Leukotoxin
MALDI-TOF MS - matrix assisted laser desorption ionization-time of flight mass spectrometry
MDR - Multidrug Resistance
mecA - gene encoding penicillin-binding protein 2a
MGE - Mobile genetic element
MIC – Minimum inhibitory concentration
MLST - Multilocus sequence type(ing)
MRSA - Methicillin-resistant *Staphylococcus aureus*
MRSP - Methicillin-resistant *Staphylococcus pseudintermedius*
MSSP - Methicillin-susceptible *Staphylococcus pseudintermedius*
PBP2a - Penicillin-binding protein 2a
PCR – Polymerase Chain Reaction
Pta - Phosphate acetyltransferase gene
purA - Adenylosuccinate synthase

Sar - Sodium sulfate symporter
SCC*mec* - Staphylococcal cassette chromosome *mec*
SEA - Staphylococcal enterotoxin A
SIG - *Staphylococcus intermedius* group (*S. delphini, S. intermedius, and S. pseudintermedius*)
SIET - *Staphylococcus intermedius* exfoliative toxin
SLV - Single locus variant
ST - Sequence Type
Spa - staphylococcal protein a
SXT - Trimethoprim- sulfamethoxazole
tetM - tetracycline resistant gene
TLV - triple locus variant
Tuf - elongation factor tu
USA - United States
WGS - Whole Genome Sequencing

CHAPTER I
Literature review

Overview

Methicillin-resistant *S. pseudintermedius* (MRSP) has generally become highly resistant to antibiotics used in the treatment of canine infections such as pyoderma, otitis externa, urinary tract infections (UTI), respiratory infections, and reproductive tract infections [1–3]. The presence of the *mecA* gene, harbored on the mobile genetic element called the SCC*mec* is responsible for the methicillin-resistant phenotype of *S. pseudintermedius* [4–6]. The *mecA* gene encodes a low-affinity penicillin-binding protein 2a (PBP2a) that confers resistance to β-lactam antibiotics [7–9]. Isolates that are MRSP are often multidrug-resistant. The term multidrug resistant refers to an organism that is resistant to at least one antimicrobial drug from three or more classes of antibiotics [10]. MRSP resistance has been detected to drugs such as macrolides (erythromycin), lincosamides (clindamycin), fluoroquinolones (marbofloxacin), tetracyclines (tetracycline), and potentiated sulfas (trimethoprim-sulfamethoxazole) [3, 11–14]. Importantly, studies on the epidemiology of human infections caused by *S. pseudintermedius* remain scarce. However, MDR in human MSSP isolates that harbored *blaZ*, *ant6-Ia*, *aph (3')-III*, *cat* $_{(Pc221),}$ *erm(B)*, and *tet(M)* resistant genes have been reported [15].

Molecular diagnostics together with whole genome sequencing (WGS) and multilocus sequence typing (MLST) have been used to detect genetically diverse strains of MRSP [16–18]. A systematic global review in 2016 reported that there are five major CCs (CC71, CC258, CC45, CC68, CC112) circulating in three continents (Europe, Asia, and North America) [19]. The review suggested that the population structure of *S. pseudintermedius* is weakly clonal and primarily driven by homologous recombination [19]. However, some countries/continents (e.g., Africa, South America, and Russia) are underrepresented due to the lack of MLST reports in these regions [19]. In the United States, studies have reported CC68 to be the predominant clonal complex [19–22]. A review of MRSP collected in 2010 in the US region revealed that most of the isolates from dogs comprised ST84, ST71, and ST68 [20]. A more recent study, published in 2020 reported ST45, ST64, ST68, ST71, ST84, ST150, ST155, and ST181 in the dog populations of the New England and Texas regions in the United States [21]. However, this study did not sample MRSP across the United States and did not identify the predominant clonal complexes. In most European countries, CC71 has always been described as the predominant clonal lineage among the MRSP isolates [19, 23, 24]. However, recent studies in European countries are reporting a decline in the detection of isolates belonging to CC71 superseded by the detection of isolates belonging to CC258 [11, 19, 25]. Furthermore, there has been an increase in the detection of new STs such as ST551, ST497, ST402, ST1053, ST1062, and ST496 in countries such as Italy, Australia, Slovenia, and France that do not belong to the major clonal complexes [12, 25–28].

The ability to harbor genes related to antimicrobial resistance and virulent genes most likely has an impact on the success of each clonal lineage. For example, in Europe, isolates belonging to CC258 showed a significantly lower prevalence of resistance to gentamicin and enrofloxacin compared to isolates belonging to CC71 and CC45 [25]. Whereas isolates belonging to CC71 are more susceptible to trimethoprim/sulfamethoxazole compared to CC258 isolates which are more susceptible to enrofloxacin and aminoglycoside [11, 20]. In France, the novel ST496 MRSP lineage detected between 2015-2016 showed systemic resistance to all veterinary-licensed antibiotics with susceptibility only present for florfenicol and fusidic acid [25]. Furthermore, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole resistance among CC71 isolates in the United States were reported to show notable differences with resistance among CC71 isolates studied in other continents [20]. Therefore, successful antibiotic treatment of MRSP canine infections could vary among Multilocus sequence types in different regions.

Most of the virulence-associated genes of *S. pseudintermedius* are generally thought to be conserved across different multilocus sequence types. Virulence-associated conserved genes such as *coa* (coagulase) and *sps*K (cell-wall anchored surface protein) have been reported among the emerging clone of ST551 isolates but not in ST71 isolates reported in Slovenia [28]. A vaccine based on immune evasive *S. pseudintermedius* proteins such as protein A (*spsQ*), leukotoxin (*lukF* and *lukS*), adenosine synthase (*adsA*), coagulase (*coa*), and exotoxin 15 (*SpEX*) could theoretically protect the host from infection [29–32]. There is a lack of information regarding how virulent factors contribute to the dissemination and emergence of successful MRSP lineages.

Antibiotic treatment failure is a serious concern in canine health. Alternatives to antibiotic treatment such as anti-virulence therapy, vaccines, bacteriophages, and quorum sensing molecules could offer successful treatment of canine infections associated with *S. pseudintermedius* [33, 34]. Therefore, detection of newly resistant strains using WGS and MLST could help identify evolutionarily conserved regions that can provide attributes contributing to the successful spread of MRSP lineages. Such analyses have been used to identify regions that could circumvent the evolution of resistance, identify antibiotic targets, and help develop inhibitory compounds [7, 35–37].

Taxonomy history of *S. pseudintermedius*

According to nomenclature and taxonomy, *S. pseudintermedius* is described as a commensal opportunistic Gram-positive bacterium belonging to the *Staphylococcus* genus [2, 38–40]. Phylogenetic analysis based on complete genome sequencing of 16S rRNA, *dna*J, *hsp*60, *rpo*B, and *sod*A genes has played an important role in distinguishing closely related species belonging to the *Staphylococcus* genus [39, 40]. History presents *Staphylococcus* and *Micrococcus* belonging to the

Stomacoccus and *Planococccus* genera as part of the Micrococcaceae family [41, 42]. Results indicating a low G/C content in chromosomal DNA suggested that the *Staphylococcus* genus is a Gram-positive bacterium belonging to the *Bacillus-Lactobacillus-Streptococcus* cluster [42, 43].

In the 19th century, Ogston *et al.* identified staphylococci in pus from abscesses, which he later termed staphylococci based on the grape clustered growth on the petri dish [44]. Ogston *et al.* used the growth of staphylococci to differentiate between cluster-forming *Micrococcus* and chain-forming *Streptococcus* [44, 45]. In the 19th century, all staphylococcal species in veterinary and human medicine were regarded as *Staphylococcus aureus* regardless of microscopic, pigment, and hemolytic variation. It was not until the late 20th century that further investigations based on improvements made in biochemical testing into the *Staphylococcus* genus clarified the interrelationships between members of the genus [46–48]. In 1884 Rosenbach *et al.*, subdivided the genus based on the culture media color [42]. *Staphylococcus aureus* formed a gold colony-forming culture whereas *Staphylococcus albus* formed white colonies [42]. Over the years, the *Staphylococcus* genus has reached a description consensus as a facultative anaerobic bacterium that displays spherical clusters in cocci that are about 0.5-1 μm in diameter [42, 49, 50]. This genus is divided into two distinct groups: coagulase-positive staphylococci and coagulase-negative staphylococci [51–53]. At the time of writing, there are 84 species and 30 subspecies cited in the *Staphylococcus* genus [54]. *Staphylococcus pseudintermedius* is a coagulase-positive *staphylococcus* together with *Staphylococcus aureus*, *Staphylococcus argenteus*, *Staphylococcus delphini*, *Staphylococcus cornubiensis*, *Staphylococcus Lutrae*, *Staphylococcus coagulans*, and *Staphylococcus scheitzeri* based on their ability to coagulate blood plasma [55, 56]. In contrast, *Staphylococcus epidermis*, *Staphylococcus ursi*, *Staphylococcus heamolyticus*, *Staphylococcus lugdunensis*, and *Staphylococcus schleiferi*, are considered coagulase-negative *Staphylococcus* due to their inability to coagulate blood plasma [53, 57].

As with the difficulty of the identification of the *Staphylococcus* genus, the identification of *S. pseudintermedius* proved to be a challenge. *Staphylococcus pseudintermedius* was first isolated in 1976 as *S. aureus var. canis* [58]. This was after smith *et al*. in 1947 described microorganisms from canines showing different characteristics and pathogenicity in biochemical tests compared to those isolated from humans and bovines [59]. The proposal for subdividing the *S. aureus* species was published in 1967 and included characteristics such as β coagulase with bovine plasma, white pigment production, white crystal violet type formation, negative fibrinolysis, beta hemolysins, and phage susceptibility [58]. In a more detailed study, Hajek *et al*. demonstrated a diverse group of coagulase-positive staphylococci in pigeons, dogs, mink, and horses [40]. They were then proposed to be placed as a new species that represented *S. intermedius* intended to indicate that these species possess some characteristics of *S. aureus* and *S. epidermis* [40]. The description made

by Hajek *et al.* showed a strong indication of *S. intermedius* as the primary staphylococcal normal flora of canines. However, the type of strain NCTC 11048 isolated from pigeons was typically used to represent *S. intermedius* in species differentiation studies [40]. The *staphylococcus intermedius* group (SIG) is comprised of closely related coagulase-positive *staphylococcus* species that include *S. intermedius*, *S. cornubiensis, S. delphini,* and *S. pseudintermedius* [39, 60, 61]. The SIG species has a 93.61% nucleotide sequence identity (highly conserved) within their core genome, which confirms their close relatedness [62].

In 2005, Deveriease *et al.* used DNA-DNA hybridization to reveal *S. pseudintermedius* as a novel pathogen distinct from *S. intermedius* [39]. Based on the investigations of SIG strains from different countries by multilocus sequence typing (MLST), *S. pseudintermedius* was confirmed to be part of the normal microbiota of dogs and cats as well as an occasional cause of zoonotic infections in humans [63]. The different body sites where *S. pseudintermedius* would mostly colonize include the nose, perineum-rectum, mouth, and groin [64–67]. Bannoehr *et al.* developed an MLST-like approach that used five gene loci (16S rRNA, *tuf, cpn60, pta,* and *agrD*) to facilitate inter and intraspecies differentiation of the SIG group from countries such as North America, Europe, and Asia [63]. The results from this study concluded that there are different host preferences of species within the SIG group. *S. pseudintermedius* is frequently isolated in canines, *S. intermedius* in horses, camels, and pigeons whereas *S. delphini* is commonly isolated in dolphins and bovines [63]. The molecular systemic classification of the staphylococci genus is important in studying the evolutionary relationship of *S. pseudintermedius* to help trace the emergence of new strains through conserved regions among the staphylococcal species.

History of Methicillin-resistant *S. pseudintermedius*

The methicillin antibiotic is a penicillinase-resistant semisynthetic penicillin that was first produced in the late 1950s to circumvent penicillin resistance in *S. aureus* [68, 69]. A year after its introduction in the treatment of *staphylococcus* infections, methicillin resistance was first observed in the UK among clinical isolates from hospitalized patients [70]. Methicillin-resistant strains are resistant to a bacterial enzyme activity called penicillinase and hydrolysis β-lactamase to prevent cell wall construction [71, 72].

Several hypotheses have been suggested for the emergence of methicillin-resistance [73, 74]. It has been hypothesized that the adaptive response following the widespread use of penicillin led to the emergence of MRSA [75]. This was followed by the introduction of methicillin into clinical practices that allowed for selective pressure of pre-existing methicillin-resistant variants [76]. Additionally, the transfer of *mecA* through horizontal gene transfer from hospital acquired-MRSA (HA-MRSA) to community acquired-MRSA (CA-MRSA) led to the increasing prevalence of

MRSA [77]. Furthermore, the use of β-lactams and cephalosporins in human and animal medicine might have contributed to the acquisition of SCC*mec* bearing *mecA* gene [78]. All veterinary staphylococcal species were shown to be methicillin-susceptible in studies that date back to the 1980s [79, 80]. However, studies in the 1990s began to report methicillin resistance and detection of the *mecA* gene among *S. pseudintermedius* of canine origin [6, 81]. Given the documented evidence in the misidentification of *S. pseudintermedius* of canine origin, it has been suggested that MRSP occurred way before the year it was first reported. A study that collected *S. intermedius* isolates from dogs between 1988 and 1992 identified methicillin resistance but the presence of *mecA* was not confirmed [81]. As with the confluence of events that provided the emergence of MRSA, there are certain possible explanations for the acquisition of the *mecA* gene by methicillin-susceptible *S. pseudintermedius*. For example, the prevalence of MRSA could have ultimately led to horizontal gene transfer of the *mecA* gene from MRSA to MSSP and intraspecies acquisition of SCC*mec* [82–84]. In addition, the continuous use of β-lactams, cephalosporins, and tetracycline to treat staphylococcal infections in human medicine, veterinary medicine, and food-producing animals could be contributing factors to the selection of MRSP [85, 86].

Concluding the exact point in time when *S. pseudintermedius* acquired methicillin resistance is a challenge given its controversial history. Perhaps previously reported MRSA of canine origin was MRSP before the mid-1970s when tested using *S. aureus* standards. Whole genome sequencing and molecular studies have informed the transfer of the *mecA* gene between different organisms [4, 87]. This has helped identify clinically relevant MRSP lineages with diverse multi-drug resistance that continue to present a challenge in the treatment of *S. pseudintermedius* infections. Since its discovery in the 1990s, the prevalence of MRSP of canine origin has continued to increase depending on each study population (Table 1-1).

Detection of MRSP

The interpretive guidelines established by the Clinical and Laboratory Standard Institute (CLSI) are used to determine MRSP [88]. Based on the CLSI guidelines, phenotypic methods such as broth microdilution and the Kirby-Bauer disk diffusion test are used by most veterinary diagnostic laboratories [89]. However, the detection of methicillin resistance in *S. pseudintermedius* from 2004 to 2010 was equivalent to those recommended for human and veterinary isolates of *S. aureus* and coagulase-negative *staphylococcus*.

After the 2008 edition of the CLSI was published, Bemis *et al.* and Schissler *et al.* evaluated the susceptibility test breakpoints to predict *mecA*-mediated resistance in *S. pseudintermedius* isolated from dogs [98, 99]. Both these studies concluded that when using the cefoxitin disk diffusion with

Table 1-1: The prevalence of MRSP reported worldwide in different years.

Reference	**Year**	**Country**	**Source**	**Prevalence**
Gortel *et al* [6]	1999	USA	Clinical isolates	0.04%
Videla *et al* [20]	2010	USA	Clinical isolates	74%
Beck *et al* [90]	2012	Canada	Bacterial pyoderma	40.5%
Onuma *et al* [91]	2012	Japan	pyoderma	11.4%
Han *et al* [92]	2015	South Korea	Healthy dogs	43%
Botoni *et al* [93]	2016	Brazil	Skin infection	34%
Gronthal *et al* [94]	2017	Finland	Clinical isolates	14%
Worthing *et al* [12]	2017	Australia	Clinical isolates	11.5%
Menandro *et al* [95]	2019	Italy	Clinical isolates	5.6%
Holmstrom *et al* [96]	2019	Brazil	Clinical isolates	43.9
Ahmed Rana *et al* [97]	2021	Bangladesh	Skin infections	6%

resistance breakpoints set at ≤24mm, the presence of *mecA* was significantly underestimated in *S. pseudintermedius* dog isolates [98, 99]. The oxacillin disk diffusion zone diameter of ≤17 mm and oxacillin minimum inhibitory concentrations of ≥0.5 μg/ml were highly correlated with the detection of *mecA* in canine *S. pseudintermedius* by polymerase chain reaction (PCR) [98, 99]. Compared to *S. aureus*, oxacillin instead of cefoxitin in *S. pseudintermedius* is the preferred surrogate of methicillin based on its sensitivity and stability [89]. The CLSI recommends the use of oxacillin and disk diffusion to determine the phenotypic expression of methicillin resistance in *S. pseudintermedius* [89]. Additionally, PCR detection of *mecA* and protein assay PBP2a, together with whole genome sequencing can be used to confirm MRSP [100, 101]. Both conventional and real-time PCR are efficient in *mecA* detection. However, real-time PCR will quantify the amount of DNA present in the sample and has been used to study the variation and regulation of *mecA* gene expression among MRSP isolates [100–103]. Furthermore, Black *et al.*, used mRNA and reverse transcription real-time PCR to study *mecA* gene expression.

The *mecA* gene

The presence of the *mecA* gene in the *S. pseudintermedius* genome in most cases will confer methicillin resistance. This gene encodes PBP2a, which takes over the transpeptidase activity (cell wall cross-linking) of the native penicillin-binding protein during cell wall synthesis [4, 90]. When treated with β-lactam antibiotics, PBP2a interacts with β-lactams at their active site serine residues causing delayed diacylation, cell wall loosening, and ultimately bacteriolysis [4]. Previous studies have suggested that the first step in the evolution of *mecA* was through the evolution of *S. scuiri mecA1* and its neighboring gene alleles in *S. vitulinus* [87, 104, 105]. The evolution of the *mecA* gene later occurred in *S. fleurettii* through vertical transmission followed by horizontal gene transfer [87, 105].

The *mecA* gene expression is regulated by a zinc-dependent metalloprotease sensor/transducer, mecR1, and transcriptional repressor, mecI [9, 106]. They are located immediately upstream from the *mecA* promoter on the mec elements and are also counter-transcribed [104]. The mecR1-mecI system is closely related to the blaR1-blaI protein triad that regulates β-lactamases expression structurally and functionally [9]. Over 60% of the sequence homology between blaI and mecI allows them to bind and suppress the other cognate promoter interchangeably during the regulation of the *mecA* gene [9]. Despite having similar recognition sequences within cleavage sites, mecR1 and blaR1 have different functions. The mecR1 can only cleave *mecI* while blaR1 can only cleave *blaI* [9]. Additionally, *mecA* induction by mecR1 takes several hours compared to several minutes by blaR1 due to its low sensitivity [9]. Studies have shown the occurrence of both *mec* and *bla* operons as co-regulators of PBP2a production for *mecA* expression in MRSP similar to MRSA [9, 107, 108]. While other studies show that some levels of the *bla* operon provides an

advantageous ability in the control of *mecA* gene expression. In a particular study, Black *et al* showed that a *mecI*-controlled system was slower to respond to oxacillin than the exclusively *blaI*-controlled system in MRSP isolates from dogs [9].

By binding to the *mecA-10* promoter sequence and *mecR1-35* sequence, the *mecI/blaI* represses the transcription of *mecA* and *mecR1-mecI* [109]. Upon the detection of external interaction with β-lactam antibiotics, the *BlaR1/mecR1* undergoes acylation, causing conformational changes that induce autoproteolytic cleavage on the cytoplasmic side of the cell membrane [109, 110]. This process leads to direct or indirect cleavage of *blaI/mecI* via proteolysis in the bacterial chromosome. Ultimately, the *mecI/blaI* repressor fails to bind to its promoter region binding site and induces *mecA* transcription and methicillin resistance [9].

The level of *mecA* production and PBP2a expression has been correlated with the MIC phenotype of MRSP and β-lactam resistance [7, 9, 111]. However, isolates harboring the *mecA* gene and are phenotypically oxacillin susceptible have been described in *S. aureus* [112–114]. The number of *S. aureus* isolates that are oxacillin-susceptible and *mecA*-positive in human and animal hospitals has increased in recent years [112, 114]. The oxacillin susceptible expression has been attributed to the *blaI* expression, loss, and truncation of *mecR1* and *mecI* as well as mutations introducing SNPs in the mec/bla promoters [115–117]. Despite the oxacillin susceptibility observed among MRSA isolates, its *mecA* gene may allow it to become highly resistant under antibiotic selection [8, 112, 118].

SCC*mec* typing

SCC*mec* elements are highly diverse mobile genetic elements (30-55kb) residing in the chromosome [72]. They are classified into types and subtypes and carry the *mecA* gene [119]. There is some evidence suggesting that the SCC*mec* is restricted to staphylococci and that similar cassettes might be interchangeable between the genera, causing recombination and deletion [120]. SCC*mec* is composed of two key components; the *mec* complex, which contains the *mecA* gene, promoter region, and its regulators (*mecI* and *mecR1*), while the *ccr* complex contains the cassette chromosomal recombinase genes that ensure cassette mobility and chromosome integration. There is an integration-specific site in the staphylococcal chromosome which serves as a target for ccr-mediated recombination and the presence of flanking direct repeat sequences containing the integration site sequence (ISS) [121]. Five classes of the mec complex (A, B, C1, C2, and E) and five ccr allotypes (*ccrAB1, ccrAB2, ccrAB3, ccrAB4*, and *ccrC*) have been identified by sequence analysis. Inside the joining areas, SCC*mec* may also incorporate insertion sequences (IS), transposons, plasmids, and housekeeping genes [119].

A hierarchical system classifies SCC*mec* elements into types designated by the roman numerals (I-X) which represents a combination of the *ccr* gene allotype and the mec complex [121]. However, the existence of composite cassettes and *pseudo*-SCC*mec* elements that do not contain *ccr* genes has hampered the assignment of nomenclature and classification of SCC*mec* elements [119, 122]. There are currently thirteen distinct structural types of SCC*mec* in *S. aureus* of which eleven are formally recognized in the database of the International Working Group on the Staphylococcal Cassette Chromosome (IWG-SCC) (https://www.sccmec.org/). While the structure of SCC*mec* elements in *S. aureus* has been demonstrated to be generally stable, SCC*mec* elements in MRSP have revealed considerable genetic variability [7, 122]. It has been suggested that the SCC*mec* cassettes are subjected to recombination and deletion of genes which could result in new variants [123, 124]. The SCCmec types in *S. pseudintermedius* are occasionally non-typeable using SCC*mec* typing systems devised for *S. aureus* [125, 126]. Several SCC*mec* elements have been reported with unique features for MRSP (Table 1-2). For example, SCC*mec* type III was previously described as SCC*mec* type II-III which represents a combination of SCC*mec* type II from *Staphylococcus epidermis* and SCC*mec* type III from *S. aureus*. Whereas the Pseudo SCCmec$_{57395}$ element contains no ccr element but appears to carry determinants of resistance to heavy metals such as arsenic, cadmium, and copper [122].

Multiplex PCR techniques have been used to amplify the mec and ccr regions by SCC*mec* types in *S. pseudintermedius* [127, 128]. This method has however presented a challenge by ignoring the SCC*mec* elements' plasticity, resulting in several non-typeable SCC*mec* elements [122, 129, 130]. Due to the size of the cassettes, sequencing of the entire nucleotide sequence is encouraged to fully define each SCC*mec* complex [127]. Advances made in whole genome sequencing allow for essential data required for SCC*mec* typing using bioinformatics tools such as SCC*mec*Finder [131]. The SCC*mec*Finder allows for the characterization of SCC*mec* elements from *S. aureus* WGS data by identifying all SCC*mec* element types (I-XIII) with subtyping of SCC*mec* types IV (2B) and V(5C2) [131]. However, there has not been a detection of SCC*mec* elements in *S. pseudintermedius* that contain the exact number and copy of genes as those described in reference *S. aureus* SCC*mec* cassettes [3, 83, 100, 132]. Using the basic local alignment tool (BLAST)-based prediction, the SCC*mec*Finder detects multiple *ccr* gene complexes [131]. BLAST-based approaches may fail to provide definitive assignments if threshold levels are too high or contigs are not sufficiently assembled. Additionally, the database only includes SCC*mec* reference elements listed by the IWG-SCC making it difficult to detect novel subtypes [131]. Overall, the detection of SCC*mec* cassette elements is essential in molecular epidemiology and could be associated with multi-drug resistance in MRSP clones.

Table 1-2: SCC*mec* elements identified in MRSP isolates.

SCC*mec* name	**Reference isolate**	**Accession**	**Mec complex**	**ccr complex**
SCC*mec* III [133]	MRSP KM1381	AM904732	A	A3/B3
SCC*mec* V_T [134]	MRSP SL/066	MQMO00000000	C2	C8
SCC*mec* VII-$_{KM241}$ [83]	MRSP KM241	AM904731.1	A	A5/B3
SCC*mec*$_{NA45}$ [135]	MRSP NA45	CPO16072.1	C1	C6
ΨSCC*mec*$_{57395}$ [122].	MRSP57395	HE984157.2	C1	No ccr
SCC*mecA*$_{I16}$ [136]	MRSPAI16	LN864705.1	A	A1B3
ΨSCC*mec*$_{KW21}$ [132]	MRSP KW21	MH713898	A	No ccr

Multidrug resistance of MRSP

Besides *mecA*, MRSP tends to contain a wide range of antibiotic-resistant genes such as *erm(B)*, *GyrA*, *tet(M)*, *dfr(G)*, *lnu(A)*, and *sat4*, which potentially makes them resistant to almost all classes of commonly used antimicrobial agents [20, 28, 95, 132]. Multidrug resistance is very common among MRSP isolates and includes resistance to antibiotics used in the treatment of pyoderma, otitis, and enterocolitis in dogs [14, 20, 24, 95]. Genotype-phenotype correlations based on MIC testing and whole genome sequencing demonstrate that MRSP can acquire and maintain multidrug and extensive drug resistance in *S. pseudintermedius* [137]. Bioinformatic evidence for the presence of different AMR determinants can be done through analysis of WGS by ARIBA, CARD, and Resfinder [7, 137, 138].

By truncating mobile genetic elements, McCarthy *et al*., demonstrated that the phenotypic differences in AMR profiles observed among MRSP isolates are due to variations in resistant genes [139]. Additionally, both vertical and horizontal gene transfers are used to acquire and maintain the AMR phenotype in MRSP due to antimicrobial selection pressure in a clinical setting [137, 138]. Multidrug resistance patterns may differ due to clonal proclivity to maintain various resistant elements or may reflect regional antimicrobial usage patterns [14]. Multidrug resistance of *S. pseudintermedius* to more than 11 antimicrobials have been observed in Northern Italy, Australia, the United States, the Netherlands, the United Kingdom, and Romania [11, 12, 20, 24, 140].

Multidrug resistance in MRSP may include resistance to tetracyclines, erythromycin, kanamycin, streptomycin, oxacillin, gentamicin, and marbofloxacin [16, 28, 95]. Lincosamides and macrolides are narrow-spectrum antibiotics that have three resistance mechanisms. These mechanisms include the inactivation of ribosomal binding sites in the 23rRNA gene caused by methylation and/or mutation. Secondly, the presence of active efflux pumps of the antibiotic encoded by the *msrA* and *msrB* genes, macrolide phosphotransferase (*mphC*) and lincosamides nucleotidyl transferase (*lnuA*) [141–144]. Finally, MRSP isolates contain lincosamides and macrolides resistant genes (*ermA*, *ermB*, *ermC*) encoded in transposons such as Tn916, and IS256 in the chromosome [3, 16, 137, 143]. Fluoroquinolones are bactericidal broad-spectrum antibiotics that inhibit nucleic acid synthesis by blocking DNA gyrase encoded by *gyrA* and *gyrB* as well as topoisomerase IV encoded by *gyr*IA and *gyrIB*. DNA gyrase *GyrA*, as well as the DNA topoisomerase, *GyrIA* of staphylococci are mutated to generate fluoroquinolone resistance [83, 91, 145, 146]. Trimethoprim-sulfamethoxazole is a broad-spectrum antibacterial agent used for the treatment of skin and soft tissue infections. The mechanism of resistance conferred by bacterial isolates includes mutation of the chromosomal dihydrofolate reductase gene (*dfrG*) located on exchangeable genetic elements. Mutations in the *folP* gene were previously associated

with resistance to sulfonamides in staphylococci. The presence of the *dfrG* gene is sufficient to predict resistant phenotypes to the combination drug trimethoprim-sulfamethoxazole [137, 143, 147]. Tetracyclines are broad-spectrum antibiotics with bacteriostatic activity that inhibits DNA translation by binding onto the 30S subunit of the ribosome [148]. When bacteria acquire the *tetM* gene through genomic islands such as Tn5801 and integrative conjugative elements such as the Tn916 located in the chromosome, tetracyclines are rendered resistant [149]. Additionally, the *tetK* gene located in plasmids has also been detected among MRSP isolates encoding efflux pumps that ultimately offer ribosomal protection from tetracycline activity [86, 148]. Chloramphenicol inhibits bacterial protein synthesis by binding it to the ribosome [150]. Resistance to this antibiotic is associated with the chloramphenicol acetyltransferase gene cat_{Pc221} located in a plasmid that also can activate efflux pump activity, inactivation by phosphotransferases, mutation of target sites, and permeability barriers [151–153]. *Staphylococcus pseudintermedius* contains genes, which encode aminoglycoside-inactivating enzymes [*aph (3′)-III*, *sat4* (streptothricin acetyltransferase) and *ant (6′)-Ia* (streptomycin adenyltransferase)] [141]. Kanamycin resistance can be conferred by the presence of the *aph (3′)-III* gene or *aac (6′)-Ie-aph (2′)-Ia* [22, 86]. While the *aac (6′)-Ie-aph (2′)-Ia* gene confers resistance to gentamycin, tobramycin, and kanamycin in canine MRSP isolates [22, 142].

Virulence factors

Methicillin-resistant *S. pseudintermedius* seems to share core virulence profiles with those identified for *S. aureus*. The most common virulence genes identified include toxins [*lukF-P*, *hlgB* (gamma hemolysin component B)], *siet* (*Staphylococcus intermedius* gene for the exfoliative toxin), *spetA* (exfoliative toxin A), *srrA* (accessory gene regulators), *sarA* (staphylococci accessory regulator A), *sarR*, *sarZ* (transcriptional regulator), cell wall anchored genes (*ebp*S, *spsC*, *spsE*, *spsH*, *spsK*, *spsA*, *spsB*, *spsG*, *spsI*, *spsM*, *spsN*, *spsQ*, *spsR), nanB* (putative sialidase toxin), *coa* (staphylocoagulase), *clpX* (ATP-dependent protease ATP-binding subunit), *hrt*A (heme efflux system ATPase HrtA), *lip* (triacylglycerol lipase), and protease *clpP* [138]. Additionally, as with *S. aureus*, *S. pseudintermedius* harbor the *nucB* and *AdsA* genes which encode cytotoxic nuclease and adenosine synthase activity leading to break down of mammalian DNA and production of cytotoxic deoxyadenosine that contributes to the persistence of infection [154]. Biofilm formation and the genes *agrA* and *agrD* have also been identified among MRSP isolated from dogs. [155]. The presence of biofilm allows the pathogen to withstand antibiotic concentrations that are more than 100-fold higher promoting tolerance and persistence of MRSP infections in dogs [33, 156].

Staphylococcus pseudintermedius proteins that have undergone attenuation could be a key component of a composite vaccine against *S. pseudintermedius* infections [157]. It has been noted that isolates of *S. pseudintermedius* exhibit genes similar to the *spa* gene in *S. aureus*. *Staphylococcus*

pseudintermedius's surface protein A is encoded by *spsQ* which binds to the Fc region of IgG leading to blocking complement fixation and opsonization [158]. Furthermore, it interacts with immunoglobulin on the surface of B cells, causing the cells to undergo apoptosis [159]. Protein A during *S. pseudintermedius* infection is secreted during the exponential growth stage. During pathogenesis, the inhibition of protein A improves phagocytosis in neutrophils, whole blood, and the canine macrophage-like DH82 cell line [158]. A series of potent toxins called two-component pore-forming leukocidins, which are made up of S (slow) and F (fast) components are released by staphylococcal species [30, 160–162]. Seven different leukocidins have been characterized for *S. aureus* compared to two components LukS-I and LukF-I of *S. pseudintermedius* [30, 160]. Once leukocidin is secreted, the innate immune system, which is the initial line of protection against pathogens, is compromised through cytotoxic effects [30].

Prothrombin is activated by *staphylococcus* coagulase, which results in fibrin deposition and makes it easier for the *staphylococcus* species to infect hosts [163]. The *S. pseudintermedius* coagulase protein is bound to C3 and immunoglobulin inhibiting the formation of opsonizing antibodies, complement, and fibrinogen that ultimately facilitates the escape of *S. pseudintermedius* from phagocytosis [164]. A sub-lethal concentration of the *S. pseudintermedius* SpEX inhibits neutrophil migration by binding to complement components, preventing complement-mediated lysis of red blood cells and killing polymorphonuclear leukocytes and monocytes [29]. Finally, adenosine synthase (*SpAdsA*) inhibits the phagocytosis of *S. pseudintermedius* by canine neutrophils and monocytes [32].

Horizontal gene transfer

The genome of *S. pseudintermedius* is a single circular chromosome with over 2000000 bp and a G+C content of 37.6% compared to an average of 32% that has been reported for other staphylococcal pathogens [165, 166]. The genome encodes 2,401 predicted protein-coding sequences and has five ribosomal operons and 58 tRNA loci [166]. Mobile genetic elements (MGE) account for over 8% of *S. pseudintermedius* genomes providing extensive evidence of MGE acquisition [167]. For example, insertion elements, transposons, staphylococcal pathogenicity island, reverse transcriptase group II introns, and putative integrated plasmids [166]. Additionally, circular genome sequence of *S. pseudintermedius* strain HKU10-03 isolated from dogs with pyoderma contained several housekeeping genes with no plasmids detected in the sequenced strain [168].

Horizontal gene transfer (HGT) is the movement and exchange of genetic material between different bacterial species and within bacterial species. It plays an important role in the evolution and spread of antibiotic-resistant genes among MRSP [169]. Similarly, HGT has driven clonal

expansion and evolution in bacterial pathogens, including *S. aureus*, *Streptococcus pyogenes,* and *Streptococcus agalactiae* [170–172]. The mechanisms of HGT in *S. pseudintermedius* are poorly understood. However, MRSP appears to have emerged through several autonomous horizontal acquisitions of resistant elements and frequent genetic exchanges that disseminate DNA to a wider population [21]. During HGT, SCC*mec* is often inserted downstream of the *orfX* which encodes an rRNA methyltransferase, at a particular location on the chromosome [173]. The vertical and HGT of MGE can be through transformation, conjugation, and transduction. In *S. aureus*, plasmid conjugation predominantly transfers genetic contents horizontally among the species compared to bacteriophage transduction by prophages which plays a role in the evolution of *S. pseudintermedius* [173]. During transformation, bacteria transfer their cellular DNA between closely related bacteria through the transfer of chromosomal proteins [174]. Whereas conjugation requires independent conjugative plasmids and transposons that replicate independently [175]. Similarly, transduction is also mediated independently by replicating bacteriophages. Bacteriophages can carry host DNA into a new host cell where it is inherited through recombination with cellular chromosomes [82, 167]. Whereas transposons can easily recombine and randomly "jump" between replicons, phages, and plasmids.

Similar to *S. aureus* and *S. epidermis*, frequent recombination may contribute to the evolution of pathogenicity and the ability of *S. pseudintermedius* to colonize its hosts. Analysis of nucleotide sequences of *S. pseudintermedius* MLST genes revealed that recombination occurs more frequently than mutation [19, 21, 28 ,176]. Additionally, the spa locus of *S. pseudintermedius* contains a plethora of horizontally transferred genes such as *bla*Z, *mecA*, *aac (6')-aph (2")*, *cat-(pC221)*, *dfrG*, *tet*(M), and *tet*(K), plasmids, prophages, transposons, CRISPR-Cas, and RM system [177]. This has been described as a recombination and horizontal gene transfer hotspot [177]. It is similar to *tbp*A and *tbp*B in *Neisseria meningitidis* and *Haemophilus influenzae* encoding surface proteins [178]. A variety of genes are thought to be carried by prophages, including those contributing to the fitness, virulence, resistance, and host adaptations of bacteria [139]. For example, MDR MRSP isolates carry a large transposable element resembling Tn5405-like elements that carry antimicrobial resistance genes *aphA3-sat-aadE-dfrG-erm*(B)-*cat* [167, 179]. Additionally, four intact prophages, namely Staphylococcal phage SpST71A, SpST71B, SpST68A, and SpST68B have been described for *S. pseudintermedius* [176]. The prophages were associated with β-lactam resistance genes by class B metal β-lactamase and virulence-associated protein E genes [176]. Additionally, a complete, competent operon consisting of *comGA*, *comGB*, *comGC*, *comGE*, *comGF,* and *comGG* genes has been identified in *S. pseudintermedius* [176]. Competent genes are essential for DNA uptake through natural transformation by competence machinery that are generally broadly investigated among *Bacillus subtilis*, *Streptococcus pneumoniae,* and *Streptococcus mutans* [174, 180–182].

Multilocus Sequence Typing

For short- and long-term worldwide epidemiology of *S. pseudintermedius*, *S. aureus, Campylobacter jejuni, Haemophilus influenzae, Streptococcus agalactiae, Streptococcus pneumoniae and Neisseria spp.*, MLST is the most useful approach in describing population genetic structure [17]. The sequence types are defined using a DNA sequence comparison of 450-500 bp internal fragments from seven housekeeping genes [183]. Housekeeping genes are highly conserved genes (not prone to recombination) that are vital for bacterial survival since they are required for the maintenance of basic cellular functions [184]. Similar to *S. aureus*, MLST of *S. pseudintermedius* is defined by 7 housekeeping genes [17, 19, 20, 118]. The housekeeping genes for *S. pseudintermedius* include phosphate acetyltransferase (*pta*), elongation factor tu (*tuf*), adenylosuccinate dehydrogenase (*purA*), formate dehydrogenase (*fdh*), sodium sulfate symporter (*sar*), CFDD chaperonin 60 (*cpn60*) and acetate kinase (*ack*) [17, 63].

A distinct sequence type is made up of a unique combination of randomly numbered alleles (Table 1-3) that is assigned by comparing the sequence data on the MLST database https://pubmlst.org/organisms/*staphylococcus*-pseudintermedius [19, 20, 25, 26]. The amount of nucleotide variations between alleles is ignored in MLST, and sequences are assigned different allele numbers regardless of whether they differ at a single or several nucleotide sites [185]. As a general principle, a single genetic event can lead to the development of a different allele by means of either a point mutation (altering only one nucleotide site) or recombination process (altering multiple nucleotide sites) [183]. MLST offers the advantage of employing many loci to reach the resolution required to reveal significant correlations among strains [185]. Sequence differences between isolates with sequence types that differ at a single locus could imply a close phylogenetic relationship compared to isolates with sequence types that differ at three or more alleles [185]. The relationship alleles share between STs can be demonstrated using geoBURST analysis.

eBURST analysis

To define the emergence of MRSP clones, MLST offers useful information for the detection of bacterial genomic diversity over a long period [7, 12, 28]. For evolutionary descendants, BURST analysis offers categorization of strains by relatedness according to their allelic profile and potential ancestral type [18, 186]. The main goal of MRSP eBURST is to identify clusters of closely related isolates within the population of *S. pseudintermedius* that are thought to have recently shared a common ancestor [12, 19, 21, 25]. The eBURST program will cluster a clonal complex using STs that differ at a single locus variant (SLV) (most stringent), double locus variant (DLV), or triple locus variant (TLV) [18, 19, 187]. Each clonal complex will have a main clonal founder, which is represented by the ST having the most SLVs [18, 187]. When an SLV of the primary founder has expanded in frequency and diversified to produce multiple of its SLVs, eBURST will

Table 1-3: Allelic variation of seven housekeeping genes across sequence types of MRSP.

ST	***ack***	***Cpn60***	***fdh***	***pta***	***pur*A**	***sar***	***tuf***
71	3	9	1	2	1	2	1
68	1	10	11	4	1	1	1
45	3	2	2	1	2	1	2
258	5	13	4	1	11	2	2
112	2	2	2	1	1	2	1
84	2	10	2	1	5	1	2
261	5	7	4	1	11	2	2
181	3	11	2	24	20	1	1

occasionally assign subgroups and subgroup founders [18, 187]. For example, eBURST was able to assign 503 STs of *S. pseudintermedius* into two major (CC71 and CC258) and two minor CCs (CC379 and CC75) in a systematic global review [19].

The BURST algorithm will also find singletons that are unrelated to any other group in the population and groupings of the STs without a predicted founder based on bootstrapping [25, 57, 188]. Interestingly, a genotype's sporadic increase in frequency due to genetic drift or natural selection is considered by the algorithm [18, 187]. This results in the diversification of the founding genotype by the accumulation of mutations and/or localized recombinational replacements, giving rise to small variations which are detected by eBURST [18, 187]. In *S. pseudintermedius*, the occurrence of several singletons and few CCs suggests the coexistence of various and separate population lineages [11, 25, 84]. MRSP has been found in correlation with several distinct lineages throughout the *S. pseudintermedius* population using eBURST [11, 189, 190]. Additionally, using eBURST, the CC71 lineage has been described as homogenous compared to the heterogenous CC258 lineage [19]. The main clonal founder indicated by eBURST typically correlates to the most prevalent ST [18]. For instance, ST71 in Europe is frequently identified among MRSP isolates, hence isolates from CC71 are classified as the epidemic European clone that has expanded globally [19, 22]. Whereas ST68 is an epidemic lineage (CC68) from North America that has expanded globally. Similarly, ST45 and ST112 represent endemic lineages (CC45 and CC112) from Asia that have dispersed worldwide [19].

The use of eBURST together with phylogenetically based analysis can help infer deep phylogenetic relationships compared to the use of eBURST alone. According to phylogenetic clustering, MRSP lineages lack a recent common ancestor and are genetically distinct from MSSP [3]. In *S. aureus*, the addition of MLST strains caused eBURST to fail to distinguish between the largest and most significant clonal complexes (CC1, CC5, CC8, CC15, and CC97) [191]. This suggested that the seven locus housekeeping genes of *S. aureus* are not enough to separate geographically dispersed clones. Similarly, eBURST of the *S. pseudintermedius* PubMLST database found only 1 major CC (CC258) consisting of all the major CCs (CC45, CC68, CC71, CC81, and CC150) [24, 192]. This was attributed to potentially incorrect links associated with high recombination to mutation ratios in the population (e.g., *Burkholderia pseudomallei*, *S. epidermis*, and *Enterococcus faecium*) [193]. Therefore, using both BURST and phylogenetic-based approaches provide better support for the clonal evolutionary model when considering rates of recombination and high clonal diversification of MRSP.

Epidemiology of MRSP

S. pseudintermedius is a commensal opportunistic pathogen and is part of the dog's normal flora. Similar to *S. aureus* and *S. epidermis* in human skin, *S. pseudintermedius* does not cause disease unless the host is immunocompromised, has alterations to its skin barrier, or both [194]. The transmission of *S. pseudintermedius* can be vertical (during birth), horizontal (between dogs), and interspecies (human colonization) [195, 196]. The carriage rate of *S. pseudintermedius* in dogs can range between 46% and 92% among healthy dogs [197, 198]. *S. pseudintermedius* is most often isolated from skin samples among dogs aged 2-4 years compared to *S. aureus* (35.1%) from skin samples most frequently identified in dogs aged 5-6 years [199].

Information provided by MLST and eBURST has played an important role in the prediction of founder strains and tracking the spread of methicillin resistance worldwide. Bannoehr *et al.* used a five gene loci approach (16S rRNA, *cpn60*, *tuf*, *pta,* and *agr*D) to distinguish SIG from various animal species that come from countries in Europe and the USA [63]. They found that *S. pseudintermedius* is characterized by 61 different STs among 89 isolates examined [63]. The eBURST analysis showed that the MRSP isolates in the study belonged to five distinct STs (ST29, ST68, ST69, ST70, ST71) that differed at two to four loci suggesting that each sequence type does not share any recent ancestor. Using the same five loci approach and PFGE, Black *et al*. found that 37 out of 38 MRSP isolates collected from the University of Tennessee College Veterinary Medicine (UTCVM) belonged to ST68 [200]. Whereas Feng *et al*. characterized MRSP in pets from South China and reported 24 different STs in which ST4, ST5, and ST95 were the dominant sequence types [201]. The findings in both studies showed that there is a broad geographic dissemination of successful clones in European countries and the USA. In 2010, Perreten *et al.* used an international multicenter study of *S. pseudintermedius* isolates from dogs in Europe, the USA, and Canada, they concluded that two clonal MRSP lineages have disseminated in Europe (ST71-J-t02-II-III) and North America (ST68-C-t06-V) [22].

The overall structure of the SIG species was revealed using sequence typing based on the four MLST loci approach [63]. However, the development of a seven MLST loci approach has offered an increase in the discriminating power of *S. pseudintermedius*. Using this approach, Solyman *et al*. identified 98 unique STs of *S. pseudintermedius* in dogs, cats, and human beings from diverse geographical regions [17]. Using the same approach, a study in Finland reported ST71, ST45, and ST402 as the major STs circulating among MRSP isolates [190]. Whereas a more extensive systematic review of MRSP published in 2016 identified five main MRSP clonal lineages disseminated worldwide [19]. Sixteen of the MRSP STs were recovered from more than one continent (ST17, ST29, ST45, ST68, ST71, ST85, ST112, ST121, ST123, ST125, ST155, ST169, ST179, ST180, ST182, ST282, and ST261). The most widely disseminated clone was ST71, isolated across

Europe, North and South America and Asia followed by ST45 found in seven countries, ST261 in 4 countries, ST68 in three countries, and ST258 in two countries [19]. Videla *et al*. studied the population genetics of *S. pseudintermedius* from a cross-section of the United States using the seven-locus MLST approach. They found 86 different MLSTs, in which CC84 contained 15 STs, CC68 contained 10 STs and CC71 contained 3 STs [20]. The reason for the success of specific MRSP clones is still unknown. However, the situation is similar to that of MRSA, in which global spread is mostly attributable to a few viable clones with a distinct geographic pattern. The presence of a small number of widespread CCs has been argued to be due to a selective advantage imparted by traits such as efficient host-to-host transmission, biofilm formation, and virulence factors [11, 19, 25, 27]. In addition, antimicrobial selection pressure perhaps paired with widespread usage of antibiotics in each region could also contribute to the dissemination of CCs lineages [19, 20]. The success of CC71 worldwide has been associated with its high ability to form biofilm and adherence [202]. Whereas some studies have suggested that the decrease in CC71 in Europe could be attributed to the presence of an immobile SCC*mec* type II-III compared to the highly mobile SCC*mec* type V reported in ST68, ST551, and ST45 [11, 203]. Furthermore, disruption of the genetic competence operon *comG* in the ST71 clone by site-specific integration of prophage, SpST71A could have disrupted the ability of ST71 strains to function in the natural uptake of foreign DNA and evolution [176]. Furthermore, genomic insights into ST45 show a high number of single nucleotide polymorphisms in their core genome which could contribute to high-level adaptability and successful host colonization [179].

Changes in the population structure and dissemination of antimicrobial phenotypes have been observed worldwide. Duim *et al*., reported that CC71 was the dominant lineage that emerged in 2004 among *S. pseudintermedius* isolates, whereas CC258, CC45, and other singletons became more frequent during the following years in the Netherlands [11]. They concluded that the lineages showed a stepwise gain and loss of phenotypic resistance over ten years that did not influence their spread. Han *et al.* identified 24 STs, including 20 new STs in samples collected from healthy dogs in Korea. The MRSP STs in this study were genetically distinct from other strains reported in Asia, North America, and Europe [204]. These results indicated that there are various genetic lineages present among healthy dogs in Korea that are not yet widely disseminated. In Australia, ST45, ST258, and ST71 were the previous dominating sequence types [12]. However, a more recent study in 2020 of MRSP samples collected between 2015 and 2019 reported a frequent detection of ST749 and ST496 with two novel STs (ST1399 and ST1400) [12, 103]. In France, ST71 is also decreasing over time and the non-ST71 population that is highly heterogeneous is superseding [25]. Two other lineages such as ST496 which is highly homogenous and ST258 which is more diverse seem to be successful in France [25]. Since ST496 emerged in Australia in 2013 but was not detected in France during that year, the Australian ST496 isolates seem to be

ancestral to the French ST496 isolates [12, 25]. Therefore, animal transportation could have played an important role in presence of ST496 in France. Menandro *et al*. detected ST261 and ST301 belonging to CC258 among clinical MRSP isolates in Italy. The presence of many closely related variants of the founder ST258 was implicated in the high evolutionary rates of ST258 compared to other STs [95]. Multidrug-resistant ST71-SCC*mec* typeII-III was the dominant lineage in Polish clinical MRSP isolates between 2007 and 2015, but ST551-SCC*mec* typeV has been detected in the majority of Polish clinical MRSP isolates since 2015 [27]. Additionally, new STs have been identified in Poland; ST680 and ST771 which are closely related to ST71 and ST551 respectively. In Finland, a diverse changing population of MRSP previously indicated a non-clonal spread [190]. The most common ST in Finland in 2010-2011 was ST71 but has since been diminished with CC258 filling the gap as a more successful lineage. Contrary to CC71, CC45 in Finland has maintained a steady proportion between 2011 and 2014 [190]. In the United States, a recent study published in 2020, reported the presence of ST150, ST68, ST71, ST84, ST155, and ST181 among *S. pseudintermedius* isolates in the New England and Texas regions [138]. The results from these studies provide the basis for the changing population and emergence of new dominant MRSP lineages worldwide. The greater diversity among the MRSP clonal lineages has been attributed to the acquisition of resistance genes by the incorporation of mobile genetic elements in the genome. Perhaps clonal spread is becoming less significant and the spread of SCC*mec* elements is more common among MRSP lineages. Hence, controlling MRSP spread may be more difficult, as it is unclear which MRSP clone will emerge.

The distribution of SCC*mec* varies significantly among the main MRSP clonal lineages. For example, SCC*mec* type II-III is associated with CC71, pseudo SCCmec_{57395} is associated with CC45, SCC*mec* type IV is associated with CC258, and SCC$mecA_{I16}$-SCC$mec_{czrAI16}$-CI is associated with the CC112. SCC*mec* type IV has been described in CC68, CC45, and CC379 [19]. EBURST analysis estimated that *S. pseudintermedius* has acquired SCC*mec* at least 48 times compared to 56 times reported for *S. epidermis* and 20 times for *S. aureus* [19]. This suggests that *S. pseudintermedius* has an increased capacity for horizontal gene transfer compared to *S. aureus* [19]. The phenotypic resistance of MRSP isolates can be correlated with genotypes identified by the presence of resistance genes and resistance-associated mutations. Antimicrobial resistance of MRSP CC258 shows more resistance to trimethoprim/sulfamethoxazole compared to enrofloxacin and gentamicin [19]. Whereas tetracycline resistance is less frequent in CC71 compared to CC258 and CC45. Chloramphenicol resistance is more frequently detected in CC45 compared to CC71 and CC258 [19]. Additionally, in the United States, tetracycline and fluoroquinolone resistance frequently occurred in CC68 compared to CC84 and CC71. Whereas chloramphenicol resistance frequently occurred in CC84 and CC71 compared to CC68. These results indicated a difference in the resistance profile of CC71 reported in European countries and CC71 reported in the United

States [188]. MRSP isolates in Korea showed resistance to tetracyclines and trimethoprim which is consistent with resistance reported in European and North American isolates [66]. Results from different countries indicate that strains belonging to CC258 seem to be less resistant compared to other successful clones [11, 19, 137]. This could suggest a variation in the carriage of resistance genes in each clonal lineage that could have an impact on the selection of therapeutic options for the treatment of MRSP infection (Table 1-4).

When compared to the ST71 and ST258 in France, ST496 isolates were the only ones that carried cell wall-associated genes *spsI* and *spsF*, genes that code for proteins capable of adhesion to the extracellular matrix of *S. pseudintermedius* [25]. Additionally, ST749 identified in Australia and ST258 in France harbored the *nanB* virulence gene that contributes to host colonization by providing carbon sources for growth, biofilm formation, and enhancing adherence [12, 25].

Staphylococcus pseudintermedius in human medicine

The incidence of *S. pseudintermedius* is clearly described in veterinary medicine, however morphological similarities, as well as diagnostic differences between *S. pseudintermedius* and *S. aureus*, could attribute to the underestimation of *S. pseudintermedius* in human medicine. Throughout the past decade, over 75% of emerging infectious diseases are of zoonotic origin with animal-human linkages. However, there is no published literature on evidence of transmission of *S. pseudintermedius* from animal to human compared to documented reverse zoonotic transmission of *S. aureus* from humans to dogs [205]. The use of MALDI-TOF in diagnostic laboratories has played a significant role in the identification of *S. pseudintermedius* as a pathogen causing infections in human medicine.

In 2006, Van Hoovels *et al*. reported the first case of *S. pseudintermedius* infection from a 60-year-old patient with ischemic cardiomyopathy and ventricle tachycardia [206]. Following the identification, *S. pseudintermedius* has been isolated in cases of dog bite wounds, individuals with skin infections, post mastoidectomy infection, onycholysis, otitis externa, sinusitis, catheter-related bacteremia, and brain abscess [207, 208]. *Staphylococcus pseudintermedius* is typically not a human colonizer and the micro-organism is not part of our normal nasopharyngeal flora. However, humans can become transient carriers if they are in close contact with an infected dog [209]. It has been suggested that old age, immunosuppressed status, and post-surgical infections are contributing factors to the isolation of *S. pseudintermedius* in the human medicine field [208]. Unfortunately, the pathogenesis of *S. pseudintermedius* in human medicine has not been described. However, with pathogenic factors of *S. pseudintermedius* in veterinary medicine being similar to *S. aureus*, *S. pseudintermedius* might exhibit similar pathogenesis to humans.

Table 1-4: Genotypic characterization of *Staphylococcus pseudintermedius* sequence types

Origin	**MLST**	**SCCmec**	**OXA**	**PEN**	**GEN/KAN**	**KAN**	**STR**	**ML**	**TMP**	**TET**	**CHL**
Europe	ST71	II-III, IV	*mecA*	*blaZ*	*Aac (6′)-Ie-aph (2′)-Ia*	*Aph (3′)-III*	*Ant (6′)-Ia*	*Erm(B)*	*Dfr(G)*	*Tet(K)*	*Catpc221*
North America	ST68	V	*mecA*	*blaZ*	*Aac (6′)-Ie-aph (2′)-Ia*	*Aph (3′)-III*	*Ant (6′)-Ia*	*Erm(B)*	*Dfr(G)*	*Tet(M)*	
Europe	ST258	IV	*mecA*	*blaZ*			*Ant (6′)-Ia*	*Erm(B)*	*Dfr(G)*	*Tet(M)*	
Asia, Oceania	ST112	IV	*mecA*	*blaZ*							
Asia	ST45	Pseudo SCCmec$_{57395}$	*mecA*	*blaZ*	*Aac (6′)-Ie-aph (2′)-Ia*	*Aph (3′)-III*	*Ant (6′)-Ia*	*Erm(B)*	*Dfr(G)*	*Tet(M)*	*Catpc221*
Australia	ST496	V(5C2&5)	*mecA*	*blaZ*	*Aac (6′)-Ie-aph (2′)-Ia*	*Aph (3′)-III*	*Ant (6′)-Ia*	*Erm(B)*	*Dfr(G)*	*Tet(M)*	*Catpc221*
Europe	ST497	ΨSCC*mec*$_{KW21}$	*mecA*	*blaZ*	*Aac (6′)-Ie-aph (2′)-Ia*	*Aph (3′)-III*	*Ant (6′)-Ia*	*Erm(B)*			*Catpc221*

Transmission in animals is usually vertical but horizontal or interspecies transmission has been described in cases of veterinary staff and dog owners in close contact with a colonized pet. A study of 13 dogs with deep pyoderma, their owners, and 13 individuals without daily dog contact showed that the same strain of *S. pseudintermedius* was found among owners and their pets [210]. However, after sampling for the second time, these carriers were no longer colonized by *S. pseudintermedius* when the dogs no longer had visible lesions. The results suggested that contact with dog lesions could be a source of transmission between dogs and owners [210]. Additionally, the strains from the dog and owners were resistant to the same antimicrobials (penicillin's, fusidic acid, macrolides/lincosamides, tetracyclines, and chloramphenicol). Similarly, Somayaji *et al.* reported that 22.2% of *S. pseudintermedius* human origin isolates are methicillin-resistant and multidrug-resistant to macrolides, sulfonamides, and fluoroquinolones [208].

Similar STs have been identified between humans and dogs. Additionally, genomic comparative analyses of ST45, ST112, and ST181 isolated from dogs and humans showed that they share similar virulence and antimicrobial resistance profiles [179]. Nasal swabs of veterinary dermatologists in Italy showed persistent colonization of the nasal cavity with ST71-SCC*mec* II-III and ST106-SCC*mec* IV [211]. In Europe, a patient with a history of recurrent rhinosinusitis and post-surgical purulent infection was treated for multidrug-resistant MRSP infection [212]. The bacteriological analysis identified MRSP ST71-t02-J containing SCC*mec* II-III. Treatment of the infection with amoxicillin, clavulanic acid, and clindamycin showed no improvement. However, the isolate was susceptible to amikacin, chloramphenicol, fusidic acid, linezolid, rifampin, vancomycin, combination quinupristin/dalfopristin and nitrofurantoin [212]. Similarly, the ST71 strain in humans showed mutations in topoisomerase genes *gyrA* and *grlA* that confer fluoroquinolone resistance [167]. However, this patient's ultimate source of MRSP infection could not be identified. A longitudinal study in Europe investigated MRSP colonization in dogs and transmission of MRSP to humans, contact animals, and the environment [213]. The results found that five households that were sampled during six months were intermittently MRSP positive for the dog, owners, and the environment. Four different STs were identified among the pet owners (ST71, ST29, ST115, ST143), in the case where owners were MRSP positive for ST29 and ST143, they were not MRSP positive when tested more than once [213].

Summary Statement

The diseases caused by *S. pseudintermedius* in dogs are clinically significant. There is a serious concern about the prevalence of MRSP and the rapid emergence of multidrug resistance. Cases of MRSP infections in dogs with no options for antibiotic treatment have already been reported. MRSP lineages seem to be dispersed worldwide. Additionally, there is an increase in reports of *S. pseudintermedius* infections in humans. A decade ago, a survey of MRSP in the United States was conducted to determine the MLST of each strain. The results indicated predominant strains such as CC68, CC71, and CC84 and

suggested a potential for the emergence of new highly resistant strains. In comparison, no studies have reported on the prevalence and predominant stains of MRSP in South Africa. Also, studies on the molecular epidemiology of *S. pseudintermedius* of human origin in the United States remain scarce. Multilocus sequence typing, geoBURST analysis, and whole genome sequencing will help characterize the major clonal complexes that are currently circulating in the United States and South Africa. Whereas antimicrobial susceptibility testing will report on the antibiotic resistance of *S. pseudintermedius*. The detection of MRSP and dissemination of major clonal lineages will provide a strong basis for the continuous surveillance and control of *S. pseudintermedius* worldwide. The detection of predominant strains and molecular epidemiology of *S. pseudintermedius* can facilitate the identification of conserved regions that can be used in the development of alternative therapeutics. Additionally, the identification of similar *S. pseudintermedius* strains in canine and human isolates can help in further studies of genetic and immunological properties that influence pathogenicity in human beings. Therefore, we hypothesize that selective pressure has driven the clonal expansion of new antimicrobial-resistant MRSP isolates and changed the spectrum of prevailing clonal complexes in the United States. Also, we hypothesize that MRSP clonal lineages have disseminated to countries such as South Africa. Finally, we hypothesize that *S. pseudintermedius* has evolved as a pathogen with the potential to cause infections in human beings.

Specific aims

1. Investigate the changes in antimicrobial resistance and population structure of MRSP isolated from dog samples across all ten geographic regions in the United States.
2. Investigate the antimicrobial resistance and molecular epidemiology of *S. pseudintermedius* isolated from dog samples submitted to a veterinary diagnostic laboratory in South Africa.
3. Investigate the antimicrobial resistance and molecular epidemiology of *S. pseudintermedius* isolated from human samples at a hospital setting in the United States.

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CHAPTER II

The detection of new clonal lineages and antimicrobial resistance characterization of Methicillin-resistant *Staphylococcus pseudintermedius* isolated from dogs in the United States.

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Abstract

Surveillance of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) is key to the detection of emerging clonal complexes, tracking antimicrobial resistance, and identifying changes that may affect resistance to alternative therapeutics. The objective of this study was to perform a phenotypic and molecular epidemiological survey to determine the antimicrobial susceptibility and genetic background of MRSP strains in the United States. Samples from 200 isolates were obtained from veterinary diagnostic facilities located in the US geographic regions sampled ten years ago. Kirby-Bauer disk diffusion was used to determine the susceptibility of 164 MRSP to 21 antimicrobials. Almost all MRSP isolates (98%) in this study were multidrug-resistant with 21% of the isolates resistant to more than 16 antimicrobials which included all antibiotics routinely used to treat *S. pseudintermedius* infections in dogs. Among the drugs generally reserved for human medicine, only one sample was resistant to linezolid and no resistance to vancomycin and rifampin was observed. Multilocus sequence typing was performed on all MRSP isolates and 78 different STs were identified. These findings revealed a remarkable change in sequence types. Whereas 10 years ago ST68, ST7, and ST84 were predominant, these strains have been supplanted by other STs, notably ST45, ST155, ST181, ST496, and ST551. ST45, ST181, and ST496 are of greatest concern because they are widespread and associated with resistance to most antimicrobials. The newly prevalent STs were most likely transported from other continents, where they were first identified. The population structure of MRSP is far more elastic than expected with new, highly resistant strains replacing the ones that predominated in the US a decade ago. Antibiotic use plays a role in selection; however, the strains that were replaced were also multidrug-resistant and other factors are likely involved. These may include metabolic efficiency and, more likely, the ability to evade their hosts' immune response via immune suppression, antigenic variation, or both.

Introduction

Staphylococcus pseudintermedius is a frequent cause of canine pyoderma, superficial skin and soft tissue infections, abscesses, urinary tract infections, and invasive diseases such as bacteremia or endocarditis in dogs [1–3]. Previous hospitalization, frequent hospital visits as well as prolonged antimicrobial

treatments are associated with risk factors contributing to MRSP infections [3–5]. Moreover, MRSP in human medicine might be underreported due to misidentification among people who acquire subclinical infections [6–9]. MRSP strains carry a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*), which harbors the *mecA* gene conferring methicillin resistance [10–16]. It is common for MRSP isolated from dogs to confer multidrug resistance to veterinary antibiotics such as tetracyclines, lincosamides, fluoroquinolone, and macrolide [14, 17–19]. *Staphylococcus pseudintermedius* can express a wide variety of virulent factors such as coagulase, hemolysins, exfoliative toxins, enterotoxins, and leukotoxins [9, 20].

The prevalence of MRSP is mainly regionally dependent [21]. For example, no MRSP isolates were identified among dogs visiting a veterinary practice in the UK in 2009 compared to 31.6% reported in Italy among independent canine and feline clinical samples in 2016 [22, 23]. The population structure of MRSP has historically been reported as clonal compared to a lack of clonality among methicillin-susceptible *Staphylococcus pseudintermedius* (MSSP) isolates [14, 24, 25]. Clonal complexes (CCs) are groups of sequence types that are closely related based on their allelic profiles and possibly share a recent ancestor [17, 25]. According to Multilocus Sequence Typing (MLST) and BURST algorithms, CC71 and CC258 dominate in Europe, CC68 is spread in North America but also found in Europe, and CC45 and CC112 are prevalent in Asia [25]. Vertical transmission and a non-clonal population shift of MRSP have led to the emergence of novel sequence types (ST) such as ST496 and ST551 in Australia [18, 26]. A recent population genomic study detected ST45, ST150, and ST181 in the New England and Texas regions of the United States [27]. Antimicrobial resistance across distinct lineages is significantly diverse [25, 28]. For example, ST45 and ST71 isolates are more likely to confer resistance to fluoroquinolones compared to the susceptibility of ST258 [17]. This suggests that antimicrobial treatment options for MRSP infections in dogs could be subject to clonal lineage involved [25].

In 2016, a study was conducted to determine the population genetics of *S. pseudintermedius* from a cross-section of the United States using a seven-locus MLST method [28]. The study detected CC68, CC71, and CC84 as the major CCs circulating among MRSP isolates. The isolates from this study showed high susceptibility to chloramphenicol compared to clindamycin, erythromycin, tetracyclines, marbofloxacin, gentamicin, and trimethoprim-sulfamethoxazole [28]. However, the identification of genes encoding resistance and virulence among the MRSP isolates was not reported in the study. Resistant genes were reported from one representative of each major clonal lineage (CC68, CC71 and CC84). All the representative lineages harbored beta-lactamase (*blaZ*) and kanamycin resistance genes *ant (6)-Ia*, aph*(3')-III*) whereas ST68 and ST71 also harbored gentamicin-kanamycin (*aac(6')-Ie-aph(2")-Ia)* and macrolide (*ermB*) resistant genes. Furthermore, the strain representing CC68 harbored lincosamides and tetracycline resistance genes *lnuA* and *tetM* respectively. The increasing MRSP prevalence reported across different veterinary diagnostic settings previously sampled in the United States suggests the

potential emergence of new clonal lineages. Therefore, we investigated the molecular epidemiology, and antimicrobial resistance of MRSP lineages from dogs across 10 regions in the United States sampled between 2019-2021. This allowed us to detect new STs in the United States, and antimicrobial resistance patterns across STs.

Results

Descriptive results

Based on oxacillin resistance, 164 isolates received across the 10 regions were confirmed as MRSP. The isolates were then used for the MLST and geoBURST analysis to detect STs and dominant clonal complexes. Additionally, antimicrobial resistance genes and virulence genes were detected.

Multi-locus sequencing and genetic relatedness

Seventy-five different STs were identified, and 26 of the STs were not previously reported, they were assigned in this study and made available in the *S. pseudintermedius* PubMLST database (http://pubmlst.org/speudintermedius). ST551 was identified most frequently (n=18), followed by ST45 (n=14), ST181 (n=13), and ST496 and ST155 in equal numbers (n=9). The geoBURST analysis resulted in 59 groups of CCs and 45 singletons based on the single locus variant (SLV) algorithm (Most of the isolates assigned in this study (ST2227, ST2226, ST2200, ST2216, ST2222) were clustered around ST298 by single and double allelic variants. ST2221 assigned in this study belonged to CC150 with a single allele variant. Only one link with four allelic variants was identified between ST1542 and ST2224 indicating a highly distant relationship.

Table 2-1). The singletons identified in the study were unrelated to any other within the SLV collection. Eight single-linked groups were identified where no founder ST could be detected. Sequence type 258 was identified as the major clonal founder of MRSP in this study. Other CCs were identified as CC150, CC2090, CC298, CC121, and CC45 for groups with more than two STs.

When the geoBURST full minimum spanning tree (MST) algorithm was utilized, 25 links at single locus variant were detected, 35 links at double locus variant (DLV), 16 links at triple locus variant (TLV), and 1 link at four locus variants (Figure 2.1). STs belonging to CC258 varied in a single allele, while ST2205 was linked using triple allele variation. ST551 seems to be distantly related to ST121 by three allelic variations whereas ST496 has a single locus variant to ST121. Most of the isolates assigned in this study (ST2227, ST2226, ST2200, ST2216, ST2222) were clustered around ST298 by single and double allelic variants. ST2221 assigned in this study belonged to CC150 with a single allele variant. Only one link with four allelic variants was identified between ST1542 and ST2224 indicating a highly distant relationship.

Table 2-1. Distribution of Clonal complexes and identified sequence types

Clonal complexes	**Sequence types**
258	2217, 277, 383, 869,258
121	2308,496, 121
45	2336, 311, 25
2090	749, 811, 257, 2090
298	2227, 2200, 298
150	757, 2209, 2221,124, 150
single link	2210-64,2212-1602,84-153, 155-433, 181-2203, 25-551, 2087-1431, 1542-17
singletons	2223,1672, 1444, 2214, 2219, 2208, 2220, 1982, 2664, 1575, 1296, 339, 2218, 2204, 2222, 2215, 2216, 2174, 2205, 2174, 71, 1672, 1692, 833, 2663, 2174, 2205, 1279, 2201, 2213, 1297, 1692, 2202, 1308, 1055, 157, 2211, 1229, 2664, 2214, 2304, 2307, 2316, 2224, 2226

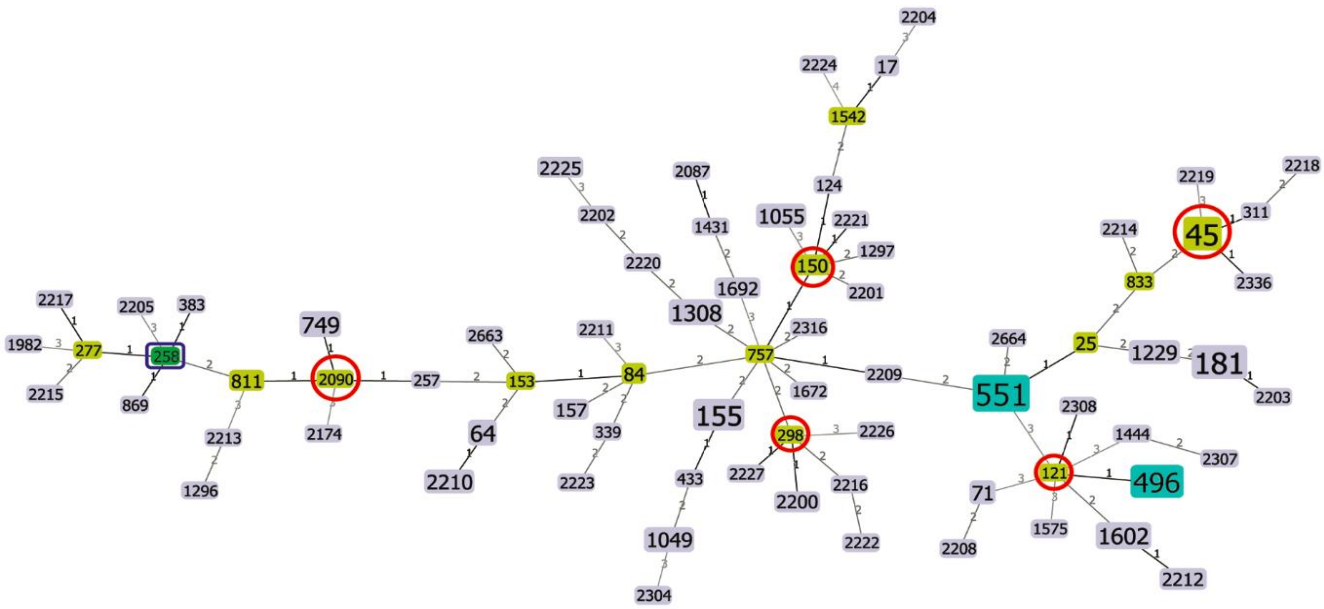


Figure 2.1: Clonal relationships of MLST in the United States in 2021.The geoBURST full MST algorithm showed links connected by SLV, DLV, TLV, and 4 link variants. Each node represents a ST. The size of the node denotes the sample size of each ST. The distance between each node is represented by 1=SLV, 2=DLV, 3=TLV, and 4= four locus variants. The predicted founder clonal complex is represented as dark green, other CCs are circled in red. The new novel STs identified are indicated as blue.

Antimicrobial susceptibility phenotype of MRSP

Almost all MRSP isolates (98%) in this study were multidrug-resistant with 21% of the isolates resistant to more than 16 antimicrobials. The majority of the MRSP isolates were phenotypically resistant to cefpodoxime (94.5%), kanamycin (91.46%), sulfamethoxazole-trimethoprim (84.76%), erythromycin (83.54%), tetracycline (84.1%), and clindamycin (82%) (Table 2-2). However, susceptibility to cephalothin (72.56%), chloramphenicol (74.39%), and fosfomycin (92.68%) were observed. None of the MRSP isolates were resistant to vancomycin and rifampin whereas only one isolate was resistant to linezolid. The most common resistance patterns identified among the MRSP isolates were marbofloxacin-clindamycin-doxycycline-erythromycin-trimethoprim-sulfamethoxazole (78%). There were no MRSP isolates that showed inducible clindamycin resistance

There were variations in antimicrobial resistance phenotype among the frequently identified STs in the study to fosfomycin, chloramphenicol, amoxicillin-clavulanate acid, cefoxitin, and cephalothin (Table 2-2). Resistance to fosfomycin occurred among ST496 (77.78%) and ST155 (11.11%) whereas chloramphenicol resistance occurred in ST45 (92.86%) and ST496 (88.89%) as well as ST155 (11.11%). Chloramphenicol resistance also occurred among the MRSP singletons (34%). Amoxicillin-clavulanate acid resistance was mostly observed among ST45 (85.71%), ST496 (77.78%), and ST551 (72.22%) compared to ST155 (11.11%) and ST181 (7.69). Cefoxitin resistance was high among all STs except only 22.22% of ST155. Resistance to cephalothin was low among ST155 (11.11%) and ST181 (7.96%) compared to the moderate resistance that occurred in 64.29% of ST45 and 55.56% of ST496. Among MRSP singleton isolates, high antibiotic resistance occurred to cefpodoxime (95.5%), kanamycin (82.2%), and tetracyclines (73.3%) whereas low antibiotic resistance was observed to amoxicillin-clavulanate (18%) and cephalothin (9%).

Antimicrobial resistance genes of MRSP

More than 90% of MRSP isolates harbored genes conferring resistance to β-lactams (*mec*A, *bla*Z) while no isolates carried the *mecC* gene (Table 2-3). ST45, ST551, and ST496 harbored *mec*A, *bla*Z, *aac (6')-Ie-aph (2")-Ia*, *aph (3')-III*, *ant (6')-Ia*, *erm*(B), *dfr*(G), *tet*M, and *catp*C221 compared to ST258 that only harbored *mec*A, *bla*Z, *dfr*G, and *tet*M genes. ST45 and ST551 were the only STs that harbored both *tet*K/*tet*M genes compared to other STs. Whereas ST749 harbored only *mecA, blaZ*, *gyrA*, and *fosB* resistance genes.

Table 2-2: Antimicrobial resistance among MRSP sequence types in 2021(n=164)

Group	**Antimicrobial**	**MRSP (n=164)**	**ST45 (n=14)**	**ST155 (n=9)**	**ST181 (n=13)**	**ST496 (n=9)**	**ST551 (n=18)**	**MRSP singletons n=45**
Lincosamides	Clindamycin	82.93%	100	100	92.31	100	100	66.6
B-lactams	Penicillin	99.39	100	100	100	100	100	100
	Amoxicillin-clavulanate acid	39.63	85.71	11.11	7.69	77.78	72.22	18
	oxacillin	100	100	100	100	100	100	100
Cephalosporins	cefoxitin	57.32	85.71	22.22	92.31	77.78	83.33	37.7
	Cefpodoxime	94.5	100	100	100	88.89	100	95.5
	Cephalothin	27.44	64.29	11.11	7.69	55.56	44.44	9
Tetracyclines	Tetracycline	84.15	92.86	100	100	100	100	73.3
	Minocycline	84.15	92.86	100	100	100	94.44	79.5
	Doxycycline	83.54	92.86	100	100	100	100	71.1
Phenols	Chloramphenicol	25.61	92.86	11.11	0	88.89	0	34
Glycopeptides	Vancomycin	0	0	0	0	0	0	0
Aminoglycosides	Streptomycin	88.41	100	100	100	100	100	79.5
	Gentamicin	80.49	100	100	92.31	88.89	88.89	62.2
	Kanamycin	91.46	100	100	100	100	100	82.2
Macrolides	Erythromycin	83.54	100	100	92.31	100	100	66.6
Fluoroquinolones	Marbofloxacin	78.05	92.86	100	100	100	94.44	53.3
Rifamycin	Rifampin	0	0	0	0	0	0	0
Phosphonic	Fosfomycin	7.32	0	11.11	0	77.78	0	0
Oxazolidinones	Linezolid	0.61	0	0	0	0	0	0
Folate inhibitor	trimethoprim/sulfamethoxazole	84.76	57.14	100	92.31	100	100	73.3

Table 2-3: Genotypic characterization among MRSP sequence types in 2021

MLST	OX	PEN	GEN/KAN	KAN	STR	ML	TMP	TET	CHL	MAR	FOS
ST45	*mecA*	*blaZ*	*aac (6')-Ie-aph (2")-Ia*	*aph (3')-III*	*ant (6')-Ia*	*erm(B)*	*dfr(G)*	*tetM/tetK*	*catpC221*	*grlA/gyrA*	*fosB*
ST155	*mecA*	*blaZ*	*aac (6')-Ie-aph (2")-Ia*	*aph (3')-III*	*ant (6')-Ia*	*erm(B)*	*dfr(G)*	*tetM*		*grlA/gyrA*	*fosB*
ST181	*mecA*	*blaZ*	*aac (6')-Ie-aph (2")-Ia*	*aph (3')-III*	*ant (6')-Ia*	*erm(B)*	*dfr(G)*	*tetM*		*grlA/gyrA*	*fosB*
ST258	*mecA*	*blaZ*					*dfr(G)*	*tetM*		*grlA/gyrA*	*fosB*
ST496	*mecA*	*blaZ*	*aac (6')-Ie-aph (2")-Ia*	*aph (3')-III*	*ant (6')-Ia*	*erm(B)*	*dfr(G)*	*tetM*	*catpC221*	*grlA/gyrA*	*fosB*
ST551	*mecA*	*blaZ*	*aac (6')-Ie-aph (2")-Ia*	*aph (3')-III*	*ant (6')-Ia*	*erm(B)*	*dfr(G)*	*tetM/tetK*	*catpC221*	*grlA/gyrA*	*fosB*
ST150	*mecA*	*blaZ*	*aac (6')-Ie-aph (2")-Ia*	*aph (3')-III*	*ant (6')-Ia*	*erm(B)*	*dfr(G)*	*tetM/tetK*	*catpC221*	*grlA/gyrA*	*fosB*
ST749	*mecA*	*blaZ*								*grlA/gyrA*	*fosB*

Antimicrobial resistance genes were determined using ResFinder for oxacillin (OX), penicillin (PEN), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), macrolides (ML), trimethoprim (TMP), tetracycline (TET), chloramphenicol (CHL), marbofloxacin (MAR), and Fosfomycin (FOS)

Virulence genes

All MRSP isolates shared a core virulence profile with a group of 24 genes (Figure **2.2**). Virulence genes that encode for adhesion factors (*adsA*), biofilm formation (*icaA, icaB, icaC*), enzymes (*lip, geh, nuc*), toxins (*lukD, lukE, sec*), and immune evasion (*AdsA*) were detected in almost all MRSP isolates. One of the isolates belonging to ST2213 assigned in this study lacked the *ica* operon, enterotoxin C, and leukotoxins but harbored hemolysin genes (*hlgB, hlgC*). Surface-associated protein genes such as *clfB* and *fnbB* were not common among MRSP isolates. Similarly, the enterotoxins encoded by *sell, selq, sea,* and *seh* were detected in low numbers. The isolate belonging to ST258 did not show any presence of cell wall-associated genes.

Discussion

The surveillance of antimicrobial resistance and epidemiological diversity of MRSP is important to understand the rapid emergence of successful clonal lineages that could have an impact on the development of therapeutics for the treatment of MRSP infections in dogs. In this study, samples were collected from diverse regions to determine new clonal complexes and antimicrobial resistance of MRSP isolated from dogs in the United States.

The high proportion of MRSP in this study is mainly due to the nature of isolates submitted from each region in the United States. Additionally, oxacillin disk diffusion test was used to predict methicillin resistance in this study. The oxacillin disk diffusion test is recommended to determine the presence of the *mec*A gene in *S. pseudintermedius* [29, 30]. Nevertheless, this method could have underestimated the proportion of MRSP in this study. For example, the *mec*A gene was detected from whole-genome sequences of 4 isolates that were MSSP based on oxacillin resistance. Previous studies have speculated that *blaI/blaR1* is indispensable in the *mec*A regulation of MRSP [31]. Therefore, the defective function of *blaI/blaR1* could explain the lack of oxacillin phenotypic resistance of *S. pseudintermedius* despite the presence of *mec*A. However, further investigations on the *mec*A region of these isolates would be necessary to demonstrate this hypothesis. Multi-locus sequence typing results suggest that MRSP isolated from dogs in the United States is undergoing a considerable amount of gene acquisition and mutations that have led to the emergence of new diverse lineages. The predominant MRSP isolates belonged to ST551, followed by ST45, ST181, ST496, and ST151. In contrast, Duim *et al.* [17] reported 39 unique MRSP sequence types that belonged to ST71, ST258, ST261, and ST256 in the Netherlands [17].

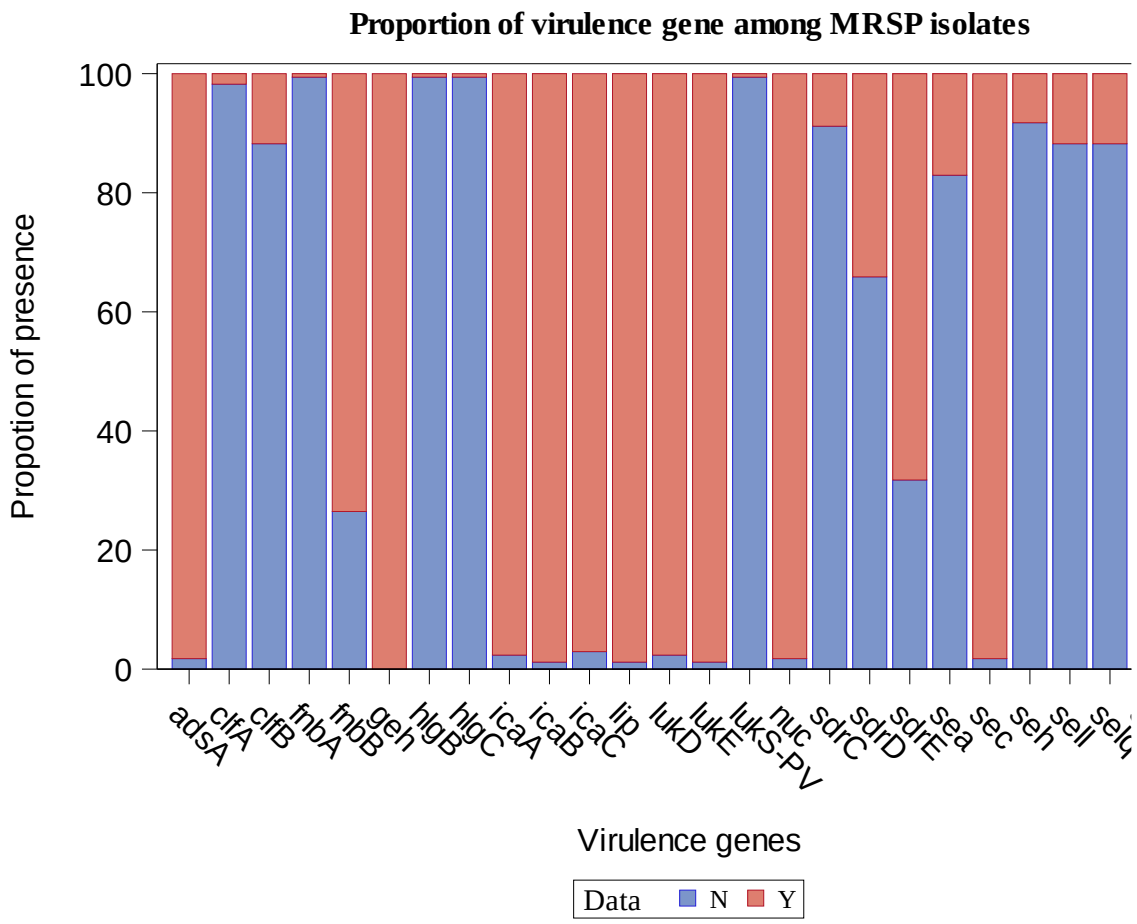


Figure 2.2: Virulence genes detected among all MRSP isolates in 2021. Clumping factor (*clfA, clfB*), fibrinogen binding protein (*fnbA, fnbB*), Intercellular adhesin (*icaA, icaB, icaC*), leukotoxin (*lukD, lukE, lukS-PV*), thermonuclease (*nuc*), ser-asp rich fibrinogen binding protein (*sdrC, sdrE, sdrD*), lipase (*geh, lip*), immune evasion (*adsA*), enterotoxin (*sea, sec, she, sell, selq*) and hemolysin (*hlgB, hlgC*).

Similarly, ST45 detected in this study was also detected in the Netherlands, Finland, France, Italy, and Australia [17, 18, 23, 32, 33]. This is the first report on the frequent detection of ST551 observed among MRSP isolates in the United States. The emergence of ST551 as a successful clone is similar to that reported in Poland and Slovenia [19, 26]. In contrast, ST258 is reported as the successful lineage in North Europe and France [18, 23, 32, 33]. However, the pathogenicity, host-host transmission, and biofilm-forming capabilities that could contribute to the observed success of these clones is unknown. This is the first study reporting the frequent identification of the novel ST496 among MRSP isolates in the United States. This ST was first identified in Australia and later reported in Italy, France, and New Zealand [18, 23, 32, 34]. The emergence of new STs in the United States could be due to the increased dissemination of horizontally transferable resistance genes and increased mobility of animals across geographical boundaries [35]. However, further studies are required to characterize the genetic diversity of ST551 and ST496 with additional genomes from dogs in the United States.

The geoBURST analysis is a globally optimized implementation of the eBURST algorithm identifying alternative patterns for several bacterial species' descent [36]. The geoBURST results in this study predicted CC258 as the major clonal founder of MRSP STs compared to ST68 which was identified 10 years ago in the United States [28]. This is similar to results in Denmark, the Netherlands, and Norway which identified CC258 as a clonal lineage filling the gap as a successful lineage while CC71 has lost its sustainability [17, 25, 37]. Other clonal complexes such as CC45 identified in this study have been identified worldwide in countries such as Asia and Europe [25]. Significantly, none of the isolates in this study were ST68, which was historically known as the major CC in the United States and dispersed to other countries in Europe and Asia [14, 25]. In comparison, a recent study reported the presence of ST68 among *S. pseudintermedius* isolates in a Texas population collected between 2017-2018 [27]. The lack of identification of ST68 in this study is astonishing and attests to the change in the population structure of MRSP in the United States. Therefore, a broader study to determine clonality and recombination rates in CC68 would be beneficial to study the genotypic evolutionary changes in this clonal lineage. The Identification of CC2090 in this study is unique, this is similar to a study in Korea that reported unique CCs compared to commonly known major clonal complexes worldwide [38]. The observed emergence of MRSP clonal lineages assigned in this study suggests that recombination could be the driver of diversity in the MRSP population in the United States [25]. This is in line with the number of heterogeneous MRSP isolates with unique characteristics found in this study.

Multidrug resistance of MRSP was observed in this study, however, all isolates were susceptible to licensed human medicine antibiotics (vancomycin, linezolid, and rifampin). This is similar to the antimicrobial susceptibility of human medicine-licensed antibiotics in Italy [39]. In

comparison, resistance to rifampin has been reported among MRSP isolates in Australia and Denmark [18, 33]. The susceptibility of MRSP isolates to rifampin and vancomycin is encouraging given its significance in human medicine and the increased risk of mutations conferring resistance during therapy [40]. The correlation between antimicrobial resistance genotype and phenotype observed in this study is similar to that reported in previous studies [27, 39, 41]. A high proportion of MRSP in this study was resistant to clindamycin (82.93%) with no resistant variation across different STs. Similarly, 83.54% of MRSP isolates were resistant to doxycycline, with no variation across all STs. The high proportion of clindamycin and doxycycline resistance observed in this study could be attributed to the presence of *erm*B and *tet*M genes among MRSP isolates. Resistance to these antimicrobials is also associated with the presence of mobile genetic elements such as Tn*5405*, Tn*5801*, Tn*916*, and IS*256* or IS*1272*-like elements [42, 43]. The high resistance observed in clindamycin and doxycycline in this study is concerning due to their significant use in the treatment of MRSP infection in dogs.

There was significant resistance to chloramphenicol (25.61%) observed among the MRSP isolates, with high resistance observed among ST45 (92.38%) and ST496 (88.89%) isolates. The resistance to chloramphenicol can be attributed to the presence of *catp*C221. These results represent the differences in the carriage of resistant genes among distinct MRSP lineages. For example, ST45 and ST551 in this study harbored more antimicrobial resistance genes, including *tet*K, compared to ST258. Presumably, this could be due to truncations of transposable elements that transport these genes in each sequence type. More studies are needed to investigate whether the presence of specific antimicrobial resistance genes plays a significant role in successful clonal lineages in the United States. The newly emerged ST496 showed resistance to fosfomycin and chloramphenicol. This is similar to the chloramphenicol resistance of ST496 from the Australian isolates [18]. However, given the emergence of ST496 and the rare use of fosfomycin for treating MRSP infections in dogs, fosfomycin resistance in this lineage should be further investigated. A high number and variety of virulence genes has been detected among the MRSP isolates. The high detection of the *sec* gene encoding for enterotoxin C among the isolates suggests that this toxin might be more associated with methicillin resistance. Similarly, the *sec* gene was mostly detected among dogs with pyoderma and chronic otitis in other studies [44].

To investigate the molecular epidemiology of MRSP in America. Samples were gathered from diagnostic facilities spread across the nation. Due to the frequent transit of dogs throughout the nation, some samples may have come from other areas. Additionally, each location was asked for a minimum of 20 MRSP, which could have resulted in selection bias due to the various MRSP detection techniques utilized in each diagnostic facility. Additionally, the minimum MRSP

samples taken in each geographic origin were insufficient to characterize relationships between the STs, CCs, and antimicrobial profiles of the isolates and their geographic origin.

Conclusion

The results from this study revealed that the population structure of MRSP in the United States is more intrinsically elastic than previously thought. The study detected new clonal complexes driving the population dynamic changes of MRSP isolated from dogs in the United States. The lack of detection of ST68 in the United States is concomitant with the detection of several MRSP isolates that are ST496 and ST551 that have been reported in other countries. The newly detected Multilocus sequence types have replaced the previously detected MDR strains of MRSP. There are limited effective, veterinary-licensed, antibiotic treatment options remaining for the treatment of MRSP in dogs. Therefore, continuous surveillance of MRSP in the United States and identification of conserved antigens common in the emergent CCs would be significant in therapeutic vaccine development.

Methodology

Bacterial isolates

MRSP isolated from dogs was acquired from private, state, and university-associated veterinary diagnostic laboratories in the United States. To collect regionally diverse samples, the states were grouped using the United States health and human services designated geographical regions. A minimum of 17 MRSP isolates was received from each region over 3 years (2019-2021). Non-duplicate, clinical isolates were received from the 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). Pure isolates of *S. pseudintermedius* were inoculated onto Columbia blood agar plates with 5% sheep blood (Remel). The isolates were identified using the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker). *Staphylococcus pseudintermedius* species assignment was made when log(score) values were ≥ 2 [30]

Phenotypic antimicrobial susceptibility

All *S. pseudintermedius* isolates were tested for their antimicrobial susceptibility by the disk diffusion method. Mueller Hinton agar plates and antimicrobial disks were obtained commercially (BD Diagnostic System, Sparks, MD). The antimicrobials tested were penicillin (P) 10µg, clindamycin (DA) 2µg, kanamycin (K) 30µg, erythromycin (E) 15µg, gentamicin (CN) 10µg,

oxacillin (OX) 1µg, tetracycline (TE) 30µg, minocycline (MH) 30µg, doxycycline (DO) 30µg, chloramphenicol (C) 30µg, cephalothin (KF) 30µg, vancomycin (VA) 30µg, streptomycin (S) 10µg, rifampin (RD) 5µg, marbofloxacin (MAR) 5µg, amoxicillin-clavulanic acid (AMC) 20µg, fosfomycin (FOS) 20µg, linezolid (LZD) 30µg, cefpodoxime (CPD) 10µg, cefoxitin (FOX) 30µg, sulfamethoxazole-Trimethoprim (SXT) 1.25/23.75ug. For inducible clindamycin resistance, the Erythromycin and clindamycin disks were placed approximately 15 mm apart. The Clinical and Laboratory Standards Institute (CLSI) guidelines at the time of the study were used for zones of inhibition interpretation [30, 45]. Additionally, clinical breakpoints from previous CLSI guidelines were used for Kanamycin, streptomycin, Cephalothin, Amoxicillin-clavulanate acid, vancomycin, and Fosfomycin [45, 46]. The intermediate susceptibility was treated as resistant for analysis in the study. Isolates that were resistant to three or more antimicrobial drug classes were defined as "multidrug-resistant".

Methicillin resistance confirmation

Isolates were screened for methicillin resistance with the Kirby Bauer disc diffusion technique using oxacillin disc. Oxacillin (1µg) is used as the predictor of methicillin resistance in *S. pseudintermedius* following guidelines of the CLSI breakpoint interpretation (S≥18, R≤17) [30]. Isolates that were oxacillin resistant were then used for further analysis.

DNA extraction and whole-genome sequencing

Isolates were inoculated onto Columbia blood agar plates containing 5% sheep blood and incubated overnight at 37 ^{O}C. Three to four bacterial colonies were suspended in 3 ml of sterile tryptic soy broth and incubated at 37 ^{O}C overnight. A commercial DNA extraction kit was used to extract DNA (UltraClean® Microbial DNA isolation kit, Qiagen). DNA concentration was measured using nanodrop. DNA libraries were constructed using a Nextera DNA sample prep kit (Illumina, Inc., USA) as per the manufacturer's protocol. The genomes were sequenced using a MiSeq and HiSeq platform (Illumina, Inc.) to produce single paired end reads of 150 bp in length.

Genome assembly and molecular characterization

Raw reads were assembled into contigs using de novo assembler SPAdes v3.13.1. STs were identified using the program pubMLST which extracts sequences of 7 housekeeping genes (*tuf*, *cpn60, pta, purA, fdh, ack*, and *sar*) [24] from contig files and compares them with *S. pseudintermedius* MLST database and the results were given by each locus (https://pubmed.ncbi.nlm.nih.gov/30345391/). The New numeric designation was assigned to alleles and STs by the curator Vincent Perreten when needed. Genome assemblies of MRSP were

screened for resistant genes using Resfinder 3.2 by applying the default cut-off settings of 60% coverage and 90% identity https://cge.cbs.dtu.dk/services/ResFinder/. All MRSP genome assemblies were screened for known virulence genes using the Virulence Factor Database (VFDB) [47].

geoBURST analysis

STs were assigned to CCs by Phyloviz 2.0 using the geoBURST algorithm default parameters https://online.phyloviz.net/ . The projected founder STs and related single-locus variations were designated as CCs using a single-locus variant algorithm. The geoBURST full MST algorithm was used to cluster STs based on locus variant links to infer the relatedness of the STs [36].

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CHAPTER III

Temporal changes in antibiotic resistance and inferences on the population structure of *Staphylococcus pseudintermedius* using Multilocus Sequence Typing Data from 2010 to 2021 in the United States.

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Abstract

Staphylococcus pseudintermedius is one of the main bacterial agents responsible for canine infections. Using multilocus sequence typing (MLST) data and geoBURST, we determined the genetic relatedness of *S. pseudintermedius* isolated from dogs in the United States. The isolates of *S. pseudintermedius* were collected over different time periods, approximately 10 years apart, which allowed for analysis of temporal changes in antimicrobial resistance and the population structure. The relationship between the Multilocus sequence types (STs) identified in 2010 and 2021 was studied using the geoBURST single-locus variant algorithm. The geoBURST analysis identified 88 clonal complexes (CC) containing 71 singletons and 8 single-linked isolates where no clonal founder could be identified. The largest clonal complex contained 34 STs, where ST174 was predicted to be the major clonal founder. Subgroup founders of this group were predicted as ST68, ST150, ST17, ST137, ST84, and ST125. Other STs that were identified as founder clonal complexes included ST45, ST258, ST152, ST2090, ST3, and ST123. The Bayesian posterior probability determined that CC258, CC45, CC84, CC68, and CC123 were not clustered with high confidence of 0.8838, 0.5131, 0.5622, 0.7105, and 0.6414 respectively with STs belonging to each clonal complex. The detection per site (rho/theta) value for all 147 STs was 27.58, suggesting that recombination occurred more frequently than mutation. Gene-wide positive selection was detected in 5 of the 7 housekeeping genes: *cpn60*, *fdh*, *pta*, *purA*, and *sar*. There was significant (p value<0.05) increase to resistance observed in clindamycin (0.0021), amoxicillin-clavulanic acid (0.0492), oxacillin (0.0003), cefoxitin (0.0456), cefpodoxime (0.0001), tetracycline (0.001), chloramphenicol (<0.0001), erythromycin (0.0001) marbofloxacin (0.00004) and gentamicin (0.0001). This study provides evidence that *S. pseudintermedius* STs between 2010 and 2021 represent numerous unrelated clones that most likely do not share a common recent ancestor. The detection of newly identified clonal lineages with an unidentified common ancestor highlights the local evolution of the *S. pseudintermedius* in the United States. These findings

expand the knowledge of the population structure of MRSP in the United States which has been affected by the dynamics of host interactions and antimicrobial resistance over time.

Introduction

Staphylococcus pseudintermedius was first identified in 2005 as a novel commensal pathogen primarily isolated from canine pyoderma and otitis externa [1–4]. A temporal trend of increasing resistance has been observed to β-lactams, bacitracin, aminoglycosides, and enrofloxacin among *Staphylococcus* isolates from dogs [5, 6]. Geographic region, specimen source, and previous antibiotic use are described as significant predictors of multi-drug resistance in *S. pseudintermedius* [5, 6]. Thus, the identification of predominant strains could help explain the spread and diversity of methicillin-resistant *S. pseudintermedius* (MRSP) within a particular region [7, 8].

Multilocus sequence typing using seven housekeeping genes (*ack, sar, purA, pta, tuf, cpn60,* and *fdh*) has been used to study the epidemiology and population-level evolution of *S. pseudintermedius* [2, 9–11]. Using eBURST analysis, MRSP clonal lineages such as CC71 and CC258 in Europe, CC68 in North America, and CC45 and CC112 in Asia have been identified [10, 12–15]. Using phylogenetic analysis, the clonal spread of MRSP has been linked to the diversity of resistance profiles due to high recombination to mutation ratios [10, 16]. Combining MLST and genomics has been important in developing the epidemiological surveillance and control strategies of infectious pathogens such as *Staphylococcus aureus, Klebsiella pneumoniae,* and *Streptococcus pneumoniae* which have public health implications [17]. Therefore, similar methods can be used to develop real-time surveillance and control strategies for *S. pseudintermedius* [11].

In France, an evolutionary population shift revealed a decline in ST71 and the emergence of ST496 over a 6-year period [18]. In the Netherlands, CC71 emerged as a dominant lineage from 2004 and onwards but currently, lineages such as CC258 and CC45 are partly replacing CC71 [19]. In Poland, the dominance of ST71 among MRSP was seen until 2015 when the emergence of ST551 in 2016 was noted [20]. However, studies elucidating the changing structure and population dynamics of *S. pseudintermedius* throughout the United States have not been reported. Therefore, the aim of this study was to examine the epidemiology and changes in population structure of *S. pseudintermedius* in the United States using MLST. Because antibiotic resistance and the population structure of *S. pseudintermedius* in the United States were investigated over two different time periods, 10 years apart, temporal changes in the population could be observed. The detection of new STs will be used to infer the population structure and evolutionary mechanisms that could be associated in the diverse expansion of *S. pseudintermedius* clones in the United States.

Results

Descriptive results

A total of 190 *S. pseudintermedius* were collected in 2010 and 185 were collected in 2021. Most of the STs (n=62) in 2010 were identified as ST68 while ST68 was not detected in 2021. Some of the STs (ST124, ST84, ST121, ST71, ST64, ST155, ST125, ST257) were similar between 2010 and 2021 (Table 3-1). None of the STs identified in 2010 were ST45, ST496, ST551, and ST258.

GeoBURST analysis of STs in 2010 and 2021

The relationship between the STs identified in 2010 and 2021 was studied using the geoBURST single-locus variant algorithm [15]. The geoBURST analysis identified 88 CC containing 71 singletons and 8 single-linked isolates where no clonal founder could be identified (Figure 3.1). The largest clonal complex contained 34 STs where ST174 was predicted to be the major clonal founder. Subgroup founders of this group were predicted as ST68, ST150, ST17, ST137, ST84, and ST125. Other STs that were identified as founder clonal complexes included ST45, ST258, ST152, ST2090, ST3, and ST123.

When using a geoBURST full minimum spanning tree (MST) algorithm, 64 links were detected at SLV, 59 links at DVL, 24 links at TVL, and 4 links at level 4 (Figure 3.2). Most of the STs assigned in 2021 (ST2202, ST2220, ST2219, ST2211, ST2216, ST2200, ST2227, ST2223) were clustered around ST84 and differed by single, double, and triple locus variants. Singletons in 2021 (ST2218 and ST2204) are related to ST17 reported in 2010 and differ by double and triple locus variants. ST2222, ST2210, and ST2663 identified in 2021 deferred to ST137 reported in 2010 by single and double locus variants. None of the STs identified in 2010 were related to ST258 identified in 2021. Whereas ST2224 assigned in this study differed from ST68 by four locus variants indicating a very distant relationship. Most isolates in 2021 were detected as ST496 and ST551 and differed from ST121 and ST25 identified in 2010 by a single locus variant respectively.

Phylogenetic analysis

The phylogenetic analysis of the concatenated STs using MrBayes [21] showed that the major STs identified by geoBURST appear as distinct phylogenetic lineages that do not share a recent common ancestor (Figure 3.3). Therefore, major group and subgroup founders were considered CC that includes STs with single locus variants as identified by geoBURST (CC45, CC68, CC84, CC121, CC123, CC137, CC258, CC174, CC125, and CC2090). Clustering of the phylogenetic tree using the Bayesian posterior probability (BPP>0.90) for CCs with more than three STs, showed that STs belonging to CCs do not share a recent ancestor (BPP<0.90).

Table 3-1: Comparison of Sequence types between 2010 and 2021

Clonal complex (CC)	**Sequence Type (ST)**	**2010 (n=190)**	**2021 (n=185)**
CC45	ST45, ST311, ST2336	0	16
CC68	ST124, ST68, ST127, ST128, ST170, ST130	62	1
CC84	ST157, ST153, ST84, ST147, ST156, ST179, ST339	16	6
CC121	ST496, ST121, ST2308	2	11
CC123	ST71, ST123, ST169	19	3
CC137	ST64, ST2210, ST2663, S T137, ST140	11	8
CC174	ST155, ST2209, ST757, ST174	2	11
CC125	ST433, ST1049, ST11, ST125, ST126, ST1055, ST131	5	5
CC2090	ST749, ST2090, ST811, ST257, ST161, ST178, ST486	3	8
CC258	ST277, ST2217, ST383, ST258, ST869	0	5

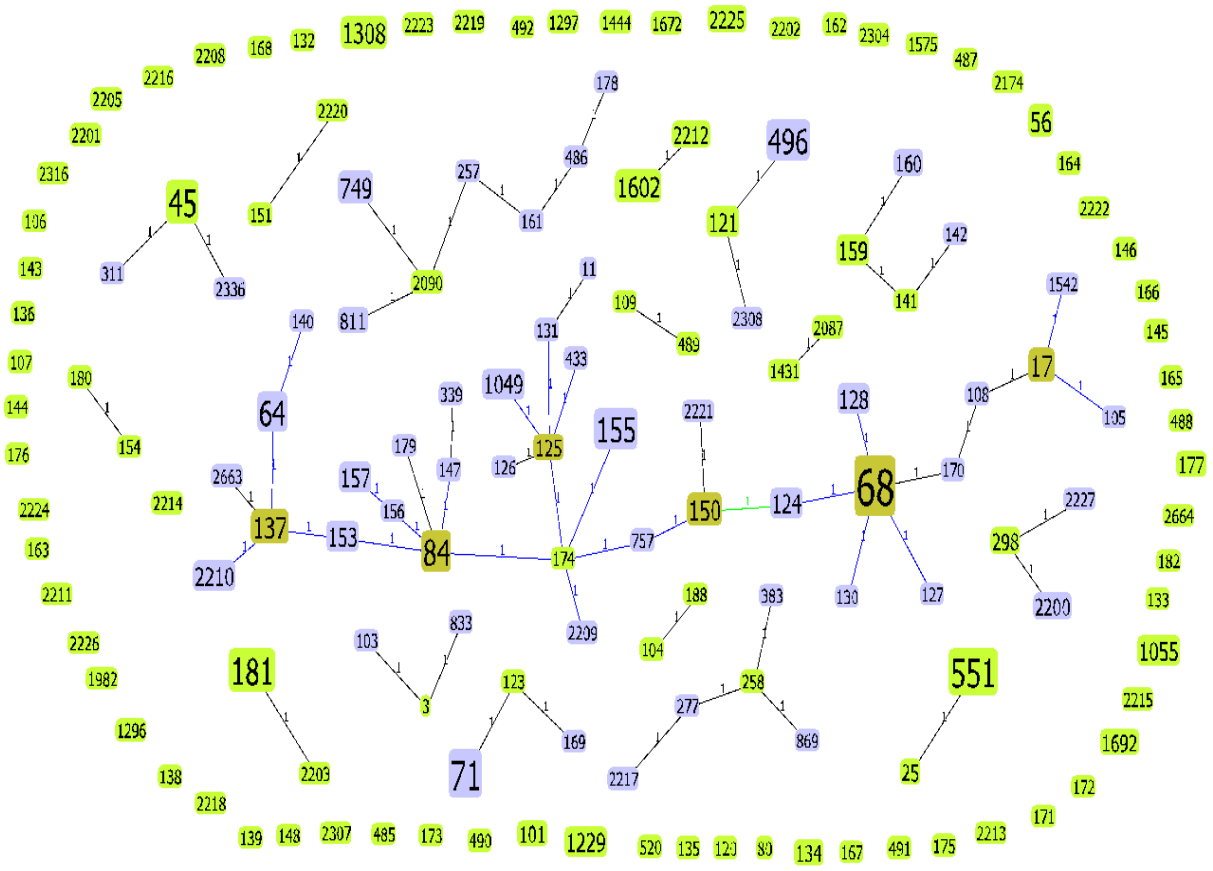


Figure 3.1: Clonal relationships of MLST sequence types in the United States (2010 and 2021). geoBURST analysis in which the branches represent single locus variants to show the relationships of the STs. Each node represents an ST. The founder STs are represented in light green while the sub founders are represented as dark green.

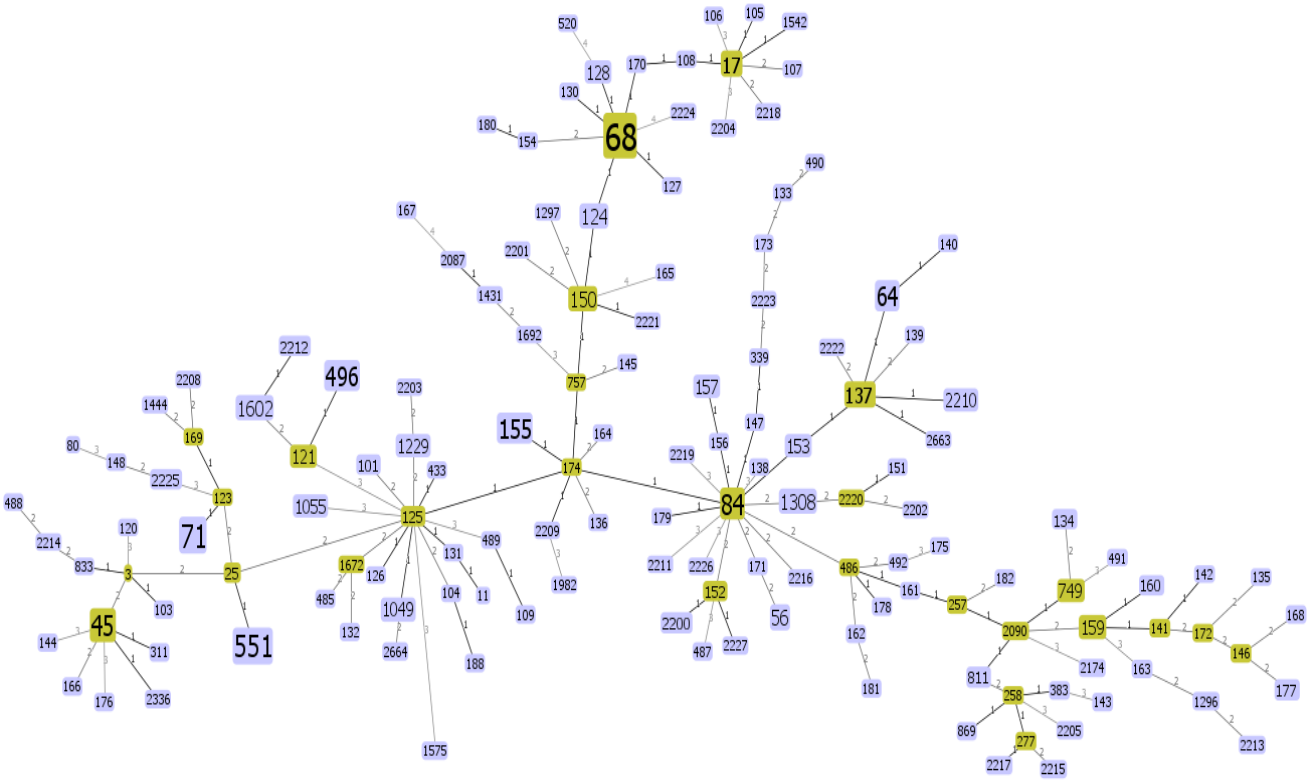


Figure 3.2: geoBURST full MST algorithm showing links in single locus variant (SLV), double locus variants (DLV), triple locus variant (TLV) and four link variants. Each node represents STs and green represents the group founder.

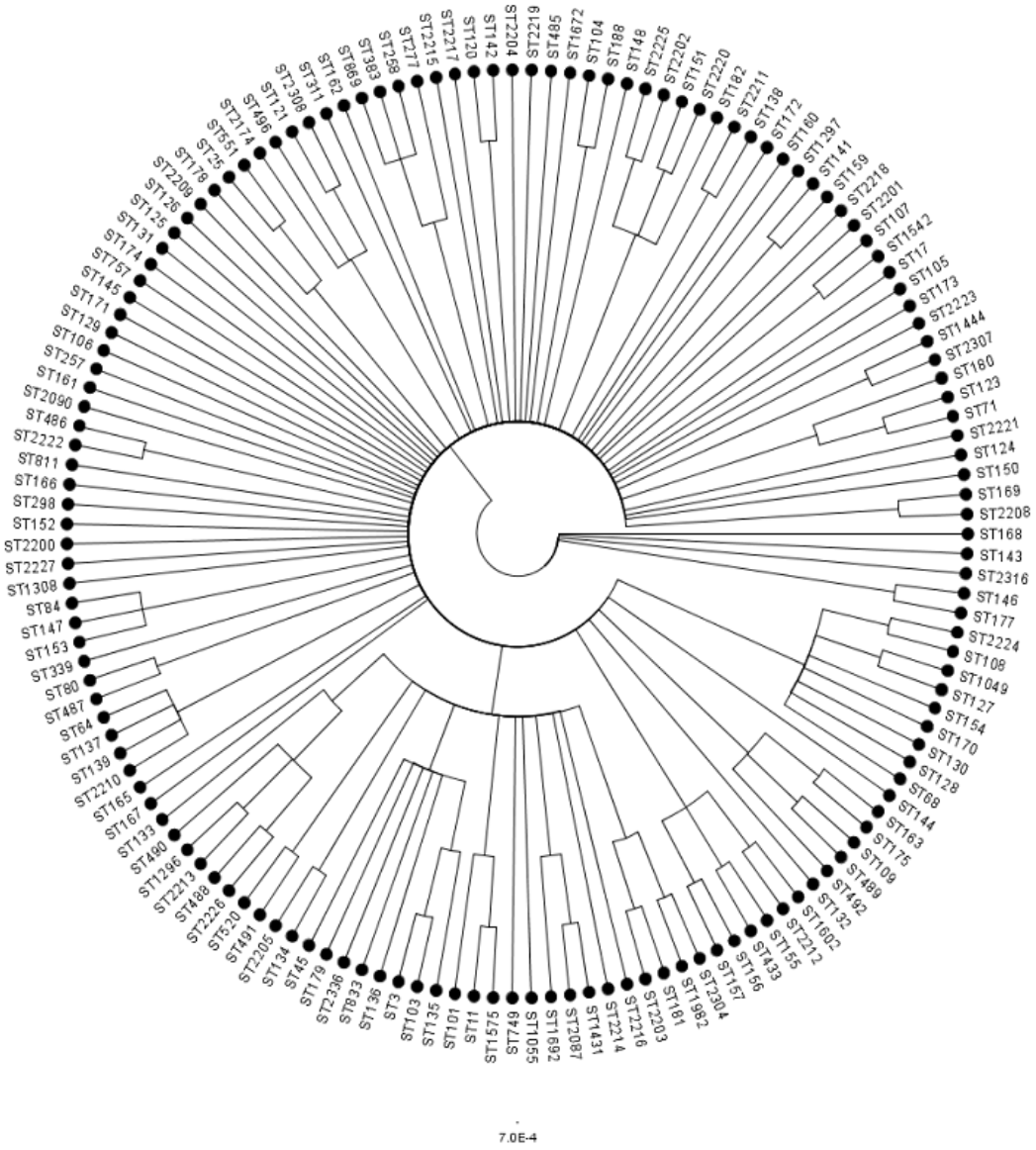


Figure 3.3: Unrooted consensus tree displaying the relationship between 147 STs of *S. pseudintermedius* in 2010 and 2021 at seven concatenated loci. There were no well-supported branches (i.e., with a Bayesian posterior probability of >0.90) for CCs with >3 STs). Each node represents an STs.

The Bayesian posterior probability for CC258, CC45, CC84, CC68, and CC123 was not clustered with high confidence 0.8838, 0.5131, 0.5622, 0.7105, 0.6414 respectively with STs belonging to the clonal complex. However, the branch for each clonal complex was well supported BPP=1. The phylogenetic clustering of ST84, ST147, and ST153 is similar to geoBURST clustering which indicated a single locus variant between the STs. For CC45, ST2336 with a single locus variant, ST3 with a double locus variant, as well as ST103 and ST833 with three locus variants were phylogenetically clustered together. Whereas CC68 was phylogenetically clustered with STs that differ in single locus variant (ST128, ST130, ST170, ST127), double locus variant (ST108), and four locus variants (ST2224). Additionally, a phylogenetic clustering of CC123 was observed with ST71 which differs in a single locus variant.

Sequence variation and recombination

To assess the general diversity of the seven MLST loci of 147 sequence types identified in 2010 and 2021, the average GC content, the number of polymorphic sites, and nucleotide diversity was calculated and shown in Table 3-2. The concatenated sequences for the seven housekeeping genes were 2944 bp in length with a diversity index of 0.004. The mean GC content of MLST gene fragments ranged from 38.0% for *ack* to 47.6% for *purA*. The nucleotide diversity index was high for *cpn60* (0.018) compared to *tuf* (0.003). The number of average polymorphic sites (9.1%) and single nucleotide polymorphism (n=39) was relatively high for cpn60. The mean genetic diversity (H): 0.6583 +/- 0.0483 was identified for all STs while *purA* represented the allele with the highest nucleotide diversity (0.00726).

The phi test is a rapid, statistically efficient test for recombination [22]. The phi test found 49 informative sites of all 147 STs, the mean value of 0.60 and p-value=0.0 indicating a significant incidence of recombination across the whole population (Table 3-3). The detection per site (rho/theta) value for all 147 STs was 27.58, suggesting that recombination occurred more frequently than mutation. The standard of associated index (IAs) value was 0.10730 ($p < 0.001$) for all 147 STs indicating a tendency towards linkage disequilibrium between alleles.

Site and Gene Wide Evidence for Positive Selection

Each housekeeping gene was first subjected to gene-wide positive selection detection. If gene-wide selection was detected with the BUSTED[S] [24] then MEME [26], a site-wise method used to detect episodic diversifying selection at a site, was employed to identify which sites explicitly were under positive selection. Gene-wide positive selection was detected in 5 of the 7 housekeeping genes: *cpn60*, *fdh*, *pta*, *purA*, and *sar*. Of those 5, signal for episodic diversifying selection at individual sites was detected in *cpn60* (site 33), *fdh* (site 40) and *pta* (sites 19 and 122)

(Table 3-4). In neither *purA,* nor *sar* was MEME able to identify any sites under episodic selection with an appropriate level of significance.

Table 3-2: Nucleotide sequence variation at each MLST Locus

Locus	**Length**	**G+C (%)**	**S/M**	**No. of polymorphic sites (%)**	**SNP**	**Nucleotide diversity**	**Genetic diversity**
ack	564	38.0	11	1.9	6	0.00530	0.7662
Cpn60	431	39.1	39	9.1	28	0.01711	0.8228
fdh	259	41.4	10	3.8	6	0.01218	0.6269
pta	492	40.8	14	2.8	12	0.00599	0.6448
purA	405	47.6	10	2.5	3	0.00726	0.7467
sar	376	41.2	5	1.3	4	0.00585	0.4824
tuf	417	39.2	2	0.5	2	0.00320	0.5185
Concatenated (147 STs)	2944	40.8	65	0.02	16	0.00424	0.6583

The length, G+C (%), S/M, number of polymorphic sites, SNP, nucleotide diversity and genetic diversity are reported. Seven housekeeping genes were tested, acetate kinase (*ack*), sodium sulfate symporter (*sar*), formate dehydrogenase (*fdh*), adenylosuccinate synthetase (*purA*), phosphate acetyltransferase (*pta*), elongation factor tu (*tuf)* and chaperonin protein (*cpn60*). As well as the concatenated ST alignment.

Table 3-3: Recombination test and linkage disequilibrium of *Staphylococcus pseudintermedius* in 2010 and 2021

Population		**Phi test**	**Recombination analysis**			**Linkage disequilibrium**	
	mean	p-value[a]	rho	theta	Rho/theta	I_A^S	p-value[b]
All(147STs)	0.6020	0.0	261.092	9.464	27.588	0.10730	<1.00 x 10-04
MRSP	0.6178	7.392E-12	420.33	10.644	39.49	0.1361	<1.00 x 10-04
MSSP	0.6469	1.789E-13	642.957	12.409	51.814	0.0284	<1.00 x 10-04

a) P-value < 0.05 is reported for the Phi test indicating presence of recombination.

b) Index of association (I_A^S) would be expected to be zero for linkage equilibrium when recombination events occur frequently.

Table 3-4: Gene wide and site-specific evidence for positive selection between the seven housekeeping genes.

GENE	BUSTED[S]	MEME sites
ack	NA	NA
cpn60	0.01178	33
fdh	0.0008427	40
pta	0.02823	19, 122
purA	5.89E-10	NA
sar	0.0445	NA
tuf	NA	NA

BUSTED[S] results reported with p-value < 0.05 and all MEME sites reported with p-value < 0.1. For BUSTED[S] the significant p-value is reported, and for MEME, when a significant p-value was identified, the specific codon site(s) under episodic diversifying positive selection in the alignment are reported. When no significant selection was detected from the test, a "NA" is reported. Seven housekeeping genes were tested, acetate kinase (*ack*), sodium sulfate symporter (*sar*), formate dehydrogenase (*fdh*), adenylosuccinate synthetase (*purA*), phosphate acetyltransferase (*pta*), elongation factor tu (*tuf)* and chaperonin protein (*cpn60*).

Antibiotic resistance of *S. pseudintermedius* in 2010 and 2021

There was no detectable difference in methicillin resistance among *S. pseudintermedius* CCs in 2010 and 2021. However, most isolates with STs belonging to CC2090 in 2010 were methicillin-susceptible while most isolates with STs belonging to CC2090 were methicillin-resistant in 2021. There was a noticeable difference between the antibiotic resistance of *S. pseudintermedius* previously reported in 2010 and the current study in 2021 (Figure 3.4). The statistical difference between antibiotic resistance of 2010 and 2021 was determined with a p-value ≤0.05. There was a significant increase observed to clindamycin (0.0021), amoxicillin-clavulanic acid (0.0492), oxacillin (0.0003), cefoxitin (0.0456), cefpodoxime (0.0001), tetracycline (0.001), chloramphenicol (<0.0001), erythromycin (0.0001) marbofloxacin (0.00004) and gentamicin (0.0001). There was no significant increase observed in cephalothin (0.6108) and sulfonamides (0.3941).

Discussion

ST68 has historically represented an endemic clone of MRSP in the United States that has spread to other countries [10, 13, 14]. In this study, we showed that most of the STs detected in 2021 are not related to ST68. In contrast, a recent study detected ST68 in New England and Texas among the dog population [27]. The phylogenetic analysis cluster of CC68 showed that STs belonging to this cluster did not arise from any recent common ancestor. Whereas geoBURST analysis showed that STs (ST128, ST124, ST130, ST1270, ST170, and ST2224) are related by single, double and four locus variants. This suggests local divergence occurring within this clonal complex. Interestingly, ST124 detected in this study has a single nucleotide difference with ST68 but was not phylogenetically clustered. This could represent the limitation of using phylogenetic analysis without geoBURST in the representation of evolutionary relationships between STs. There is a lack of research in the evolutionary origin of *S. pseudintermedius* ST68 in the United States. Therefore, elucidating the lack of detection of ST68 in 2021 compared to 2010 is challenging. However, new STs were detected in 2021 compared to 2010, perhaps ST68 has low competitive fitness relative to new STs such as ST496 and ST551 detected in 2021 [18, 20, 28, 29].

Both geoBURST and MrBayes phylogenetic analyses support that the population of *S. pseudintermedius* in 2010 and 2021 is found with numerous unrelated clones throughout the population. As many as 71 singletons were observed in 2010 and 2021 where the majority of STs were found only once. Furthermore, most of the STs in the 2010 and 2021 populations are not related, suggesting that this population of *S. pseudintermedius* in the United States is non-clonal. Despite the lack of evidence for a recent common ancestor, the clustering performed by MrBayes agreed to some extent with the results from geoBURST.

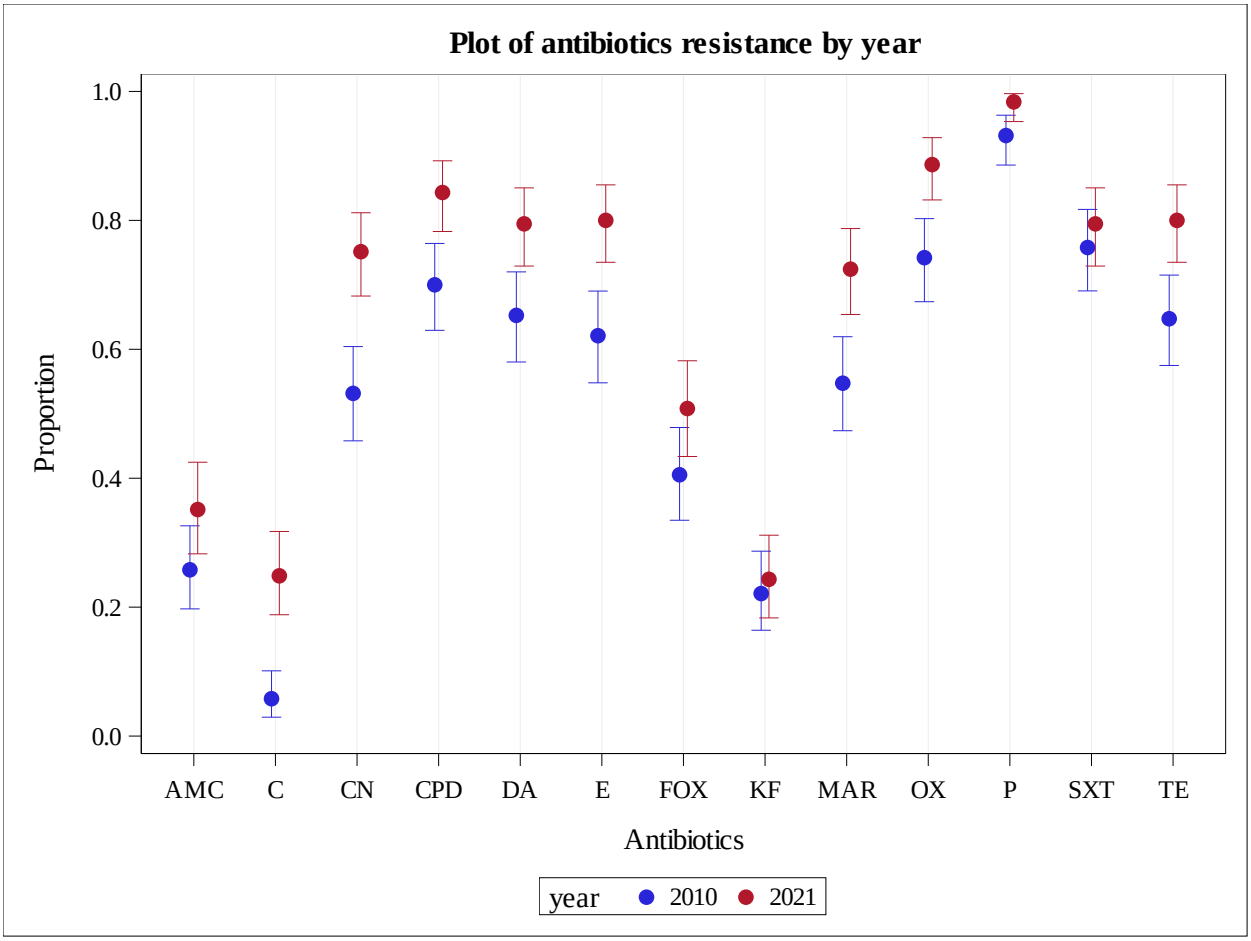


Figure 3.4: Proportion and 95% confidence intervals of each antibiotic in 2010 and 2021 were compared for amoxicillin clavulanic acid (AMC), chloramphenicol (C), gentamicin (CN), cefpodoxime (CPD), clindamycin (DA), erythromycin (E), cefoxitin (FOX), cephalothin (KF), marbofloxacin (MAR), oxacillin (OX), penicillin (P), trimethoprim-sulfamethoxazole (SXT) and tetracycline (TE).

However, geoBURST was the most suitable model for inferring evolutionary relationships between STs in 2010 and 2021 as described by Feil *et al*. [15]. Additionally, this could indicate the coexistence of distinct and diverse lineages that have emerged independently or transported from other countries into the *S. pseudintermedius* population in the United States [10]. The population genetics of *S. pseudintermedius* in 2010 and 2021 showed high levels of recombination to mutation ratios (rho/theta=27.588) suggesting that recombination has contributed to the diversification of the sequence types. In comparison, highly clonal species such as *S. aureus* show low recombination to mutation ratios with distinct clonal lineages that have diversified from phylogenetically well-supported founders [15, 30, 31]. However, recombination was not significant to break linkage disequilibrium between the housekeeping genes of *S. pseudintermedius* (IAs=0.10730) consistent with a clonal population structure. This can be supported by the lack of recombination breakpoint detection in each housekeeping gene. A systematic global epidemiological study reported high recombination rates and a weak index of association between MLST of *S. pseudintermedius* indicating a weakly clonal population [10]. Furthermore, the *S. pseudintermedius* phylogeny based on the seven-gene MLST loci previously showed an inadequately supported star-like phylogeny due to lack of variation and high rates of recombination at the loci [11].

The strains in 2010 and 2021 exhibited greater nucleotide polymorphism at housekeeping genes, possibly related to host adaptation and use of antibiotics. In genomes, housekeeping genes tend to be highly conserved and evolve relatively slowly [32]. For a cell to exist, these genes are essential for maintenance of basic cellular functions [32]. For those reasons, housekeeping genes have been used in analyses to understand the epidemiology and pathogenesis of *S. aureus* and *S. pseudintermedius* infections [10, 13, 33]. However, Andrei *et al*. suggested that the introduction of new MRSA strains had resulted in linkage of major clonal complexes (CC1, CC5, CC8, CC15 and CC97) due to a lack of discrimination using the seven loci MLST database [33]. We tested for gene-wide positive selection in the 7 housekeeping genes of *S. pseudintermedius* and found evidence for positive selection in 5 of the 7 housekeeping genes (*cpn60, fdh, pta, purA,* and *sar*). Specifically, these genes are associated with protein folding, biofilm formation, energy requirement fulfillment, purine nucleotide biosynthesis, and synthesis of cell-wall and extracellular proteins [34–37]. Within those 5 housekeeping genes we identified specific sites under positive, diversifying selection. Solyman *et al*. previously reported low numbers of nonsynonymous substitution in *fdh*, *ack*, *sar* and *purA* suggesting the genes are under purifying selection [9]. There were only 2 sequenced *S. pseudintermedius* genomes with no studies reporting on nonsynonymous /synonymous substitution ratios within *S. pseudintermedius* at the time of the study. Our findings of evidence for positive selection are important for several reasons. Firstly, using housekeeping genes for analyses based on the premise that their sequences are under high conservation

(purifying, negative selection), might be incorrect. Secondly, reporting the sites with signals of episodic, diversifying positive selection provides an opportunity to examine how alterations at these sites might impact the structure and host adaptability of these proteins. One limitation of this selection inference is that we used MLST data to identify signals, therefore using the entire length of the protein-coding sequence may alter the inference of the selection identified.

Temporal changes of *S. pseudintermedius* antimicrobial resistance could indicate a decreasing or increasing temporal trend depending on the study [5, 6, 38]. The results of this study showed a significantly increasing resistance trend to β-lactam antibiotics (amoxicillin clavulanic acid, cephalothin, oxacillin and penicillin) among *S. pseudintermedius* in 2010 and 2021. Similarly, Conner *et al* reported an increasing temporal trend to β-lactams among staphylococcus canine specimens submitted to the University of Kentucky Veterinary Diagnostic Laboratory between 1993-2009 [6]. The observed statistical increase in β-lactam resistance of *S. pseudintermedius* in the United States is not unexpected due to the significant increase in the detection of MRSP between 2010 and 2021. A statistically significant increase in chloramphenicol was observed, similar to results reported among MRSP and MSSP isolated from canine specimens submitted to a veterinary diagnostic laboratory in Tennessee between 2006 and 2017 [5]. This could be implicated to selective pressure caused by increased use of chloramphenicol for treatment of MRSP canine infections [5, 39]. An increasing trend in tetracycline resistance was observed in this study compared to a significant decreasing trend in canine staphylococcus specimens isolated in Kentucky between 1993-2009 [6]. The increasing trend in tetracycline observed in the study could be due to the increased use of doxycycline encoded by *tetM*/*tetK* genes that have been reported among MRSP strains [40]. Therefore, developing alternative therapeutics such as phage therapy and vaccines is important in successfully treating MRSP canine infections [1].

There are important limitations to consider when interpreting the data in this study. The samples in 2010 and 2021 were obtained from every region in the United States. A mix of MRSP and MSSP was obtained and included in the study. The collection of isolates is naturally biased towards MRSP. Only MRSP isolates were requested from each geographic region. For identification of *S. pseudintermedius*, MALDI-TOF MS was used for isolates in 2021 compared to polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based on *MboI* restriction site in the *pta* gene. However, these differences would not have affected the results on the population structure of *S. pseudintermedius* in the United States. Additionally, the CLSI interpretive guidelines used at each time of the study could have an influence on the difference in proportions of antimicrobial resistance reported in 2010 and 2021.

Conclusion

Staphylococcus pseudintermedius in the United States is evolving to represent a more non-clonal population structure. Sequence types in 2021 are not phylogenetically related to STs previously reported in 2010 and most likely do not share any recent common ancestor. High recombination rates to mutation ratios indicate that the STs in 2010 and 2021 have the same probability of diversifying into a large population by recombination compared to mutation. It is unclear which MRSP lineages will successfully arise as the dominant lineage in the United States given the local evolution and introduction of new STs occurring among the *S. pseudintermedius* population. The changes in the population structure and diversity of *S. pseudintermedius* isolated from dogs could imply host adaptability that may lead to different host colonization by successful STs.

Methodology

Data extraction and review approach

A database was created using excel to record each isolate's species, region, year, antibiotic susceptibility, and STs. The susceptibility data were based on the interpretation criteria used in each study. The data available in the *S. pseudintermedius* MLST database (27 April 2022) was incorporated for some analysis in the study. SAS 9.4 (SAS Institute Inc., Cary, NC, USA) statistical package was used for statistical analysis of the *S. pseudintermedius* population. The statistical significance of each antibiotic resistance was performed using a chi-square test of independence and P value < 0.05 was considered statistically different.

Population structure analysis and Phylogenetic relationships

The software geoBURST [15] on Phyloviz [41] using clustering rules for linking STs as eBURST [42] was used to infer the relatedness of STs between 2010 and 2021 [41]. Clonal complexes were defined as the predicted founder STs and linked single locus variants. The geoBURST full MST algorithm was used to cluster STs based on locus variant links to infer the evolutionary relatedness of STs at default settings [42]. Phylogenetic reconstructions were performed using Bayesian Inference (BI) by seven-gene MLST alignments obtained in the *S. pseudintermedius* PubMLST database [9]. The phylogenetic tree was inferred using 147 concatenated MLST sequences of isolates in 2010 and 2021 by MrBayes v3.1.2 [21]. The Markov Chain Monte Carlo (MCMC) of two runs with 5,000,000 generations using an GTR+Γ Nucleotide substitution evolution model and a sampling frequency of 100 was used. Subsequently, 50% of the runs were discarded as burn-in. The parameter convergence of the Bayesian phylogenetic inferences was analyzed using the potential scale reduction factor (PSRF) meaning the PSRF parameters approaching 1.0 indicate convergence between run 1 and run 2. Finally, the average deviation of split frequency should be between 0.01 and 0.05 as the two runs converge onto a stationary

distribution. The Bayesian posterior probability of a given cluster (i.e., the proportion of trees supporting a given branch) was used to evaluate the confidence level in the assigned cluster; the clusters with confidence level > 0.90 were considered well supported.

Sequence diversity analyses and recombination analyses

The G+C content, variable sites or mutations, number of polymorphic sites, average pairwise nucleotide, number of singletons (SNPs) and nucleotide diversity were calculated using DnaSP version 6.12.03 [43]. The pairwise homoplasy index (phi) test implemented in SplitTree4 for recombination was performed, and a p-value of <0.05 indicated that recombination existed [44]. The LDhat program implemented in the Recombination Detection Program (RDP) v.4.97 was used to calculate the per-site rho/theta based on the concatenated sequences of seven loci [45]. The MCMC was set at 5000000 iterations, rho starting at 30 and MCMC burn-in of 500000. The parameters rho and theta represent the rates of recombination and mutation respectively. Linkage disequilibrium from allelic data was evaluated by calculating the standardized index of association (IAs) using LIAN v3.735 [46] in a web interface http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query. The null hypothesis of complete linkage disequilibrium (IAs>0; the presence of linkage disequilibrium or clonality) was tested by using the MCMC iterations with 10000 iterations on the allelic profile. If there was linkage equilibrium based on frequent recombination, the expected value of IAs is zero suggesting no association between alleles at different loci, if IAs are statistically significant from zero ($p < 0.05$), alleles suggest genetic linkage with rare recombination.

Gene Wide and Site Wise Selection

A precursor to performing selection analyses is to identify genetic recombination. Selection analyses can be confounded by recombination signals, where multiple tree topologies can represent a single gene. To identify alignment-wide recombination breakpoints within the housekeeping genes codon-aware alignments the Genetic Algorithm for Recombination Detection (GARD) method [23] was applied. GARD is based on the Akaike Information Criterion with a correction for small sample sizes (AIC-c). If there is no improvement in AIC-c in the model that considers a recombination breakpoint relative to the AIC-c of the model that does not consider recombination, then there is no evidence for recombination. Phylogenetic trees were reconstructed for each gene for the entire alignment using RAxML [47] under the GTR + Γ nucleotide substitution model. The codon-aware alignment concomitant with the reconstructed tree would serve as input for the selection analysis downstream. We performed gene-wide and site-specific selection tests based on the dN/dS (nonsynonymous/synonymous) rate ratio estimation implemented in the HyPhy software package v.2.5.32 [48] to test for evidence of positive selection acting on MLST data from housekeeping genes in *Staphylococcus*

pseudintermedius. We used the BUSTED[S] [24] and BUSTED[S]-MH methods [25] to identify evidence of gene-wide positive selection and the MEME method [26] to detect episodic diversifying selection pressure at individual sites.

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CHAPTER IV

The molecular epidemiology and antimicrobial resistance of *Staphylococcus pseudintermedius* canine clinical isolates submitted to a veterinary diagnostic laboratory in South Africa.

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Abstract

Staphylococcus pseudintermedius is an important cause of clinical infections in small-animal-veterinary medicine. Evolutionary changes and the introduction of new strains have been observed among *S. pseudintermedius* strains in some European countries and the United States, however, there are limited or no studies on antimicrobial resistance, genetic diversity, virulence factors, and clonal complexes of methicillin-resistant *S. pseudintermedius* (MRSP) in South Africa. South Africa differs from other countries geographically and in practices related to dispensing antimicrobials. Therefore, this study aimed to determine the molecular epidemiology of *S. pseudintermedius* in South Africa. Non-duplicate, clinical isolates from dogs were obtained as convenience samples from four provinces in South Africa. The disk diffusion oxacillin test was used to determine methicillin resistance and antimicrobial susceptibility. We used Resfinder and the CARD database to detect antimicrobial resistance genes. Virulence genes were identified using ARIBA on the virulence factor database and BLASTN on Geneious prime. The geoBURST was used to study relationships between multilocus sequence types and to identify clonal relationships. Finally, the maximum likelihood phylogeny was determined using randomized accelerated maximum likelihood (RAxML). Twenty-three isolates were confirmed as *S. pseudintermedius* of which 14 were MRSP. In addition to β-lactam antimicrobials, MRSP isolates were resistant to tetracycline (85.7%), doxycycline (92.8%), kanamycin (92.8%), and gentamicin (85.7%). The isolates harbored antimicrobial resistance genes (*tetM, ermB, drfG, cat, aac (6')-Ie-aph (2")-Ia, ant6-Ia,* and *aph3-III*) and virulence genes (*AdsA, geh, icaA*, and *lip)*. Eight new multilocus sequence type (STs) were discovered in this study (ST2228, ST2229, ST2230, ST2231, ST2232, ST2318, ST2326 and ST2327) and previously reported multilocus sequence types including ST45, ST71, ST181, ST551 and ST496 were also detected. SCC*mec* type IVg was identified among ST301, ST261 and ST496. The geoBURST and phylogenetic analysis suggests that the isolates in South Africa are likely genetically related to isolates identified in other countries. This is the first study

reporting on the detection of MRSP in South Africa. Highly resistant strains (ST496, ST71, and ST45) were reported that could present challenges in the treatment of canine infections in South Africa. Hence, molecular epidemiological studies could help inform on antibiotic treatment options to target the predominant MRSP strains in South Africa.

Introduction

In dogs, *Staphylococcus pseudintermedius* is a commensal bacterium that affects the skin and mucosa and can transiently colonize human beings [1–4]. There is an increasing prevalence of MRSP globally and they have become a threat to the successful treatment of infections in small-animal-veterinary-medicine [1, 5–7]. Methicillin resistance in *S. pseudintermedius* is attributed primarily to the *mecA* gene harbored within a mobile genetic element called SCC*mec* which encodes penicillin-binding protein 2a (PBP2a) [8–10]. The PBP2a allows for cell wall biosynthesis in the presence of most β-lactams antibiotics, thereby inducing resistance [11, 12]. *Staphylococcus pseudintermedius* is the most common staphylococcal species isolated from dogs in South Africa [13]. Yet, the extent of methicillin resistance occurrence in *S. pseudintermedius* isolated from dogs has not been investigated in this country.

Multi-locus sequence typing (MLST) is a well-established method used to identify dominant MRSP lineages and geographical patterns of the clonal spread of *S. pseudintermedius* worldwide. As a result, CC71/ CC258 in Europe, CC68 in the United States, and CC45/CC112 in Asia have been established as dominant clonal complexes in the past [5]. Africa has not been described in terms of prevalent MRSP lineages. In 2012, Jung-Ho Youn *et al.* reported ST39, ST200, ST54, ST204, ST18 among *S. pseudintermedius* isolates from a veterinary hospital in Zambia [14]. In Botswana, ST885 to ST890 were obtained from nasal swab samples collected from healthy dogs in 2018 [15]. In South Africa, MLST profiles of *S. pseudintermedius* isolated from dogs are lacking.

There are significant regional differences in patterns of antimicrobial resistance and virulence factors associated with distinct clonal lineages. For example, a low percentage of tetracycline and chloramphenicol resistance was reported from isolates belonging to the CC71 lineage in North America compared to isolates belonging to the CC71 lineage in Europe [1, 16]. In France, ST258 MRSP isolates present a higher susceptibility to gentamicin, sulfonamides and fluoroquinolones compared to isolates belonging to ST71 [16]. In Australia, ST496 isolates harbor SCC*mec* type Vt whereas ST71 in Europe is associated with SCC*mec* type III [5, 17]. Additionally, ST749 in canine Australian isolates carries the *nanB* gene for sialidase, while ST496 in France carries the genes for surface adhesion virulent *spsI* and *spsF* [16, 17]. In South Africa, Qekwana *et al.* reported multidrug resistance and increasing levels of resistance to fluoroquinolones and sulfonamides among *S. pseudintermedius* isolated from dogs at a veterinary teaching hospital [18]. In Zambia,

Jung-Ho Youn *et al.* reported high penicillin and tetracycline resistance among *S. pseudintermedius* isolates implicating *tetM* and *blaZ* genes [14]. There is limited data on the prevalence of antimicrobial resistance and virulence factors of MRSP isolated from dogs in South Africa.

Recent studies in various countries show a changing population structure among *S. pseudintermedius* because of the acquisition of SCC*mec* and the international movement of animals [5, 16, 19]. A dearth of information prevents us from understanding how the population structure of *S. pseudintermedius* has changed over the years in the African continent. Whether MRSP STs are arising from horizontal gene transfer from methicillin-susceptible bacteria in South Africa or evolving from MRSP introduced from other countries is an important question. The aims of this study were to determine antimicrobial susceptibility of MRSP, to identify SCC*mec* elements, resistant genes, and virulence genes, as well as to determine MLST and compare STs in South Africa with STs reported globally.

Results

Isolate Collection

Based on MALDI-TOF MS, twenty-three isolates were confirmed as *S. pseudintermedius* and distributed geographically as shown in Table 4-1. The isolates represent four provinces in South Africa (Gauteng, Kwazulu-Natal, Western Cape, and Eastern Cape) that were submitted for diagnostics at the Vet diagnostic laboratory. Nine of the isolates were methicillin susceptible (MSSP) and 14 of the isolates were MRSP. Seventeen different STs were identified in the isolates from South Africa. Eight *S. pseudintermedius* alleles were unique and were assigned multilocus sequence types (STs) in this study (ST2232, ST2229, ST2228, ST2231, ST2230, ST2318, ST2326, ST2327). Five of the MRSP isolates belonged to ST496, ST1308, ST45, ST181, and ST1111. Whereas seven of the MSSP isolates were previously reported on the pubMLST database (ST301, ST551, ST261, ST121, ST496, ST71, ST181, and ST1431). SCC*mec* types were not detected in most of the isolates, regardless of the presence of the *mecA* gene. SCC*mec* type Ivg(2B) was detected in ST301, ST261 and ST496 isolates and SCC*mec* type V was identified in ST496 and ST1431.

Antibiotic resistance

All isolates were resistant to at least one antibiotic whereas 87% of the isolates were multidrug resistant (MDR) (i.e., resistance to 3 or more classes of antimicrobials).

Table 4-1: Characteristics of 23 *Staphylococcus pseudintermedius* isolated from canine clinical samples submitted to a veterinary diagnostic laboratory in South Africa, 2021.

Isolate	**Age of Dog (Year)**	**Collection Site**	**Location of Collection (City)**	**Methicillin Resistance**	**Sequence types**	**SCC*mec* type**
20VMG1528	5y	Post-op	Johannesburg	S	301	type Ivg(2B)
20VMG1536	6y	Skin	Stella	S	551	-
20VMG1580	7y	Ear	Randburg	S	261	type Ivg(2B)
21VMG0143	2y	Skin	Randburg	R	496	-
21VMG0271	7y	Skin	Randburg	R	1308	-
21VMG0402	6y	Hock-hygroma	Johannesburg	R	2232	-
21VMG0561	8y	Skin	Port Elizabeth	S	2230	-
21VMG0581	5y	Wound	Johannesburg	R	2229	-
21-VMG0567	5y	Post-op	Johannesburg	S	121	-
21-VMG0568	8y	Skin ulcers	Johannesburg	S	496	type V(5C2)
21VMK0081	3y	Mastitis	Pietermaritzburg	S	71	-
21VMK0268	8y	Toe	Umvoti	R	261	-
21VMK0323	4 months	Pyoderma	Durban	R	2228	-
0445	N/A	N/A	N/A	R	2326	-
21VMK0532	8y	Pyoderma	Salt Rock	R	45	-
21VMG0811	2y	Post-op	Johannesburg	R	2231	-
0536	N/A	N/A	N/A	S	181	-
21-2683	N/A	Lip fold	Cape Town	S	1431	type V(5C2&5)
21-3244	5y	Wound	Cape Town	R	496	type Ivg(2B)
21-3512	3y	Pyoderma	Cape Town	R	1111	-
21-5383	2y	Post-op	Cape Town	R	496	-
21-3929	2y	Cystitis	Cape Town	R	181	-

Table 4.1. continued

Isolate	**Age of Dog (Year)**	**Collection Site**	**Location of Collection (City)**	**Methicillin Resistance**	**Sequence types**	**SCC*mec* type**
21-5495	2y	Nail Bed infection	Cape Town	R	2228	-

Table 4-2: Antimicrobial resistance patterns of *Staphylococcus pseudintermedius* isolated from canine clinical samples submitted to a veterinary diagnostic laboratory in South Africa, 2021.

		***S. pseudintermedius*(n=23)**			**MRSP(n=14)**			**MSSP (n=9)**		
		Percent (%)	**95%CI[a]**		**Percent (%)**	**95% CI[a]**		**Percent (%)**	**95% CI[a]**	
Group	**Antibiotic**		**Lower**	**Upper**		**Lower**	**upper**		**Lower**	**Upper**
Lincosamides	Clindamycin	39.1	40.9	80.8	57.1	16.9	68.7	11.11	68.4	100
Penicillins	Penicillin	95.6	87.1	100	100	100	100	88.89	68.4	100
	Amoxicillin-clavulanate acid	21.7	61.4	95.2	35.7	39.2	89.3	0	100	100
	oxacillin	60.8	19.2	59.1	100	100	100	0	100	100
Cephalosporins	cefoxitin	17.4	67.1	98.1	28.5	47.7	95.1	0	100	100
	Cefpodoxime	60.8	19.1	59.1	100	100	100	0	100	100
	Cephalothin	17.4	67.1	98.1	28.5	47.7	95.1	0	100	100
Tetracyclines	Tetracycline	69.5	11.6	49.2	85.7	67.3	100	44.4	23.1	88
	Minocycline	43.4	36.2	76.7	57.1	83.1	31.2	22.2	50.6	100
	Doxycycline	69.5	11.6	49.2	92.8	79.3	100	33.3	35.8	97.5
Phenicols	Chloramphenicol	17.4	67.1	98.1	21.4	57.1	100	11.1	68.4	100
Glycopeptides	Vancomycin	0	100	100	0	100	100	0	100	100
Aminoglycosides	Streptomycin	91.3	79.7	100	85.7	67.3	100	100	100	100
	Gentamicin	56.5	23.2	63.7	85.7	67.3	100	11.1	68.4	100
	Kanamycin	78.2	61.4	95.1	92.8	79.3	100	55.6	23.1	88

Table 4.2. continued

		S. pseudintermedius(n=23)			MRSP(n=14)			MSSP (n=9)		
		Percent (%)	**95%CI[a]**		**Percent (%)**	**95% CI[a]**		**Percent (%)**	**95% CI[a]**	
Group	**Antibiotic**		**Lower**	**Upper**		**Lower**	**upper**		**Lower**	**Upper**
Macrolides	Erythromycin	39.1	40.9	80.8	57.1	16.9	68.7	11.1	68.4	100
Fluoroquinolones	Marbofloxacin	34.7	45.7	84.6	57.1	16.9	68.7	0	100	100
Rifamycin	Rifampin	0	100	100	0	100	100	0	100	100
Phosphonic	Fosfomycin	17.4	67.1	98.1	28.5	47.7	95.1	0	100	100
Oxazolidinones	Linezolid	0	100	100	0	100	100	0	100	100
Folate inhibitor	Trimethoprim-sulfamethoxaole	73.9	8.14	44	85.7	67.3	100	55.6	11.9	76.9

[a]95% CI = 95% confidence interval

Most of the isolates were resistant to penicillin (95.6%), streptomycin (91.3%), kanamycin (78.2%), trimethoprim-sulfamethoxazole (73.9%), and cefpodoxime (60.8%) (Table 4-2). The level of tetracycline and doxycycline resistance among the isolates was similar (69.5%) compared to 43.3% of minocycline. Low resistance was observed to cephalothin (17.4%), chloramphenicol (17.4%), fosfomycin (17.4%) and cefoxitin (17.4%). None of the isolates were resistant to vancomycin, linezolid, or rifampin. All MRSP isolates were resistant to penicillin and cefpodoxime. MRSP isolates exhibited high proportions of resistance to tetracycline (85.7%), doxycycline (92.8%), kanamycin (92.8%), streptomycin (85.7%) and trimethoprim/sulfamethoxazole (85.7%) compared to high susceptibility (82.7%) observed to fosfomycin, cephalothin, cefoxitin and chloramphenicol. In comparison, MSSP isolates showed high susceptibility to minocycline (77.8%), doxycycline (66.7%), tetracycline (55.6%), streptomycin (100%) and penicillin (88.8%).

Antibiotic resistant genes

The *blaZ* gene which encodes for narrow spectrum beta-lactamases was detected in all but one isolate (Table 4-3). Sixteen of the *S. pseudintermedius* isolates harbored *tetM* resistance genes whereas one isolate harbored *tetK*. The *dfrG* gene conferring trimethoprim-sulfamethoxazole resistance phenotype was detected in 14 of the isolates in the study. Most (11/23) of the isolates in this study harbored *erm(B)* genes. Chloramphenicol acetyltransferase genes (*catpC221*) were detected in six isolates. Isolates harbored *aac (6')-Ie-aph (2")-Ia* (14/23), *ant6-Ia* (9/23) and *aph3-III* (9/23) that confer resistance to acetyltransferase, nucleotidyl transferase and phosphotransferase, respectively.

Among isolates that harbored the *blaZ* genes (n=22), only 4 isolates were resistant to penicillin's and 3 isolates were resistant to cephalosporins (Table 4-4). Additionally, among isolates that harbor the *mecA* gene (n=14), only 8 isolates expressed oxacillin resistance. Tetracycline resistance is attributed to the presence of *tetM* gene, among isolates that harbor the *tetM* gene (n=16), only 6 isolates were resistant to all tetracyclines (tetracycline, minocycline, and doxycycline). Among isolates harboring the bifunctional enzyme *aac (6')-Ie-aph (2")-Ia* (n=14), 7 isolates were phenotypically resistant to aminoglycosides. The *erm(B)* gene was detected among 11 isolates, but only 3 of these isolates were erythromycin resistant. Whereas 11 isolates harbored *dfrG* and were resistant to trimethoprim-sulfamethoxazole. Additionally, none of the isolates that harbored the *catpC221* were resistant to chloramphenicol.

Virulence genes

All the isolates were positive for immune evasion (*adsA*), exfoliative toxin (*speta*) and intracellular adhesion gene (*icaA and icaD*) (Figure 4.1). Whereas the *nanB* gene was detected in 9 isolates.

Table 4-3: Antimicrobial resistance genes of *Staphylococcus pseudintermedius* isolated from canine clinical samples submitted to a veterinary diagnostic laboratory in South Africa, 2021.

Isolate	[a]**MDR-1**	***aac (6′)-Ie-aph (2″)-Ia***	***ant6-Ia***	***aph3-III***	***blaZ***	***catpC221***	***dfrG***	***ermB***	***mecA***	***tetK***	***tetM***
20VMG1528											
20VMG1536											
20VMG1580											
21VMG0143											
21VMG0271											
21VMG0402											
21VMG0561											
21VMG0581											
21VMG0567											
21VMG0568											
21VMK0081											
21VMK0268											
21VMK0323											
0445											
21VMK0532											
21VMG0811											
0536											
21-2683											
21-3244											
21-3512											
21-5383											
21-3929											

Table 4.3. continued

Isolate	[a]**MDR-1**	***aac (6′)-Ie-aph (2″)-Ia***	***ant6-Ia***	***aph3-III***	***blaZ***	***catpC221***	***dfrG***	***ermB***	***mecA***	***tetK***	***tetM***
21-5495											

[a]MDR-1= multi-drug resistance (resistance to 3 or more antibiotic class)

Table 4-4: Resistance genes and antimicrobial resistance phenotypes of *Staphylococcus pseudintermedius* isolated from canine clinical samples submitted to a veterinary diagnostic laboratory in South Africa, 2021.

Isolates	**Resistance genes**	**Antimicrobial Resistant Phenotype**
4	*blaZ,*	Penicillin, amoxicillin-clavulanic acid, oxacillin
3	*blaZ*	Cefpodoxime, cefoxitin, and cephalothin
8	*mecA*	Oxacillin
6	*tetM*	Tetracycline, minocycline, doxycycline
7	*aac (6')-Ie-aph (2")-Ia*	Streptomycin, kanamycin, gentamicin
11	*dfrG*	Trimethoprim-sulfamethoxazole
3	*ermB*	Erythromycin

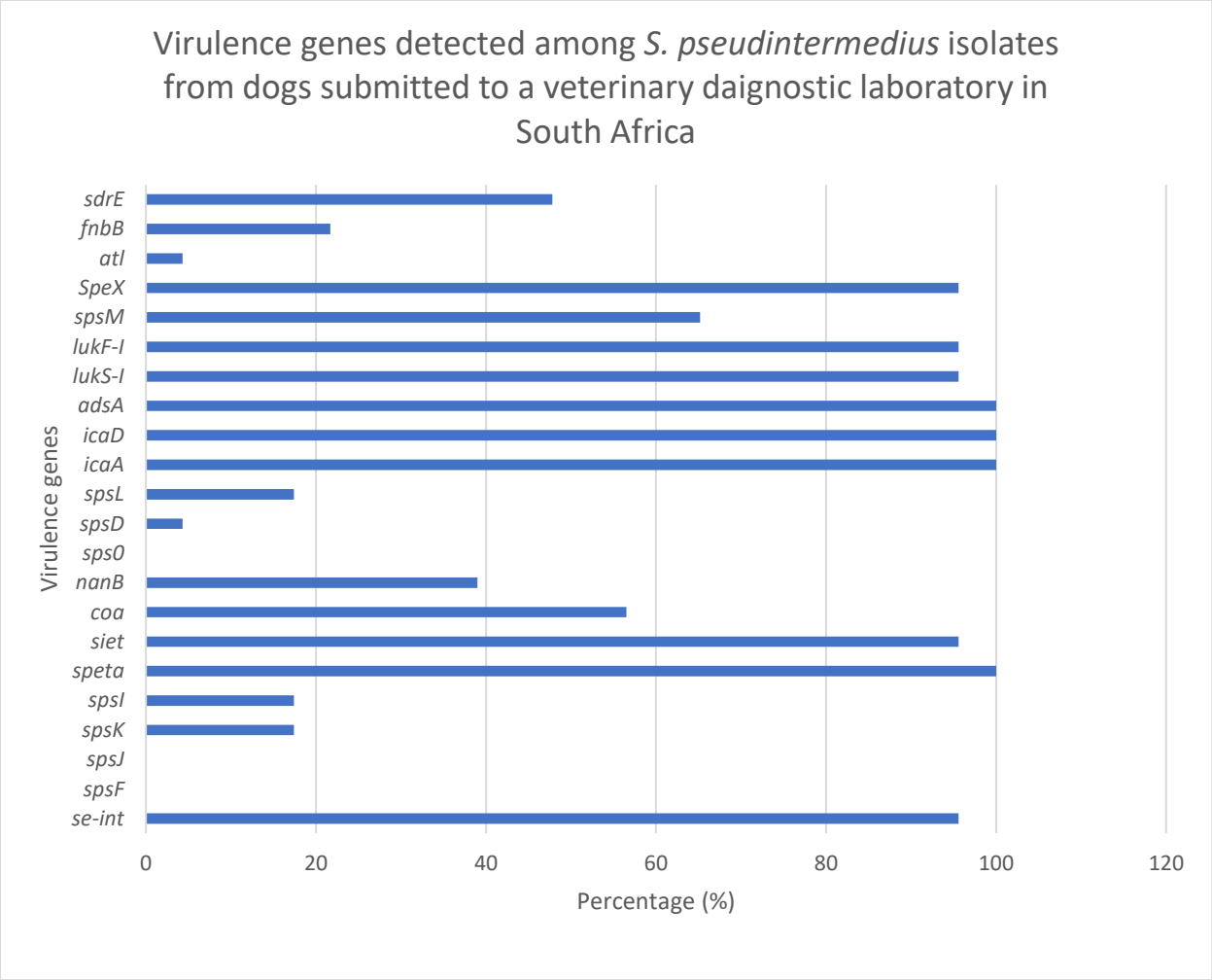


Figure 4.1*:* Virulence genes detected among *Staphylococcus pseudintermedius* isolates submitted to a veterinary diagnostic laboratory in South Africa, 2021. *Staphylococcus pseudintermedius* cell wall anchored proteins genes (*spsM, spsL, spsD, spsO, spsI, spsK, spsF*), fibrinogen binding protein gene (*fnbB*), Intercellular adhesion (*icaA, icaB*), leukotoxin (*lukF-I, lukS-I*), ser-asp rich fibrinogen binding protein (*sdrE,*), immune evasion (*adsA*), exfoliative toxin (*se-int, SpeX, speta, siet*) and exoenzyme (*coagulase, nanB*).

The *seh* and *seg* canine enterotoxin gene was only identified in MRSP isolates while the *atl* and *spsD* were identified among MSSP isolates. Isolates belonging to ST2228 and ST181 harbored the *spsI* gene compared to ST496. The ST71 isolate in this study did not harbor the *lukS-I* and *lukF-I* virulent genes compared to other isolates in the study. Compared to ST551, ST496 harbored *sdrE, nanB,* and *spsM* virulent genes.

GeoBURST analysis

All entries available in the pubMLST database in June 2022 were grouped using the goeBURST method to assess the clonal relationship of STs observed in South Africa. No clonal complex could be identified from these STs because none of the CCs contained more than 3 STs with a single locus variant (SLV=1). The isolates representing the assigned STs (ST2232, ST2231, ST2228, ST2229, ST2230) were singletons and were not related to the STs in the database These STs were part of a branch located very far from the other isolates indicating a distant evolutionary relationship. To further investigate clonal relationships, the geoBURST full MST algorithm was used for STs identified in Kenya, Botswana, and South Africa with major STs identified in the United States (ST68), Europe (ST71 and ST258), and Asia (ST45). Four links were observed at level 1, 3 links at level 2, 19 links at level 3, and 6 links at level 4 (Figure 4.2). None of the links connected STs in this study with those identified in Botswana and Kenya. Whereas STs assigned were linked to ST71, ST68, ST496, ST551, and ST181. For example, ST2326 assigned in this study had a single locus variant to ST71 while ST2318 had a double locus variant to ST496. None of the isolates assigned in this study were closely related to ST45 and ST258.

Genomics and phylogenetic analyses

Analysis of the *S. pseudintermedius* genomes in PATRIC showed an average genome size of 2571404.8bp (range 2180042bp to 2842167bp) with an average of 2657 genes annotated (range 2378 to 2991) (

Table 4-5). A phylogenetic tree that included 15 *S. pseudintermedius* previously sequenced genomes and the 23 strains in this study was generated (Figure 4.3). The phylogenetic analysis showed that the clustering of some isolates identified in this study was not monophyletic, with 21VMG0402 representing ST2232 assigned in this study separated from the other isolates.

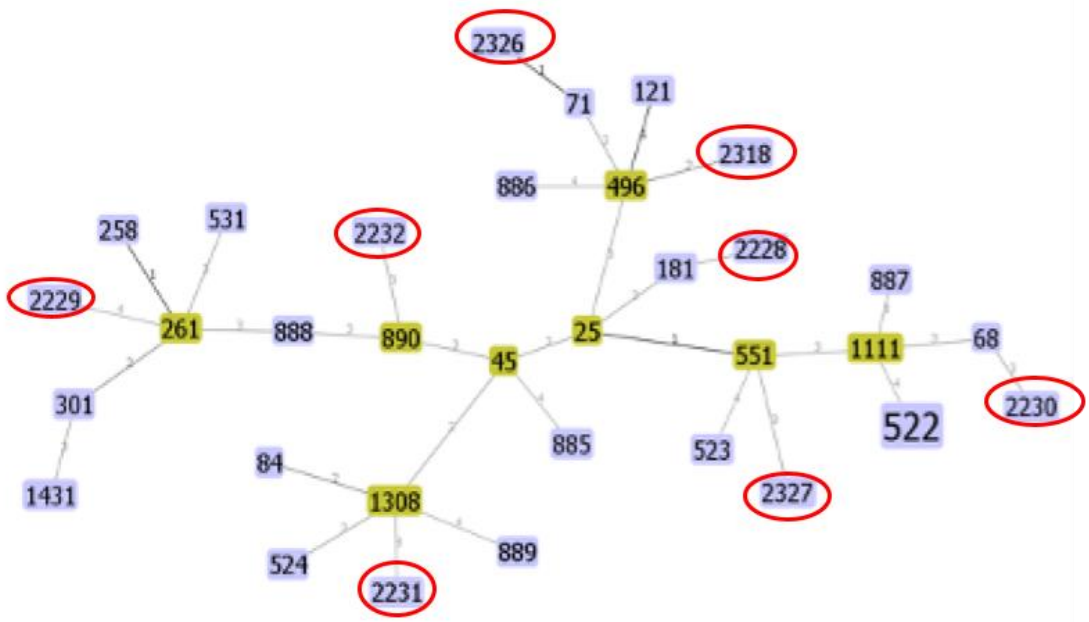


Figure 4.2*:* Clonal relationship of MLST using isolates identified in South Africa and major STs identified worldwide. The geoBURST full MST algorithm showed links connected by SLV, DLV, TLV and 4 link variants. Each node represents a ST. The size of the node denotes the sample size of each ST. The distance between each node is represented by 1=SLV, 2=DLV, 3=TLV and 4=four locus variants. The predicted founder clonal complexes are represented as green. STs identified In South Africa are circled in red.

Table 4-5: Genomic characterization of *Staphylococcus pseudintermedius* isolated from canine clinical samples in South Africa, 2021.

Isolate	Genome ID	Contigs	Genome length (bp)	GC % content	protein encoding genes	CDS
21VMK0081	283734.2901	537	2566833	37.3	1949	2991
21VMG0143	283734.2902	513	2536780	37.3	2166	2869
21VMK0268	283734.2903	74	2433078	37.5	1611	2378
21VMG0271	283734.2904	118	2652971	37.3	1764	2636
21VMK0323	283734.2905	109	2842167	37	1799	2853
21VMG0402	283734.2906	115	2457080	37.5	1611	2397
0445	283734.2907	144	2596460	37.3	1944	2624
21VMK0532	283734.2908	95	2671983	37.3	1993	2627
21VMG0536	283734.2909	126	2664368	37.3	2026	2676
21VMG0561	283734.2910	21	2603918	37.4	1908	2504
21VMG0567	283734.2911	116	2687989	37.3	2053	2676
21VMG0568	283734.2912	311	2601928	37.3	2111	2785
21VMG0581	283734.2913	98	2522001	37.5	1890	2462
21VMG0811	283734.2914	84	2685478	37.3	2034	2672
20VMG1528	283734.2915	142	2623199	37.4	2024	2663
20VMG1536	283734.2916	289	2782246	37.1	2141	2877
20VMG1580	283734.2917	275	2524757	37.4	2040	2668
21-2683	283734.2918	253	2658551	37.3	2141	2807
21-3244	283734.2919	72	2550718	37.4	1933	2485
21-3512	283734.292	128	2637389	37.3	1996	2627
21-3929	283734.2921	27	2524647	37.5	1871	2431
21-5383	283734.2923	754	2180042	37.5	2098	2767
21-5495	283734.2922	102	2686798	37.2	1997	2643

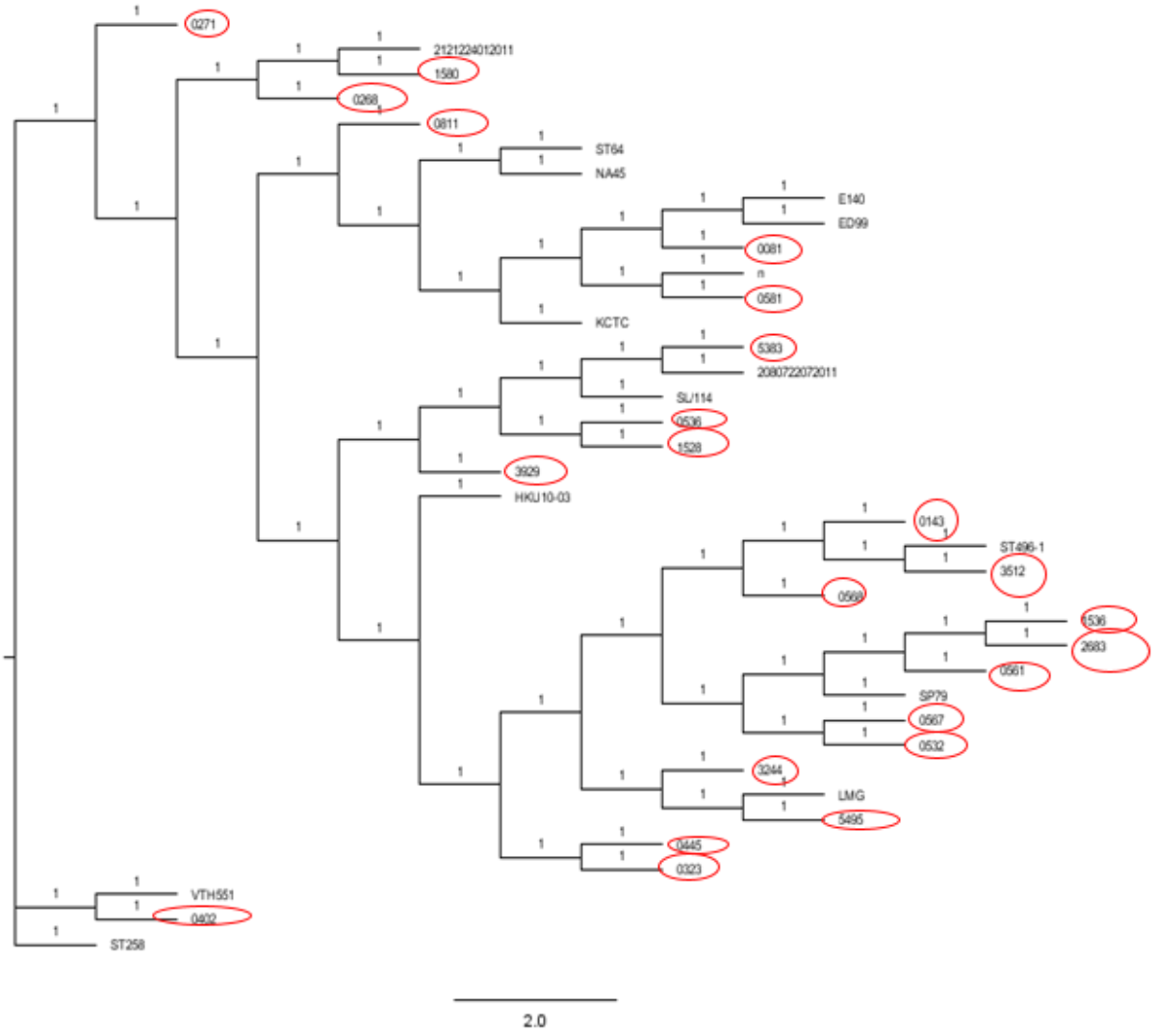


Figure 4.3: Phylogenetic tree based on the time-reversible nucleotide substitution model and four gamma categories for rate heterogeneity of *S. pseudintermedius* isolates. The phylogenetic tree includes isolates of the present study (circled in red) and selection of previously sequenced genomes representing phylogenetic diversity found across the species.

Discussion

Methicillin-resistant *S. pseudintermedius* is the predominant cause of clinical pyoderma in dogs. The prognosis of infection is dependent on the antimicrobial resistance profile and virulence factors which are associated with the multilocus sequence type [10, 20–22]. In this study, we investigated the molecular epidemiology of *S. pseudintermedius* isolated from clinical dog samples submitted to a veterinary diagnostic laboratory in South Africa.

All isolates in this study were resistant to at least one antimicrobial, 87% were MDR, whereas 60.8% were MRSP. The occurrence of MRSP reported in this study is higher than the 14% reported among clinical isolates of dogs in Finland [23]. In comparison, Videla *et al*. reported a higher prevalence of MRSP (74%) among clinical isolates in the USA [1]. A comparable difference in the population size is likely to explain the variation of MRSP in each study. In Finland, MRSP was determined among 1958 clinical *S. pseudintermedius* isolates collected from private clinics and veterinary teaching hospitals [23]. Whereas in the USA, Videla *et al.* reported MRSP among dog samples submitted from 10 geographic regions in the USA [1]. Therefore, due to the sample size in this study, the proportion of MRSP and MSSP is not a representation of the distribution of *S. pseudintermedius* in the South African dog population. However, the high proportion of MRSP detected in this study suggests that the detection of MRSP in South Africa is concomitant with the global MRSP trends.

Resistance to antimicrobials was higher among MRSP compared to MSSP isolates. In addition to β-lactam resistance, MRSP isolates in this study were resistant to tetracyclines, aminoglycosides, folate inhibitors, macrolides, and lincosamides. This is similar to results reported among MRSP isolates in Argentina and Finland [7, 24]. In a previous study, Qekwana *et al*. reported a significant increase in the proportions of resistant isolates to trimethoprim-sulfamethoxazole, clindamycin, and orbifloxacin between 2007 and 2012 in South Africa. Canine antibiotic resistance patterns in South Africa may be influenced by veterinary prescription practices. Therefore, more studies illustrating the implications of antibiotic use and MRSP in South Africa are important.

Similar to other studies, the genes responsible for penicillin, tetracyclines, erythromycin, and trimethoprim-sulfamethoxazole (*blaZ*, *tetM*, *ermB*, and *dfrG*) were found to be the predominant genes encoding resistance in *S. pseudintermedius*. Resistance to aminoglycosides was associated with the presence of the adenyl nucleotidyl transferase gene (*ant(6)-Ia*), phosphotransferase gene (*aph(3′)-III*), and the acetyltransferase/phosphotransferase gene (*aac(6′)-Ie-aph(2′)-Ia*) in all isolates except five that only harbored the *aac (6′)-Ie-aph (2′)-Ia* gene. Other studies have reported a strong correlation between the resistant phenotypes and resistance genes detected among *S. pseudintermedius* isolates [7, 16, 25]. However, in this study, a good correlation was not observed.

For example, none of the isolates that were chloramphenicol resistant harbored the *cat* gene. Furthermore, only three isolates harbored the *ermB* gene and were resistant to erythromycin. It has been suggested that inconsistencies between resistant genes present among susceptible isolates could be attributed to insertion, deletion, or mutation in the gene involved [26]. Therefore, further studies with a high number of isolates could help analyze the promoter sequences of genes showing susceptibility despite an intact resistance gene.

The detection of virulence genes among *S. pseudintermedius* isolates in this study is similar to those reported in other studies [16, 27–29]. There were no lineage associated virulence genes identified in this study. However, compared to ST551, ST496 isolates harbored *sdrE* (cell wall adhesins), *nanB* (putative sialidase toxin), and *spsM* (cell wall anchor protein). The *nanB* gene could contribute to colonization by offering a carbon source for growth, forming biofilms, or increasing adherence if receptors on the host are exposed [30]. The *spsM* cell wall protein and *sdrE* have been associated with mediating binding, invasion, and degradation of epithelial cells [30–32]. Whereas the isolate belonging to ST71 in this study did not harbor the leukocytes genes (*LukS-I* and *LukF-I*) that are responsible for the destruction of canine polymorphonuclear leukocytes (PMNs) by pore formation and cell lysis [33]. The success of *S. pseudintermedius* lineages has previously been associated with variation in the surface proteins. Therefore, more studies analyzing the functional expression of virulence genes among canine *S. pseudintermedius* isolates could inform on the successful lineages in South Africa.

The analysis by geoBURST could not identify a main clonal founder and clonal population structure of MSSP and MRSP isolates in South Africa. In previous studies, MRSP isolates were associated with fewer clonal clones than MSSP isolates, which had relatively less clonal expansion [1, 5, 19, 25]. The diversity observed in this study suggests that the *S. pseudintermedius* population structure isolated from dogs in South Africa could be non-clonal. In comparison, Dos Santos *et al*. concluded that the population of *S. pseudintermedius* was weakly clonal due to the presence of recombination among STs worldwide [5]. The PubMLST had not previously reported ST2232, ST2231, ST2228, ST2229 and ST2230. These clones represent locally evolved clones since they were located on a very distant branch compared to STs in the database. However, ST496, ST71, ST551, ST45 and ST181 detected in this study have been reported worldwide [5, 16, 19, 34]. These clones could have been transported from the countries they were first identified in. ST496 was identified as a novel clonal lineage in Australia in 2018 whereas ST551 was first identified in Poland in 2016 [19, 35]. Of concern, ST496 has shown significant antibiotic resistance to veterinary antibiotics and contains virulence genes such as *spsI* and *spsF* associated with adhesion to the extracellular matrix [16]. The isolate in this study representing ST551 harbored *tetK* which encodes tetracycline resistance through efflux pumps. This is a unique feature that has been identified for ST551 among MRSP isolated from dogs in Poland and Slovenia [34, 35]. Hence determining clinical

implications of dominant *S. pseudintermedius* strains in South Africa could help in the successful treatment of canine infections.

There are important limitations to this study. The population size was very limited to detect important information on the clonal complexes of *S. pseudintermedius* in South Africa. The sample size was also not sufficient to study the characteristics of MRSP in each clonal complex. The population of the isolates used in this study came from samples submitted to one bacteriology laboratory. Therefore, the results of this study should not be generalized to the canine population in South Africa.

Conclusion

This is the first report addressing the phenotypic and genotypic characterization of canine *S. pseudintermedius* isolates submitted to a veterinary diagnostic laboratory in South Africa. The results from this study suggest the presence of a highly diverse clonal population structure of *S. pseudintermedius.* The detection of STs such as ST71, ST45, ST551, and ST496 shows that these lineages have disseminated worldwide. Multidrug resistance and the presence of MRSP observed in this study could be a major threat to canine health. The collection of *S. pseudintermedius* and MRSP in Africa is underrepresented compared to worldwide continental reports. Despite the challenges associated with specimen collection of *S. pseudintermedius* in Africa, information on the molecular epidemiology and antibiotic resistance of MRSP in Africa is vital to obtain a universal understanding of this organism.

Methodology

Bacterial Isolates and Species verification

Non-duplicate, clinical isolates from dogs were obtained as convenience samples from a veterinary diagnostic laboratory in South Africa, Gauteng. Dog samples were obtained from Johannesburg, Middleburg, Cape Town, Port Elizabeth, Pietermaritzburg, and Randburg. Clinical isolates were taken from post-operative infection, cystitis, otitis, wound, abdominal fluids, left front limb, pyoderma, and hock hygroma. Isolates were then shipped to the University of Tennessee Veterinary College, bacteriology lab in transport media for further analysis. *Staphylococcus pseudintermedius* isolates were inoculated onto Columbia blood agar plates containing 5% sheep blood (Remel) and incubated overnight at 37 °C. The isolates were identified as *S. pseudintermedius* using MALDI-TOF MS, Bruker [36].

Antimicrobial Susceptibility

Antimicrobial susceptibility tests were performed on all confirmed *S. pseudintermedius* isolates. A disk diffusion method was used and interpreted as recommended by the most current Clinical

and Laboratory Standards Institute (CLSI) guidelines available at the time of the study [37]. The antimicrobials tested were penicillin (P) 10µg, clindamycin (DA) 2µg, kanamycin (K) 30µg, erythromycin (E) 15µg, gentamicin (CN) 10µg, oxacillin (OX) 1µg, tetracycline (TE) 30µg, minocycline (MH) 30µg, doxycycline (DO) 30µg, chloramphenicol (C) 30µg, cephalothin (KF) 30µg, vancomycin (VA) 30µg, streptomycin (S) 10µg, rifampin (RD) 5µg, marbofloxacin (MAR) 5µg, amoxicillin-clavulanic acid (AMC) 20µg, fosfomycin (FOS) 20µg, linezolid (LZD) 30µg, cefpodoxime (CPD) 10µg, cefoxitin (FOX) 30µg, sulfamethoxazole-Trimethoprim (SXT) 1.25/23.75µg. For inducible clindamycin resistance, the erythromycin and clindamycin disks were placed approximately 15 mm apart. The intermediate susceptibility was treated as resistant for analysis in the study. Isolates that were resistant to three or more antimicrobial drug classes were defined as "multidrug-resistant".

Whole Genome Sequencing

Isolates were inoculated onto Columbia blood agar containing 5% sheep blood and incubated overnight at 37 ^{0}C. Three or four bacterial colonies were suspended in 3 ml of sterile tryptic soy broth, incubated at 37 ^{0}C overnight, and a commercial kit (UltraClean® Microbial DNA Isolation Kit, Qiagen) was used for DNA extraction. The concentration was measured by QubitTM assay. Sequencing libraries were constructed using a Nextera DNA sample prep kit (Illumina, Inc., USA) according to the manufacturer's instructions. The genomes were sequenced using a MiSeq platform (Illumina, Inc.) with a single-end read length of 150 bp at the University of Tennessee Immunology Laboratory. Sequences were assembled using spades. Annotation and analysis of the genomes were performed using the PATRIC software [38].

Molecular epidemiology

ResFinder (genomic epidemiology.org), CARD (Comprehensive Antimicrobial Resistance Database, https://card.mcmaster.ca/) databases, and PATRIC were used to identify resistance genes. Virulence genes were detected using ARIBA to query the Virulence Factor Database (VFDB), additionally, the Geneious prime was used for BLASTN analysis of virulence genes [39]. Genomes that carry the chromosomal cassette SCC*mec* were detected using the SCC*mec*Finder v.1.226 at default settings [40].

Population structure analysis

The sequence type (ST) of each isolate was determined by multilocus sequence typing of seven housekeeping genes as we described previously [41]. The sequence types were assigned by comparison with the allele sequences available in the PubMLST database. Isolates with new combinations of alleles were submitted to the MLST database curator Vincent Perreten for assignment. Using entries from the global PubMLST *S. pseudintermedius* database, we ascertained the clonal relationships of the sequence types obtained in this study through goeBURST

clustering analysis on PHYLOViZ [42]. To further investigate the ST's relationships compared to the major STs identified in the United States, Europe, and Asia (ST258, ST45, ST71, ST68), Botswana (ST888, ST885, ST887, ST886, ST889) and Kenya (ST590, ST591, ST592, ST593, ST594) together with STs identified in the South African data.

Phylogeny

The Global protein families (PGFams) in PATRIC were used to identify 655 protein families from genes that were present as a single copy per genome of *S. pseudintermedius* in South Africa and previously reported genomes [38]. For each of the chosen genes, both the gene (nucleotide) and encoded protein (amino acid) sequences were used. MUSCLE was used to align protein sequences and nucleotide sequences [43]. The sequences were concatenated into one alignment using a phylip formatted file. The maximum likelihood phylogeny was then generated using Randomized Accelerated Maximum Likelihood (RAxML) with a general time-reversible nucleotide substitution model and four gamma categories for rate heterogeneity [44]. The resulting newick file was viewed in FigTree v1.4.4.

Statistical analysis

All proportions and 95% confidence intervals in this study were performed using SAS ® 9.4 (SAS Institute Inc., Cary, NC, USA).

Table 4-6: Genomes used for phylogenetic analysis in addition to isolates in this study.

Strain	**GenBank Accession**	**Genome Length(bp)**	**Contigs**	**Country**	**ST**
KCTC 43134	CP045084	2611104	1	South Korea	585
HKU10-03	CP002439	2617381	1	Hong Kong	308
E104	LAWU00000000	2757000	49	Germany	N/A
SP79	AP019372	2509706	1	Japan	2141
ED99	CP002478	2572216	1	Germany	25
LMG 22219	MLGE00000000	2523112	50	United States	N/A
NA45	CP016072	2841212	1	United States	84
ST496-1	QQPB00000000	2745362	467	Australia	496
ST64-4	QEJG00000000	2681612	463	Australia	64
E140	ANOI00000000	2769458	1	Denmark	71
2080722072011	PEOJ00000000	2571729	101	Netherlands	45
2121224012011	PEPS00000000	2650237	98	Netherlands	258
SL/114	MQND00000000	2590176	51	Sri Lanka	45
ST258 1	QEJD00000000	2620567	1441	Australia	258
VTH551	RJQE00000000	2637925	32	United States	258

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CHAPTER V

Antimicrobial resistance and molecular epidemiology of human *Staphylococcus pseudintermedius* isolates.

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Abstract

There has been an increase in the emergence of human infections associated with *S. pseudintermedius* colonization in immunocompromised and elderly patients. An important public health question is whether the organism is zoonotic or adapted to produce disease in humans. Hence, this study characterized the antimicrobial susceptibility and molecular epidemiology of human *S. pseudintermedius* isolates. Human isolates were compared with previously reported canine isolates in terms of antimicrobial resistance, virulence, mobile genetic element profiles, and population structure. Most of the human isolates were methicillin-susceptible *S. pseudintermedius* with broad antimicrobial susceptibility. Compared to human isolates, the canine isolates consistently had higher overall levels of antimicrobial resistance. The carriage of antibiotic-resistant genes: *aac (6')-Ie-aph (2")-Ia*, *ant6-Ia*, *aph (3')-III*, *blaZ*, and *erm(B)*, which confers resistance to aminoglycosides, β-lactams, and erythromycin respectively, were significantly (p-value<0.05) different between canine and human isolates. Additionally, virulence genes encoding cell wall-associated proteins; *spsK, spsI, spsD*, and *spsL* were significantly (p-value<0.05) different between canine and human isolates. Carriage of genes for intact prophages, plasmid BacP222, and competence (*comk,* and *comGA*) were associated with both canine and human isolates. Whereas carriage of genes associated with Pre25-like mobile genetic elements was mostly associated with canine isolates. For the first time, ST150, ST811, ST822, and ST862, previously reported in canine isolates, were identified among human *S. pseudintermedius* isolates. Based on geoBURST analysis, most human *S. pseudintermedius* isolates reported on the pubMLST are singletons and represent a non-clonal population structure. The clonal complex (CC) 1279 was identified as the main clonal founder of all human *S. pseudintermedius* isolates available on the pubMLST database compared to CC309 identified in canine isolates. In conclusion, this study suggests that *S. pseudintermedius* has evolved to become a human pathogen. Continuous surveillance of drug resistance and strain typing of human *S. pseudintermedius* isolates is important.

Introduction

Staphylococcus pseudintermedius is a Gram-positive commensal bacterium that is frequently isolated as an opportunistic pathogen in dogs. Humans have not been described as the natural host of *S. pseudintermedius* [1–3]. However, *S. pseudintermedius* has been reported in immunocompromised and elderly human patients [4]. *Staphylococcus pseudintermedius* has also been reported to be significantly more common among dog owners compared to non-dog owners [5, 6]. Most *S. pseudintermedius* human infections are associated with dog bite wounds [1, 7, 8]. In addition, sino nasal, and urinary tract infections have been reported [9, 10].

Implementation of MALDI-TOF and whole genome sequencing has facilitated the identification of *S. pseudintermedius* from humans [11–13]. Similar sequence types (ST) have been detected between canine and human isolates. Studies have shown the colonization of pets and owners with MRSP ST71, ST29, ST115, and ST143 in Europe [14]. In Thailand, ST45, ST112, and ST181 were identified among MRSP isolated from clinical human samples [15, 16]. In the Netherlands, CC241 was identified among methicillin-susceptible *S. pseudintermedius* (MSSP) human isolates [17]. Additionally, nasal swabs of veterinary dermatologists in Italy showed persistent colonization of the nasal cavity with ST71-SCC*mec* II-III and ST106-SCC*mec* V [18, 19].

Due to its invasiveness and virulence, *S. pseudintermedius* can cause serious infections in humans [1, 20]. For example, the *Staphylococcus intermedius* (most *S. intermedius* are now considered as *S. pseudintermedius*) exfoliative toxin (*SIET*) gene and leukotoxin genes (*lukF/S*) were identified among elderly patients with bullous skin lesions using routine methods for *S. aureus* [21]. Multidrug resistance (MDR) with the carriage of resistance genes such as *aac (6')-Ie-aph (2")-Ia, ant6-Ia, aph (3)-III, blaZ, cat, dfrG, erm(B), tetM, and lnuA* has been identified among human MSSP isolates [17]. In addition, mobile genetic elements (MGE) such as plasmids and prophages have been reported among human *S. pseudintermedius* isolates [15–17]. The frequent acquisition of host-associated genes through horizontal gene transfer (HGT) has enabled *S. aureus* to adapt to different hosts multiple times throughout its evolutionary history [22, 23]. Therefore, the aim of this study was to examine the molecular epidemiology, antimicrobial resistance, and virulence profiles of human isolates compared to canine isolates to provide insights into the host adaptability of *S. pseudintermedius* to the human host.

Results

Genomic features of *S. pseudintermedius* isolated from human samples submitted to a diagnostic laboratory.

Human *S. pseudintermedius* isolates were obtained from a diagnostic bacteriology laboratory. Most of the isolates were MSSP except two that were MRSP. Most MSSP isolates were obtained from skin samples, and some were from the bone and sputum. The genome size of the isolates was between 2.4-2.7 Mbp for all strains. The general genomic features are presented in Table 5-1. The genome annotation showed an average of 2,569 protein-coding sequences (CDS) and an average of 1,961 proteins with functional assignments.

Protein Subsystem Distribution

The subsystem and gene distribution of *S. pseudintermedius* human isolates is presented in Figure 5.1. The most common subsystem class was responsible for metabolism with over 7705 genes, including genes responsible for carbohydrates, lipids, energy, nucleotide, and amino acid pathways. There were 3241 genes that were detected as responsible for protein processing whereas 1462 genes were reported for stress response, defense, and virulence. The annotation of the isolates revealed several roles (Figure 5.1). FtsB, FMN-dependent isopentenyl, L33p protein, N-acetyl-L, L-diaminopimelate deacetylase, transcriptional regulator, PTS system, ribulokinase, sulfite reductase, translation initiation factor 3, ydbs transmembrane, ydbt transmembrane and SpyDAC protein were conserved among all the isolates as predicted from their gene sequences. Some genomes (4/15) harbored the methyl-directed repair DNA adenine methylase and the Arginyl-tRNA synthetase (5/15). The role of the genes encoding sulfur carrier protein, sulfite reductase [NADPH] flavoprotein alpha-component, and the transmembrane protein ydbT could not be identified in the Type Strain (*S. pseudintermedius* LMG 22219).

Antimicrobial resistance phenotypes and genes

Broad antimicrobial susceptibility was observed among the *S. pseudintermedius* isolates (Table 5-2). Four of the isolates (WU6, WU7, WU11, and WU14) were susceptible to all antimicrobials tested and harbored none of the resistant genes. All isolates were susceptible to amoxicillin-clavulanic acid, linezolid, vancomycin, and rifampin. Multidrug resistance (MDR) (i.e., resistance to 3 or more classes of antimicrobials) was identified in 6/15 (40%) of the isolates. In addition to β-lactams and *blaZ* resistance gene carriage (10/15), isolates were also resistant to aminoglycosides and harbored the *aac (6')-Ie-aph (2")-Ia*, *aph (3')-III* and *ant6-Ia* resistant genes (6/15).

Table 5-1: Genomic features of *staphylococcus pseudintermedius* among human isolates

Feature	**WU1**	**WU2**	**WU3**	**WU4**	**WU5**	**WU6**	**WU7**	**WU8**	**WU9**	**WU10**	**WU11**	**WU12**	**WU13**	**WU14**	**WU15**
Genome size (bp)	2,489,284	2,711,210	2,651,366	2,459,001	2,577,501	2,469,628	2,485,910	2,632,832	2,529,944	2,694,104	2,563,213	2,606,129	2,586,513	2,499,140	2,690,314
GC content (%)	37.58	37.3	37.45	37.72	37.51	37.7	37.58	37.4	37.53	37.16	37.49	37.56	37.45	37.61	37.3
CDS	2,390	3,386	2,670	2,394	2,536	2,353	2,387	2,602	2,467	2,661	2,496	2,593	2,510	2,453	2,639
Repeat regions	5	6	0	0	0	24	17	13	13	0	0	16	18	0	29
Rrna	6	4	6	7	4	5	4	6	6	6	6	5	6	6	5
Trna	59	27	68	57	59	57	59	59	59	57	59	59	59	57	59
hypothetical protein	543	860	618	533	600	508	552	614	565	733	595	598	600	558	638
functional protein	1,847	2,526	2,052	1,861	1,936	1,845	1,835	1,988	1,902	1,928	1,901	1,995	1,910	1,895	2,001

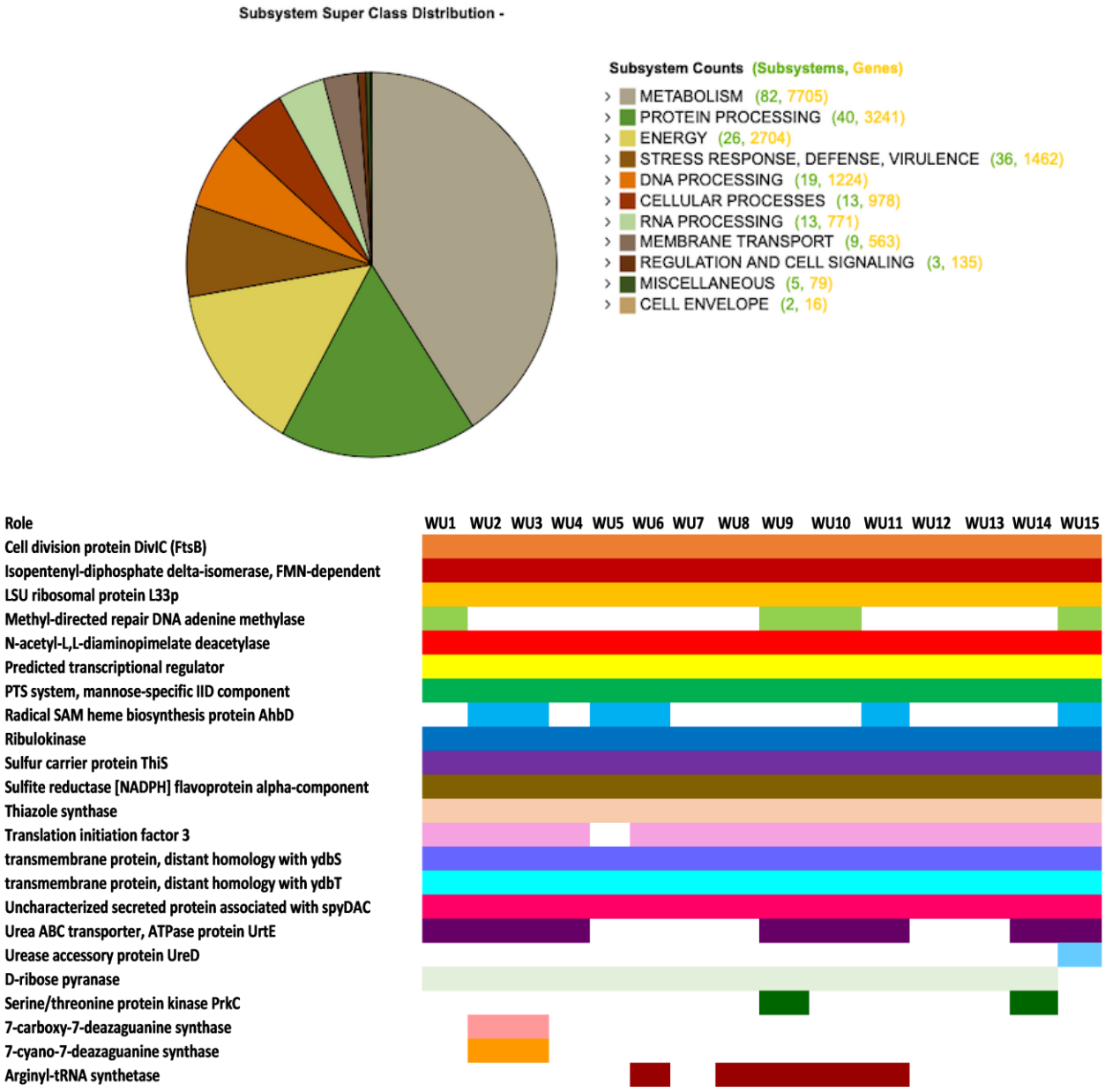


Figure 5.1: Protein subsystem distribution of human *S. pseudintermedius* isolates produced by PATRICs protein family sorter and the proteome comparison of the human isolates to a *S. pseudintermedius* type strain (LMG 22219).

Table 5-2: Resistant phenotypes, genes, sequence types and agrD typing of *Staphylococcus pseudintermedius* human isolates submitted to a diagnostic laboratory.

Isolate	Resistance Genes	Resistance Phenotype	Sequence type	agrD type
WU.SIG.1	-	S	2206	type II
WU.SIG.2	*aac (6')-Ie-aph (2")-Ia, aph (3')-III, blaZ, dfrG, mecA, tetM*	C, DA, E, OX, P, K, S	150	type IV
WU.SIG.3	*aac (6')-Ie-aph (2")-Ia, blaZ, mecA*	KF, CN, P, TE, K, S, FOX, CPD, DO, MH	811	type II
WU.SIG.4	*blaZ*	P, S	822	type II
WU.SIG.5	*ant6-Ia, aph3-III, blaZ, dfrG, ermB, tetM*	DA, E, TE, SXT, FOS, K, S, DO, MH	155	type IV
WU.SIG.6	-	-	2303	type II
WU.SIG.7	-	-	1506	type I
WU.SIG.8	*aac (6')-Ie-aph (2")-Ia, ant6-Ia, aph3-III, blaZ, cat, dfrG, ermB, tetM*	C, DA, E, CN, P, TE, SXT, K, S, DO, MH	862	type IV
WU.SIG.9	*blaZ*	P, SXT	2207	type IV
WU.SIG.10	*aac (6')-Ie-aph (2")-Ia, blaZ, tetM*	CN, P, TE, K, DO, MH	2207	type I
WU.SIG.11	-	-	2207	type III
WU.SIG.12	*blaZ*	P, S	2207	type II
WU.SIG.13	b*laZ, tetM*	P, TE, DO, MH	2207	type III
WU.SIG.14	-	-	2207	type II
WU.SIG.15	*aac (6')-Ie-aph (2")-Ia, aph3-III, blaZ, ermB, mecA, tetM*	DA, E, CN, MAR, OX, P, TE, SXT, K, S, FOX, CPD, DO, MH	181	type IV

C (chloramphenicol), DA (clindamycin), E (erythromycin), OX (oxacillin), P (penicillin), K (kanamycin), S (streptomycin), DO (doxycycline), MH (minocycline), FOX (cefoxitin), TE (tetracycline), CN (gentamicin), MAR (marbofloxacin), SXT (sulfamethoxazole trimethoprim).

Isolates that were resistant to tetracycline, doxycycline and minocycline harbored the *tetM* gene (6/15). However, one isolate harbored *tetM* without phenotypic resistance to tetracyclines. The isolates were categorized into agr group I 13% (n=2), 40% (n=6) in agr group II, 13% (n=2) in agr group III, and 33% (n=5) in agr group IV. Most of the STs were assigned as ST2207. This is the first report of the detection of ST822, ST150, ST811 and ST862 among human *S. pseudintermedius* isolates. The MRSP isolates that were sequenced belonged to ST181 and ST150.

The comparison of canine and human isolates for antibiotic resistance

The canine isolates consistently had higher overall levels of antimicrobial resistance compared to the human isolates (Table 5-3). Statistically significant ($p<0.05$) differences were noted for all antibiotics except tetracycline, minocycline, doxycycline, cephalothin and chloramphenicol. Additionally, statistically significant differences ($p<0.05$) were not observed for antibiotic resistance genes *dfrG*, *cat*, *tetM* and *tetK* between the human and canine isolates (Table 5-4).

Comparison of virulence genes in canine and human isolates

Overall, the human and canine isolates seem to share similar virulence genes profiles (Table 5-5). Both human and canine isolates harbor *spsM, icaA, icaD, speta, lukF-I* and *lukS-I*. In comparison both the canine and human isolates did not harbor the *spsJ* resistant gene. Statistically ($p<0.05$) significant differences were observed for the genes *spsK, spsI, spsD*, and *spsL*, associated with cell wall surface proteins. Additionally, the genes *clpP*, *coa* and *expB* associated with cell wall clumping, coagulase and exfoliative toxin were also statistically ($p<0.05$) significantly different.

Mobile genetic elements

Regarding HGE, 46.6% (7/15) of the isolates showed >90% identity but low query coverage for the gene encoding the bacSp222 plasmid. Similarly, (53.3%) 8/15 of canine isolates harbor the same plasmid (Canine isolates). The pRE25-like chromosomal element carrying resistance genes *ermB, catPC221, aph (3')-III, ant6-Ia, sat4* and a toxin antitoxin system was identified among 6/15 (40%) of the isolates. In comparison, almost all canine isolates 86.6% (13/15) harbor genes associated with the Pre25-like chromosomal element (Supplementary). Both the canine and human isolates showed high sequence identity (>90% and >90% coverage) of the competence genes, *comGA* gene and *comk* associated with natural genetic transformation.

Table 5-3: Comparison in antimicrobial resistance between human and canine isolates

Group	Antibiotic	Human Isolates	Canine isolates	P-value[a]
Lincosamides	Clindamycin	26.6	80	0.0034
B-lactams	Penicillin	60	100	0.0062
	Amoxicillin-clavulanic acid	0	46.6	0.0025
	oxacillin	13.3	100	<.0001
Cephalosporins	cefoxitin	13.3	60	0.008
	Cefpodoxime	13.3	100	<.0001
	Cephalothin	6.67	26.67	0.1416
Tetracyclines	Tetracycline	40	66.6	0.1432
	Minocycline	40	66.6	0.1432
	Doxycycline	40	66.6	0.1432
Phenicols	Chloramphenicol	13.3	20	0.6
Glycopeptides	Vancomycin	0	0	-
Aminoglycosides	Streptomycin	53.3	86.6	0.04
	Gentamicin	26.6	73.3	0.0106
	Kanamycin	40	86.6	0.008
Macrolides	Erythromycin	26.6	80	0.0034
Fluoroquinolones	Marbofloxacin	6.6	60	0.0019
Rifamycin	Rifampin	0	0	-
Phosphonic	Fosfomycin	6.67	13.3	0.5428
Oxazolidinones	Linezolid	0	0	-
Folate inhibitor	SXT	26.6	86.6	0.0009

a) P-value<0.05 indicates statistically significant differences

Table 5-4: Presence of resistance genes in human and canine *Staphylococcus pseudintermedius* isolates

Resistant gene	**Human isolates**	**Canine isolates**	**p-value[a]**
blaZ	60	100	0.0062
cat	6.6	33.3	0.0679
dfrG	26.6	66.6	0.281
ermB	20	80	0.001
mecA	20	100	<.0001
tetK	0	20	0.0679
tetM	40	66.6	0.1432
aph3_III	26.6	80	0.0034
ant6_Ia	13.3	73.3	0.0009
aac (6′)-Ie-aph (2″)-Ia	33.3	80	0.0099

a) P-value<0.05 indicates statistically significant differences

Table 5-5: Virulence genes in human and canine *Staphylococcus pseudintermedius* isolates

Virulence gene	Human isolates	Canine isolates	p-value[a]
spsK	46.6	100	0.001
spsI	66.6	100	0.0143
spsM	-	-	-
spsD	20	93.3	<.0001
spsJ	-	-	-
spsL	73.3	100	0.0317
spsF	6.6	0	0.309
clpP	33.3	100	0.0001
icaA	-	-	-
icaD	-	-	-
speta	-	-	-
spEX	86.6	100	0.1432
siet	93.33	100	0.3091
lukF-I	-	-	-
lukS-I	-	-	-
nanB	40	33.3	0.7048
coa	60	100	0.0062
sec-canine	20	13.3	0.6242
expA	0	13.33	0.1432
expB	0	26.6	0.0317

a) P-value<0.05 indicates statistically significant differences

siet exfoliative toxin, *spetA* (exfoliative toxin A), exotoxin (*SpeX*), cell wall anchored genes *spsL, spsM, spsD, spsF, spsK, spsJ*, *nanB* (exoenzyme), *coa* (staphylocoagulase) lukF-I and luks-I (leukotoxins), *icaD* and *icaA* (ica operon of biofilm formation), *clpP* (clumping factor P).

Prophages were investigated in all human isolates (Table 5-6). Intact prophages were detected in 10/15 (66%) of the isolates of which 2 isolates (WU.SIG.12 and WU.SIG.3) showed 2 intact prophages. The length of intact prophages ranged from 19.2kb to 66.3kb. Additionally, the GC% content of intact prophages ranged from 34.25% to 38.99%. Among the canine isolates (Supplementary), intact prophages were determined in 66% (10/15) of which of the isolates (UCD5, UWI3, CU3 and TX19) harbored 2 intact prophages. The length of the intact prophages ranged from 30.2kb and 61.7k and the GC% ranged from 34.25% to 36.96%.

geoBURST analysis of human isolates

The geoBURST analysis included all available *S. pseudintermedius* human isolates reported on the PubMLST (https://pubmlst.org/organisms/staphylococcus-pseudintermedius) as well as STs identified in this study. A total of 325 human isolates have been reported on the database. geoBURST revealed that the *S. pseudintermedius* human isolates are highly diverse, with 229 STs reported as singletons (Figure 5.2). Out of 308 STs, ten groups were formed that had more than 2 STs by the single locus variant (SLV) algorithm. ST1297 was determined as the main clonal founder (CC1297) with eight STs belonging to this CC. ST2303 assigned in this study was also determined as the CC of another group with only 2 STs (ST1888 and ST2526) belonging in this CC. The GeoBURST full minimum spanning tree (MST) algorithm identified 51 links at level 1, 184 links at level 2, 69 links at level 3 and 3 links at level 4 (Supplementary). The isolates assigned in this study (ST2207) were connected to ST2460 by three-locus variants. ST155 was identified as a group founder connected to STs by DLV. The ST150 which has been reported among canine isolates was part of the largest clonal complex with a SLV to ST2534. In comparison, the canine *S. pseudintermedius* PubMLST database with 2435 STs showed 67 groups that had more than 2 STs by SLV (Supplementary). The main clonal founder was identified as ST309 with 1270 STs belonging to this clonal complex.

Phylogenetic analysis between human and canine isolates

A maximum likelihood phylogenetic tree was constructed using PGFams sequence alignments. A close phylogenetic relationship between human and canine isolates was observed (Figure 5.3). However, the human isolates were widely distributed across the tree. Importantly, the isolates belonging to canine TX19 and human WU.SIG.5 seem to arise from a common recent ancestor and belong to ST155. Similarly, UWI3 and WU.SIG.3 were clustered together and belong to ST811. Human isolates identified as ST2207 assigned in this study did not seem to cluster together phylogenetically.

Table 5-6: Detection of genes associated with mobile genetic elements of *Staphylococcus pseudintermedius* human isolates.

Isolate	**plasmid *bacSp222***	***pRE25***	***comGA***	***comK***	**Prophages**
WU.SIG.1					Incomplete
WU.SIG.2					Intact
WU.SIG.3					Intact
WU.SIG.4					Intact
WU.SIG.5					Intact
WU.SIG.6					Incomplete
WU.SIG.7					Incomplete
WU.SIG.8					Intact
WU.SIG.9					Incomplete
WU.SIG.10					Incomplete
WU.SIG.11					Intact
WU.SIG.12					Intact
WU.SIG.13					Intact
WU.SIG.14					Intact
WU.SIG.15					Intact

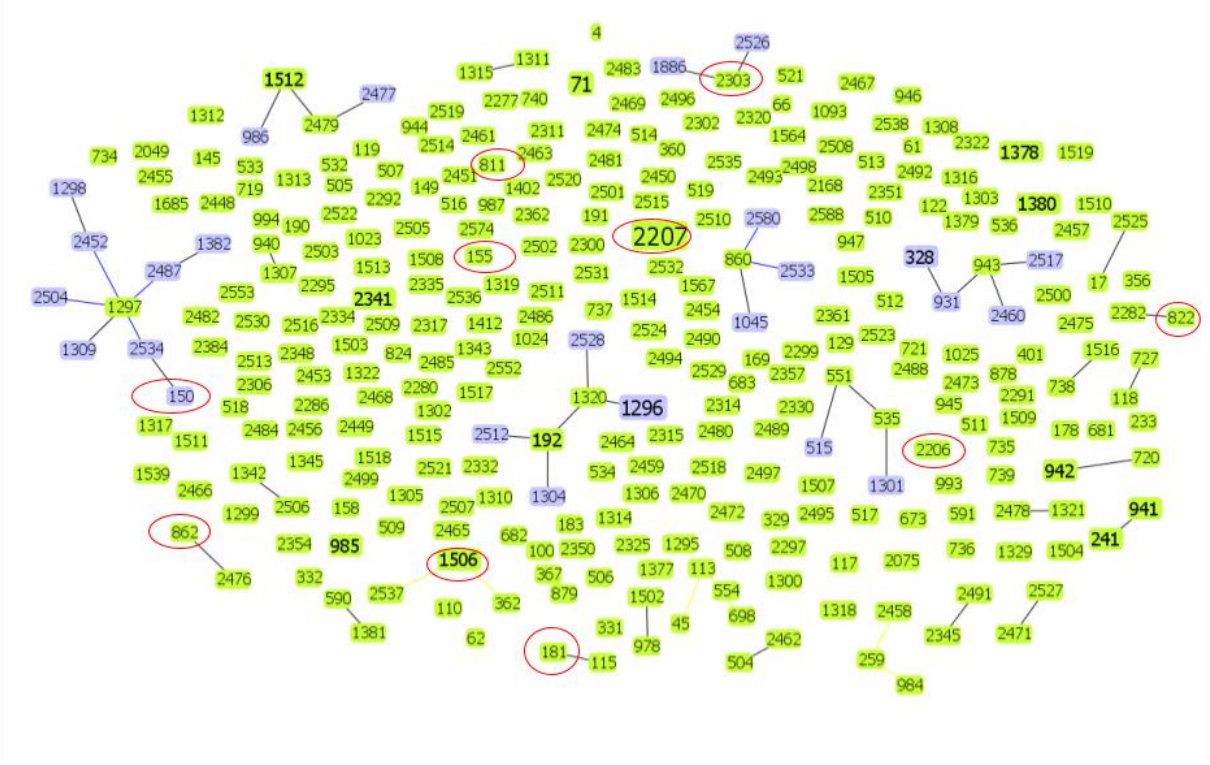


Figure 5.2: geoBURST single locus variant algorithm of all STs identified in this study and human STs reported in the pubMLST database. Each node represents a ST, of which the node circled in red represent STs identified in this study. Green nodes represent the predicted founder of each group. The ST reported for human *S. pseudintermedius* are highly diverse and do not seem to share similar allelic variation.

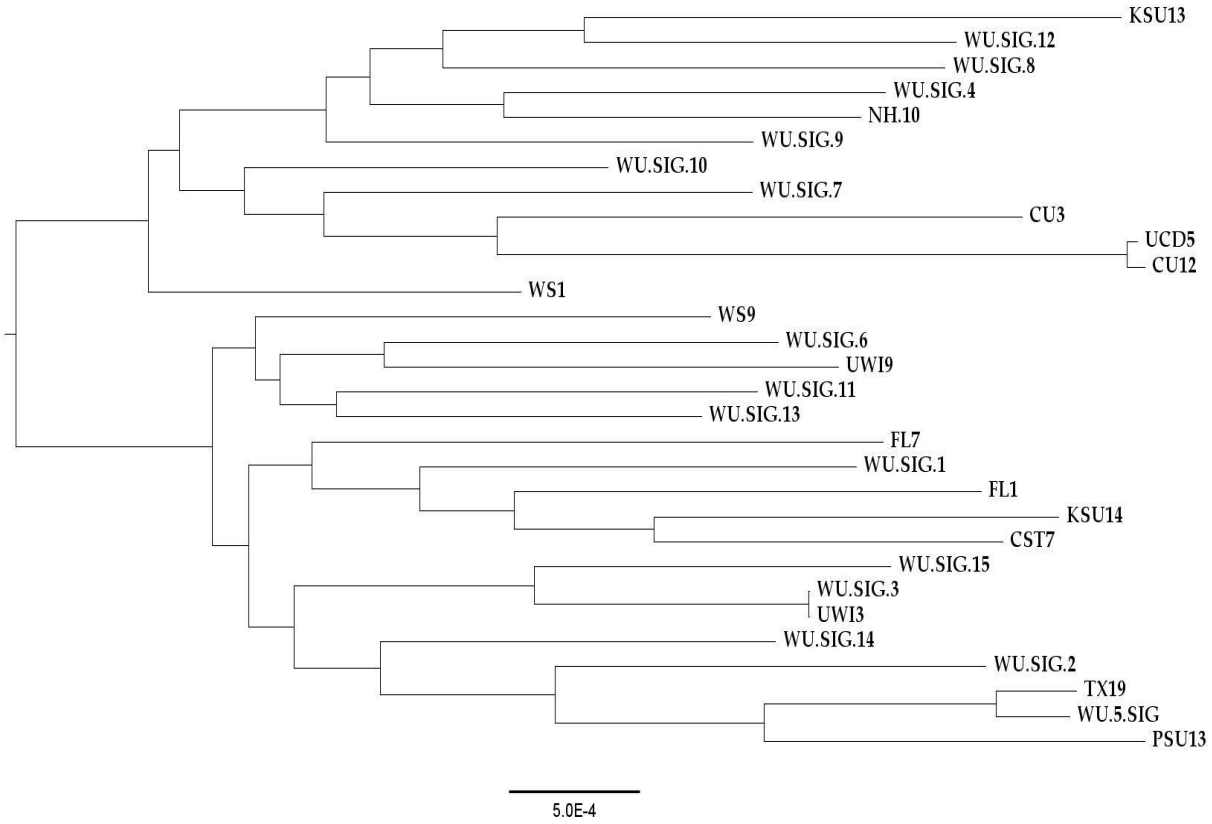


Figure 5.3: Maximum likelihood phylogenetic tree of *S. pseudintermedius* human and canine isolates based on protein global families (PGFams). The scale bar represents the number of nucleotides substitutions per site.

Discussion

Few studies have reported on the genetic characteristics of *S. pseudintermedius* isolates originating from human infections. In this study, the genomic analysis revealed over 1462 specialty genes that are responsible for stress response, defense, and virulence of *S. pseudintermedius.* Importantly, the annotation revealed that the sulfur carrier protein (ThiS), the sulfite reductase [NADPH] flavoprotein alpha-component, and the transmembrane protein (ydbT) have roles that could not be identified in the type of strain (*S. pseudintermedius* LMG 22219). The ydbT transmembrane protein is shown to be responsible for antibiotic resistance and stress response in *Bacillus subtilis* [24]. Additionally, The *BacSp222* structural gene in *S. pseudintermedius* is flanked by two ORFs that each encodes hypothetical proteins that have significant homology to *Bacillus subtilis* YdbS and YdbT proteins [25]. The plasmid gene *BacSp222* was identified among both *S. pseudintermedius* human and canine isolates in this study. The BacSp222 is a multifunctional peptide that kills Gram-positive bacteria and demonstrates significant cytotoxic activities towards eukaryotic cells [24, 25]. Wegener *et al*. suggested that the plasmid BacSp222 could offer an adaptive advantage to the MDR CC241 clone composed of mainly human *S. pseudintermedius* isolates [17]. Although no statistically significant differences in the carriage of plasmid gene *BacSp222* were observed in this study, it would be worth investigating the clinical implications of the ydbT transmembrane protein and BacSp222 plasmid among canine and human *S. pseudintermedius* isolates.

Most of the *S. pseudintermedius* isolates in this study were MSSP and belonged to ST2207. Similarly, Wegener *et al*. reported MSSP among human isolates in the Netherlands [17]. The MRSP isolates detected in the study belonged to ST150 and ST181, previously reported among MRSP canine isolates in the United States [26, 27]. Additionally, ST1279 identified as the main clonal founder among human isolates has also been identified in canine isolates in the PubMLST database. This indicates that canine and human *S. pseudintermedius* share similar STs as previously reported [15, 16, 19, 28].

Using SLV, the geoBURST analysis with all human STs in the pubMLST database predicted 10 clonal complexes with more than 2 multilocus sequence types. There was no geographical clustering of the STs assigned in this study as they were scattered on the geoBURST tree. This suggests that the population structure of human Multilocus sequence types is highly nonclonal and represents strains that are not related to each other. The lack of clonality among MSSP isolates observed in this study has been reported among MSSP isolates from humans and dogs [17, 29, 30]. In contrast, a highly clonal population structure has been observed for *S. aureus* with a low recombination-to-mutation ratio as well as MRSP isolates of canine origin [31]. Similar Multilocus sequence types (ST155 and ST811) in canine and human isolates were clustered on the

phylogenetic tree. Similarly, Multilocus sequence types (ST45, ST71, ST181) representing major clonal lineages in canine isolates have been reported in human *S. pseudintermedius* isolates [16, 19, 32]. This suggests that *S. pseudintermedius* has adapted as a human pathogen. However, MRSP was not commonly identified among human lineages compared to canine lineages. Therefore, continuous surveillance of the MSSP clonal lineages will be important to understand the emergence of MRSP lineages in human *S. pseudintermedius* isolates.

Canine and human isolates were susceptible to antimicrobials used exclusively in human medicine. Similarly, susceptibility to rifampin, linezolid, and vancomycin have been observed among canine isolates in the United States and Slovenia [33]. Some human isolates were susceptible to all antimicrobials tested. It is therefore possible to use susceptible antibiotics to treat human MSSP infections, which has been reported in the past [34]. There were statistically significant differences found between canine and human isolate's carriage of *blaZ, ermB, mecA, aph3-III, ant6*-Ia, and *aac (6')-Ie-aph (2")-Ia*. In comparison, Wegener *et al*. had previously reported no statistically significant differences between dog and human isolates in the carriage of resistant genes [17]. The difference in the carriage of resistant genes could be attributed to the detection of *pRE25*-like mobile element genes that carry the *erm(B), catpC221, aph (3')-III, ant6-Ia* and *sat4* genes mostly in canines compared to human isolates. The pRE25-like mobile element is a conjugative and mobilizing multi-resistant plasmid that has been identified among 52.2% of MDR *S. pseudintermedius* isolated from canine origin in Korea [35]. Additionally, Wegener *et al*. reported that the pRE25-like element among CC241 isolates mostly represented MSSP human isolates. The presence of the pRE25-like element in MSSP lineages most likely confers MDR which could limit the availability of antibiotics to treat MSSP human infections. Additionally, it could contribute to the dissemination of resistance genes among MSSP canines and human isolates through HGT. Therefore, given the zoonotic potential and the horizontal transfer of AMR genes, AMR surveillance is important for both veterinary and human drugs.

Overall, the human and canine isolates seem to share similar virulence gene profiles. Statistically ($p<0.05$) significant differences were observed for genes *spsD*, and *spsL* associated with cell wall surface proteins and exfoliative toxin gene *expB*. Pickering *et al*. had previously demonstrated that the spsL protein has evolved the ability to bind canine fibrinogen with higher strength than human fibrinogen [36]. This can be attributed to different fibrinogen binding mechanisms observed between spsL and spsD proteins [37]. The spsL protein forms weak, diverse interaction with a lack of clustering to Fn, allowing the pathogen to attach to the integrin through moderate adhesion and detach from the host cell surface enabling the colonization of new sites. Invasion and clustering of integrins are favored by the formation of SpsD-Fn nanodomains on the bacterial cell surface with a highly strong binding affinity [37]. The exfoliative toxin (*expB*) has been suggested to play a role in bullous skin lesions found in human patients similar to the role of skin

infections in dogs [21]. More studies will require a high number of canine and human isolates to confirm the host association and pathogenesis of each gene among human patients.

There is strong evidence that prophages carry genes that contribute to the fitness, virulence, resistance, and adaptations of bacteria to their host [38–40]. The prophage sequences in this study were investigated using PHASTER to quantitate and identify the intact prophage regions in each genome [41]. Both canine and human isolates harbor intact prophages. Using the same method, Brooks *et al*., detected four intact prophages that were identified in ST71 and ST68 *S. pseudintermedius* isolates of canine origin [42]. The results suggest that bacteriophage-mediated DNA transfer (transduction) could be a major route of HGT among *S. pseudintermedius* canine and human isolates. Comparative genomics of *S. aureus* has shown that colonization of human hosts by an *S. aureus* pig isolate can occur due to the repeated transfer of bacteriophages [43]. Furthermore, evidence that *S. pseudintermedius* has genes (*comGA* and *comK*) encoding comGA and comK competence transcription factors were reported in this study. The *comGA* gene encodes an ATPase enzyme required for the assembly of pilin subunits and the formation of the pseudopilus structure during transformation [38, 44]. Brooks *et al*, previously reported a complete comG operon with an integrated prophage among *S. pseudintermedius* isolates of canine origin [42]. However, in this study, we did not detect the complete operon consisting of comGD, comGC, comGE, comGF, and comGG. Additionally, the functional expression of the competent operon by the transcriptional activator protein comk was not determined. Therefore, natural genetic competence and transduction as mechanisms of HGT and host adaptation should be further investigated among *S. pseudintermedius* human and canine isolates.

Conclusions

The prevalence of *S. pseudintermedius* in humans is gradually increasing, and future studies representing a large population of human isolates will be important to demonstrate the pathogenesis *S. pseudintermedius* infections. This study found that canine and human isolates share closely related phylogenetic relationships and virulence genes. Antibiotic options remain for the treatment of MSSP infection in humans compared to MRSP canine infections. Several intact prophages and plasmids were detected among the isolates indicating that HGT of resistance and virulence genes among canine and human *S. pseudintermedius* isolates could occur. It is therefore important in public health to monitor MSSP human clonal lineages.

Methodology

Bacterial Isolates and Species verification

Fifteen canine isolates were selected randomly from isolates within the previously described collection (Chapter II). The number of isolates was limited to 15 due to the availability of human

isolates and the MLST profile that has been commonly described among canine isolates worldwide. For human isolates, non-duplicate isolates were obtained as convenience samples from Washington University in Saint Louis, Missouri. Isolates were then shipped to the University of Tennessee Veterinary College, bacteriology lab for further analysis. *Staphylococcus pseudintermedius* isolates were inoculated onto Columbia agar plates containing 5% sheep blood (Remel) and incubated overnight at 37°C. The isolates were identified as *S. pseudintermedius* using MALDI-TOF MS (Bruker) [45].

Antimicrobial Susceptibility

Antimicrobial susceptibility tests were performed on all confirmed *S. pseudintermedius* isolates. A disk diffusion method was used and interpreted as recommended by the most current Clinical and Laboratory Standards Institute (CLSI) guidelines available at the time of the study [46, 47]. The antimicrobials tested were penicillin (P) 10µg, clindamycin (DA) 2µg, kanamycin (K) 30µg, erythromycin (E) 15µg, gentamicin (CN) 10µg, oxacillin (OX) 1µg, tetracycline (TE) 30µg, minocycline (MH) 30µg, doxycycline (DO) 30µg, chloramphenicol (C) 30µg, cephalothin (KF) 30µg, vancomycin (VA) 30µg, streptomycin (S) 10µg, rifampin (RD) 5µg, marbofloxacin (MAR) 5µg, amoxicillin-clavulanic acid (AMC) 20µg, fosfomycin (FOS) 20µg, linezolid (LZD) 30µg, cefpodoxime (CPD) 10µg, cefoxitin (FOX) 30µg, sulfamethoxazole-Trimethoprim (SXT) 1.25/23.75µg. For inducible clindamycin resistance, the Erythromycin and clindamycin disks were placed approximately 15 mm apart. The intermediate susceptibility was treated as resistant for analysis in the study. Isolates that were resistant to three or more antimicrobial drug classes were defined as "multidrug-resistant".

Whole Genome Sequencing

Three to four bacterial colonies were suspended in 3 ml of sterile tryptic soy broth, incubated at 37 C° overnight, and a commercial kit (UltraClean® Microbial DNA Isolation Kit, Qiagen) was used for DNA extraction. The concentration was measured by QubitTM assay. According to the manufacturer's instructions, sequencing libraries were constructed using a Nextera DNA sample prep kit (Illumina, Inc., USA). The genomes were sequenced using a HiSeq and Miseq platform (Illumina, Inc.) with a single end read length of 150 bp. Sequences were assembled using Spades 3.15.5 [48]. Annotation and analysis of the genomes were performed using the PATRIC software by the RAST tool kit (RASTtk) [49, 50]. The genomes were submitted to NCBI.

Virulence genes and mobile genetic elements of human and canine isolates

Using the ResFinder (genomic epidemiology.org) and CARD (Comprehensive Antimicrobial Resistance Database, card.macmaster.ca) databases, PATRIC was used to identify resistance genes by comparing the gene content to the phenotypes they produced [50]. Identification of regions with significant virulence and mobile genetic element genes was performed using the

BLAST option with Geneious prime (http://www.geneious.com/) (sequence identity>90, query coverage>80 query coverage) (Table 5-7). BLAST was similarly used to determine the agr group on the genomes using previously published sequences for *agrD* alleles EU157356.1, EU157391.1, EU157400.1 and EU157402. Additionally, prophages were predicted using the PHASTER web server [41].

GeoBURST and Phylogenic analysis

Multilocus sequence typing was performed using the 7-gene MLST scheme (*ack, cpn60, fdh, pta, purA, sar*, and *tuf*) [51] on the *S. pseudintermedius* PubMLST website (https://pubmlst.org/organisms/staphylococcus-pseudintermedius). In cases of new sequence types, the alleles for MLST were assigned an ST number by the curator of the PubMLST database, Vincent Perreten [51]. Additionally, STs available on pubMLST of human and canine origin were obtained (assessed October 6, 2022). To study the relationship between the identified STs in this study and globally available human and canine isolates, the Phyloviz 2.0 using geoBURSRT single locus variant (SLV) and minimum spanning tree (MST) algorithm was used [52]. The clonal complexes were classified where STs share at least 6 out of 7 identical alleles and the primary founders have the greatest number of single-locus variants. To visualize the minimum spanning trees generated from canine and human STs, the GrapeTree web browser application was used [53]. The Global protein families (PGFams) in PATRIC were used to build a maximum likelihood tree by Randomized Accelerated Maximum Likelihood (RAxML bootstrapping) [50, 54]. MAFFT was used for amino acid and nucleotides alignments [55]. The resulting newick file was viewed using Figtree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Statistical analysis

Statistical differences between canine and human isolates were performed using SAS 9.4 by utilizing the Pearson's chi-square test for statically significant p-value (<0.05).

Supplementary

Table S1. Canine isolates used in this study: Canine isolates

Supplementary F1 GeoBURST full minimum spanning tree algorithm of human STs from this study and the pubMLST database: Supplementary

Supplementary F2, GeoBURST full minimum spanning tree algorithm of canine isolates available on the pubMLST database: Supplementary

Table 5-7: Virulent genes and mobile genetic elements tested in the study

Characteristic	**Accession number**
Se-int	AB116378.1
spsD	WP_214548619.1
speta	ADX77621
siet	CAR57917.1
coa	VED70381.1
icaA	MH800295.1
icaD	MH800297.1
lukF-I	WP_014613567.1
lukS-I	AM850056.2/ WP_014613568.1
nanB	VED69667.1
Sec-canine	U91526.1
spsL	NZ_CP065921.1
SpeX	WP_225549882.1
Plasmid BacSp222	CP011490.1
pRE25-like element	MK775653.1
spsK	WP_233276534.1
spsI	ADX77057.1
spsJ	WP_127504874.1
spsF	WP_214546210.1
clpP	CP098634.1
expA	JN604832.1
expB	AB569087.1
spsM	WP_250882748.1

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CHAPTER VI

The detection of *mecA*-positive oxacillin susceptible *Staphylococcus pseudintermedius* among canine isolates in the United States.

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Abstract

This study examined *mecA*-positive, oxacillin-susceptible *Staphylococcus pseudintermedius* isolates obtained from canines. Isolates were screened for methicillin resistance using the Kirby-Bauer disk diffusion method with oxacillin disks. A standard PCR assay was performed to detect the *mecA, mecI, mecR1 and mecA* promoter genes. A commercial colony test was used to detect PBP2a among *S. pseudintermedius* isolates. Additionally, genome assemblies of *S. pseudintermedius* were screened for *mecA* regulatory apparatus using the BLASTN NCBI algorithm. The *mecA* gene was successfully amplified from all six of the examined isolates whereas the *mecI* and *mecR1* regions were not detected. The *mecA* promoter region was examined in 4 isolates that also produced PBP2a. SCC*mec* was identified in only two isolates. The *blaI* repressor and *blaR1* inducer genes were detected in all isolates. Overall, the result from this study suggests that the bla regulators are important in the control of the oxacillin-susceptible phenotype in *mecA*-positive *S. pseudintermedius*.

Introduction

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) isolated from dogs with pyoderma has become increasingly common [1–4]. MRSP lineages with multidrug resistance (MDR) have expanded geographically throughout the world [5–7]. Methicillin resistance is associated with the *mecA* gene located on a mobile genetic element staphylococcal chromosome cassette *mec* (SCC*mec*) [8–10]. The *mecA* gene encodes the penicillin-binding protein 2A (PBP2a) that expresses low affinity to the β-lactam binding site [1, 11, 12]. The *mecA* gene expression is regulated by the transducer mecR1 and transcriptional repressor mecI [13, 14]. Both the *mecI*/*mecR1* and *blaI*/*blaR1* configurations in methicillin-resistant *Staphylococcus aureus* (MRSA) significantly affected the rate and nature of *mecA* induction [9, 15]. During the expression of *mecA*, *mecI*/*blaI* repress transcription of *mecA* and *mecR1-mecI* by binding the *mecA* and *mecR1* promoter sequence [16, 17]. Whereas BlaR1/mecR1 induces *mecA* transcription during the interaction with β-lactam antibiotics by autoproteolytic cleavage of blaI/mecI [14, 16]. Mutations in the mec operator region can affect the ability of the repressors to bind appropriately leading to differential *mecA* expression [13, 16]. In methicillin resistant *S. pseudintermedius*, bla regulatory control is essential

in the expression of methicillin resistance and successful clonal expansion [14]. Furthermore, there is evidence that *S. pseudintermedius* PBP4 is directly associated with the dissociated oxacillin and cefoxitin phenotype [18].

The occurrence of *mecA*-positive *S. aureus* that are oxacillin susceptible (OS-MRSA) has been described in clinical isolates around the world as well as in animals and animal products [19, 20]. Additionally, many deletions of the *mecI* and varying levels of truncations in the *mecR1* have been described [21–23]. Therefore, the goal of this study was to investigate oxacillin susceptible *mecA* positive *S. pseudintermedius*.

Results

Detection of *mecA*, *mecA* regulatory regions, PBP2a and SCC*mec*

Using the Kirby-Bauer oxacillin disk diffusion test and *mecA* PCR, four isolates were identified that were *mecA* positive and oxacillin susceptible and two isolates that were *mecA* positive and oxacillin resistant (Table 6-1). Two of the oxacillin-susceptible isolates, FL10 and FL14, produced non-detectable amounts of PBP2a. Additionally, the *mecA* promoter region was also not detected among these isolates. The *blaZ* gene and its accessory genes, *blaI* repressor and *blaR1* inducer, were detected among all isolates. Additionally, SCC*mec* was identified in FL10 (SCC*mec*_type_V) and FL14 (SCC*mec*_type_IVg(2B)).

The *mecA* promoter region of *S. pseudintermedius* isolates in the study

The promoter sequences for isolates CST9, KSU1, FL15 and UWI6 were all similar. A single base polymorphism was detected in the *mecA* promoter (TATACT to TATATT) when compared to the reference *mecA* promoter sequence (Figure 6.1).

Discussion

The patterns of *mecA* expression of *S. pseudintermedius* observed in this study are similar to those previously reported in *S. aureus* isolates from rhinosinusitis patients and bovine mastitis [19, 20, 24]. The results of this study show that PCR-based detection of *mecA* may inaccurately identify MRSP isolates that are oxacillin susceptible. Previous studies have suggested that the use of PCR for the detection of *mecA* gene is a fast, accurate and valuable diagnostic tool in areas where MRSA is endemic [25–27]. Additionally, *mecA*-positive staphylococcal strains are considered resistant to all β-lactam antibiotics due to coding for PBP2a. In this study, the isolates were all resistant to penicillin but susceptible to cephalothin and amoxicillin clavulanate acid. The PBP2a assay in this study was not able to detect PBP2a in two *mecA*-positive oxacillin susceptible isolates.

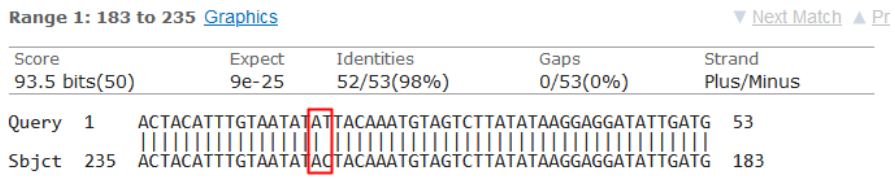


Figure 6.1: *mecA* promoter region of isolates. The single point mutation is represented within the red box.

Table 6-1: The description of *mecA*-positive oxacillin susceptible *Staphylococcus pseudintermedius*

Isolate	**Zone of Inhibition**	**Oxacillin susceptibility**	***mecA* PCR**	***mecA* promoter**	**PBP2a**	***blaZ***	**SCC*mec***	***mecI***	***blaI***	***blaR1***	***mecR1***
FL.10	24	S		-	-			-			-
FL.14	26	S		-	-			-			-
CST.9	20	S					-	-			-
KSU.1	26	S					-	-			-
FL.15	6	R					-	-			-
UWI6	6	R					-	-			-

In a previous study, the Alere PBP2a test accurately detected *mecA*-positive isolates although cefoxitin induction was required prior to testing [28]. Therefore, a combination of phenotypic and genotypic routine detection of MRSP canine isolates should be considered in diagnostic laboratories.

The oxacillin susceptible isolates had an oxacillin zone of inhibition that is greater than eighteen mm following guidelines of the CLSI breakpoint interpretation (S≥18, R≤17) [29]. The *mecA* gene did not confer oxacillin resistance among *mecA*-positive isolates. Differential expression of *mecA* has been observed in isolates with complete regulatory complements (mecI/mecR1 and blaI/blaR1) and those with incomplete or absent regulatory complements [14, 22, 23]. The sequence of the *mecA* promoter region was similar among four isolates with a single point mutation that could influence *mecA* transcription. In previous studies, point mutations created a perfect palindrome in the *mecA* promoter -10 region containing MecI and BlaI binding sites [30]. In *S. aureus*, a high level of expression of *blaI* is responsible for the repression of *mecA* rendering oxacillin-susceptible MRSA without mec regulators [23]. Using whole genome sequences, *blaI*/*blaR1* was detected in all isolates. These results suggest that a functional *blaI*/*blaR1* could be efficient in the expression of *mecA* in *S. pseudintermedius*. The *mecI*/*mecR1* was not detected in all isolates. Similarly, Black *et al*, reported the absence of *mecI* and *mecR1* genes among *mecA* and *blaZ* positive *S. pseudintermedius* isolates [14]. Previous studies in *S. aureus* have suggested that the genes *mecR1* and *mecI* are truncated or lost in all SCC*mec* types except types II, III, VIII and XI [31, 32]. It is important to identify *mecA*-positive oxacillin susceptible isolates since these isolates can become highly resistant under antibiotic selection. It is therefore crucial to understand how *blaI* affects *mecA* expression in isolates that are oxacillin susceptible.

Typing of the SCC*mec* has been a challenge in *S. pseudintermedius* because of their heterogeneity. The SCC*mec*Finder can identify SCC*mec* elements (I to XIII) using a BLAST and K-mer-based approach for *S. aureus* [33]. Using this method, SCC*mec*_type_V and SCC*mec*_type_IVg(2B) were identified from whole genome sequences of *mecA*-positive oxacillin susceptible isolates in this study. However, when using the same database for *S. aureus*, contradicting predictions and additional complexes of the SCC*mec* elements were detected for FL10. Whereas it was not possible to predict any whole SCC*mec* cassette with a template coverage of at least 40% in CST9 and KSU1. There is evidence of vast diversity in structural organization and genetic content of SCC*mec* elements in *staphylococcu*s species [34, 35]. Hence, SCC*mec* in *S. pseudintermedius* are occasionally non-typeable using SCC*mec* typing systems devised for *S. aureus* [36, 37]. Therefore, additional studies and a database for detecting and characterizing SCC*mec* elements in *S. pseudintermedius* are essential.

Conclusions

Isolates that are *mecA*-positive and oxacillin susceptible may go undiagnosed using routine phenotypic tests of *S. pseudintermedius*. The results from this study suggest that the bla regulators are important in the control of oxacillin-susceptible phenotype in *mecA*-positive *S. pseudintermedius*. Perhaps the detection of bla complex by PCR should be included in the identification of *mecA*-positive oxacillin susceptible isolates.

Methods

Oxacillin susceptibility

Isolates were screened for methicillin resistance with the Kirby-Bauer disc diffusion technique using oxacillin disks. Oxacillin (1µg) was used as the predictor of methicillin resistance in *S. pseudintermedius* following guidelines of the CLSI breakpoint interpretation (S≥18, R≤17) [29].

mecA gene PCR

A standard PCR assay as previously described was performed [26] using specific primers (Table 6-2) and sequences were obtained from Eurofins Genomics Sequencing Data and Analysis.

PBP2a culture colony test

Isolates were inoculated onto Columbia agar plates containing 5% sheep blood and incubated overnight at 37 ^{O}C. The Clearview PBP2a *S. aureus* culture colony test was used for rapid detection of PBP2a without induction among *S. pseudintermedius* isolates per manufacturer's instructions [38].

Genome sequencing and assembly

Isolates were inoculated onto Columbia agar plates containing 5% sheep blood and incubated overnight at 37 ^{O}C. Three to four bacterial colonies were suspended in 3 ml of sterile tryptic soy broth and incubated at 37 ^{O}C overnight. A commercial DNA extraction kit was used to extract DNA (UltraClean® Microbial DNA isolation kit, Qiagen). DNA concentrations were measured using nanodrop. DNA libraries were constructed using a Nextera DNA sample prep kit (Illumina, Inc., USA) as per the manufacturer's protocol. The genomes were sequenced using a MiniSeq platform (Illumina, Inc.) to produce single paired end reads of 150 bp in length. Raw reads were assembled into contigs using the SPAdes v3.13.5 de novo assembler [39].

Table 6-2: Primers for conventional PCR

***mecA* promoter**	**Primers**
Forward	5′-CGGACGTTCAGTCATTTCTACTTC-3′
Reverse	5′-ACACCTTCTACACCTTATCAC-3′
mecI	
Forward	5′-AATGGCGAAAAAGCACAACA-3′
Reverse	5′-GACTTGATTGTTTCCTCTGTT-3′
mecR1	
forward	5′-TGGTATTTGGTTTAGTGAA-3′
Reverse	5′-GATTAGGTTTAGGCATTGA-3′
mecA	
Forward	5′-GGTCGGTAATGAAGAAGTCAA-3′
Reverse	5′-TTCTGCTACCGTCTCATCAAAG-3′

Genetic detection

Genome assemblies of MRSP were screened for *mecA* and *blaZ* resistant genes using Resfinder 3.2 by applying the default cut-off settings of 60% coverage and 90% identity https://cge.cbs.dtu.dk/services/ResFinder/. The SCC*mec*Finder was used to detect SCC*mec* elements using default cut-off settings of 60% coverage [33]. The isolates were aligned using the BLASTN algorithm with NCBI for the *mecI* (QNT12818.1), *mecR1* (QNT12817.1), *BlaI* (USK10129.1) and *blaR1* (USK10128.1). AliView was used to evaluate the sequence homology of the gene sequences.

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CHAPTER VII

CONCLUSION

Antibiotic and topical treatments are often used to treat MRSP infections in dogs. However, due to the emergence of resistant strains treatment options for MRSP infections in dogs are rapidly diminishing. The main objective of this doctoral project was to provide surveillance of the molecular epidemiology of *S. pseudintermedius* and provide information that can be used to address the need for new therapeutic strategies to control canine staphylococcal infections. In a previous study, conducted on samples collected ten years ago in the United States, it was found that clonal complexes CC84, CC71 and CC68 are circulating among the MRSP isolates. There has not been an in-depth analysis in the surveillance of MRSP in the United States since the previously reported data in 2010. Thus, this study elucidates changes in the population structure of *S. pseudintermedius* in the United States and provides a basis to understand the stability of the dominant strains. Also, there has not been any report on the presence of MRSP and clonal complexes in South Africa. The changes in the population structure have not been shown to affect *S. pseudintermedius* antigenic and antimicrobial resistance profile as well as host adaptability. Hence, the molecular epidemiology of *S. pseudintermedius* isolated from human samples is important. The first objective of this study was to perform the molecular epidemiology of MRSP to study the temporal changes in population structure and antimicrobial resistance in the United States. Methicillin resistant *S. pseudintermedius* collected across 10 regions in the United States were analyzed using the disk diffusion method, whole genome sequencing, MLST and geoBURST. The key findings in Chapter II identified ST258 as the major clonal founder of MRSP compared to ST68 detected in 2010. In the United States, new sequence types (ST551, ST45, and ST496) with high antibiotic resistance were detected that are driving the changes in the MRSP population structure. The next step was to understand the bacterial population genetics and evolution of *S. pseudintermedius* in the United States. By using MLST data reported from the same regions 10 years ago, we were able to analyze temporal changes in *S. pseudintermedius* over time. The key findings (Chapter III) showed that there has been a shift in the population structure of MRSP in the United States over a ten-year period. GeoBURST and phylogenetic analysis showed that the population in 2010 is genetically diverse to the population in 2021. High recombination rates to mutation ratios indicated that the isolates in 2010 and 2021 have the same probability of diversifying into a large population by recombination compared to mutation. The strains in 2010 and 2021 demonstrated greater nucleotide polymorphism and positive selection at housekeeping genes. Furthermore, the *S. pseudintermedius* isolates collected in 2021 showed significantly higher antimicrobial resistance compared to isolates collected in 2010. The lineage information obtained in this study should be useful to target the development of new therapeutics and prophylactics towards the most commonly occurring strains of MRSP in the United States. Furthermore, the

expansion of predominant lineages could be linked to multidrug resistance profiles and host adaptability. The expansion of the predominant ST identified in the United States can be seen by the detection of similar STs (ST496, ST551, ST71, ST181, and ST45) among dog isolates in South Africa (Chapter IV). The isolates that were assigned STs (ST2232, ST2231, 2228, ST2229, and ST2230) represent isolates geographical unique to South Africa. This was the first study that reported on the prevalence of MRSP among dogs in South Africa. The result in this chapter informs on the ability of MRSP lineages to spread worldwide. It remains an important question, as to how these MRSP lineages arise, whether through HGT from MSSP lineages or through transportation from continents such as North America, Europe, or Asia. The result from this chapter informs on the need for control and surveillance of MRSP in South Africa. Not only are *S. pseudintermedius* lineages geographically dispersed, but they also seem to have adapted to the human host. The results in Chapter V showed that the canine and human isolates share a close phylogenetic relationship. These isolates seem to have similar STs (ST150, ST155, ST181, ST811, ST822, and ST862). Additionally, isolates have similar virulence genes profiles such as *spsM, icaA, icaD, lukF-I,* and *lukS-I*. The human isolates however show high antimicrobial susceptibility compared to canine isolates. But the presence of MGE indicates that HGT of resistance and virulence genes could lead to dissemination of highly resistant and pathogenic strains of *S. pseudintermedius* in humans. The detection of MRSP has important public health implications. In Chapter VI, oxacillin susceptible MRSP isolates were detected. *Staphylococcus* species with *mecA* gene have been linked to MRSP emergence, antibiotic resistance increase, and host adaptability. Therefore, the detection of *mecA* gene among *S. pseudintermedius* isolates is important. The results in this study suggest that the bla regulators are important in the control of oxacillin-susceptible phenotype in MRSP. Therefore, implementation of bla complex in diagnostic laboratories is important.

It is evident from the results in this study that the population structure of *S. pseudintermedius* is changing over time. It is unclear which *S. pseudintermedius* lineage will become successful in the United States. Data representing the prevalence and epidemiology of MRSP in the African continent is still lacking. Whether MSSP in human isolates will become adapted to MRSP through HGT and antibiotic selective pressure remains to be seen. Additionally, oxacillin susceptible MRSP presents a challenge in detection of MRSP in human and animal medicine. The detection of new lineages and host adaptability may present changes in conserved characteristics of *S. pseudintermedius* that can impact the development of alternative therapeutics.

VITA

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