WHOLE-GENOME SEQUENCING, QUORUM SENSING, AND BIOFILM FORMATION OF *STAPHYLOCOCCUS PSEUDINTERMEDIUS*

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2019

Major Subject: Biomedical Sciences

ABSTRACT

Staphylococcus pseudintermedius is a major opportunistic canine pathogen that has been associated with an increasing number of human infections. As an opportunist, *S. pseudintermedius* causes a wide range of infections, predominantly of skin and soft tissue but also device/implant related infections and systemic infections such as bacteremia. Increasing methicillin and multiple-drug-resistance in the species complicate treatment of *S. pseudintermedius* infections, which are often already difficult to treat due to the bacteria's ability to produce biofilms that hamper effective use of antimicrobial drugs. Biofilm formation and virulence factor expression in *Staphylococcus* is regulated, in part, by the accessory gene regulator (*agr*) operon. Polymorphisms in this operon result in four distinct *agr* groups (I, II, III, and IV) in *S. pseudintermedius*. In *S. aureus*, *agr* groups are associated with infection type, virulence factor carriage, and phylogenetic relationships. This operon has shown promise as a therapeutic target in *S. aureus*. Similar relationships remain largely unexplored in *S. pseudintermedius*. Furthermore, the pathogenic differences in infections caused by *S. pseudintermedius* between canines and humans and the characterization of isolates causing human infections in general are substantively lacking. Biofilm characterization was performed on 710 clinical isolates from healthy and diseased canines. We found that the majority of clinical isolates from dogs produce biofilm, while a type of biofilm utilizing slime was found to be associated with isolates causing disease. In order to explore the associations of *agr* groups of *S. pseudintermedius*, whole-genome-sequencing was performed on 160 isolates collected from four groups (pyoderma infections, surgical infections, urinary tract infections, and healthy colonization). We found that *agr* group II isolates were significantly associated with healthy colonization rather than disease, and were less likely to be multiple-drug-resistant or carry multiple toxin genes. The predominant methicillin-resistant sequence types were identified for the

geographic region (Texas) including ST64, St68, ST71, ST84, ST150, and ST155. Four isolates from human bacteremia infections and four from canine bacteremia infections were sequenced and complete, or near-complete, genome assemblies were achieved and published, allowing for the examination and comparison of virulence factors associated with both human and canine invasive infections. While statistical significance was not seen with the small sample size, pan-genomic analysis did allow us to identify genes unique to the isolates causing human infections.

ACKNOWLEDGEMENTS

I would like to thank my committee chair and advisor, Dr. Sara Lawhon, and my committee members Dr. Höök, Dr. Cai, and Dr. Cohen for their support throughout my Ph.D. I would also like to thank Dr. Andrew Hillhouse and Mr. Kranti Konganti for their patience and support with the sequencing and bioinformatic analysis performed throughout my Ph.D. Furthermore, I extend my gratitude to Drs. Carey-Anne Burnham, Jennifer Dien Bard, Romney Humphries, and Lars F. Westblade for access to strains and advice for Chapter V. Finally, I want to express my deep appreciation for all the emotional support and laughter provided by my labmates Elena, Laura, and Mary; my family Ed, Kim, and Natasha; my boyfriend Ryan; and Alyssa.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of advisors Associate Professor Sara Lawhon, Associate Professor James Cai, and Professor Noah Cohen of Texas A&M University, along with Professor Magnus Höök of the Texas A&M Health Science Center Institute of Biosciences and Technology Center for Infectious and Inflammatory Diseases.

The statistical analyses for Chapter III and IV were conducted by Professor Noah Cohen of the Department of Large Animal Clinical Sciences. Library preparation, DNA quality control, MinION sequencing, and Illumina MiSeq sequencing of the bacterial isolates detailed in Chapters IV and V were performed by Research Assistant Professor Andrew Hillhouse of the Department of Veterinary Pathobiology in the Molecular Genomics Workspace.

All other work conducted for the dissertation was completed by the student independently.

Funding sources

Funding for this project was provided by Veterinary Orthopedic Society and the Morris Animal Foundation Pilot Data Grant D15CA-833: Quorum Sensing in *Staphylococcus pseudintermedius*. Sara Little was supported by a Texas A&M Diversity Fellowship and also received the CVM Graduate Trainee Grant. The contents of this work are solely the responsibility of the authors and do not necessarily represent the official views of the Morris Animal Foundation or the Veterinary Orthopedic Society. Funding for all laboratory supplies and support were provided by the laboratory funds of Associate Professor Sara D. Lawhon. Dr. Lawhon receives funding from FDA through grants FD004638 and FD006171 for clinical diagnostic work which partially supported the creation of the *Staphylococcus pseudintermedius* isolate bank.

NOMENCLATURE

AGR	Accessory gene regulator
AIP	Autoinducing peptide
BHIB	Brain heart infusion broth
BIGSdb	Bacterial Isolate Genome Sequence database
BUSCO	Benchmarking Universal Single-Copy Orthologs
CC	Clonal complex
CRA	Congo red assay
CVA	Crystal violet microtiter plate assay
MALDI-TOF	Matrix-assisted laser desorption and time-of-flight mass spectrometry
MDR	Multiple-drug-resistant
MGE	Mobile genetic element
MLS	Macrolides, lincosamides, and streptogramins
MLST	Multi locus sequence type
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
MSSP	Methicillin-susceptible *Staphylococcus pseudintermedius*
NCBI	National Centre for Biotechnology Information GenBank database
PATRIC	Pathosystems Resource Integration Center
PFGE	Pulsed-field gel electrophoresis
PIA	Polysaccharide intercellular adhesin
rMLST	Ribosomal multi locus sequence type

SCC*mec*	Staphylococcal cassette chromosome *mec*
SIG	*Staphylococcus intermedius* group
ST	Sequence type
TIGSS	Texas A&M Institute for Genome Sciences and Society
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VMTH	Veterinary Medical Teaching Hospital
WGS	Whole genome sequencing

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CHAPTER I

INTRODUCTION

Background

In *Staphylococcus pseudintermedius* very little is known about the *agr* groups and their associations with virulence factors, types of infection, and phylogenetic relationships. The complex nature of biofilm regulation and its association with infection and colonization of healthy animals requires further investigation; whether biofilm formation is associated with *agr* group, as the *agr* operon is known to influence biofilm dispersion, is unknown. *Staphylococcus pseudintermedius* is increasingly being found in human infections; the characterization of such isolates and investigation into whether there are phenotypic or genetic pathogenic differences between isolates infecting these two species are needed. To address these questions, the dissertation is divided into 3 aims, each comprising a chapter of the dissertation.

Specific Aims

AIM 1: Measure biofilm forming capabilities of 710 *S. pseudintermedius* isolates collected from canines presenting with and without clinical disease to the Texas A&M University Veterinary Medical Teaching Hospital (VMTH), and classify them based on biofilm production.

AIM 2: Complete whole-genome sequencing and assembly of 160 clinical isolates. Determine the *agr* type of 160 isolates collected from dogs presenting with surgical infections, pyoderma, urinary tract infections, and healthy dogs that presented to the VMTH. Determine the

multi locus sequence type (MLST) of all clinical isolates sequenced. Determine whether *agr* type is associated with disease type, MLST, or biofilm production.

AIM 3: Complete whole-genome assembly of 8 bacteremia isolates (four canine and four human) using a combination of long- and short-read sequencing technologies. Determine whether there are any describable differences between canine or human isolates regarding *agr* type, MLST, and virulence factors.

CHAPTER II

LITERATURE REVIEW

Overview of *Staphylococcus pseudintermedius*

Staphylococcus species are well known both as commensal bacteria and as significant pathogens in humans and animals. These gram-positive, nonmotile bacteria cause infections ranging from mild skin infections to bacteremia, necrotizing pneumonia, and toxic shock. As antibiotic resistance spreads, challenges to both human and animal health are increasing. In 2017, following the emergence of vancomycin -intermediate and -resistant strains, the World Health Organization labeled methicillin-resistant *Staphylococcus aureus* (MRSA) as a high-priority pathogen. In the past twenty years, health care-associated infections, especially infective endocarditis and prosthetic device infections, have been increasing alongside an epidemic of community-associated skin and soft tissue infections caused by drug resistant strains of *S. aureus* (163). Concurrently, *Staphylococcus* infection and antimicrobial resistance in animal species is increasing, posing concerns not only for the health of our pets and livestock, but for increased treatment costs and potential human infections (95, 136). *Staphylococcus pseudintermedius* is a coagulase-positive, opportunistic pathogen that colonizes many different species of animals and is a major pathogen in dogs. It is the primary cause of canine pyoderma and the culprit of a wide range of infections, including bacteremia, device-related, and post-surgical infections. Increasing resistance to antimicrobial agents has been observed, with methicillin-resistant *S. pseudintermedius* (MRSP) becoming a widespread challenge to antimicrobial treatments. While rare, human infections caused by *S. pseudintermedius* have been observed and, as bacterial

identification methods improve, more isolates are being identified as agents of human infection, including those previously misidentified as *S. aureus* (14, 21, 85, 94, 143, 156, 187, 188).

Population Structure

Staphylococcus pseudintermedius is part of the normal flora of dogs. It is a commensal that is cultured at high frequencies from the nares, oropharynx, and perineum of dogs. Cutaneous microbiome analysis shows this species is present in low numbers at most body sites in all dogs tested and constitutes 90% of staphylococci isolated from healthy carriers and from dogs with underlying skin disease (46, 61, 67). The majority of dogs appear to be persistent carriers, and such carriage is associated with higher numbers of *S. pseudintermedius* at the colonization sites (9, 68). The genetic diversity of strains isolated from different anatomical sites of the same dog is high (12, 46, 68, 128, 129, 139). This diversity is lower in dogs afflicted with atopic dermatitis, where isolates from different body sites are more likely to be related or identical using pulsed-field gel electrophoresis (PFGE) (46). In dogs with infected lesions of atopic dermatitis, superficial pyoderma, or otitis externa, the majority of the isolates from infection sites are either closely related or identical to isolates found in the mucosa (46, 128, 139), suggesting a link between colonization and infection similar to that seen in humans with *S. aureus* (182).

Unlike *Staphylococcus aureus*, the population structure of *S. pseudintermedius* is extremely heterogeneous. There is a great variability of PFGE patterns observed in isolates from both healthy and diseased dogs (46, 124). Over the past decade, methicillin-resistant and multi-drug resistant strains have emerged worldwide through the horizontal transfer of resistance plasmids into successful methicillin-susceptible *S. pseudintermedius* (MSSP) lineages, forming large MRSP clonal complexes (CCs) that dominate specific geographic regions (45). These MRSP

lineages are an exception to the otherwise high diversity of *S. pseudintermedius* isolates, although significant diversity has been discovered within the CCs (151).

Multi Locus Sequence Typing

Multi locus sequence typing (MLST) is the predominant DNA sequenced-based technique used for analysis of population structure and epidemiological trends of *S. pseudintermedius* worldwide. Prior to 2005, any non-pigmented, coagulase-positive, hemolytic staphylococci isolated from dogs were considered *Staphylococcus intermedius*, which included staphylococci colonizing a wide range of animals including dogs, horses, skunks, weasels, raccoons, bears, minks, camels, and pigeons (66). High diversity within *S. intermedius* led investigators to hypothesize multiple species were being categorized together; after thirty years, *S. intermedius* was determined to be made up of three distinct species: *Staphylococcus delphini* (which can further be divided into groups A and B), *Staphylococcus intermedius*, and *Staphylococcus pseudintermedius*; known collectively as the *Staphylococcus intermedius* group (SIG) (8, 42, 140). In 2007, Bannoehr *et al.,* proposed the first 5-loci MLST scheme for SIG isolates, which determined two major MRSP clones at that time: ST68 in North America and ST71 in Europe (126). In 2013, Solyman *et al.,* published the first species specific MLST scheme, consisting of 7 genes, for *S. pseudintermedius* with a publicly available database on the online Bacterial Isolate Genome Sequence Database (BIGSdb) genomics platform (http://pubmlst.org/spseudintermedius/) (151).

In 2016, a comprehensive review of published, sequence-typed *S. pseudintermedius* isolates determined that MRSP represented 76% (n = 1087) of isolates characterized by the 7-gene MLST scheme and found high genotypic diversity among isolates (130). The high representation

demonstrates reporting bias toward MRSP isolates, which are more clinically relevant yet make up a minority of *S. pseudintermedius* isolates. The Pires dos Santos review found that MRSP isolates are associated with a variety of different sequence types (STs) that are often clustered with the more genetically heterogeneous MSSP STs. The major group and subgroup founders (ST45, ST68, ST112, ST258, *etc*.) appeared as distinct phylogenetic lineages that do not share a recent common ancestor and were categorized into CC including the founder and single, double, and triple locus variants. At the time of the review, the majority of reported MRSP isolates were recovered from Europe (70.6%), followed by 22.4% from Asia and only 6.2% from North America. The most widely disseminated clone was found to be ST71, which successfully spread across 14 countries in 3 continents. CC71 and CC258 were most frequently found in Europe (93% and 98% respectively), while CC68 was the most common complex in North America (66.7%) and CC45 and CC112 were the most common in Asia (59.5% and 48.9% respectively).

Ribosomal MLST (rMLST) examines the allelic variation in 53 ribosomal genes to type any genus and species of bacteria. Most *Staphylococcus* spp. contain two copies of the *rpsN* and *rpmG* genes from this scheme, which are consequently excluded for typing (n = 51 genes for *Staphylococcus* rMLST) (79, 105). This is a newer method of typing than MLST, and there are limited rMLST reported for *S. pseudintermedius* in BIGSdb, with some predominant rMLST correlating with major ST; rMLST 17490 (ST71), 17491 (ST68), and 48490 (ST84).

Commensal to Pathogen: Virulence Regulation

Regulatory systems

Staphylococcus pseudintermedius is a commensal colonizer of most dogs and does not act as a pathogen until the resistance of its host is lowered and the skin barrier impacted, often by

predisposing factors (e.g. atopic dermatitis, immunosuppressive disorders, and medical or surgical procedures) (180). *Staphylococcus pseudintermedius* virulence is tightly regulated by several systems in order to coordinate the shift from commensal to pathogen. One of the main regulatory quorum sensing systems of *Staphylococcus* virulence is the accessory gene regulatory system (*agr*). While this is not the only regulatory system in *Staphylococcus* species, *agr* is considered a main global regulator of virulence. It regulates over 70 genes, many of which have been shown to be virulence factors, as well as directly impacting several other global regulators (55).

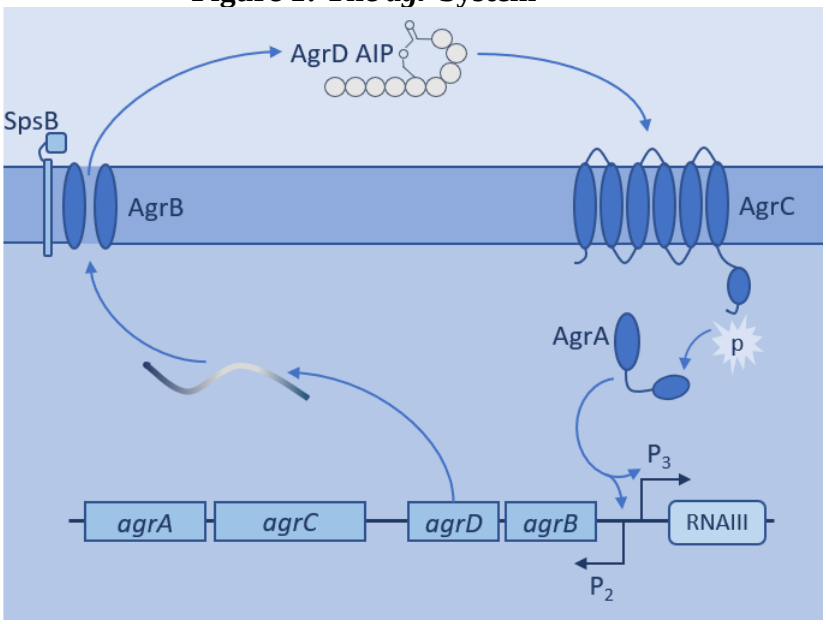
In *S. aureus,* the *agr* system not only has direct effects on virulence through activation and repression of toxins and cell adhesion molecules, as will be described in the next section, but also indirect effects by interacting with other regulatory systems. The mRNA effector molecule of *agr*, RNAIII, directly binds to the *rot* global regulator and results in the upregulation of exoproteases, lipase, urease, enterotoxins, leukocidins, alpha-toxins, methicillin resistance, and downregulation of protein A and oligopeptide permease among other surface proteins (19, 134). SarR and SarS are regulators that repress certain virulence genes, and RNAIII binds to several Sar proteins and acts as an anti-repressor (5). RNAIII also represses *spa*, which codes for the surface protein A, and mediates the inhibition of translation and the degradation of stable *spa* mRNA by RNase III. In a similar dual action manner, RNAIII binds to the mRNA of *coa*, which codes for staphylocoagulase, a virulence factor promoting the clotting of human plasma, and represses the synthesis of the molecule as well as degrading the mRNA (28). The *mgrA* global regulator in *S. aureus* affects over 350 genes involved in virulence, including antibiotic resistance, autolysis, and biofilm formation; RNAIII binds to and stabilizes the regulatory molecule of this system (65). This list is hardly exhaustive; *S. aureus* has at least 16 two-component regulatory systems identified so far, with many interactions between them. Furthermore, these regulatory systems respond to a wide range

of environmental stimuli, including oxidative stress, host serum, nutrient availability, iron, glucose, and hemoglobin (74, 106, 116, 135, 144, 159, 190). As a result, gene regulation in Staphylococcal species consists of a multitude of overlapping and interacting feedback networks (5). The *agr* system is perhaps the most well understood and one of the few that has also been well defined in *S. pseudintermedius*. It is also the focus for most therapeutic intervention studies targeting quorum sensing systems in *S. aureus*, which suggests it has potential as a therapeutic intervention in *S. pseudintermedius* as well.

The accessory gene regulator system

The *agr* locus generates two divergent primary transcripts, RNAII and RNAIII, which are transcribed in opposite directions under the control of the P2 and P3 promoters, respectively. The P2 promoter directly controls the transcription of four proteins necessary for the quorum sensing mechanism: AgrA, AgrB, AgrC, and AgrD, while P3 controls the transcription of genes affecting virulence. The system is summarized in Figure 1.

Figure 1: The *agr* System



The two-component *agr* quorum sensing system. The *agr* operon encodes the AgrD propeptide, the AgrB transmembrane protein which removes the AgrD C-terminus and cyclizes the peptide into a lactone ring structure called the autoinducing peptide (AIP), the AgrC transmembrane sensor kinase which is autophosphorylated in response to binding by AIP, and the response regulator AgrA which acts at promoters P2 and P3 to induce transcription of RNAII and RNAIII which regulates biofilm formation and virulence factors.

The AgrD propeptide consist of 45 amino acids and is the precursor of the final AIP. The propeptide consists of the N-terminal amphipathic leader, a center 7-12 amino acid region that will form the final AIP molecule, and a C-terminal region. The N-terminal leader localizes the propeptide to the cytoplasmic membrane, where the transmembrane protein AgrB will remove the C-terminal tail and cyclize the remaining peptide. In *S. aureus*, the AIP region contains 8-9 amino acids, with a cysteine in the 5th position from the carboxyl terminal and will form a thiolactone ring structured AIP (109). In *S. pseudintermedius*, the AIP region contains 9 amino acids with a 5th position serine, resulting in a lactone ring structured AIP (29). Following cyclization, the AgrD peptide is released from AgrB, transported to the outer face of the cellular membrane, and the N-terminal is removed by type I signal peptidase SpsB to form the final AIP (84). At sufficient density, the AIP molecule is detected by the receptor kinase AgrC (98). Receptor binding of AIP

results in autophosphorylation of AgrC, which activates the response regulator AgrA via trans-phosphorylation. AgrA forms a phosphorylated homodimer to induce the transcription of P2 and P3, with a higher affinity for P2 (54, 88).

The P3 promoter controls the upregulation of secreted virulence factors and down regulation of surface proteins, such as protein A encoded by *spa*, via RNAIII (148). RNAIII also acts as a messenger RNA and encodes the *hld* gene for delta-toxin (δ-haemolysin) as well as increasing the production of proteases and capsule synthesis (75, 76, 113, 114).

In *S. aureus*, the *agr* operon positively regulates various toxins and virulence factors while negatively regulating surface proteins and consequently enhancing biofilm detachment. The *agr* system is activated by AIP during the transition from exponential growth to the stationary phase. During the initial stage of infection, cell density is low and the *agr* system is repressed, allowing the production of adhesive cell surface proteins to promote colonization. This repression of the system is necessary for biofilm formation, and enhanced adhesion is seen in mutants with nonfunctional *agr* systems (173). As the cellular density increases, AIP production passes the threshold and activates the *agr* system, resulting in the production of exoproteins and membrane-damaging toxins that aid in biofilm detachment, spread, and upregulation of bacterial cells (20, 93, 173).

Autoinducing peptides: alleles of agrD

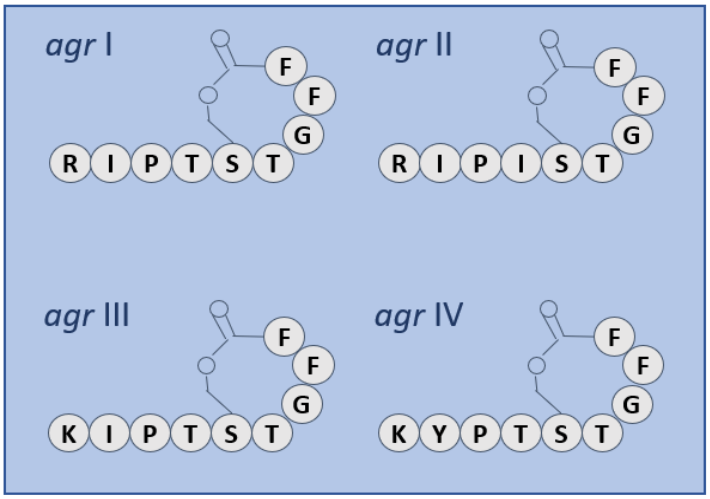
Polymorphisms in the AIP region of AgrD and corresponding regions of the AgrB and AgrC proteins have resulted in the classification of different *agr* groups in staphylococcal species. Four AIP allelic variants have been identified in *S. aureus* with many more identified in other staphylococci species. Four variants (I – IV), distinct from those of other *Staphylococcus* species,

have been identified in *S. pseudintermedius* (8, 159). The four *agr* groups of *S. aureus* demonstrate competitive inhibition to AgrC binding (54, 78, 118). Similar cross-inhibition and cross-activation has been demonstrated in other species of *Staphylococcus,* as well as between different species, suggesting that these interactions may be involved with niche competition and colonization (90, 120).

Two hypotheses for *agr* diversity have been proposed. The first suggests that *agr* interference may result in isolated bacterial populations due to decreased genetic exchange, niche competition, and evolutionary diversification; this is supported by the frequent association of the *agr* alleles with specific genotypes. The second hypothesis suggests that *agr* diversity may result in QS systems with differing signaling properties and consequent virulence regulation, supported by the fact that there are differences in the timing and magnitude of P3 signal generation and unique cell density response points for the four *agr* alleles in *S. aureus* dependent on the variability of the *agrBDCA* genes (54).

In *Staphylococcus pseudintermedius*, four unique *agr* alleles have been identified, as shown in Figure 2: RIPTSTGFF (I), RIPISTGFF (II), KIPTSTGFF (III), and KYPTSTGFF (IV) (8, 159). Relationships between these four *agr* types, types of infections, and virulence factor carriage are poorly understood in this species. Further, while inter- and intra-species cross interference effects have been shown to exist with *agr* alleles of *S. aureus* and *S. epidermidis*, these have not been well examined for *S. pseudintermedius agr* alleles. Downregulation of *S. aureus* RNAIII by *S. pseudintermedius* AIP-containing supernatant has been observed, but the *agr* groups of these isolates were unknown (24).

Figure 2: *S. pseudintermedius* AIP Peptides



S. pseudintermedius has 4 unique *agrD* alleles resulting in 4 distinct, lactone-ring structured autoinducing peptides.

Virulence Factors

Biofilms and adhesion molecules

One of the major virulence factors in staphylococci is the ability to form biofilms: layers of densely packed cells embedded in an extracellular matrix of secreted polysaccharide intercellular adhesion (PIA), proteins, and DNA that allow the bacteria to adhere to surfaces, including implanted devices and catheters. Bacterial cells embedded in biofilms benefit from enhanced antimicrobial resistance, potentially due to decreased metabolic activity and cell division, both of which are targets of many antibiotics (32). Bacteria embedded in biofilms can withstand antibiotic concentrations up to 1000-fold higher than their planktonic counterparts (26) and have a higher resistance to host immune responses and chemotherapies (96, 123). As 80% of chronic bacterial infections are biofilm associated, this is a major clinical concern (39). In fact, there is almost a direct correlation between the emergence of *S. epidermidis* as a pathogen and its biofilm-dependent capacity to colonize indwelling medical devices (172). Disrupting biofilms in

S. aureus re-sensitizes MRSA to β-lactam antibiotics, an important step to improving treatment of these infections (191). While biofilm formation is less well-characterized in *S. pseudintermedius,* recent studies have shown that the majority of *S. pseudintermedius* isolates from canine infections are able to produce biofilms (150, 155).

Staphylococcal biofilms initially form through adherence of the bacterial cells to a biotic or abiotic surface followed by growth-dependent accumulation of cells. This results in multiple layers of bacterial cells embedded in a slime matrix. Initial attachment often involves the secretion of PIA, which is a main component of the slime matrix. PIA-producing isolates are known as slime-forming isolates. The production and secretion of PIA is controlled by the *icaADBC* operon, and mutations in the operon result in inability or reduced capacity to form biofilms in both *S. aureus* and *S. epidermidis* (35, 69). This operon is tightly regulated, and co-expression of *icaA* and *icaD* results in increased PIA production (2). These genes are found in a majority of staphylococcal isolates; in *S. pseudintermedius*, *icaA* and *icaD* homologs are found in 78% and 91%, respectively, of isolates examined (25, 150).

PIA-independent biofilms also exist in staphylococcal species and there are many other important biofilm molecules, including autolysins (Atl and AtlE), accumulative-associated protein (Aap), biofilm associated protein (Bap), and Bap homologue protein (Bhp) that offer alternative, non-PIA associated mechanisms for biofilm formation in *S. aureus* and *S. epidermidis* (30, 36, 47, 51, 164). The most prevalent group of surface proteins that are involved in staphylococcal adhesion are known as microbial surface component recognizing adhesive matrix molecules (MSCRAMMs), consisting of two adjacent IgG-folded domains that allow them to adhere to components of the host's extracellular matrix, which is crucial not only for biofilm formation, but also for the invasion of host cells (100, 112). MSCRAMMS of *S. pseudintermedius* are less well

characterized than those in *S. aureus*; the *S. pseudintermedius* surface proteins A-R (SpsA-SpsR) are a collection of predicted cell-wall-anchored surface proteins. SpsD and SpsL (homologous to *S. aureus* FnBPA and FnBPB) adhere to fibrinogen, fibronectin, and, for SpsD, cytokeratin and are expressed during infection. Further, SpsD, SpsL, and SpsO are known to mediate adherence to canine corneocytes (9). In 2016, a comparative study reported *S. pseudintermedius* as the only staphylococcal species that significantly adheres to human fibronectin and invades the human osteosarcoma-derived MG-63 cell line with higher rates than *S. aureus*, mediated through SpsD and SpsL (103, 127).

Biofilm regulation

Staphylococcal biofilms have a complex system of regulation that is impacted by *agr* and other global transcriptional regulators, including the staphylococcal accessory regulator (*sarA*), as well as environmental cues. Similarly, biofilm dissemination involves active mechanisms to return bacteria to a planktonic state, using both environmental cues and multiple regulatory systems.

The exact nature of the relationship between *agr* and biofilm is complex. This relationship has been most comprehensively studied in *S. aureus*, but there is significant conflict in the literature. In some strains (RN6390B), biofilms can only be formed after *agr* has been deleted or suppressed (13, 166), while in other strains *agr* is required. *Staphylococcus aureus*'s glucose-induced biofilm formation is downregulated by *agr* when grown on polystyrene plates (173), but *agr* was found to both promote and inhibit glucose-induced biofilm on polystyrene in another study (31). When the level of AIP signal is artificially controlled, researchers found that the entire biofilm could be dispersed in both *S. aureus* and *S. epidermidis* (20, 175). The *agr* system is generally considered necessary for biofilm dispersal, allowing the bacteria to spread to new sites

during an infection; close examination of *S. aureus* biofilms show pockets with activated *agr* systems, resulting in regions of the biofilm with active cells leaving the biofilm to colonize a new site (175, 189). Additionally, bacteria dispersing from biofilms show high levels of *agr* activity, while bacteria embedded in biofilms show repressed *agr* systems (189). This is due in part to *agr* activation resulting in the transcription of proteases important to biofilm dispersal and inhibition (20).

Antimicrobial resistance

Methicillin and β-lactam Resistance: Methicillin was introduced in 1960 to treat infections caused by Gram-positive bacteria that produced beta-lactamase or penicillinase, an enzyme that rendered penicillin inactive. The first methicillin-resistant *S. aureus* was identified in 1961. Because of significant side effects of therapy with methicillin such as interstitial nephritis, alternatives were developed and methicillin has been replaced by other drugs, however the description "methicillin-resistant" has remained in use to describe resistance to all penicillin-family drugs. Resistance is conveyed by *mecA*, which codes for alternative penicillin-binding protein PBP2a and is located on the mobile genetic element (MGE) staphylococcal cassette chromosome *mec* (SCC*mec*), and *blaZ*, encoding a β-lactamase that can be carried on a plasmid or chromosomally integrated. Both of these resistance genes can be horizontally transferred between species of *Staphylococcus* (180). Prior to 1999, the reported frequency of MRSP in canine isolates was under 5% (59). The first *mecA* positive strain was reported in a canine isolate from the US in 1999 (59) and has since been increasingly reported worldwide and recognized as a serious health problem (81, 180). There are a limited number of MRSP clones spread worldwide, similar to the distribution and dissemination of MRSA (62). MRSP is widespread, and many, if

not the majority, of MRSP isolates are multiple-drug-resistant (MDR) (56, 57, 181), with resistance in nearly all classes of antibiotics and often mediated by resistance genes carried by MGE (82). There has been an increasing trend of resistance to penicillinase-labile penicillins since the 1980s in MSSP, likely as a result of the spread of *blaZ* (111). Cephalosporin remains one of the only classes of β-lactams for which no resistance genes have been seen (181).

Tetracyclines: Tetracyclines (including doxycycline, minocycline, and tigecycline) function by inhibiting protein synthesis by reversibly binding to the 30S ribosomal subunit and preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. There are several tetracycline resistance genes (*tet* genes) found in bacteria, which function as either efflux pumps or as ribosomal protection, and the majority are associated with conjugative or mobile genetic elements. In *Staphylococcus*, *tet*(K) and *tet*(L) code for efflux pumps while *tet*(M) and *tet*(O) code for ribosome protective proteins, with *tet*(M) and *tet*(K) being the most common (82, 126). The *tet*(M) gene confers resistance to all tetracyclines (16) while *tet*(K) does not confer minocycline resistance; one study found 26% of isolates were doxycycline resistant but minocycline susceptible, with 69% of MRSP isolates susceptible to minocycline (179).

Macrolides, Lincosamides, and Streptogramins (MLS): Macrolides (including erythromycin, roxithromycin, azithromycin, and clarithromycin) act by inhibiting protein synthesis via binding to the 50S ribosomal unit with a variety of targets and efficiency. Lincosamides and streptogramins both bind to the 23S area of the 50S bacterial ribosomal subunit, interfering with protein synthesis and consequently preventing replication. In *S. pseudintermedius*, *mrs*(A) acts as an ABC transporter that can export macrolides and streptogramin B antibiotics while *erm*(A), *erm*(B), and *erm*(C) encode rRNA methylases conferring resistance to macrolides, lincosamides, and streptogramin B antibiotics. The *erm*(B) gene is most common, and tends to be

constitutively expressed (58, 126). The *Inu*(A) gene in *S. pseudintermedius* codes for a lincosamide nucelotidyl-trasnferase, which is less common and often carried with *erm*(B). These genes are often carried on MGE.

Chloramphenicol: A bacteriostatic antimicrobial, chloramphenicol inhibits protein synthesis by binding to the 23S rRNA of the 50S ribosomal subunit and preventing peptidyl transferase activity. Resistance to chloramphenicol is less common, but still clinically relevant in *S. pseudintermedius,* where cat_{pC221} is the most common resistance gene, encoding a chloramphenicol acetyltransferase that is carried on 3-4 kb plasmids (60, 145, 146).

Aminoglycosides: Aminoglycosides inhibit protein synthesis by binding to the 30S ribosomal subunit in bacteria. In *S. pseudintermedius*, *ant(6')-Ia* encodes a streptomycin adenyltransferase that is encoded on plasmids and found chromosomally integrated and conveys resistance to streptomycin (18), while *aac(6')-Ie-aph(2')-Ia* encodes an aminoglycoside acetyltransferase and is found on MGE. Another common aminoglycoside resistance gene is *aph(3')-III,* which is a transposon encoded aminoglycoside phosphotransferase. Frequency of each of these resistance mechanisms varies with one study finding that 75% (24/32) of amikacin resistant isolates carried *aph(3')-IIIa* followed by *aac(6')/aph(2'')* in 12% (4/32) of isolates and *ant(4')-Ia* in 3% (1/32) of isolates (57).

Trimethoprim: Binding to dihydrofolate reductase, trimethoprim inhibits bacterial DNA synthesis by preventing the synthesis of thymidine. The gene *dfrG* is often carried on a plasmid and encodes a dihydrofolate reductase that is resistant to trimethoprim binding. This resistance gene is frequently seen in MRSP isolates (126).

Other Resistances: This summarizes the most common resistances in *S. pseudintermedius* that will be examined in the following chapters, but resistance to fluoroquinolones, rifampicin, mupirocin, and others are also seen in this species (6, 41, 56, 107, 126, 142).

Toxins

Staphylococcal species have a plethora of toxins that fall into three main groups: exfoliative, membrane damaging, and superantigens. In *S. aureus*, these toxin genes can be horizontally transferred between *Staphylococcus* isolates and species and are often found on large MGE including pathogenicity islands, integrated prophages, or plasmids (1). *Staphylococcus pseudintermedius* infections in both canines and humans are often severe as a consequence of tissue necrosis (178). Isolates show virulent behavior on non-professional phagocytic cells including invasion, persistence, and cytotoxicity in canine keratinocytes, human epithelial cells and human osteoblasts (102, 127). Secreted virulence factors including delta-toxin and Luk-I leukocidin induce cytotoxic effects, although fewer toxins have been identified and confirmed compared to *S. aureus* (102).

Exfoliative Toxins: The staphylococcal exfoliative toxins consist of secreted serine proteases that may facilitate infection by cleaving the cell-to-cell adhesion molecule desmoglein (Dsg-1) in the skin (77). These toxins are involved in cutaneous infections in mammals, such as bullous impetigo and staphylococcal scaled-skin syndrome. Four exfoliative toxins have been identified in *S. pseudintermedius*, but their potential for horizontal gene transfer is undetermined. The *speta* toxin gene is found almost universally among *S. pseudintermedius* isolates (10, 15, 34). Of similarly high carriage is the *siet* toxin (10, 15, 33, 34), which has also been found to induce cell rounding in cultured epithelial cells (161). The clinical impact and biological relevance of this

toxin is still under dispute, as dogs injected with SIET developed clinical signs similar to pyoderma in one study (161), while a later study found no such effects (72). The last two exfoliative toxin genes, *expA* and *expB*, have more variable carriage (15). Formerly *exi*, the *expA* gene encodes the toxin EXPA, found to cause intraepidermal splitting in mouse and canine skin (52, 72). Similarly, the EXPB toxin was found to cause intraepidermal splitting and degradation of Dsg-1 in beagle dogs (73).

Membrane Damaging Toxins: Several staphylococcal toxins, including α-hemolysin, leukocidins, and phenol-soluble modulins are pore-forming, causing damage to the cellular membrane of host cells that leads to cellular lysis and tissue necrosis (63). Leukocidins in *S. aureus* are associated with recurrent soft tissue infections and necrotizing pneumonia, contribute to immune system evasion by lysing a variety of immune cells, and are generally encoded on prophage (63). In *S. pseudintermedius*, the bicomponent Luk-I leukocidin, encoded by *lukS* and *lukF*, is found in the vast majority of isolates and displays high cytotoxicity for polymorphonuclear neutrophils (131). Recent research suggests this toxin exhibits a tropism for myeloid cells expressing the CXCR2 receptor (102). Delta-toxin, encoded by *hld*, is a phenol-soluble modulin found in the vast majority of strains that may act as a surfactant and limit the aggregation of cells into a biofilm (173). It is secreted at high levels by clinical strains *in vitro* and is cytotoxic to non-professional phagocytic cells (specifically osteoblasts) in a dose-dependent manner (102). The phenol-soluble modulin PSMε is similarly highly represented, secreted, and cytotoxic to osteoblasts (102). In contrast, BacSp222 is a bacteriocin-like toxin that has been found only rarely in *S. pseudintermedius* and that lyses other Gram-positive bacteria (184).

Superantigens: Superantigens indiscriminately stimulate T-lymphocytes through the activation of major histocompatibility complex class II molecules, bypassing the need for antigen

presentation and resulting in a flood of cytokines. This cytokine storm can cause shock and organ failure in hosts. Two possible enterotoxin superantigens have been identified in *S. pseudintermedius*: sec_{canine} and se-int (44, 53). While the clinical importance of se-int remains unknown, sec_{canine} has been shown to induce vomiting and T-cell proliferation (44, 70).

Virulence profiles of clonal complexes

The major clonal complexes of MRSP display differences in virulence gene carriage patterns, with some CCs displaying conserved virulence gene profiles (CC45) while others show higher diversity (CC71 and CC258), suggesting that the distribution of resistance and toxin genes are correlated both with clonal spread as well as horizontal gene transfer within this species (126, 181). While antibiotic resistance profiles have been well examined in the three largest CC (CC45, CC71, and CC258), profiles of other CC have not displayed significance or have not been well examined. Toxin gene carriage and biofilm formation within clonal complexes have also not been well established.

Isolates of ST71 generally display high resistance; a multicenter study found that 74% of MRSP isolates belonged to ST71, and of those 87% were resistant to at least 6 classes of antimicrobials (126). ST71 isolates typically carry *tet*(K) if they demonstrate doxycycline resistance, while *tet*(M) is rarely seen in this group. In contrast, non-CC71 lineages were significantly associated with carriage of *tet*(M) alone (130). Tetracycline resistance overall was lower in CC71 than isolates of CC258 and CC45 (130). One study found 15 different resistance patterns within isolates of this CC, and additionally determined ST71 to be the most likely to demonstrate discrepancies between antimicrobial resistant genotypes and phenotypes (181). CC71

isolates were also found to produce significantly more biofilm compared with the other major groups (117).

Isolates of CC258 were found to display fewer resistance patterns with high variability (126). Isolates of ST258 generally show lower prevalence of resistance to gentamicin and enrofloxacin compared to the other groups, and have a higher prevalence of resistance to trimethoprim and sulfamethoxazole (130). Chloramphenicol and tetracycline resistance were also lower within this CC (130).

In contrast to CC258 and CC71, strains in CC45 display a very conserved pattern of resistance gene carriage of *ant(6')-Ia, aac(6')-Ie-aph(2')-Ia, aph(3')-III, ermB, dfrG, tet*(M), *catPc221*, *blaZ*, and *mecA*. Consequently, CC45 isolates show remarkably higher prevalence of resistance to chloramphenicol (95%) compared with either CC71 (43%) or CC258 (13%) (130). Few other CC have been studied for virulence profiles; CC68 generally demonstrates resistance to doxycycline, minocycline, and tetracycline via carriage of *tet*(M) (126).

Cross-Interference of the *agr* System and Therapeutic Potential

With the increasing difficulty of treating highly-resistant staphylococcal infections, the manipulation of quorum sensing systems has become a promising area of therapeutics (86). The cross-inhibiting AIPs of *Staphylococcus* species offer a natural reservoir of molecules to test for potential clinical use. The *agr* groups are often defined by mutual inhibition, within and between species that results in a unique type of competition and bacterial interference where the *agr* regulon is blocked by binding of a competing AIP rather than induced (78). This is especially relevant for species that frequently co-inhabit environments, such as *S. aureus* and *S. epidermidis* on the human skin; all *S. aureus* groups are sensitive to inhibition by the *S. epidermidis* AIPs, except for *S. aureus*

agr group IV, which is also the only group able to inhibit the *S. epidermidis agr* system in return. The impact of this competition has been suggested as the reason for why *S. epidermidis* predominates over *S. aureus* on human skin (118). A more recent study found that the AIP molecules of 37/52 staphylococcal isolates representing 17 different species could inhibit *S. aureus agr*, with *S. schleiferi* AIP molecules the most potent inhibitors against all 4 *agr* groups of *S. aureus*. When these AIPs were synthetically recreated, they could completely abolish *agr* induction of *S. aureus* (24).

The ability to understand and exploit this natural avenue of competition is apparent when the effects are demonstrated *in vivo.* When *S. schleiferi* and *S. aureus* species were injected into moth larvae in a co-colonization competition, the expression of *S. aureus* virulence factors was suppressed and the infection was far less severe, with a significant increase in survival (24). Directly inhibiting the *agr* system has been found to block abscess formation (185), decrease skin lesion size and halt the progression of skin infections (38, 109), and improve host defenses in murine models (38, 158). In a mouse model of inflammatory skin disease mimicking atopic dermatitis, an *agr*-inhibitor molecule resulted in reduced production of delta-toxin (an important virulence factor impacting cutaneous inflammation) and decreased skin inflammation (7). In contrast to antimicrobial treatments, no induction of resistance or tolerance has been seen in this approach (158).

No studies on cross-inhibition or virulence attenuation have been performed with the four *S. pseudintermedius* AIPs and *S. pseudintermedius* infections.

Human Infections of *S. pseudintermedius*

While humans have not been recognized as a typical host for *S. pseudintermedius* and any colonization is often transient, nasal passage colonization of humans can occur for prolonged periods (50, 168). Transmission between dogs and owners is possible, and has been reported more frequently with veterinary staff having a higher prevalence of *S. pseudintermedius* colonization than the general population (40, 50, 64, 67, 104, 140, 160, 168). Several studies have confirmed that the isolates carried by veterinary staff or dog owners are the same as those found on the dogs (8, 50, 64, 124, 152, 167). This suggests *S. pseudintermedius* is a zoonotic pathogen, however cases of apparent human-to-human infection have been described in a Swedish hospital (154) and recent studies on human colonization and infections have discovered that the association with dogs in human cases is as low as 10% (94, 188). In 2010, a case of human-human transmission through a saliva contaminated syringe resulting in skin abscesses was also documented (85).

One of the major complications of recognizing *S. pseudintermedius* as a human pathogen has been the underreporting of cases due to frequent misidentification of *S. pseudintermedius* as *S. aureus.* Until recently, *S. pseudintermedius* was not included in the databases of commercial identification systems (169) and similarities in morphology and biochemical characteristics resulted in rates of misdiagnosis ranging from 13% (21) to 21% (188). These errors are significantly reduced when species identification is performed with matrix-assisted laser desorption and time-of-flight mass spectrometry (MALDI-TOF) (188). Accurate identification is key because the cefoxitin surrogate for methicillin-resistance testing in *S. aureus* often fails to detect methicillin-resistance in *S. pseudintermedius* (14, 143, 187). Misidentification of *S. pseudintermedius* can therefore complicate treatment of human infections.

Staphylococcus pseudintermedius is most frequently associated with skin and soft tissue infections as well as animal bite wounds, but can cause a wide range of infections in humans including bacteremia, otitis externa, cardiomyopathy, and endocarditis (21, 152, 169). A Canadian study of 24 cases found the median age of patients to be 61 with a 75% majority of the cases being a skin/soft tissue infection. Of the 17 isolates cultured from these cases, 6 were MRSP and MDR and belonged to ST71 and ST181; the remainder were MSSP of varying STs (152). The pattern of a majority of human clinical isolates being MSSP is reflected in several other studies, where 1/13 (21), 12/80 (188), and 0/4 were found to be MRSP (101), resulting in the majority of human isolates attributed to new STs rather than the major MRSP CCs. One study examined toxin carriage in 13 *S. pseudintermedius* isolates that had been misdiagnosed as *S. aureus* and found universal carriage of *LukS/F-I, siet*, and *se-int* and low carriage of *expA* (2/13) and *expB* (1/13) (21). Many infections occur in patients who are diabetic or immunocompromised (152, 188).

CHAPTER III

BIOFILM FORMATION AND PHENOTYPIC CHARACTERIZATION OF CLINICAL ISOLATES[1]

Introduction

Biofilm formation is an important component of *Staphylococcus* virulence, allowing the pathogen to colonize surfaces of implants, catheters, and other medical devices. The vast majority of tested *S. pseudintermedius* isolates are biofilm formers, with 96% producing moderate to strong biofilms (25, 150). A vital component of biofilm formation is the ability of bacteria to produce slime (PIA) resulting in a viscous extracellular polysaccharide layer (35, 69). The purpose of this study was to phenotypically characterize the biofilm forming capabilities of our *S. pseudintermedius* collection (n = 710) through testing for slime production and overall biofilm production. We hypothesized that most isolates would be biofilm and slime producing, and that there would be a difference in slime production and overall biofilm production between diseased and healthy dogs.

Slime production can be qualitatively determined using the Congo red biofilm assay (CRA): Congo red dye binds to polysaccharides secreted by colonies growing on plates containing the dye along with sucrose to encourage biofilm formation. Slime producing colonies will turn black to indicate secretion of polysaccharides, while non-slime forming colonies will remain red. This assay is often implemented as a quick screening method for large numbers of isolates. However, as biofilms produced by *Staphylococcus* can be protein based and form without

[1] Reprinted with permission from Little SV, Bryan LK, Hillhouse AE, Cohen ND, Lawhon SD. 2019. Characterization of *agr* groups of *Staphylococcus pseudintermedius* isolates from dogs in Texas. mSphere 4. Copyright 2019 Little *et al.* Creative Commons 4.0 International License.

the extracellular polysaccharide layer (115), this assay can result in false negatives for biofilm production. Furthermore, the evaluation is qualitative and variation in the literature on positive and negatives suggest this test is not the most reliable; for a quantitative measure of overall biofilm production, the crystal violet microtiter plate test (CVA) has been employed (87, 108). This assay uses crystal violet, a dye which is most commonly known for its use in Gram staining, which binds to peptidoglycans and sugar-containing molecules, including DNA. By growing the bacteria under biofilm-inducing conditions, carefully rinsing non-adherent cells, dying adhered cells and biofilm, and then resuspending the dye and measuring the optical density, a quantitative measure on overall biofilm production can be obtained.

Materials and Methods

Bacterial isolates and demographics

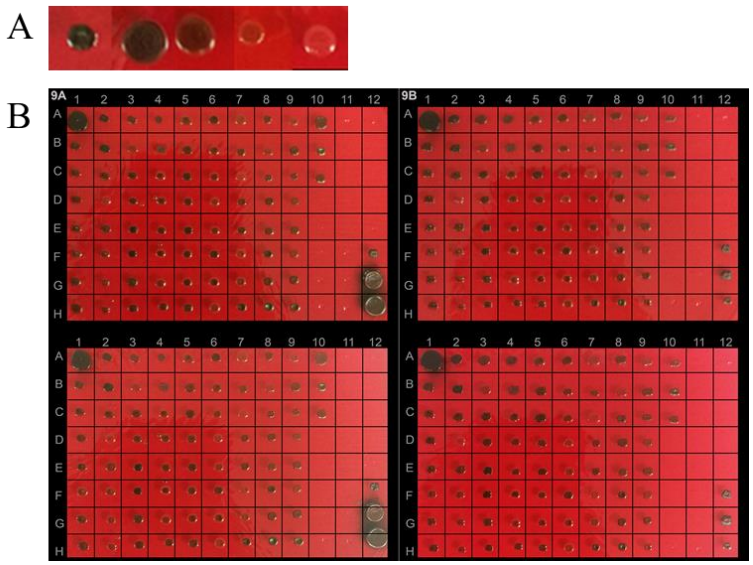
Staphylococcus pseudintermedius strains collected between 2007 and 2016 were acquired from the clinical microbiology lab of a referral veterinary hospital, post-species identification. Isolates were collected from canine patients, both healthy (n = 182) and diseased (n = 528), and stored in glycerol lysogeny broth (LB) at -80°C at the time of isolation. Diseased isolates were residual patient diagnostic samples collected from canines with various conditions and culture sources, of which skin (n = 319) and urine (n = 81) were the most common. Healthy isolates were collected for a staphylococcal screening study by culturing the nares and perineal skin of dermatologically healthy canines attending evaluations for orthopedic surgery. All sampling conformed to the ethical guidelines and standards of care for the hospital. The Institutional Animal Care and Use Committee and Clinical Research Review Committee approved the collection of isolates from the healthy dog group (AUP 2010-068).

For this study, plate cultures were grown on trypticase soy agar (TSA) with 5% sheep blood (blood agar; BBL, USA) and incubated aerobically at 37°C for 24 hours. Liquid cultures were made by single-colony inoculation of tryptic soy broth (TSB; BD, USA) incubated aerobically and shaken overnight. ATCC *S. epidermidis* strain 12228 and ATCC *S. aureus* strain 25923 were used as negative and positive controls, respectively, for biofilm production.

Phenotypic characterization of slime production by Congo red assay

To assess isolate ability to produce slime as characterized by polysaccharide intercellular adhesin (PIA) production, isolates were cultured on Congo red agar plates. Plates were prepared as previously described (2, 4). In brief, 37 g brain heart infusion broth (BHIB; BD, USA), 13.5 g agar (Teknova, USA), 36 g sucrose (VWR, USA), and 0.8 g Congo red (VWR, USA) per 1 L of media was autoclaved for 15 minutes at 121°C and poured into plates. After inoculation, plates were incubated at 37°C for 24 hours, brought to room temperature, assessed, photographed, and then left to incubate overnight at room temperature and assessed again at 48 hours. Slime production was determined by the color and texture of the colony surface based on a 5-point scale system adjusted from Aricola, *et al.:* smooth, very red (0), red (1) and bordeaux (3) colonies are non-slime-producing, and rough, almost black (4) and black (5) colonies are slime-producing (2, 4). This scale and a plate example are visualized in Figure 3. It should be noted that some negative strains (including ATCC 12228) may produce a black coloration on the surrounding agar while the colonies themselves are red. Plates were grown in biological and technical duplicate.

Figure 3: Congo Red Assay



The Congo Red Assay utilized a 5-point scale (A) where, from left to right, smooth, very red colonies (0), smooth, red colonies (1), and bordeaux colonies (2) were negative for slime production, and rough almost black (3) and black (4) colonies were positive. An example of a technical duplicate of the plates at two time points (B) demonstrates the qualitative nature of the assay.

Phenotypical characterization of biofilm production by crystal violet microtiter plate assay

Overall biofilm production was assessed by the ability of the isolates to adhere to a 96-well, polystyrene microtiter plate (Nunc-Immuno, USA), using protocols adjusted from Stepanović *et al.* (157). Cultures were grown on blood agar plates for 24 hours at 37°C and single colonies were then transferred to deep well plates containing 1 mL of TSB. Liquid cultures were grown, shaken, overnight at 37°C. Cultures were then diluted 1:200 in TSB containing 1% glucose (Mallinckrodt Chemicals, USA) to encourage biofilm production in flat bottomed, non-tissue culture treated microtiter plates. Following 24 hours of incubation at 37°C, the suspension cultures were removed and wells gently washed with ddH2O to remove non-adherent cells. Adherent cells and biofilm were stained with 0.1% crystal violet dye per well for 15 minutes. The excess dye was removed, wells rinsed with ion-free water, and the plate allowed to air dry. Biofilm-bound dye was resuspended in 95% ethanol (Koptec, Decon Labs, USA) and the absorbance of each well read at

OD570. The values of each plate were adjusted by subtracting the average of the blank control wells (TSB broth only). Cutoffs for biofilm production was established using a previously established 4-point scale (157), where 0 is non-adherent ($OD \leq OD_{control}$), 1 is weakly adherent ($OD_{control} < OD \leq 2*OD_{control}$), 2 is moderately adherent ($2*OD_{control} < OD \leq 4*OD_{control}$), and 3 is strongly adherent ($4*OD_{control} < OD$). Microtiter plate assays were performed in technical and biological triplicate.

Statistical analyses

All analyses for the biofilm characterization study were performed using SAS software. To assess for relationships between disease status and biofilm, the isolates were categorized as diseased or healthy. Slime production was categorized as slime-producing (1) or non-slime producing (0). Overall biofilm production was categorized as 0, 1, 2, or 3 (corresponding to the grading scale of non-adherent, weakly adherent, moderately adherent, and strongly adherent). These categorical variables were compared using chi-squared or Fisher's exact test when expected cells had counts fewer than 5. A P value less than 0.05 was considered significant.

Results

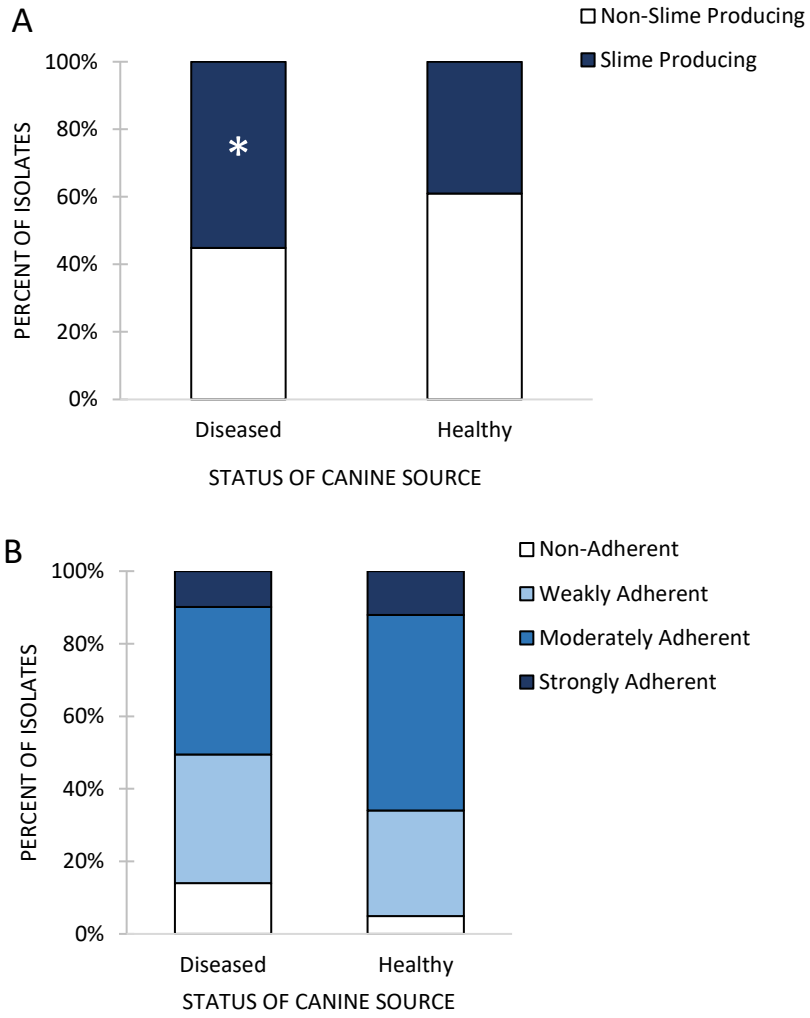
Biofilm assays were performed on clinical isolates (n = 710) from unique dogs (Table 1). Slime-production, as determined by CRA, was found in approximately half of the isolates (51%, n = 362). The proportion of slime-producing isolates in diseased dogs (55%, 291/528) was significantly (P = 0.0002) greater than that of isolates from healthy dogs (39%, 71/182) (Figure 4).

Table 1: Biofilm Assay Results

	CRA Negative *Non-Slime Producing*	**CRA Positive** *Slime Producing*	**CVA 0** *Non-Adherent*	**CVA 1** *Weakly Adherent*	**CVA 2** *Moderately Adherent*	**CVA 4** *Strongly Adherent*
Total Collection (n = 710)						
Total	348 49%	362 51%	83 12%	240 34%	313 44%	74 10%
Healthy	111 32%	71 20%	9 11%	53 22%	98 31%	22 30%
Diseased	237 68%	291 80%	74 89%	187 78%	215 69%	52 70%
CRA Negative	-	-	37 45%	118 49%	154 49%	39 53%
CRA Positive	-	-	46 55%	122 51%	159 51%	35 47%

Percentages have been rounded and may not total to 100%.

Figure 4: Biofilm Production of Clinical Isolates



Results of biofilm assays on the 710 clinical isolates from diseased dogs and healthy dogs. The proportion of slime producing dogs was significantly higher (*) in diseased dogs than healthy dogs.

The CVA $OD_{control}$ was 0.3317 (±0.1818). Overall biofilm production, measured by CVA, showed a majority of isolates (78%, 553/710) produced weak or moderate biofilms. Eighty-three isolates (12%) were non-adherent while only 74 (10%) were strongly adherent. There was no significant difference in the distribution of overall biofilm production between slime-producing and non-slime-producing isolates. Overall biofilm production differed significantly between

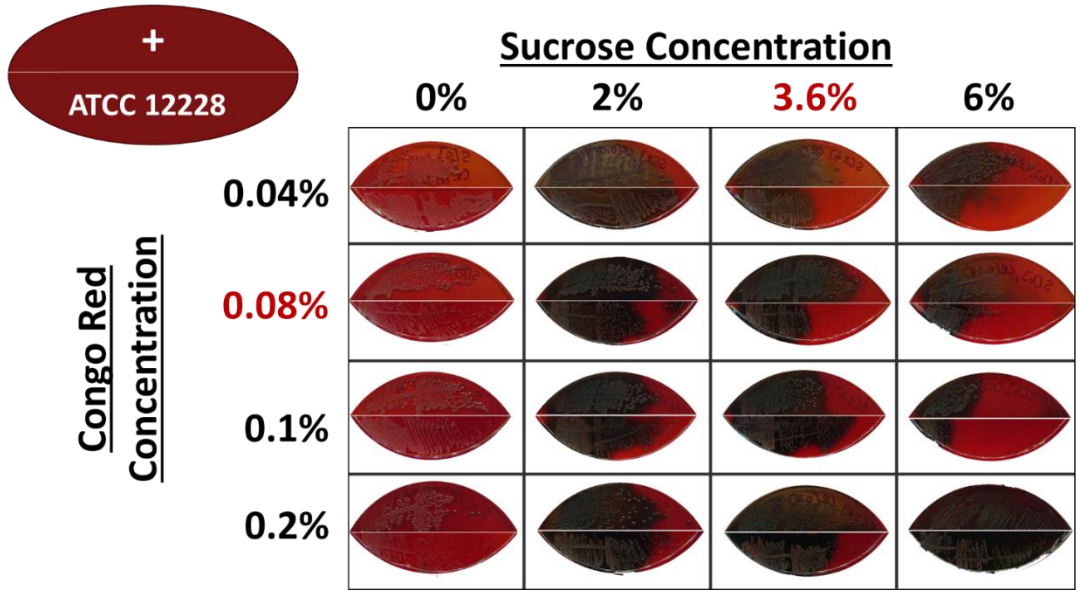
healthy and diseased sourced isolates (P = 0.0014), which was largely attributable to a high proportion of non-adherent isolates in the diseased category.

There was no significant association between overall biofilm production measured by CVA and slime production as measured by CRA, with similar distributions of CVA scores between CRA negative and positive isolates.

Discussion

Slime-production was only seen in half of isolates, which is lower than expected (25). Slime production occurred in a significantly higher proportion in isolates from diseased dogs than isolates from healthy colonization, perhaps highlighting the importance of slime in clinical disease. However, the Congo red test has several drawbacks; in part, it underestimates the total biofilm-producing capability in *Staphylococcus* spp. due to PIA-independent biofilms and highly variable induction conditions. The genes required for slime production in *Staphylococcus*, *icaA* and *icaD* in particular, are nearly universal across isolates and yet we do not see slime production in all isolates using the standard Congo red protocol. This phenomenon has been widely acknowledged across literature in *S. aureus*, supporting sensitive and varied growth conditions required for slime production (25, 35, 170). Furthermore, there is variation in the color of the single ATCC strain negative control across literature even with identical culture conditions (27, 71, 83, 192, 194). As such, the scale in this study was altered to include bordeaux colonies as negative for slime production rather than intermediate, as dark red and bordeaux colonies of ATCC 12228 and negative controls are seen both in literature (27, 71, 83, 192, 194) and in this study (Figure 5), even when conditions were matched to those studies that produced bright red colonies.

Figure 5: Negative Slime Control Color Variation



Congo red plate results for the negative control strain ATCC 12228 compared to a positive strain under varying plate compositions. The standard dye and sucrose concentration are indicated by red (0.08% CR, 3.6% sucrose), at which concentrations ATCC 12228 grows dark red colonies on a black plate discoloration.

Consequently, while Congo red is useful for quick, large-scale characterization, more weight is given to CVA as an accurate measure of biofilm production (87, 108). According to CVA, the majority of the isolates (88%) were biofilm producers, which agrees with the current literature on biofilm production in *Staphylococcus* clinical isolates and highlights the importance of biofilm formation for the colonization of *Staphylococcus* (136, 155).

CHAPTER IV

ASSOCIATIONS BETWEEN DISEASE TYPE, VIRULENCE FACTORS, MLST, AND *AGR* GROUP[2]

Introduction

While associations between *agr* type and infection type have been found in *S. aureus* and *S. epidermidis*, no such associations have been thoroughly investigated in *S. pseudintermedius*. Four known *agr* AIPs have been found unique to *S. pseudintermedius*, consisting of 9 amino acids containing a serine at position 5 and processed to form a lactone ring between that serine and the terminal end (RIPTSTGFF (I), RIPISTGFF (II), KIPTSTGFF (III), KYPTSTGFF(IV)). The purpose of this study was to determine the *agr* type of 160 clinical isolates from 4 different infection source groups (healthy dogs, urinary tract infections, surgical infections, and pyoderma) and examine the relationship(s) between *agr* type, infection, MLST, and virulence factors such as biofilm formation, antimicrobial resistance gene carriage, and toxin genes. We hypothesized that there would be associations between infection source and *agr* type as well as virulence factors and *agr* type. Due to the clonal nature of MRSP, we also hypothesized that there would be associations between MLST and virulence factors and *agr* type, despite the horizontal transfer of virulence factors on MGEs.

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Materials and Methods

Bacterial isolates and demographics

Out of the 710 clinical isolates described in Chapter III, 40 were randomly selected from the disease groups pyoderma, urinary tract infection, and healthy colonization. The number was limited to 40 due to availability of surgical infection isolates in the collection (n = 40), all of which were included, resulting in a total of 160 isolates sequenced for this analysis.

DNA extraction, library preparation, and sequencing

Isolates were grown from source freezer stock on blood TSA for 24h at 37ºC and subcultured twice. A single colony was inoculated into brain, heart infusion broth and grown for 8 hours and 1 mL inoculum was used for DNA extraction via MasterPure™ Gram Positive DNA Purification Kit, (Epicentre, Lucigen, USA) per their standard protocol. DNA was quantified using the Life Technologies Qubit high sensitivity dsDNA assay and all samples were normalized to 100 ng of DNA in a total volume of 14 ul. Sequencing libraries were prepared by hand using the Bioo Scientific NEXTflex Rapid DNA-Seq Kit per their standard protocol. Prepared libraries were checked with the Qubit high sensitivity dsDNA assay to determine concentration and the Agilent TapeStation D1000 HS to determine the average fragment size of the prepared libraries. All samples were normalized to 4 nM and pooled together for sequencing. The 4 nM pool was diluted to 20 pM and sequenced on the Illumina MiSeq 300 x 300 cycle v3 sequencing kit. All run data and FASTQ files were uploaded to BaseSpace (Illumina) for downstream analysis.

Genome assembly and annotation

The Illumina MiSeq reads were assembled using SPAdes de novo genome assembly (version 3.6.2) on the Texas A&M Institute for Genome Sciences and Society (TIGSS) computing cluster, with the following parameter: --careful. The resulting assemblies were annotated using the Rapid Annotation using Subsystem Technology tool kit (RASTtk) on the Pathosystems Resource Integration Center (PATRIC) (177).

Typing agr

In order to determine *agr* group, BLAST was used on the assembled genomes using previously published sequences for the four *agrD* alleles (GenBank EU157356.1, EU157391.1, EU157400.1, and EU157402.1). In the case of alleles that did not match the four published types, the sequence was checked in the original reads and then the *agrD* sequences were extracted and purified for Sanger sequencing by Eton Bioscience using PCR primers previously published for *agrBD* (29).

Virulence factors

Biofilm production and slime production were measured as described in Chapter III. Carriage of *icaA* and *icaD*, genes essential to PIA/slime production, was checked by PATRIC's BLASTP, searching for genomic features using previously published primers (150). BLAST was similarly used to identify toxin carriage of known *S. pseudintermedius* toxin genes (*speta* (UniProt ADX77621), *siet* (UniProt CAR57917.1), *expA* (UniProt BAI49625.1), *expB* (UniProt BAJ23893.1), *Luk-S/F* (131), *sec-canine* (44), *se-int* (53), *BacSp222* (184). ResFinder 2.1 (August 2018, 193) was used to check for antimicrobial gene carriage for the following antimicrobial

resistance gene categories: aminoglycoside; β-lactam, colistin; fluoroquinolone; fosfomycin; fusidic acid; glycopeptide; macrolide, lincosamide, and streptogramins (MLS); nitroimidazole; oxazolidinone; phenicol; rifampicin; sulphonamide; tetracycline; and trimethoprim. The presence or lack of *mecA* (UniProt B1GVE2) was confirmed by BLAST.

MLST

Multilocus sequence typing was performed using the 7-gene (*tuf, cpn60, pta, purA, fdh, sar, ack*) MLST scheme (MLST-7) utilized by the *Staphylococcus pseudintermedius* MLST database (https://pubmlst.org/spseudintermedius/; curator Vincent Perreten) (151). Potential new alleles were identified in our genomes via BLASTP and the FASTA sequences submitted to the database for allelic determination and final ST assignment. Grouping of isolates into CC was performed using the PHYLOViZ analysis (49), including all *S. pseudintermedius* isolates deposited in the database as of August 2018. Due to a lack of resolution in MLST-7, a 53-gene ribosomal multilocus sequence typing (rMLST) method was also used to type the isolates, utilizing the database curated by Keith Jolley (https://pubmlst.org/rmlst/) (79).

Statistical analyses

All analyses for the biofilm characterization study were performed using SAS software. Significant associations were assessed using chi-squared or Fisher's exact test when expected cells had counts fewer than 5. Data were analyzed using contingency tables (k x k, as indicated for the specific comparison: some tables were 2 x 2 layouts, whereas others had more than dichotomous levels). Significance for analysis was set at $P < 0.05$.

Results

Prevalence and distribution of agr *groups*

All but two *S. pseudintermedius* WGS isolates had 100% identity to one of the four known *agrD* alleles. One exception contained a silent mutation in the AIP region, the other contained a missense mutation in the C-terminus region resulting in a lysine instead of glutamic acid. As neither of these mutations impact the final AIP protein structure, they were categorized accordingly.

Eleven isolates (7%) were classified as *agr* group I, 50 (31%) as *agr* group II, 44 (28%) as *agr* group III, and 55 (34%) as *agr* group IV.

Table 2: WGS Biofilm Assay Results

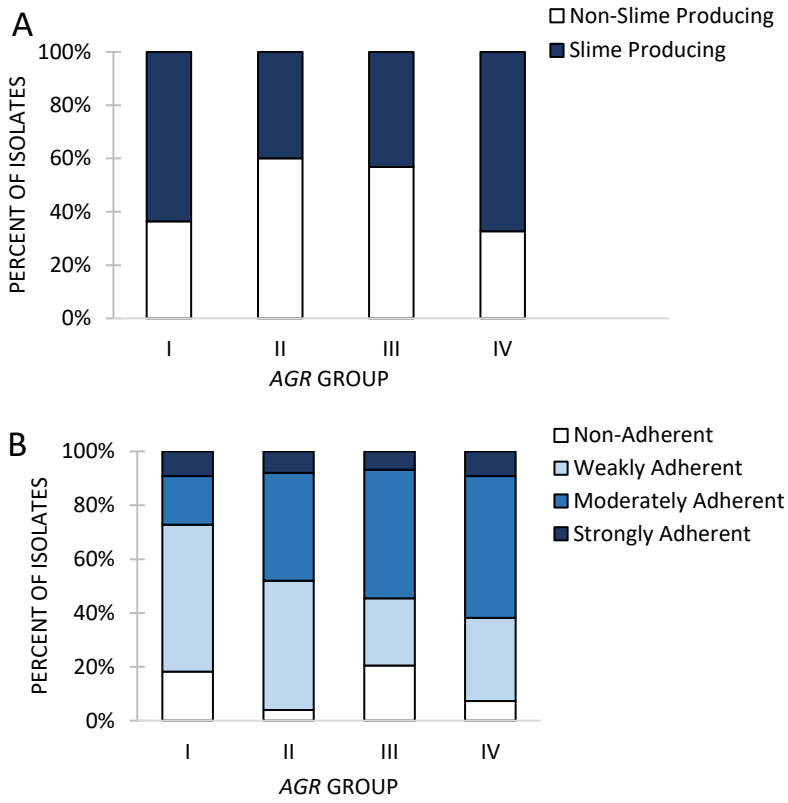
	CRA Negative *Non-Slime Producing*	**CRA Positive** *Slime Producing*	**CVA 0** *Non-Adherent*	**CVA 1** *Weakly Adherent*	**CVA 2** *Moderately Adherent*	**CVA 4** *Strongly Adherent*
Sequenced Isolates (WGS, n = 160)						
Total	77 48%	83 52%	17 11%	58 36%	72 45%	13 8%
Healthy	23 30%	17 20%	2 12%	15 26%	21 29%	2 15%
Pyoderma	19 25%	21 25%	3 18%	12 21%	18 25%	7 54%
Urinary Tract	15 19%	25 30%	4 24%	18 31%	14 19%	4 31%
Surgical	20 26%	20 24%	8 47%	13 22%	19 26%	0 0%
CRA Negative	-	-	10 59%	28 48%	33 46%	6 46%
CRA positive	-	-	7 41%	30 52%	39 54%	7 54%
MRSP	16 21%	36 43%	9 53%	15 26%	23 32%	5 38%
MSSP	61 79%	47 57%	8 47%	43 74%	49 68%	8 62%

Biofilm assay results. Percentages have been rounded and may not total to 100%. Adapted from Little *et al.* 2019 (99).

Biofilm production

Biofilm results for the 160 WGS isolates are given in Table 2 (99). Approximately half of the isolates were slime-producing and there was no significant association of slime production as measured by CRA with the disease groups or methicillin resistance as determined by presence of *mecA*. For overall biofilm formation as determined by CVA, a majority of isolates formed biofilms (89%; 143/160), of which most (91%; 130/143) were weak to moderate. No significant associations were seen between overall biofilm production and disease group, slime-production, or methicillin resistance. Slime-production was significantly less associated with *agr* II and III compared to *agr* I and IV (P = 0.0415, Figure 6, A). There was no significant difference between overall biofilm production in the four *agr* groups (Figure 6, B).

Figure 6: Biofilm Production by *agr* Group



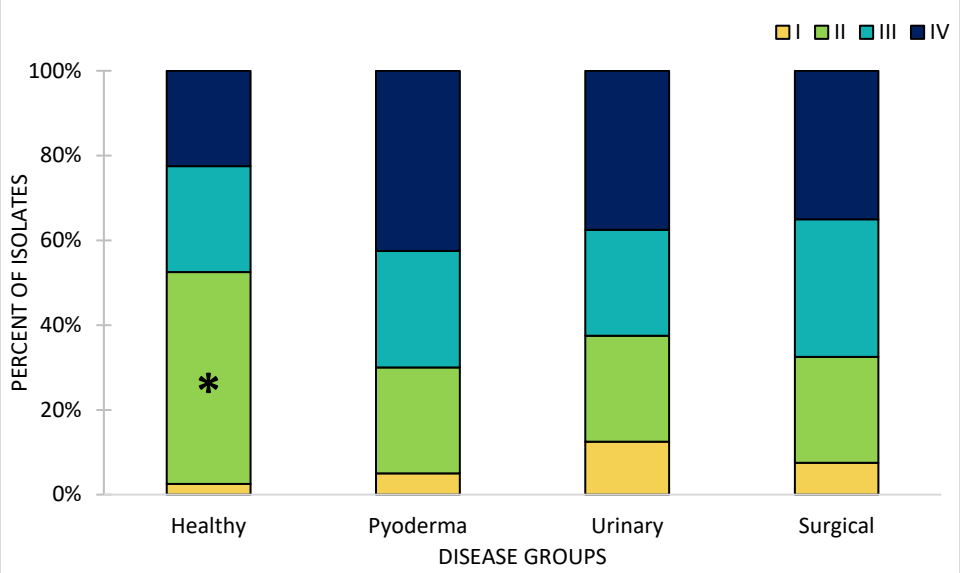
Slime-production (A) and overall biofilm production (B) across *agr* groups. Slime production was significantly less associated with groups II and III (A).

The majority (98%; 156/160) of isolates carried both *icaA* and *icaD* for PIA/slime production. Of the four that did not, one was slime positive and formed a moderate biofilm, one was slime positive and formed a strong biofilm, one was slime negative and formed a moderate biofilm, and one was negative for both slime and biofilm production.

Disease associations of agr *groups*

The distribution of *agr* groups differed significantly between healthy and diseased dogs (P = 0.025) Figure 7. Group II isolates were significantly more common in healthy dogs than diseased dogs (P = 0.0058). No further significant associations were seen between *agr* groups and disease and the disease groups of healthy, pyoderma, urinary tract, or surgical infections.

Figure 7: The *agr* Distribution of Disease Groups



The distribution of *agr* groups across the four disease groups. Group II was significantly more common in isolates from healthy colonization.

Resistance gene carriage of agr *groups*

Resistance gene carriage across *agr* groups is described in Table 3. The majority of WGS isolates were methicillin-susceptible (68%; n = 108/160) as determined by the lack of *mecA*. Methicillin resistance was identified for 45% (n = 5) of *agr* group I isolates, 18% of group II (n = 9), 34% of group III (n = 15), and 42% of group IV (n = 23). Group II isolates were significantly (P = 0.0025) less likely to be MRSP. Isolates from healthy dogs were significantly (P = 0.0002) less likely to carry *mecA* than disease isolates. The *mecA* cassette was found in 8% of healthy isolates (n = 3), 48% of pyoderma isolates (n = 19), 30% of urinary isolates (n = 12), and 45% of surgical isolates (n = 18). There was a slightly significant difference in distribution of MRSP among the three diseased groups, with urinary tract infections being less likely to be MRSP than pyoderma and surgical infections (P = 0.0471).

Sixty-four isolates carried genes for aminoglycoside resistances (*aac(6')-aph(2''), ant(6)-la,* or *aph(3')-III*), but there was no significant association between *agr* types and aminoglycoside resistance. There was a significant association between *agr* groups and macrolide, lincosamide, and streptogramin (MLS) resistance gene carriage (*erm(B) and inu(A))*, with the proportion of gene carriage significantly less in *agr* group II isolates (P = 0.0171). Similarly, the proportion of tetracycline resistance carriage (*tet(K)* and *tet(M)*) was significantly (P = 0.0057) less in group II. For trimethoprim resistance, the proportion of isolates that carried the resistance gene *dfrG* among *agr* groups III or IV was significantly (P = 0.0005) higher than that for isolates of groups I or II. There was no association seen between *agr* group and β-lactam resistance gene carriage, as most isolates (n = 139) carried *blaZ*. No association was seen between *agr* group and chloramphenicol resistance, as most isolates (n = 152) did not carry *cat(pC221)*.

Table 3: Virulence Factor Carriage Across *agr* Groups

	***agr* Group I n = 11**	***agr* Group II n = 50**	***agr* Group III n = 44**	***agr* Group IV n = 55**
Toxin Gene Carriage				
expA	0%	8%	9%	22% **
expB	27%	20%	7% *	4% *
sec – canine	0% *	2% *	14%	16%
speta	100%	100%	100%	100%
siet	100%	100%	100%	100%
luk – S/F	100%	100%	100%	100%
se – int	100%	100%	100%	100%
Antimicrobial Resistance and Gene Carriage				
mecA	45%	18% *	34%	42%
Aminoglycoside	45%	34%	32%	51%
Beta-lactam	82%	84%	82%	95%
Chloramphenicol	0%	4%	9%	4%
MLS	45%	18% *	34%	45%
Tetracycline	55%	36% *	61%	69%
Trimethoprim	9% *	12% *	32%	44%
MDR	45%	16% *	34%	47%

Virulence factor carriage. * indicates *agr* group is significantly less likely to carry, ** indicates *agr* group is significantly more likely to carry.

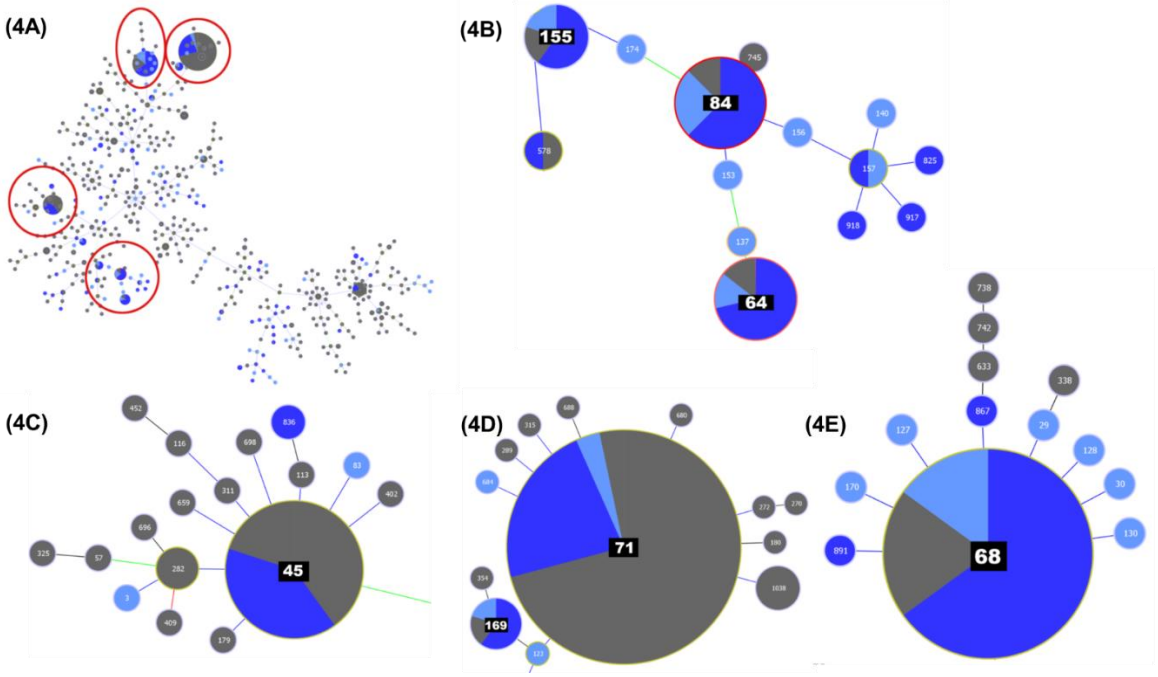
There was a significant association between *agr* group and multiple-drug-resistance (MDR) as defined by resistance to three of the five classes of antibiotics other than β-lactams. Group II isolates were significantly (P = 0.0045) less likely to be MDR and groups II or III were significantly (P = 0.0052) less likely to be MDR than groups I or IV.

Only 16 isolates were susceptible to all drug classes. Of these, 2 (13%) were *agr* group I, 7 (44%) were *agr* group II, 6 (38%) were *agr* group III, and 1 (6%) was *agr* group IV.

Toxin carriage of agr *groups*

Toxin carriage is summarized in Table 3. There was universal carriage of the *speta*, *siet*, *luk-S/F,* and *se-int* toxin genes. Twenty isolates carried *expA* and 17 carried *expB,* with 1 isolate carrying both genes. Relative to the other *agr* groups, *agr* group IV isolates were significantly more (P = 0.0199) likely to carry *expA*. The overall distribution of *agr* groups by *expB* carriage also differed significantly, as isolates of *agr* groups III and IV were significantly less (P = 0.0037) likely to carry *expB* than isolates of groups I and II. Sixteen isolates carried *sec-canine*, with *agr* groups III and IV being significantly more (P = 0.0126) likely to carry *sec-canine* than groups I and II. Finally, one isolate carried the gene for the bacteriocin-like peptide BacSp222.

Figure 8: Phylogenetic Relationships of North American Isolates



PHYLOViZ (goeBURST) visualization of genetically related groups and their founders through 7-gene MLST as of August 2018. Dark blue indicates the isolates from our lab, including the 160 WGS study, light blue indicates other North American isolates, grey indicates non-North American isolates. Node size is linearly related to the number of isolates of that ST submitted to PubMLST *S. pseudintermedius* database. All isolates in the database are included in (A), with the major founder groups circled in red as follows: 84 (B), 45 (C), 71 (D), and 68 (E).is significantly more likely to carry.

Phylogenetic relationships and clonal complexes based on MLST

MLST determined 91 new allele combinations, 31 new alleles, and 113 new strain types submitted to the *S. pseudintermedius* pubMLST database. PHYLOViZ analysis (49) in August 2018 suggested the major *S. pseudintermedius* clonal complexes (CC) represented in this study and in all submitted North American isolates to be CCs 45, 64, 68, 71, 84, 150, and 155 (Figure 8). The highest represented STs in our isolates were STs 64, 68, 71, 84, 150, and 155. Significant associations were seen between ST64 and *agr* group II ($P = 0.012$, 5/5 isolates were *agr* II), between ST68 and *agr* IV ($P < 0.0001$, 13/13), between ST71 and *agr* III ($P = 0.0005$, 7/7), and between ST 84 and *agr* I ($P < 0.0001$, 5/5); ST150 and ST155 had too few isolates ($n = 3$ each) to see significance, but all members were *agr* group IV. Characteristics of these groups are summarized in Table 4.

Table 4: Characteristics of Major MLST Groups

MLST	*agr* Type	Disease Group	Slime Formation	Overall Biofilm Production	Drug Resistances	Toxin Carriage
64 (n = 5)	**5 *agr* II ***	1 Healthy 2 Pyoderma 1 Urine 1 Surgical	2 Slime Forming 3 Non-Slime Forming	0 Non-Adherent 3 Weakly Adherent 2 Moderately Adherent 0 Strongly Adherent	**5 *mecA* (MRSP)*** **5 Aminoglycoside*** **5 Beta-lactam*** **2 Macrolide*** **3 Tetracycline*** **1 Trimethoprim*** **1 MDR***	0 *expA* 0 *expB* 0 *sec-canine*
68 (n = 13)	**13 *agr* IV***	0 Healthy 2 Pyoderma 6 Urine 5 Surgical	11 Slime Forming 2 Non-Slime Forming	3 Non-Adherent 3 Weakly Adherent 6 Moderately Adherent 1 Strongly Adherent	**13 *mecA* (MRSP)*** **13 Aminoglycoside*** **13 Beta-lactam*** **13 Macrolide*** **13 Tetracycline*** **13 Trimethoprim*** **13 MDR***	0 *expA* 0 *expB* 0 *sec-canine*
71 (n = 7)	**7 *agr* III***	0 Healthy 1 Pyoderma 1 Urine **5 Surgical***	1 Slime Forming 6 Non-Slime Forming	**4 Non-Adherent*** 0 Weakly Adherent 3 Moderately Adherent 0 Strongly Adherent	**6 *mecA* (MRSP)*** **7 Aminoglycoside*** **7 Beta-lactam*** **7 Macrolide*** **4 Tetracycline*** **7 Trimethoprim*** **7 MDR***	0 *expA* 0 *expB* 0 *sec-canine*
84 (n = 5)	**5 *agr* I***	0 Healthy 2 Pyoderma 1 Urine 2 Surgical	5 Slime Forming 0 Non-Slime Forming	1 Non-Adherent 2 Weakly Adherent 1 Moderately Adherent 1 Strongly Adherent	**5 *mecA* (MRSP)*** **5 Aminoglycoside*** **5 Beta-lactam*** **5 Macrolide*** **4 Tetracycline*** **1 Trimethoprim*** **5 MDR***	0 *expA* 1 *expB* 0 *sec-canine*
150 (n = 3)	3 *agr* IV	1 Healthy 2 Pyoderma 0 Urine 0 Surgical	2 Slime Forming 0 Non-Slime Forming	0 Non-Adherent 3 Weakly Adherent 0 Moderately Adherent 0 Strongly Adherent	**3 *mecA* (MRSP)*** **3 Aminoglycoside*** **3 Beta-lactam*** **2 Macrolide*** **3 Tetracycline*** **3 Trimethoprim*** **3 MDR***	0 *expA* 0 *expB* **3 *sec-canine****
155 (n = 3)	3 *agr* IV	1 Healthy 1 Pyoderma 0 Urine 1 Surgical	3 Slime Forming 0 Non-Slime Forming	0 Non-Adherent 0 Weakly Adherent 3 Moderately Adherent 0 Strongly Adherent	0 *mecA* (MRSP) **3 Aminoglycoside*** **3 Beta-lactam*** **2 Macrolide*** **3 Tetracycline*** **2 Trimethoprim*** **2 MDR***	**3 *expA**** 0 *expB* **3 *sec-canine****

Significance indicated by results in bold marked by *.

The proportion of isolates outside of these main STs was significantly lower (P = 0.0162) in diseased dogs compared to healthy dogs. ST was significantly associated with surgical infections – in particular, ST71 isolates were significantly (P = 0.0111) more likely to be derived from surgical cases. STs also differed significantly (P = 0.0014) by slime production, however there was no evidence that individual STs were significantly associated with slime production. The distribution of overall biofilm production by CVA differed significantly (P = 0.0032) between ST71 and the other types, with a higher proportion of isolates being non-adherent.

ST was significantly associated (P < 0.0001) with MDR, with STs 64, 68, 71, 84, 150, and 155 being more likely to be MDR than other STs and all but 150 being significantly more (P < 0.0001) likely to carry *mecA* than the other types. These STs were also more likely to carry resistance genes to aminoglycosides (P < 0.0001), β-lactam (P = 0.0043), MLS (P < 0.0001), tetracycline (P = 0.0003), and trimethoprim (P < 0.0001). Overall, MRSP isolates were more likely to be MDR (P < 0.001) and ST associated with methicillin-resistance were significantly (P < 0.0001) associated with MDR.

For toxin carriage, there was a significant (P = 0.0142) association of ST with *expA* carriage; ST155 isolates were significantly more (P = 0.0017) likely to be *expA* positive. No association was seen with *expB* carriage, but ST155 and ST150 were significantly more (P = 0.0017) likely to carry *sec-canine*.

Most of the WGS isolates were from unique rMLST groups. The most common rMLST was 17491 (n = 9), 8 of which were ST 68. The next most common was 63159 (n = 3) and 63162 (n = 3).

Discussion

The majority of WGS isolates were biofilm producing (89%), with the majority of those being weakly- and moderately- adhering. This is similar to what we saw in Chapter III, agreeing with literature showing that the vast majority of *S. pseudintermedius* clinical isolates are biofilm producing (136, 155). Again, slime production was seen in approximately half of the isolates. All but four WGS isolates (98%; 156/160) contained the required *icaA* and *icaD* genes for PIA production, which suggests that, similar to what has been seen in *S. aureus*, isolates may have the *ica* locus but may still fail to produce slime *in vitro* due to sensitive growth conditions (25, 35, 141, 170). These results impress the importance of a combination of phenotypic and genotypic methods for examining biofilm formation in *S. pseudintermedius*.

The global population structure of *S. pseudintermedius* has been well examined; a 2016 review of 58 studies published in the previous decade determined five major MRSP CCs worldwide: CC71, CC258, CC68, C45, and CC112 (130). However, the eBURST analysis at that time assigned the isolates into only two major (CC71 and CC258) and two minor (CC379 and CC75) clonal complexes (130). Our goeBURST analysis of the 1047 isolates present in the *S. pseudintermedius* PubMLST database at the time of this study, including our 160 isolates, identified only one major CC comprising all of the major STs: CC258. This suggests that MLST lacks the power to separate the groups, a problem also recently observed in *S. aureus* (37).

Worldwide, ST71 is the most predominant MRSP lineage, followed by ST45, ST258, ST261, ST112, ST265, ST68, ST169, and ST181 (130). A 2018 study of 190 canine isolates from North America determined the region's major STs to be ST68, ST71, and ST84 (171). While our study also identified ST68, ST71, and ST84 as major North American STs, we discovered ST64 to be similarly highly represented. Unpublished data including an additional 32 sequenced isolates

suggests seven major North American STs are represented in our clinical isolates: ST45 (n= 6), ST64 (n = 5), ST68 (n = 13), ST71 (n = 7), ST84 (n = 5), ST150 (n = 3), and ST155 (n = 3), which is the first time ST45 has been found to be a major ST in the United States. It should be noted that this is not representative of North America as a whole, as sample collection was limited to Texas.

MRSP STs are known to be associated with virulence factor carriage (multidrug-resistance) and *agrD* alleles; indeed, in the previous 5-gene MLST scheme, *agrD* was one of the genes used to determine ST (8). Each of the major STs identified in this study is associated with a specific *agr* group, highlighting a lack of assortative recombination within these specific ST in our collection. While this is a small sample set from which to draw conclusions, this may suggest that, similar to *S. aureus*, radiation preceded clonal diversification and recombination played a lesser role in *agr* distribution (186).

Biofilm associations are less well understood; previously, ST71 was identified as one of the strongest biofilm formers of any MRSP lineage (11). For overall biofilm formation, ST71's distribution of CVA differed significantly from other types, but it was due to a slight majority of ST71 isolates being non-adherent while the rest were moderately adherent, in contrast to previous studies. Interestingly, ST71 was associated with surgical infections where biofilm and slime production may be important, suggesting that the conditions required for *ica* activation are both complex and sensitive, with potential variation between isolates (138). These main groups were also all more likely to be multiple drug resistant, highlighting their clinical importance.

The majority of our clinical isolates belonged to new, unique STs less closely related to the main six MRSP STs per goeBURST. These groups were associated with a higher proportion of healthy colonization and were less likely to be MDR. Most were MSSP, which is consistent with the known lack of clonality among MSSP isolates (17, 171). MSSP represents a lesser studied, but

still clinically relevant group that comprises the majority of *S. pseudintermedius* isolates. Due to their lack of clonality and unique STs, associations between MSSP STs and virulence factors are difficult to determine, but associations between *agr* group and infection and virulence profiles can still be examined.

The prevalence of *agr* groups in our clinical collection is similar to that seen in the only other classification of over 100 *S. pseudintermedius* isolates into *agr* groups, with *agr* I being the least prevalent group while *agr* II, III, and IV are similarly distributed at approximately 30% (8). Within our collection, *agr* group II was found to be more highly associated with commensal carriage and methicillin susceptibility and less likely to be slime-producing, MDR, or carry *expA* or *sec-canine,* while being more likely to carry *expB*. In contrast, *agr* groups I and IV were more likely to be slime-producing and MDR. Previously, *agr* III was determined to be more strongly associated with MRSP (33) than the other *agr* groups, but we did not see that in our collection. The lack of association with pyoderma, surgical, or urinary tract infections is not necessarily indicative of a lack of association of *agr* group and infections; such associations may be based upon severity of infection and other infection types that we were unable to examine in this study.

We found that healthy dogs are less likely to carry MRSP isolates than diseased, but this result is likely in part due to selection bias in the diseased group of dogs, as most dogs are only cultured if an infection is not responding to treatment. The surgical disease group was cultured at first appearance of problems without any prior treatments, but the urinary tract disease group and pyoderma disease group were potentially subject to this referral hospital bias. Healthy dogs were candidates for orthopedic surgery; while not entirely healthy, they did not present any visible skin problems and were cultured without any treatment discrimination.

Understanding the clinical associations of *agr* groups is important as demands for alternative methods of treatment grow due to increased antimicrobial resistances. The *agr* system is an alternative target for therapeutics in *S. aureus*, where inhibition of the *agr* system has demonstrated effects on virulence *in vivo* including increased survival, blocked abscess formation, decreased lesion size, hindered progression of skin infections, and improved host defense in murine models (24, 38, 122, 158, 185). The judicious use of antimicrobials in veterinary practice is an attempt to limit the spread of resistances to classes of drug considered important or of last resort in human medicine. For this reason, vancomycin is not used in small animals at the veterinary hospital from which these isolates were collected. As MRSP resistant to all drugs used for canine patients are frequently encountered in our patient population, there is a strong need for alternative therapies to treat resistant and pervasive infections. Disruption or interference with the quorum sensing system of *S. pseudintermedius* to reduce virulence is a potential avenue that would not apply selective pressures for antibiotic resistance. Furthermore, *agrD* alleles are known to act within and between species as potent agr inhibitors, and those of S. pseudintermedius, while not comprehensively examined, have demonstrated effects against S. aureus (24). Examining the cross-interference potential of the four *S. pseudintermedius* AIPs will be important, not only as a natural reservoir for potential therapeutics but also to understand the effects of co-colonization and potential clinical implications of patients colonized by *S. pseudintermedius* alongside *S. aureus* or *S. epidermidis*.

CHAPTER V

COMPLETE WHOLE-GENOME HYBRID ASSEMBLY AND GENOME COMPARISON OF CANINE AND HUMAN BACTEREMIA ISOLATES OF *S. PSEUDINTERMEDIUS*

Introduction

As of January 2019, there are only seven complete *S. pseudintermedius* genomes published on the National Centre for Biotechnology Information GenBank database (NCBI) (HKU10-03, ED99, NA45, 081661, 063228, G3C4, SP79) with 43 scaffolded partials and 90 contig submissions - in comparison, *Salmonella enterica* has 630 complete genomes with almost 3,000 scaffolded partials and 10,000 contig submissions. The goal of this experiment was to use two complementary sequencing techniques in order to try and complete the genomes of 8 *S. pseudintermedius* isolates to contribute to NCBI. The ability of staphylococci to invade the host and spread through the body via blood suggests the presence of important virulence factors (43, 48, 119). For this reason, sequenced isolates collected from patients with bacteremia may help future identification of important virulence factors. As *S. pseudintermedius* has recently been acknowledged as a human pathogen in addition to a major canine pathogen, whole genome sequencing of isolates from both species may provide valuable insights into the pathogenesis of the organism in humans.

We randomly selected four isolates from canine bacteremia cases to compare to four available isolates from human bacteremia cases. Complete genomes of these isolates may provide important information regarding potential differences in strains that infect humans. Isolates were sequenced using Oxford Nanopore's MinION technology, a long-read sequencing technology, and Illumina MiSeq. Long-read sequencing offers improved resolution of complex regions of the

genome (such as repeats or duplications) while short reads provide highly accurate but more fragmented region coverage. The accuracy of the MinION sequencing is lower than that of MiSeq, with an error rate ranging between 14-30% (133, 165). We compensated for this increased error rate with both high coverage levels for the MinION sequencing and by using the more accurate MiSeq sequences to correct the genome. In order to determine the best assembly algorithm that is bold enough to complete the genome while maintaining accuracy, we assembled the genomes through the MinION reads alone, MiSeq reads alone, and utilized two hybrid assembly algorithms and assessed the resultant assemblies for completeness. While we did not expect to see statistically significant differences at this sample size, we hypothesized that we would see descriptive differences in virulence factor carriage between human and canine isolates and potentially between their pan-genomes.

Materials and Methods

Bacterial isolates

Four canine bacteremia isolates were selected randomly from bacteremia isolates within the previously described clinical isolate collection (Chapter III). Four human bacteremia isolates were available to us from a collection of human isolates from the Ronald Reagan UCLA Medical Center, Los Angeles, CA and the Centers for Disease Control and Prevention, Atlanta, GA (187). The identification of the human isolates was performed according to the standard operating procedures of the submitting institutions and further confirmed at a central laboratory via a nucleic-acid-based assay. All isolates were stored and cultured as previously described in Chapter III.

DNA extraction, library preparation, and sequencing

For Illumina sequencing, a 1 mL aliquot of culture grown in BHIB for 8 hours at 37ºC was used for extraction. Samples were lysed in a Qiagen Tissue Lyser set to 30Hz for 30 seconds using the Macherey-Nagel Bead Tubes Type B and lysis buffer from the NucleoMag Tissue DNA Kit. Following the manual lysis, DNA was isolated following the manufacturer's protocol (Macherey-Nagel). Genomic DNA quality was verified on a genomic DNA Tapestation run (Agilent) and samples were quantified with the Qubit fluorometer. DNA sequencing libraries were prepared using the Illumina NexteraDNA Flex Library Preparation Kit following the manufacturer's protocol (Illumina). Libraries were pooled at equimolar concentrations and then sequenced on an Illumina MiSeq V2 2x250 sequencing kit.

For MinION sequencing, DNA was similarly extracted and purified, with the additional requirement of the DNA sample being approximately 20 ng/µL or higher concentration and the addition of lysostaphin during the lysing incubation. MinION libraries were prepared for sequencing on the Oxford Nanopore MinIon following the manufacturer's protocol for 1D PCR Barcoding of Genomic DNA using the SQK-LSK108. Genomic DNA quality was verified on a genomic DNA TapeStation run (Agilent) confirming high quality (>60 kb) DNA. Samples were quantified using High Sensitivity dsDNA Qubit assays on the Qubit 2.0 fluorometer (ThermoFisher). DNA samples were normalized to 22 ng/µL and sheared with a Covaris g-Tube to generate a ~8kb DNA fragment. After dA-tailing and adapter ligation, specific barcodes were added to each sample by PCR amplification using the NEB LongAMP Taq 2x Master Mix following conditions: 1 cycle - denature 3 minutes at 95°, 13 cycles – denature 15 seconds at 95°, anneal 15 seconds at 62°, extension for 10 minutes at 65°, then a final extension for 10 minutes at 65° followed by a 4° hold. After barcoding, all samples were pooled and prepared for loading onto

the MinIon Flow Cell. Data collection was performed by the MinKNOW software, utilizing the following workflow: NC_48Hr_Sequencing_Run_FLO_MIN106_SQK-LSK108.py. MinION sequencing data was basecalled using Albacore Sequencing Pipeline Software version 1.2.4.

Genome assembly and annotation

Four separate assemblies for each isolate were made and assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO) version 3, which provides a quantitative measure for the genome assembly, gene set, and transcriptome completeness (149, 176). BUSCO was run using the Firmicutes OrthoDB version 9 dataset. The assemblies were also checked by MLST and rMLST to ensure no errors with input reads were made, and to compare gene presence and completeness. The original MinION and MiSeq reads were assembled separately and assessed, and then two hybrid assembly approaches were made using Unicycler and Canu Polishing as described below.

MinION reads were assembled using Canu version 1.5 (89) with the following parameters: stopOnReadQuality=false, minReadLength=500, genomeSize=2.6m, useGrid=false. MiSeq reads were assembled using SPAdes as previously described in Chapter III.

The first hybrid assembly approach utilized Pilon version 1.22 (174) to improve the MinION assembly using MiSeq reads. The MinION Canu assembled FASTA file was provided as the input draft genome, along with BAM files generated by Bowtie2 (default parameters; 91, 92) of the MiSeq reads mapped to the MinION genome. Pilon uses read alignment analysis to recognize inconsistencies between the MinION genome and MiSeq reads and will correct single bases, small and large indeps, fill gaps, and correct misassemblies and then output an improved FASTA assembly. Pilon was run three times, to examine improvement between successive

polishing runs. Each subsequent run used the previous Pilon polished assembly genome as input, along with the BAM files for MiSeq reads mapped to the input genome.

The second hybrid assembly utilized the Unicycler 0.4.3 pipeline (183). Unicycler functions by assembling the MiSeq reads into a highly accurate 'assembly graph' made up of contigs and their interconnections. It then incorporates the MinION long reads to find the optimal paths through the graph (*i.e.* constrained scaffolding), therefore producing a completed assembly with lower missassembly rates. Unicycler was run in --bold mode for these assemblies, where the quality cutoff is reduced to increase the chance of completing the assembly.

Completed hybrid assemblies were scaffolded using Medusa version 1.6 (22) with three input reference genomes: ED99 (GenBank Accession No. CP002478) and the two single-contig polished assemblies of 51-092 and 53-060.

Typing and gene carriage

After assembly, *agr* type, MLST, and virulence gene carriage were examined as described in Chapter III. Ribosomal MLST was determined using the rMLST database (https://pubmlst.org/rmlst/) as part of the BIGSdb platform (80). New rMLST alleles were submitted for inclusion to the database (curated by Keith Jolley). Phylogenetic relationships of the 8 isolates based on MLST were visualized using PHYLOViZ as previously described in Chapter III.

Genome comparisons and pan-genome analysis

Pan-genomes for all the isolates, human isolates, and canine isolates separately were calculated using Roary version 3.11.2 (121). The input GFF3 files for Roary were generated using

Prokka version 1.13.3 (147), with the scaffolded and complete genomes as inputs. Pan-genome comparisons were run using querey_pan_genome with set one consisting of the canine isolates and set two consisting of the human isolates.

A Newick phylogeny tree file was generated using the Roary output core_gene_alignment.aln file as input for RAxML version 8.2.10 (153), for maximum-likelihood based phylogenetic inference. A pan-genome frequency plot, presence and absence matrix against phylogenetic tree, and a pie chart visualization of the pan genome were generated using roary_plots.py.

Analysis of the gene_presence_absence.csv file produced by Roary in conjunction with a traits file (canine/human, slime production, overall biofilm production, and *agr* type) was conducted using Scoary version 1.6.14 to determine significant associations between accessory genes and traits (23).

Proteome comparisons were generated using PATRIC's Proteome Comparison Service, which performs protein sequenced-based genome comparison using bidirectional BLASTP as previously described (177). Protein family analysis was similarly performed using PATRIC's Protein Family Sorter tool to generate clustered heatmaps and to summarize gene pathways.

Results

Genome assemblies

Assembly data is described in Table 5. The Illumina MiSeq sequencing resulted in an output of paired-end read sets containing approximately 1.5 million reads/isolate of 250 bp resulting in approximately 300X coverage. MinION sequencing resulted in an output of read sets containing 100 mb to 500 mb per sample, resulting in 40X – 200X coverage. MinION Canu

assemblies resulted in the fewest contigs, with two complete, single-contig assemblies (51-092 and 53-060) and the rest ranging from 5 to 29 contigs. MiSeq assemblies contained far more contigs (66 to 111). Unicycler assemblies were intermediate between the two, containing 1 to 58 contigs, while the Canu + Pilon hybrid assemblies preserved the low contig number from the MinION assemblies. Scaffolding using Medusa resulted in genomes with 1 to 16 scaffolds for Unicycler assemblies and 1 to 3 scaffolds for the Canu + Pilon polished assemblies. The Canu + Pilon polished genomes were consequently the most contiguous.

In terms of quantitative measures of completeness, BUSCO scores were very low in the original MinION assemblies (9.9% to 21.6%), while MiSeq were extremely high (99.5% to 99.6%). Unicycler assemblies preserved the range of MiSeq BUSCO scores. Pilon polishing the MinION assemblies increased the BUSCO score dramatically, with the first run bringing the scores to 96.6 - 99.6%. Successive runs of Pilon polishing increased the scores to similar values found in Unicycler, with the majority reaching 99.6%. Canu + Pilon BUSCO scores increased until the number of fragments reached 0 for all assemblies after the third Pilon run. One genome, 53-063, had a 0.1 higher BUSCO score with Canu + Pilon than Unicycler, while 49-044 and 46-057 both had lower BUSCO scores for Canu + Pilon assembly versus Unicycler (by 0.8 and 0.3 respectively).

Table 5: Bacteremia Genome Assembly Data

Assembly	Contigs	Scaffolds	BUSCO	MLST Completeness	rMLST Completeness
46_57					
MiSeq	111		99.5%	7/7	51/51
MinION	13		13.3%	1/7	5/51
Unicycler	14	2	99.5%	7/7	51/51
Canu + Pilon	13	2	99.2%	7/7	51/51
49_44					
MiSeq	96		99.5%	7/7	48/51
MinION	20		9.9%	0/7	3/51
Unicycler	31	7	99.5%	7/7	51/51
Canu + Pilon	20	1	98.7%	7/7	51/51
50_21					
MiSeq	66		99.6%	7/7	51/51
MinION	7		16.8%	1/7	8/51
Unicycler	11	1	99.6%	7/7	51/51
Canu + Pilon	7	2	99.6%	7/7	51/51
51_92					
MiSeq	66		99.5%	7/7	48/51
MinION	1		21.6%	2/7	8/51
Unicycler	1	1	99.6%	7/7	51/51
Canu + Pilon	1	1	99.6%	7/7	51/51
53_58					
MiSeq	90		99.6%	7/7	49/51
MinION	24		17.2%	2/7	9/51
Unicycler	22	3	99.6%	7/7	51/51
Canu + Pilon	24	2	99.6%	7/7	51/51
53_60					
MiSeq	66		99.5%	7/7	51/51
MinION	1		17.2%	6/7	9/51
Unicycler	1	1	99.6%	7/7	51/51
Canu + Pilon	1	1	99.6%	7/7	51/51
53_63					
MiSeq	91		99.5%	7/7	46/51
MinION	29		10.3%	1/7	5/51
Unicycler	29	16	99.5%	7/7	51/51
Canu + Pilon	29	3	99.6%	7/7	50/51
53_88					
MiSeq	69		99.6%	7/7	50/51
MinION	5		16.8%	2/7	7/51
Unicycler	5	1	99.6%	7/7	51/51
Canu + Pilon	5	1	99.6%	7/7	51/51

Assembly data for the four different assembly methods. Isolates 46_57 – 51_92 are canine isolates, 53_58 – 53_88 are human isolates. Base MiSeq and MinION assemblies were not scaffolded as they were not to be used in endpoint analyses.

For sequence typing, the MinION assemblies had low yields of identifiable genes. The rest of the assemblies could consistently be identified as their expected MLST in all assemblies. When using rMLST to examine ribosomal *S. pseudintermedius* genes, MinION assemblies performed similarly poorly while MiSeq, Unicycler, and Canu + Pilon assemblies were all complete enough to identify the vast majority of genes. Four new alleles were identified, submitted, and assigned types resulting in 4 new rMLST profiles for *S. pseudintermedius*.

As the most contiguous with high BUSCO scores, the Canu + Pilon hybrid assemblies were submitted to GenBank (Project accession PRJNA521119, genome accession numbers listed in Table 6) and used for the subsequent analyses.

Table 6: Bacteremia Published Genome Data

Isolate	Source	BUSCO (%)	N50	Genome Size (bp)	GenBank Accession	SRA MinION Accession	SRA MiSeq Accession
46_57	Canine	99.20	638705	2561987	SEZZ00000000	SRR8538958	SRR8538959
49_44	Canine	98.70	385638	2583863	CP035743	SRR8538960	SRR8538961
50_21	Canine	99.60	1401107	2527337	SEZY00000000	SRR8538954	SRR8538955
51_92	Canine	99.60	2512263	2512263	CP035742	SRR8538956	SRR8538957
53_58	Human	99.60	238709	2655352	SEZX00000000	SRR8538952	SRR8538953
53_60	Human	99.60	2615859	2615859	CP035741	SRR8538946	SRR8538947
53_63	Human	99.60	191852	2628670	SEZW00000000	SRR8538948	SRR8538949
53_88	Human	99.60	1396228	2593641	CP035740	SRR8538950	SRR8538951

Characteristics and accession numbers of genomes of *S. pseudintermedius* bacteremia isolates published to GenBank.

Virulence factors

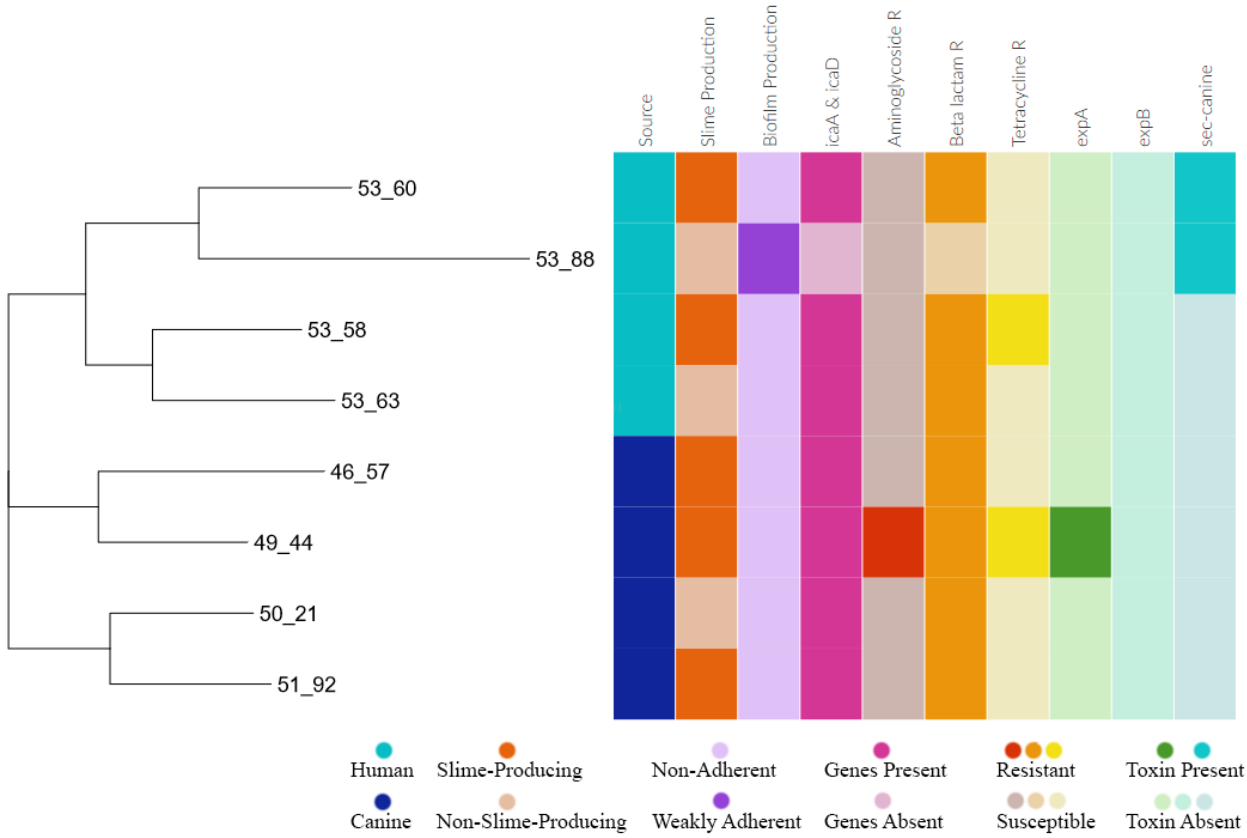
Virulence factors of the 8 bacteremia isolates are described in Figure 9. For biofilm production, 63% (5/8) were slime-producing, with 3/4 of the canine isolates and 2/4 of the human

isolates being slime producers. All of the isolates except 53-088 carried *icaAD* for slime-production. All isolates except for the human isolate 53-088 were found to be non-adherent when examining overall biofilm production via CVA. 53-088 was weakly adherent.

All isolates carried the toxin genes *se-int, Luk-I, siet,* and *speta*. None carried *expB*. The canine isolate 49-044 alone carried *expA,* while human isolates 53-060 and 53-088 both carried *sec-canine*.

All isolates were MSSP, as determined by the lack of *mecA*. Only 49-044 carried aminoglycoside resistance genes (both *ant(6)-Ia* and *aph(3')-III*). All isolates except 53-088 carried *blaZ* for β-lactam resistance, and both 53-058 and 49-044 carried *tet*(M) for tetracycline resistance.

Figure 9. Characterization of Bacteremia Isolates



Characteristics of bacteremia isolates organized by maximum likelihood phylogenetic tree. No significance was seen.

Sequence typing, agr, *and relatedness*

None of the bacteremia isolates were *agr* group I; 2 of the canine isolates and 1 human isolate were *agr* group II; 1 canine isolate and 2 human isolates were *agr* group III; and 1 canine and 1 human isolate were *agr* type IV. All 8 were unique MLST and they were also all unique rMLST (Table 7). The Newick phylogeny tree (Figure 9) suggests the 4 human isolates are more closely related to each other than any of the canine isolates, and vice versa. PHYLOViZ analysis (Figure 10) showed a more complex relationship when relatedness was based solely upon MLST and included all known STs submitted to the MLST database.

Table 7: Typing Characteristics of Bacteremia Isolates

Isolate	Source	*agr* Group	MLST	rMLST	Methicillin Resistance
46_57	Canine	II	815	65964	MSSP
49_44	Canine	IV	856	65100	MSSP
50_21	Canine	III	1335	102351	MSSP
51_92	Canine	II	865	65103	MSSP
53_58	Human	III	824	65109	MSSP
53_60	Human	II	879	65101	MSSP
53_63	Human	III	878	102352	MSSP
53_88	Human	IV	1045	102353	MSSP

Mulitlocus sequence typing, *agr* group, and MRSP/MSSP status of bacteremia isolates.

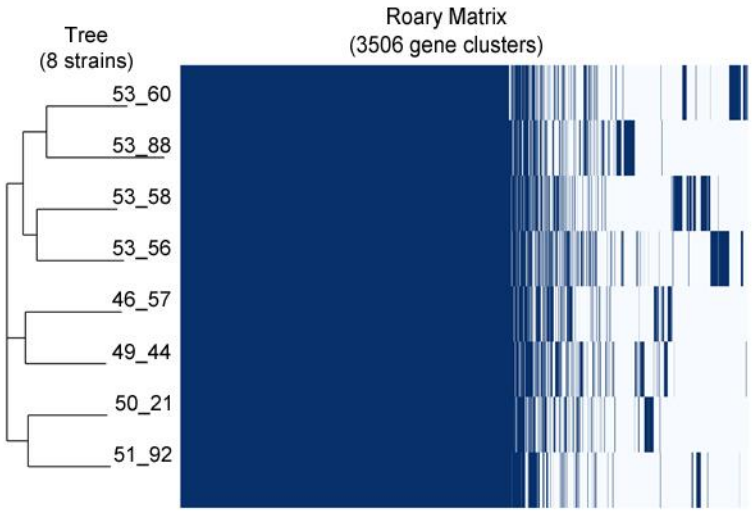
Figure 10: Phylogenetic Relationships of Bacteremia Isolates

A complex phylogenetic tree showing the more complex nature of how canine (blue) and human (purple) isolates are related based on all known MLST (via PHYLOViZ).

Pan-genome analysis

Pan-genomes represent the non-redundant genes present in a given dataset, consisting of the core genome (genes shared by all genomes in the dataset), accessory genome (absent in some genomes), and species- or strain-specific genes (present only in 1 genome). The core genome generally consists of genes involved in essential cellular processes, while accessory and strain-specific genes are often involved with niche adaptation (110, 162).

Figure 11: Roary Gene Clusters

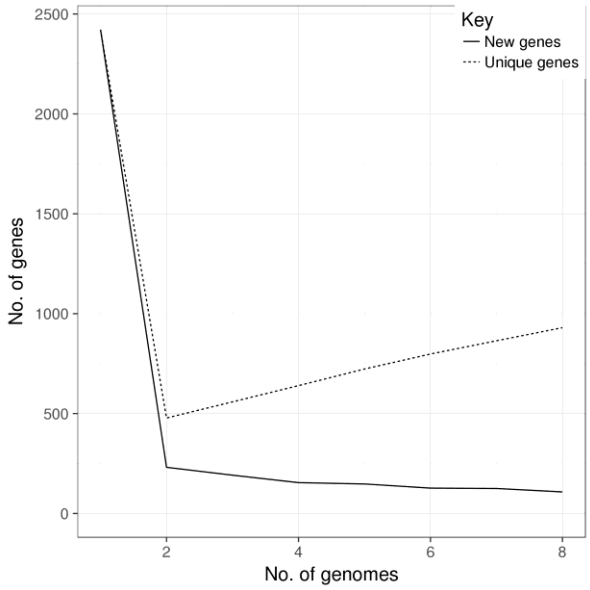


Roary output of gene groups shared between isolates, visualized by Roary_Plots. Isolates in descending order on the tree are 53_60, 53_88, 53_58, and 53_56 (human isolates); 46_57, 49_44, 50_21, and 51_92 (canine isolates).

Pan-genomic analysis of the 4 canine isolates alone found a core genome of 2,107 genes with 724 shell genes (genes present in 15% - 95% of isolates) for a total of 2,831 genes. The human isolates had a core genome of 2,088 genes with 1077 shell genes for a total pan-genome of 3,165. When analyzed together, the core genome of the 8 bacteremia isolates consisted of 2,032 genes, with 544 shell genes and 930 cloud genes (present in 0% - 15% of isolates) for a pan-genome of

3,506 genes. The gene clusters shared between the isolates were visualized using Roary_Plots and can be seen in Figure 11, while the number of new and unique genes found in the genomes is visualized in Figure 12. The most common core genes were hypothetical proteins (n = 577).

Figure 12: Core Gene Output



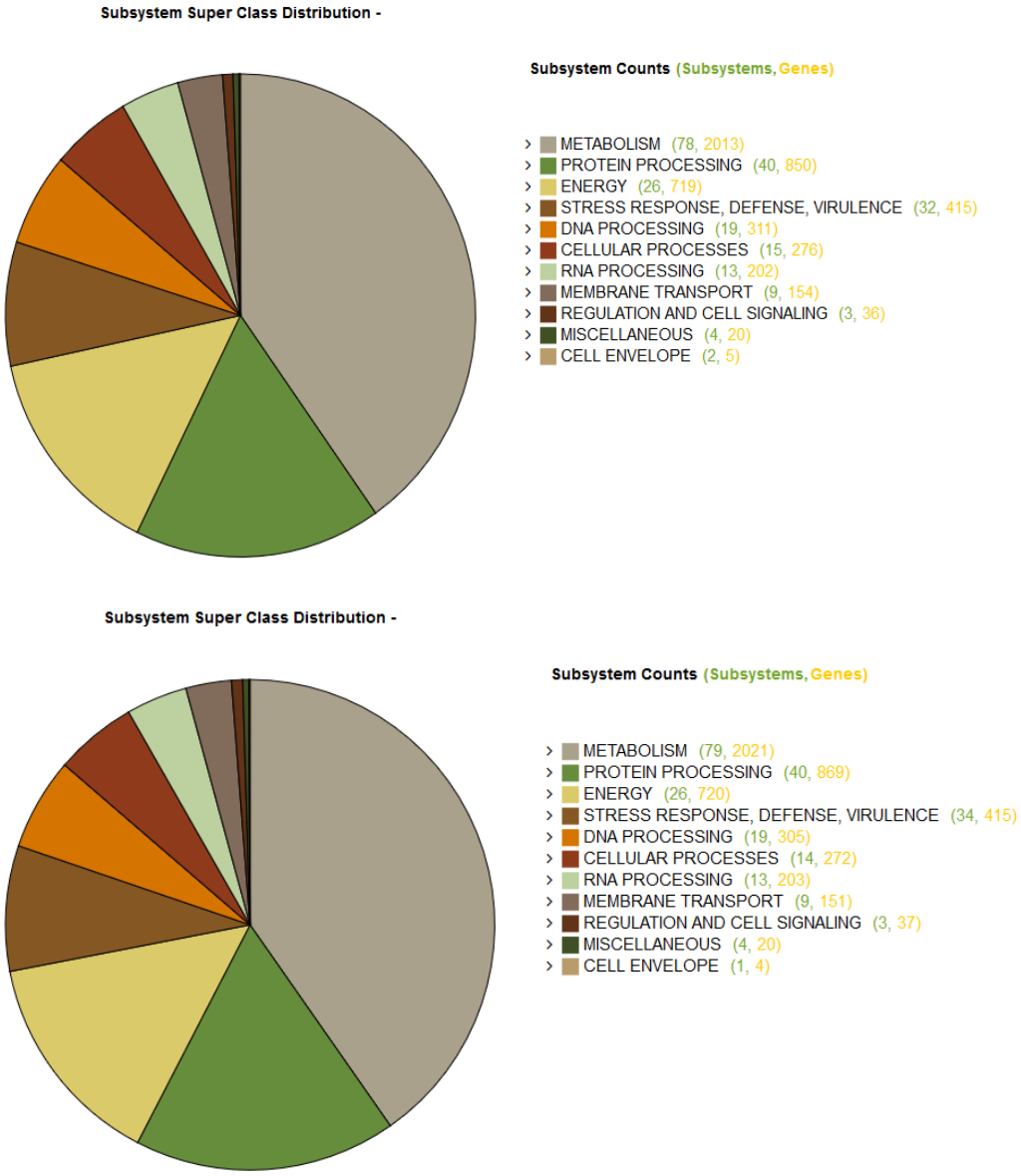
Roary output of genes shared between isolates, visualized by Roary_Plots, demonstrating the high amount of core genes shared between them as a marked decrease (seen from moving from 1 genome to 2 genomes) of new and unique genes identified when analyzing each genome.

The most common subsystem class of genes of the core pan-genome was metabolism, followed by protein processing, energy, and stress response, defense, and virulence. The subsystem distributions between human and canine isolates were similar (Figure 13). There were 2,487 genes found in both canine and human isolates, with 339 accessory genes found unique to the 4 canine isolates and 680 accessory genes unique to the 4 human isolates. Of these unique accessory genes, the majority (n = 306 for canine and n = 624 for human) were found in only 1 isolate from either group. From canine isolates, 24 genes were found in 2/4 isolates, 9 in 3/4 isolates, and none in all

4. From the human isolates, 52 genes were found in 2/4 isolates, 4 in 3/4 isolates, and none in all

4. The majority of these genes were hypothetical proteins.

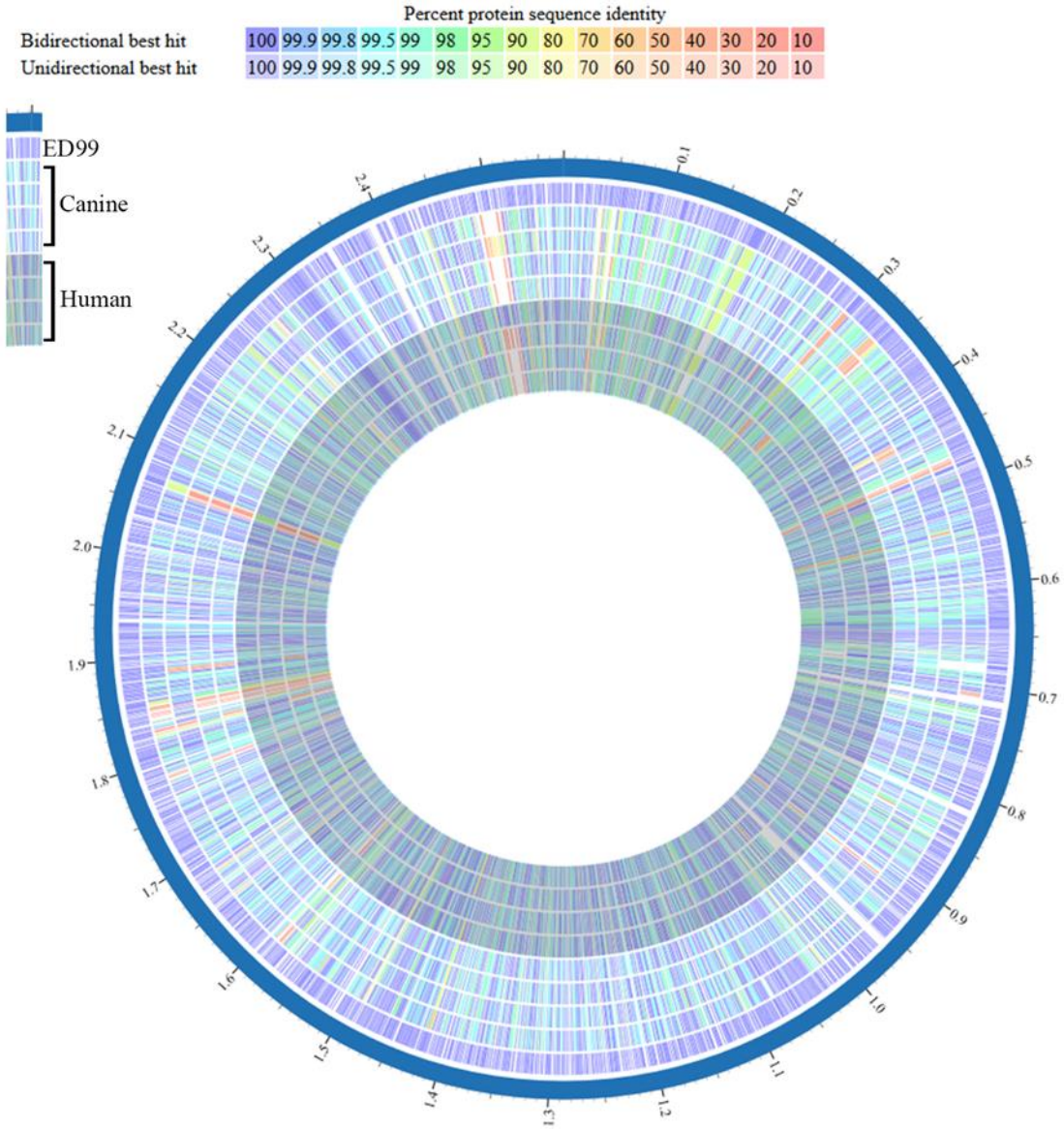
Figure 13: Protein Subsystem Distribution



Subsystem of human isolates (top) and canine isolates (bottom), produced by PATRIC's Protein Family Sorter.

No major regions of difference between human and canine isolates were apparent using a proteome comparison (Figure 14).

Figure 14: Proteome Comparison



Proteome comparison of human and canine isolates when compared to reference strain ED99. While regions of difference were apparent between our 8 isolates and ED99, major regions unique to canine vs human were not observed. Figure adjusted from PATRIC's Proteome Comparison Service.

For canine isolates, the 9 genes found in 3/4 isolates encoded 5 hypothetical proteins, a group II intron-encoded protein LtrA, an IS256 family transposase ISBsu2, a lipase, and a lactococcin-G-processing and transport ATP-binding protein. NCBI's Blastp analysis showed two of these hypotheticals had 100% identity to two *S. pseudintermedius* group II intron reverse transcriptases/maturases (GenBank BBH74133.1 and PXA19680.1) and one was most closely identical (98%) to a bacteriocin found in another *S. pseudintermedius* (GenBank WP_110165491.1).

For genes found in 3/4 of the human isolates, 3 encoded hypothetical proteins and 1 encoded a glycosyltransferase. NCBI's BLASTP found that one hypothetical protein had 100% identity to a *S. pseudintermedius* trypsin-like serine protease (GenBank WP_037543863.1) and another had 100% identity to an IS110 family transposase (GenBank WP_096548892.1).

No genes were found to be significantly associated with the source (human vs canine) of the isolates according to Scoary's statistical analysis, with the Bonferroni adjusted P value. Similarly, no genes were found significantly associated with *agr* group given the small sample size of the groups (0 I, 3 II, 3 III, 2 IV), although several genes were identified with a significant naïve P value due to being carried in all isolates of the respective trait and none outside (or vice versa) – while not to be considered statistically significant, these are potential genes of interest.

Of these, 4 genes were found only in *agr* group II isolates, which included the type II *agrB* allele and 3 hypothetical proteins (Blastp suggests these include *agrC* and *agrD*). Three proteins - *agrB* of other types and 2 hypothetical proteins suggested to be *agrD* alleles and a glutamyl endopeptidase - were found in all isolates outside of agr group II and none from group II. For *agr* group III a hypothetical gene was found in all 3 members of the group and none of the other isolates, and was suggested by Blastp to be the *agrC* III allele. Twelve genes of interest were found

in *agr* group IV; 2 genes found only outside group IV including a hypothetical protein and *rbsD_2*, a D-ribose pyranase found in all isolates outside of this group, and 10 genes found only within this group including 9 hypothetical proteins and an IS3 family transposase ISHahy5. Blastp analysis suggests the 9 hypothetical proteins include *agrC* and *agrD* of type IV, a nucleotidyltransferase, and an ATP-grasp domain-containing protein.

No genes were significantly associated with overall biofilm production or slime production; however, one hypothetical protein was found in all slime-producing isolates and *fnbA*, the fibronectin-binding protein A, was found in all non-slime-producing isolates and none of the slime-producing isolates.

Discussion

The combination of MinION and MiSeq reads allowed for genomes to be assembled into accurate and highly contiguous assemblies. MiSeq assemblies alone result in highly accurate, but very fragmented assemblies, while using them in conjunction with MinION maximizes the benefits of long-read sequencing technology (fewer contigs) and compensates for higher error rate. Unicycler and Canu + Pilon hybrid assemblies both can result in similarly near-complete genomes per BUSCO. Unicycler is a single-input pipeline that requires far less time and effort to run, while repeated Pilon polishing and BAM generation is more time consuming. However, Pilon polishing retains the low contig number from the MinION assemblies. While Unicycler did reduce the contigs from the number in MiSeq assemblies, it did not manage to match the number from the original or polished MinION assemblies, even when run on Bold mode. While both hybrid assembly strategies have advantages, for more contiguous and still highly scored assembles, we found Canu + Pilon to be superior.

Pan-genome analysis of these genomes identified approximately 300 accessory genes unique to the canine isolates and 700 to the human isolates, although the vast majority were hypothetical proteins found only in 1 isolate from each group. Combined with the small sample size, there were only a handful genes that were of note as they appeared unique to each source group and in the majority (3/4) of isolates from that group. Most of these genes were involved in mobile genetic elements, with 3 of the 9 proteins found unique to canine isolates encoding group II intron proteins and 1 encoding a transposase, and 1 of the 4 proteins found unique to the human isolates being a transposase. Two more genes found in canine sourced isolates were related to bacteriocin carriage (1 suggested/uncharacterized bacteriocin and one gene encoding the bacteriocin lactococcin G processing and export protein). Of potential note was the unique carriage of a glycosyltransferase by three of the four isolates from humans. Prokka annotation attributed the gene to the Gtf1 family, members of which are required to export GspB, a protein that mediates binding of *Streptococcus gordonii* to human platelets (132), and homologues have been found in *S. aureus* and *S. epidermidis* strains (UniProtKB A0A0U1MTH8 and A0A0H2VKQ1). Blastp matches it 100% to a teichoic acid biosynthesis protein carried in a canine *S. pseudintermedius* strain of a different MLST (ST84, strain NA45, NCBI Protein Annotation # ANQ80832.1) and >99% to a glycosyltransferase carried in *S. aureus*. The clinical implications of the carriage of this protein are currently unclear and the carriage was not statistically significant within this sample collection, however it is worth investigating the carriage of this gene and the related system proteins in a larger selection of *S. pseudintermedius* isolates from human infections.

Due to the small sample size, it is not surprising that few significant differences were found. Results of the Scoary analysis found no statistical significance in gene carriage between source or *agr* group with Bonferroni adjusted p values, although the program did identify genes that were

found only in certain *agr* groups, mainly their respective *agrBCD* alleles. Of potential interest was that the fibronectin-binding protein *fnbA* was only found in non-slime producing isolates and was in none of the slime-producing isolates, although 3/4 of the non-slime producing isolates carried *ica* operon for slime-production. Significant joint-carriage of *fnbA* and the *ica* operon has been seen in *S. aureus* (97), and a wide range of *S. aureus* isolates (40 - 80%) carry *fnbA* (3, 195), so this result is unexpected. As mentioned in previous chapters, *ica* expression and biofilm production is complex and subject to environmental conditions resulting in many *ica*-operon-containing isolates not producing slime and consequently reading as negative on CRA, while still producing biofilms via CVA. In this case, only 53-088, which lacked the *ica* operon, produced any overall biofilm on CVA. It is possible that the non-slime-producing isolates apart from 53-088 would produce slime in environments similar to *in vivo* colonization that were not tested in this study. Published literature has found higher carriage of *fnbA* in specific *S. aureus* infections, including auricular, orthopedic, endocarditis, and prosthetic hip and knee joint infections (3, 125, 137, 195). Given the small sample size, the implications of *fnbA* carriage only being seen in slime-negative isolates of bacteremia infections are unclear.

The pan-genomic analysis of these 8 genomes suggest that there is merit in analyzing the pan-genomes of larger sample sizes of isolates from human infections and canine infections, as differences are suggested even with this small sample size that cannot be fully elucidated without higher statistical power. As more human *S. pseudintermedius* infections are being identified, larger collections will allow for higher powered analysis that may be important in understanding the similarities and differences of *S. pseudintermedius* virulence and pathogenesis across these two species.

CHAPTER VI

CONCLUSIONS

Staphylococcus pseudintermedius is an important pathogen causing skin and a wide range of infections in both canines and humans. As its recognition as a significant human pathogen is recent, far less has been elucidated regarding the pathogenesis and virulence of *S. pseudintermedius* as compared to *S. aureus* and *S. epidermidis*. Understanding the epidemiology of the *agr* operon, which is an essential component of virulence regulation, and its associations with different types of infections, resistance, and virulence factors, provides valuable information to microbiologists and clinicians. The *agr* operon is a promising target for alternative treatment in *S. pseudintermedius* as increased antimicrobial drug resistance in this species make treatment challenging. Alternative therapies are of particular interest as judicious use of antimicrobials in veterinary medicine prevents using antibiotics considered drugs of last resort in human medicine. There were three aims of this study focused on improving our understanding of *S. pseudintermedius* virulence, the *agr* system, and infections in canines and humans. The findings are summarized as follows.

AIM 1: Measure biofilm forming capabilities of 710 *S. pseudintermedius* isolates collected from canines presenting with and without clinical disease to the VMTH, and classify them based on biofilm production.

We measured both overall biofilm production (via CVA) and slime production (via CRA) in 710 clinical isolates from unique dogs. Approximately half of the isolates (51%, n = 362) produced slime in the tested conditions, with slime production being significantly higher in isolates

from diseased dogs (55%) compared to healthy (39%). The majority (88%) of isolates produced biofilm per CVA, with most (78%) producing weak or moderate biofilms while only 12% were found to be non-adherent. The presence of overall biofilm production in the majority of isolates highlights the importance of biofilm to staphylococcal colonization. The lower number of slime-producing isolates, despite the fact that the vast majority of *S. pseudintermedius* isolates contain the *ica* operon for slime production, demonstrates the complex and highly regulated nature of biofilm production in these species. It is therefore important not only to perform a combination of phenotypical tests such as CVA and CRA, but to also take into account the genetics as well as conditions (nutrients, open systems, or microcosms) for testing isolates of interest. While these more involved biofilm assays and conditions can be impractical for large-scale characterizations such as the one performed here, biofilm production is too complex to be fully understood using the CVA and CRA assays alone.

AIM 2: Complete whole-genome sequencing and assembly of 160 clinical isolates. Determine the *agr* type of 160 isolates collected from dogs presenting with surgical infections, pyoderma, urinary tract infections, and healthy dogs that presented to the VMTH. Determine the MLST of all clinical isolates sequenced. Determine whether *agr* type is associated with disease type, MLST, or biofilm production.

WGS and genome assembly of the 160 canine isolates of *S. pseudintermedius* allowed us to identify the MLST of all isolates, including 113 new strain types. Our phylogenetic analysis using PHYLOViZ suggested the major North American MRSP clonal complexes to be CC 45, 64, 68, 71, 84, 150, and 155. This is the first time CC45 has been identified as a major group in the United States. The relationship between biofilm production (slime and overall) and MLST is,

unsurprisingly, complex, with ST71 previously being identified as the most slime-producing ST, while our study suggested it was significantly less associated with overall biofilm production while also being associated with surgical infections. These MRSP CCs were also all more likely to be MDR, but the majority of our isolates belonged to new ST groups and were MSSP. Due to a lack of resolution found using MLST, future studies should incorporate rMLST as the 53-gene scheme offers higher resolution of the relationships between STs and CCs.

The *agr* group of all 160 isolates was determined, with 11 isolates (7%) belonging to *agr* group I, 50 (31%) to group II, 44 (28%) to group III, and 55 (34%) to group IV. While the major MRSP STs are associated with a single *agr* group, *agr* groups were not associated with STs (i.e., all ST68 isolates are *agr* group IV, but not all isolates in *agr* group IV are ST68). The only significant association between *agr* group and disease group (healthy, pyoderma, urine, surgical) was that *agr* group II was more likely to be associated with healthy colonization rather than disease. No group had significant associations with overall biofilm production, although slime-production was significantly more associated with *agr* groups I and IV compared to II and III. Group II isolates were also less likely to carry MLS resistance genes, tetracycline resistance genes, and trimethoprim resistance genes – and, overall, group II isolates were less likely to be MDR. In contrast, group IV not only had increased association with slime production, but also with trimethoprim resistance gene carriage as well as carriage of the exfoliative toxin gene *expA* and, along with group II, were more likely to carry the toxin gene *sec-canine.*

Further study is required with isolates from both more specifically defined infections and infections with graded severity to better clarify the relationships between *agr* group and infection type. Investigation of the four *agr* alleles and their ability (or lack thereof) to cross-interfere with

each other and the consequent clinical impact of *agr* interference will be an important next step in assessing possible therapeutic avenues targeting the *agr* system in *S. pseudintermedius*.

AIM 3: Complete whole-genome assembly of 8 bacteremia isolates, four canine and four human, using a combination of long- and short-read sequencing technologies. Determine whether there are any describable differences between canine or human isolates regarding *agr* type, MLST, and virulence factors.

Four separate assemblies, including 2 hybrid assemblies, were performed for each isolate. The Canu + Pilon hybrid assembly was the most contiguous and scored approximately 99% for completeness via BUSCO, while Unicycler hybrid assemblies scored similarly high yet were far more fragmented. For our purposes of publishing complete (if possible) genomes, Canu + Pilon hybrid assembly proved to be the most successful.

There were no describable differences in *agr* type between human and canine bacteremia isolates, and they all had different sequence types. Seven of the eight isolates were found to be non-adherent without discernable biofilm formation, while one human isolate produced a weak biofilm. There were no clear differences in toxin carriage, slime production, or antimicrobial resistances between human and canine bacteremia isolates.

Interestingly, the Newick phylogeny tree generation suggests the human and canine isolates are more closely related to the others within their respective source groups, however this was not reflected in MLST phylogeny and may not be seen in a larger sample size. The pan genomes of the two groups did differ, mainly due to accessory gene carriage, but none of the gene differences were significant between human and canine genomes.

The majority of the genes found unique to human or canine isolates that were seen in 3/4 of the genomes were associated with mobile genetic elements. Statistical analysis of the pan-genome via Scoary did not find any significant associations with human or canine source, but did find genes of interest associated with slime-production and *agr* type. This suggests that pan-genomic analysis could provide valuable insight and find significant gene associations with characteristics such as those examined in Chapter IV, where a larger sample size would increase the statistical power. Further study is needed with a larger sample size of isolates to examine potential differences and genes of importance in human infections compared to canine infections.

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