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Whole-genome sequencing of *Staphylococcus pseudintermedius* isolated from nasal swabs of healthy dogs in Pennsylvania

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ABSTRACT

In this study, a genomic investigation of *Staphylococcus pseudintermedius* isolated from the nasal passages of healthy dogs was conducted to identify the presence of virulence and antimicrobial resistance genes and to study the ability of *S. pseudintermedius* to form biofilms. Eighteen isolates regrown from stock cultures maintained by the Bacteriology Section of the Penn State Animal Diagnostic Laboratory were characterized using whole-genome sequencing. Biofilm production was examined using a crystal violet staining method. Identified sequence types included MLST188, MLST241, MLST527, MLST764, and MLST1296, along with several unknown sequence types, reflecting diversity in the collection. Similar ranges of virulence genes, encoding enzymes, toxins, adhesions, and regulatory systems, that influence the ability of *S. pseudintermedius* to cause opportunistic infection were detected in the isolates. Particularly, great diversity in frequency was observed in *S. pseudintermedius* surface protein genes, markedly: *spsF*, *spsO*, *spsP*, *spsQ*, and *spsR*. A wide array of antimicrobial resistance genes was detected in this study that correlated with the phenotypic resistance of isolates, highlighting the concern of *S. pseudintermedius* in human and veterinary health. The most predominant antimicrobial resistance gene was the *blaZ* gene. In addition, four isolates were methicillin-resistant and encoded the *mecA* gene. In terms of biofilms, the *ica* operon was observed in most isolates; however, the relationship between the amount of biofilm formation and the presence of the *ica* operon is unclear. *Staphylococcus pseudintermedius* isolates from the nasal passages encoded for similar virulence and antimicrobial genes as isolates collected from healthy dogs and dogs with atopic dermatitis. This suggests that in addition to the skin, the nasal passages could be an important source for the transmission of *S. pseudintermedius*.

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INTRODUCTION

In recent years, the rapid emergence of genome-based sciences has enabled an increased understanding of the relationship between host and microbial genomes. This knowledge can be applied to preventing infection and modifying the bacterial population to protect and promote the health of animals, humans, and the environment.

Humans and animals alike are colonized by millions of diverse bacteria along bodily systems, such as the skin, gastrointestinal tract, reproductive organs, and respiratory system, known as the microbiome. These bacteria may show tropism to specific host species or host tissues and play a crucial role in moderating host health. For example, commensal bacteria inhabiting the skin surface replace the colonization of potentially harmful bacteria, produce antimicrobial products to destroy competing bacteria, and communicate with the host immune system (Khan et al., 2019).

Many areas of the skin seem inhabitable by bacteria because it is salty, acidic, and dry (Weese, 2013). Compared to the human gut microbiome, human skin does contain fewer microorganisms per unit of space (Weese, 2013). However, the skin is inhabited by a diverse assortment of bacteria that vary by body site, depending on local conditions like moisture, hair coverage, and temperature (Weese, 2013).

Bacterial skin infections are caused by a disruption in the normal relative proportions of bacteria composing the microbiome (Bradly et al., 2016). A reduction in microbial diversity inhibits the ability of the microbiome to shield the host and prevent the infection of new pathogenic bacteria (Tang et al., 2020). During this imbalance, a dominant bacterial species may

produce toxins, enzymes, or other factors that contribute to infection by modifying the microenvironment or damaging the skin (Bvm, 2009). As the number of pathogenic bacteria increases relative to other species, the more severe the infection becomes (Bradly et al., 2016).

Staphylococcus pseudintermedius is the most common cause of opportunistic bacterial skin infections in dogs. Although it is a normal inhabitant of the skin microbiome, relocation of cells to non-resident sites, nosocomial infection during surgeries, or immune suppression can lead to secondary infections, such as urinary tract infections, ear infections, or pyoderma (Zukancic et al., 2020). In recent years, methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has rapidly emerged as a threat to animal and human health and spread to many regions globally. The emergence of MRSP, its ability to cause opportunistic infection, and its zoonotic public health risk signifies the importance of disease prevention and genetic surveillance of the species.

The objectives of this study include:

1. To perform a genomic analysis of *Staphylococcus pseudintermedius* isolates obtained from nasal swabs of healthy dogs in Pennsylvania.
2. To examine the virulence and antimicrobial genes and identify prophages of *S. pseudintermedius* isolates.
3. To study the ability of *S. pseudintermedius* isolates to create biofilms.

REVIEW OF LITERATURE

1. Bacterial Species Associated with the Canine Epidermis

The most common bacteria found on canine skin include *Porphyromonas*, *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Corynebacterium* species and genera within the Neisseriaceae and Moraxellaceae families (Bradly et al., 2016). Although the same taxa can be found on both healthy and infected skin, the proportions of bacteria differ. Comparison of the skin microbiome of dogs with canine atopic dermatitis (cAD) and that of healthy dogs revealed that dogs with cAD experienced an increased relative abundance of *Staphylococcus* species on all areas of the skin compared to healthy dogs. Likewise, dogs with cAD experienced a decreased relative abundance of *Porphyromonas* species in the ear and armpit regions and an increase in the relative abundance of *Corynebacterium* in the groin region (Bradly et al., 2016). Bacteria found on infected skin are often the same as those found on healthy skin, indicating that bacteria associated with cAD are opportunistic pathogens (Weese, 2013).

Examples of skin infection-causing bacteria include *S. pseudintermedius*, *S. aureus*, *S. schleiferi*, *P. aeruginosa*, *E. coli*, and *Proteus* ssp. *Staphylococcus* species frequently cause skin infections, which are often secondary infections to atopic dermatitis caused by flea allergy dermatitis, demodicosis, or hypothyroidism (Fitzgerald, 2009).

The *Staphylococcus* genus is comprised of facultative anaerobic, gram-positive, catalase-positive, non-motile cocci that appear as clusters under a microscope. There are at least 45 different species within the genus and 24 subspecies. Staphylococci are frequent inhabitants of the skin and are well accustomed to its salty and acidic conditions. The staphylococci that are

considered pathogenic produce coagulases; however, not every *Staphylococcus* is coagulase-positive, such as *S. epidermidis*. Coagulase-negative Staphylococci may cause infection, but usually in hosts with altered immune responses (Bond and Loefler, 2012).

1.1. Staphylococcus intermedius group

Staphylococcus intermedius was first characterized by Hajek in 1976 from pigeon, dog, mink, and horse hosts. Until recently, it was accepted that *S. intermedius* was the predominant causative agent of skin infections in these animals. However, Meyer and Schleifer (1978) showed that there was significant genotypic diversity among *S. intermedius* isolates of separate biotypes, suggesting the presence of more than one staphylococcal species. In addition, Fitzgerald (2009) found that *S. intermedius* isolates showed host tropism to specific species using evidence from ribotype clusters and that co-evolution of *S. intermedius* with specific host species had occurred over time. *Staphylococcus delphini* was isolated from dolphins and first described in 1988, and *Staphylococcus pseudintermedius* was first described in 2005 after molecular characterization of cat, dog, horse, and parrot isolates (Bond and Loefler, 2012). These species previously considered to be *S. intermedius* were found to be distinct and have separate host tropisms. These findings resulted in the grouping of *S. intermedius*, *S. delphini*, and *S. pseudintermedius* as closely related species of the *Staphylococcus intermedius* group (SIG).

After the reclassification of the species, *S. pseudintermedius* was found to be the most common cause of skin infections in dogs, and other SIG members were not found on dogs. Based on these findings, it is now accepted that all canine isolates previously thought to be *S.*

intermedius are likely to be *S. pseudintermedius* unless proven otherwise by sequencing methods (Bond and Loefler, 2012).

2. Epidemiology of *S. pseudintermedius*

Staphylococcus pseudintermedius is frequently isolated from dogs with and without skin disease, constituting about 90% of staphylococci isolated from dogs (Bannoehr and Guardabassi, 2012). *Staphylococcus pseudintermedius* is a part of the normal microflora and has a preference to colonize mucocutaneous sites, such as the mouth, nose, and anus. Actions such as licking may relocate cells to regions where it is not resident and cause potential infection (Bvm, 2009). Nearly 69% of *S. pseudintermedius* isolates obtained from skin lesions were found to be closely related or identical to isolates obtained from the mucosa of the same dog (Bannoehr and Guardabassi, 2012). Likewise, 94% of isolates from skin lesions on dogs with pyoderma were identical to isolates from carriage sites- skin cells and hair- on the same dog (Bond and Loefler, 2012). This highlights the potential of *S. pseudintermedius* to act as an opportunistic pathogen and cause infection when paired with favorable predisposing factors.

Staphylococcus pseudintermedius is not frequently found to cause infection on human skin. However, it is possible that many *S. pseudintermedius* infections in humans are not reported due to misdiagnosis. Approximately 90% of *S. pseudintermedius* isolates tested on rapid latex slide agglutination tests used to screen for *S. aureus* will result in a negative test because it does not produce clumping factors or cell-bound proteins. This lack of sensitivity causes *S. pseudintermedius* to be categorized as a coagulase-negative staphylococcus (CNS) despite being coagulase-positive (Robb et al., 2017). Likewise, *S. pseudintermedius* and *S. aureus* have similar

morphologies, so they can be difficult to distinguish using regular microbiological procedures (Somayaji et al., 2016).

Although *S. pseudintermedius* is not commonly a human pathogen, there are reports of infection occurring in humans. For example, a retrospective study reviews clinical *S. pseudintermedius* infections of individuals with confirmed contact with dogs. Most individuals had a skin and soft tissue infection; however, these infections in humans must be differentiated from infections caused by *S. aureus* or other organisms which can provoke similar clinical conditions such as skin and ear infections and prosthetic joint infections (Somayaji et al., 2016).

Zoonotic transmission of *S. pseudintermedius* is reported in a study conducted by Guardabassi et al. (2004), which collected samples from humans and their dog and subtyped the *S. pseudintermedius* isolates using pulse-field gel electrophoresis (PFGE). The study revealed that half of the individuals with dogs were found to carry *S. pseudintermedius*, compared to the control group where only one individual without contact with dogs carried *S. pseudintermedius*. In addition, 6 of the 7 dog-owning individuals carrying *S. pseudintermedius* were infected with the same strain as their pet (Guardabassi et al., 2004). Transfer can occur through physical contact, such as bite wounds, or potentially the environment (Fitzgerald, 2009, Guardabassi et al., 2004).

The reports of *S. pseudintermedius* causing skin infection and sharing phenotypic characteristics with a known human pathogen indicate the pathogenic potential of *S. pseudintermedius* in humans. In addition, the combination of contact with dogs, whether it be pet-ownership or via a dog bite, and infection of *S. pseudintermedius* suggest zoonotic transmission (Somayaji et al., 2016).

The endurance of *S. pseudintermedius* has also been studied in household environments by Røken et al. (2022). It was found that all 7 households containing dogs infected with methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) harbored detectable quantities of MRSP in the food bowl, sleeping place, floor, kitchen, and bathrooms. In addition, indirect transmission is suggested by the discovery of MRSP in areas not within usual contact of dogs. Upon the dog's recovery from infection and cleaning of the environment, MRSP was still found in some households after 5 weeks, but not after 10 weeks (Røken et al., 2022). Despite continuous exposure of pet owners to their dog and a contaminated environment, only one individual tested positive for MRSP, and one individual tested positive for MSSP (Røken et al., 2022).

3. Characterization of *S. pseudintermedius*

3.1. Phenotype-based Identification Methods

Staphylococcus aureus and members of the *Staphylococcus intermedius* group are well-documented for their production of coagulases and are grouped as coagulase-positive *Staphylococcus* (CoPS) species (Bond and Loefler, 2012). CoPS species produce free or bound coagulase enzymes, which coagulate rabbit blood plasma (Barrow and Feltham, 1993, 21-45). Testing for coagulase production is most accurate using a tube method in which rabbit plasma is inoculated with a loopful of the isolate and incubated for 1-6 hours. The formation of an irreversible clot is indicative of coagulase production. For ease of use and quicker results, the slide test is another frequently used method to test for coagulase production; however, the

presence of protein A or clumping factor A bound to the surface of *S. pseudintermedius* may provide incorrect results (Bannoehr and Guardabassi, 2012).

Table 1. shows the phenotypic characteristics that may be used to differentiate CoPS species of canine and feline origin. Commonly observed factors include acetoin production (determined by the Voges-Proskauer reaction), and mannitol, trehalose, and lactose fermentation in aerobic conditions. *S. aureus* can most notably be distinguished from members of the SIG through the Voges-Proskauer reaction. CoPS species other than *S. aureus* do not produce acetoin during fermentation (Barrow and Feltham, 1993, pp. 50-93). In addition, *S. aureus* ferments trehalose, lactose, and mannitol. This can be distinguished from *S. delphini*, which does not ferment trehalose, and *S. intermedius*, which does not ferment mannitol. In addition, *S. pseudintermedius* is unable to ferment mannitol, and the fermentation of lactose can be variable (Bond and Loefler, 2012).

Table 1. Characteristics used for phenotypic identification of coagulase-positive staphylococcal species isolated from dogs and cats (Taken from Bond and Loefler et al., 2012).

	Staphylococcus sp.				
Test	***aureus***	***pseudintermedius***	***intermedius***	***delphini***	***schleiferi* ssp. *coagulans***
Hemolytic effect	+	+	+	+	+
Clumping factor	+	variable	variable	-	-
Tube coagulase	+	+	+	+	+
Voges-Proskauer reaction	+	weak	-	-	+
DNase	+	+	+	weak	+
Trehalose	+	+	+	-	-
Lactose	+	variable	+	+	-
Mannitol	+	-	-	+	variable

It is difficult to distinguish *Staphylococcus pseudintermedius* from other members of the SIG through routine phenotypic methods. All three species produce hemolysins and coagulases. Detecting for fermentation of mannitol or trehalose sugars can distinguish *S. pseudintermedius* and *S. intermedius* from *S. delphini* because the latter ferments mannitol and does not ferment trehalose in aerobic conditions. Based on these biochemical reactions, it is difficult to distinguish *Staphylococcus pseudintermedius* from other members of the SIG using only routine phenotypic methods. Therefore, a combination of accurate biochemical and enzymatic tests is required to correctly identify SIG species based on phenotypic characteristics (Bond and Loefler, 2012).

3.2. Proteomic-based Identification Methods

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a method used to identify bacterial cells based on their protein composition and comparison to an internal database. In a study, conducted by Savage et al. (2017), comparing the efficacy of MALDI to other common identification procedures used in veterinary medicine, such as ARIS and API, MALDI was able to correctly identify 93.9% of isolates obtained from bull tank milk. For catalase-positive, Gram-positive cocci, MALDI correctly identified all 45 (100%) isolates correctly to the genus level and 44 (97.7%) isolates to the species level. The only misidentification was *S. chromogenes* identified as *S. piscifermentans* (Savage et al., 2017). Therefore, MALDI suggests an accurate and effective method to identify *Staphylococcus* species.

MALDI-TOF has been used to distinguish *S. pseudintermedius* from other members of the SIG with variable efficacy. Silva et al. (2015) compared the identification of SIG isolates via

MALDI-TOF to biochemical tests, using PCR as a gold standard. All *S. pseudintermedius* isolates were identified as a SIG member, but only 24.5% of isolates were correctly identified, and the remaining 75.5% were classified as *S. intermedius*. It is important to note that the recent renaming of *S. intermedius* may have contributed to these results due to limited knowledge in the machine's database. In addition, only 21 out of 49 *S. pseudintermedius* isolates could be correctly identified using biochemical tests, further showing its insufficiency in characterizing SIG isolates (Silva et al., 2015).

Updating the MALDI database has shown increased efficacy in the identification and differentiation of *S. pseudintermedius* from other SIG members. In a study demonstrating this, 17 isolates initially and incorrectly identified as *S. intermedius* were reidentified correctly as *S. pseudintermedius* after supplementing the database with increased references of SIG from several locations and species (Murugaiyan et al., 2014). More recent studies have demonstrated that MALDI is sufficient in identifying *S. pseudintermedius* at the species level (Pérez-Sancho et al., 2020).

MADLI-TOF has the benefit of being cost-effective because it is quick, uses reusable targets, and requires few reagents and materials (Savage et al., 2017). However, the MALDI database must be updated and well informed to make accurate identifications.

3.3. PCR-based Identification Methods

The gold standard for identifying *S. pseudintermedius* was developed by Sasaki et al. in 2010 and involves multiplex PCR of the thermonuclease (*nuc*) gene. The study evaluated 374 *Staphylococcus* strains of 7 different species: *S. aureus, S. intermedius, S. schleiferi* ssp.

coagulans, *S. pseudintermedius*, *S. hyicus*, and *S. delphini* group A and B. Primers designated for each *Staphylococcus* species were used to mark the *nuc* gene locus in the extracted DNA from each isolate. The reaction mixture was amplified via thermal cycling and the resulting DNA fragments and the identity of the species were interpreted through electrophoresis (Sasaki et al., 2010). The *nuc* gene was selected for this method because it is well conserved but has sequence diversity among *Staphylococcus* isolates. The range of nucleotide identity of the nuc gene in coagulase-positive species and closely related coagulase-negative species is 60-95.9% (Sasaki et al., 2010). Therefore, the *nuc* gene provides enough of a difference between species to give accurate identification. Multiplex PCR of the nuc gene was found to be 99.8% sensitive and 100% specific (Sasaki et al., 2010).

The first diagnostic method used to differentiate *S. pseudintermedius* from *S. aureus* and other members of the SIG was reported by Bannoehr et al. in 2009 and is based on the principle that *S. pseudintermedius* has a unique *MboI* restriction site on the *pta* gene. The *pta* gene (320bp) is amplified through PCR, the mixture is subsequently incubated with the *MboI* restriction enzyme, and the resulting material is interpreted using agarose gel electrophoresis. *S. pseudintermedius* has one *MboI* restriction site on the *pta* gene, so 2 fragments (213bp and 107bp) appear on electrophoresis after digestion. This contrasts with *S. intermedius* and *S. delphini*, which do not have a *MboI* restriction site on the *pta* gene, and only one fragment (320bp) appears on electrophoresis after digestion. Lastly, *S. aureus* has a different *MboI* restriction site on the *pta* gene than *S. pseudintermedius*, so fragments of size 156bp and 164bp appear on electrophoresis after digestion (Bannoehr et al., 2009). Differentiation based on the *MboI* restriction site on the *pta* gene has been used numerous times to distinguish members of the SIG. For example, a study reclassifying 59 previously considered *S. intermedius* isolates and

comparing them to Rep-PCR analysis found that 94% were *S. pseudintermedius* and 6% were *S. intermedius* (Mališová et al., 2018).

3.4. Whole Genome Sequencing

Recently, whole genome sequencing (WGS) has emerged as a useful technique in molecular epidemiology to determine the entire genetic sequence of a microbial species. During the process, a library is created by fragmenting DNA into segments and tagging them. PCR is used to amplify the segments many times before the sequence is determined in a short-read sequencer. Depending on the platform, complementary DNA fragments are synthesized in parallel to result in a sequence (Coleman and Humphreys, 2019). Online resources are used for the analysis of sequenced data. WGS can be conducted in an increasingly cost-effective and rapid manner, and it has many applications. One application includes the typing of a pathogen to detect its relatedness to other isolates collected from the same animal, another species, or from other regions.

Single nucleotide polymorphisms (SNP) originate from single nucleotide variations that become fixed in the population. These can occur due to point mutations, insertions, deletions, duplications, horizontal gene transfer, or rearrangements. SNPs can be studied by comparing sequence reads to a reference genome to detect differences and relatedness between isolates (Coleman and Humphreys, 2019).

Multilocus sequence typing (MLST) is a method used to assess the genetic relatedness of isolates of the same species based on seven housekeeping genes. Changes in the sequence of each gene provides a new allele, and various combinations of alleles can be used to designate a

sequence type (ST) (Humphreys & Coleman, 2019). STs are significant in epidemiologic analysis because they can provide insight into a microbe's virulence, antimicrobial resistance, or worldwide distribution. For example, out of the major *S. pseudintermedius* lineages ST71 is found in Europe, ST68 is found in North America, and ST45 is found in Asia. However, there are over 2,000 recorded STs in the PubMLST database (Zukancic et al., 2020). MLST poses several limitations, such as overlooking other components of the genome. The characterization of an isolate can be further resolved using core-genome MLST (cgMLST) or whole-genome MLST (wgMLST). The core genome contains genes that are present in many isolates of the same species, and cgMLST uses predetermined core-genome genes to compare isolates. Similarly, wgMLST uses both the core genome and accessory genome obtained from WGS to compare isolates on a gene-by-gene case (Coleman and Humphreys, 2019). These two methods increase the accuracy of MLST by expanding the set of analyzed genes.

4. Virulence Determinants of *S. pseudintermedius*

Staphylococcus pseudintermedius encodes many genes to produce a wide range of virulence factors, including coagulases, nucleases, adhesions, toxins, and biofilms. Staphylococcal toxins include superantigens (enterotoxins), exfoliative toxins, and cytotoxins (leukocidins and hemolysins). Some virulence genes are encoded in the core genome and are common in all strains of *S. pseudintermedius*; however, other genes are obtained through horizontal gene transfer and are sometimes lineage specific (Zukancic et al., 2020).

Coagulases: Staphylococcus pseudintermedius produces a coagulase protein, encoded by the *coa* gene, that converts fibrinogen into fibrin and bypasses the normal coagulase cascade

reaction. This promotes the development of a fibrin shield that reduces the opsonization of the bacteria and hinders the ability of the immune system to detect and destroy the bacteria. The *S. pseudintermedius* coagulase protein is also able to bind canine IgG and C3, further disrupting the complement pathway (Sewid et al., 2018).

Nucleases: Both *S. aureus* and *S. pseudintermedius* produce nucleases that are involved in similar pathways to cause infection. Nucleases (NucB in *S. pseudintermedius*) degrade host DNA by targeting neutrophil extracellular DNA traps (NETs). NETs normally bind to pathogens to prevent their spread until they can be destroyed by neutrophils. However, the destruction of NETs allows *S. pseudintermedius* to escape this mechanism and produce bacterial cytotoxins or spread in the host instead (Bünsow et al., 2021).

Adhesions: The attachment of *S. pseudintermedius* to host extracellular matrix molecules, like fibrinogen, fibronectin, elastin, and collagen, is mediated by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Bannoehr et al., 2011). Each Staphylococcal species has a unique collection of MSCRAMMs, which provides evidence of host-tropism for each species (Bannoehr et al., 2012). In *S. pseudintermedius*, there are 18 genes encoding cell wall anchored proteins named *Staphylococcus pseudintermedius* surface proteins (Sps) (Bannoehr et al., 2011). Three of these proteins have been found to be significant contributors to the pathogenicity of the organism, SpsL, SpsD, and SpsO. Both SpsD and SpsO are involved in attachment to canine corneocytes, which may initiate skin infections. SpsD shows variation in its adherence to corneocytes based on individual dogs and across breeds, potentially explaining why some breeds, such as bull terriers and boxers, experience more skin infections (Bannoehr et al., 2011). The production of IgG with cognate receptors to SpsL and SpsD provides evidence of their role during infection (Bannoehr et al., 2011).

Exfoliative toxins: Exfoliative toxins are toxins produced by many Staphylococcal species, such as *S. aureus, S. hyicus,* and *S. intermedius,* that digest desmoglein (Dsg) in the skin. The destruction of Dsg interferes with the keratinocytes that maintain cell-cell adhesion and results in gross signs such as erythema and skin vesicles. *S. pseudintermedius* contains four genes responsible for exfoliative toxin production, *siet*, *speta*, *exp*A, and *exp*B. The *siet* and *speta* genes are frequently found among isolates (Bergot et al., 2018); however, the role of SIET in pathogenesis is under dispute, and the role of *speta* is not well characterized (Banovic et al., 2017). *Exp*A, previously named *exi,* and *exp*B are found in about a quarter of *S. pseudintermedius* isolates obtained from dogs with a skin infection and found much less frequently in healthy dogs (Iyori et al., 2010). These genes are homologous to the exfoliative toxins produced by other *Staphylococcus* species, and *exp*B contains the same conserved triad of amino acids found in other exfoliative toxins known to digest Dsg1 (Iyori et al., 2010). Interestingly, ExpA was not found to digest human Dsg1 by Iyori et al. (2011).

Leukocidins: Leukocidins are bi-component toxins categorized as pore-forming toxins that kill leukocytes with immense cell tropism and host specificity (Spaan et al., 2017). Leukocidins are composed of an S and F protein subunit. The S subunit is responsible for recognizing host cells and binding to receptors. Once bound, the F subunit induces pore formation and the release of iron and other divalent cations necessary for the cell to maintain homeostasis, resulting in cell death (Abouelhair et al., 2018). Pathogenic strains of *S. pseudintermedius* produce the toxin, Luk-I, in notable amounts. A study conducted to understand the secretome of *S. pseudintermedius* reported that canine polymorphonuclear leukocytes were lysed by Luk-I within 30 minutes at a concentration of 200ng of LukS-I and LukF-I (Abouelhair et al., 2018). This demonstrates the ability of *S. pseudintermedius* to weaken the host's immune

system through Luk toxins. The leukocidin genes, *LukS-I* and *LukF-I*, were found to be located on an incomplete prophage and are conserved among isolates (Abouelhair et al., 2018).

Hemolysins: S. pseudintermedius produces both alpha- and beta-hemolysins, and cause hemolysis in both sheep and rabbit red blood cells (Bannoehr and Guardabassi, 2012).

5. Biofilms

The formation of biofilms is an important virulence factor of staphylococci infection. Biofilms are a complex network of bacterial cells surrounded by an extracellular polymeric matrix composed of polysaccharides, proteins, and DNA. The formation of biofilms is described in four stages: attachment, proliferation, maturation, and dispersal. Bacterial cells can attach to biotic or abiotic surfaces and proliferate at this site. During maturation, these cells continue to divide and form a 3-dimensional structure. Eventually, cells will begin to detach from the matrix and disseminate in the host to colonize new regions of the body (Andrade et al., 2022). Biofilms provide several advantages to bacterial cells, such as enhancing cell-cell communication via quorum sensing and reducing metabolism. These two mechanisms provide increased tolerance to antibiotics, evasion of the host immune response, and serve as a hotspot for horizontal gene transfer (Singh et al., 2013).

Staphylococcus pseudintermedius has been shown to produce biofilms to varying degrees in several studies (Little et al., 2019; Wang et al., 2022). Biofilm formation may be influenced by the presence of the *ica* operon, which partly encodes polysaccharide intercellular adhesion (PIA), the extracellular matrix of the biofilm (Singh et al., 2013). Studies of *S. epidermidis* indicate that the *icaA* and *icaD* genes increase the production of PIA, but *ica*-independent biofilm formers

have been reported in *S. aureus* isolates (Singh et al., 2013). A study in *S. aureus* speculates that the absence of elastin-binding proteins combined with environments high in Zn^{2+} are also factors favorable for biofilm formation (Nakakido et al., 2014).

The accessory gene regulatory (*agr*) system regulates quorum sensing through autoinducing peptides (AIP) in staphylococci. AgrD is a precursor to AIP that is processed and exported from the cell by ArgB. Through two-component kinase signaling involving AgrC and AgrA, AIP is detected in neighboring cells. Through positive feedback, the production of proteases and toxins is increased, and expression of surface proteins is decreased (Little et al., 2019). *S. pseudintermedius* can be sorted into four distinct *agr* types based on the AIP produced (Little et al., 2019).

Biofilm formation has presented many issues in hospital settings by producing surgical site infections, leading to patient complications, prolonged hospitalization, and increased costs and frustrations to pet owners (Singh et al., 2013). A study examining the potential of *S. pseudintermedius* to form biofilms on various suture types found that multifilament cotton sutures were highly susceptible to bacterial colonization because of the large contract surface. This is in contrast to monofilament structures, like polypropylene, yet biofilms were still created on these surfaces to a lesser degree (Pesset et al., 2022). Biofilms of *S. pseudintermedius* are also able to grow on stainless steel in vitro, indicating its potential to grow on implants (Singh et al., 2013). These factors highlight the potential for *S. pseudintermedius* to create nosocomial infections.

6. Resistance of *S. pseudintermedius* to Antimicrobials

Staphylococcus pseudintermedius has been reported to be resistant to numerous classes of antimicrobials- many of which are commonly used in veterinary medicine- including beta-lactams, tetracyclines, macrolides, lincosamides, chloramphenicol, aminoglycosides, and trimethoprim (Kadlec and Schwarz, 2012). Resistance can be acquired through the integration of resistance genes from transposons into the bacterial genome (Kadlec and Schwarz, 2012). In addition, increased antimicrobial use has been linked with resistance. For example, clindamycin resistance was significantly greater in methicillin-susceptible isolates obtained from dogs with pyoderma and previously treated with antimicrobials within 12 months compared to dogs not treated with antimicrobials within 12 months (VanDamme et al., 2020).

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) is resistant to all beta-lactam drugs, such as penicillin and cephalosporins, and is often associated with multidrug resistance (Kadlec and Schwarz, 2012). MRSP can also be considered as analogous to methicillin-resistant *Staphylococcus aureus* (MRSA) seen in human hospitals because MRSP is responsible for nosocomial infections in veterinary clinics (Kadlec and Schwarz, 2012). For these reasons, MRSP has emerged as a major concern for animal and human health due to its ability to cause opportunistic infection and its resistance to many antimicrobials.

6.1. Antimicrobial Resistance Genes of S. pseudintermedius

Traditional antimicrobial susceptibility testing involves culture-based laboratory tests that determine how well a bacterial species can grow in the presence of an antimicrobial. Antimicrobial resistance is often encoded in the genome as well and can be detected using

sequencing methods with increasing efficacy (Boolchandani et al., 2019). Tyson et al. (2021) evaluated both the phenotypic and genotypic resistance of nearly 7,000 isolates and found a 98.4% correlation between phenotypic resistance and the presence of the gene coding for resistance. This indicates that whole-genome sequencing can be a tool for the identification of resistance; however, it does have a limitation of potentially reporting resistant isolates as susceptible (Tyson et al., 2021).

There are several antimicrobial resistance genes for each antimicrobial drug class. Beta-lactams are most significant to *S. pseudintermedius* due to the prevalence of MRSP. The *mecA* gene encodes alternative penicillin-binding protein (PBP2a), which inhibits the action of methicillin. It is also located on a mobile genetic element known as the staphylococcal cassette chromosome *mec* element (SCC*mec*) (Kadlec and Schwarz, 2012). The *blaZ* gene codes for beta-lactamase, which is an enzyme that destroys penicillin (Kadlec and Schwarz, 2012). Tetracycline resistance is created by genes that encode efflux pumps, *tet(K)* and *tet(L),* or ribosome protective proteins, *tet(M)* or *tet(O)* (Kadlec and Schwarz, 2012). Macrolides and lincosamide resistance are coded by *erm(A), erm(B), and erm(C),* which create 16S rRNA methylases (Kadlec and Schwarz, 2012). Chloramphenicol resistance is coded by the cat_{pC221} gene which prevents acetylation (Kadlec and Schwarz, 2012). Trimethoprim resistance, associated with folate pathway inhibitors, is predicted by the *dfrG* gene (Kadlec and Schwarz, 2012). Lastly, resistance to aminoglycosides is conferred by several genes: *aac(6')-Ie-aph(2')-Ia, aph(3")-III, and amt(6)-Ia* (Kadlec and Schwarz, 2012).

There is great variation in the types of antimicrobial genes and combination of genes in *Staphylococcus pseudintermedius* isolates, as demonstrated by Tyson et al. (2021) in a large genomic study looking at the genotypic and phenotypic resistance of isolates collected from

across the United States and Canada. Isolates were either found to have resistance genes to few drug classes (1-2) or many drug classes (6-7); however, the study found specific combinations of resistance genes. For example, every isolate with the *catA* gene (n=52) also had the *erm(B)* gene. In addition, nearly half of the isolates with the *mecA* gene also had *aac(6')-Ie-aph(2'')-Ia, ant(6)-Ia/aph(3')-IIIa, erm(B), blaZ, tet(M),* and *dfrG* genes (Tyson et al., 2021). Multidrug resistance in *S. pseudintermedius* complicates treatment options, so genomic monitoring of antimicrobial resistance genes and susceptibility testing is necessary to inform treatment plans.

MATERIALS AND METHODS

1. Collection of *S. pseudintermedius* isolates

Staphylococcus pseudintermedius isolates were obtained from a previous study conducted by Zemanek (2019) characterizing the nasal bacterial microflora of healthy household dogs. Isolates were stored in a bacterial culture stock repository at -80 ° C. Eighteen isolates of *S. pseudintermedius* isolated from nasal swabs of healthy dogs were selected for this study and were not associated with disease conditions in the previously surveyed dogs. The *S. pseudintermedius* isolates were sub-cultured on Tryptic Soy Agar (TSA) plates with 5% sheep blood (Remel, Inc.) and grown overnight at 37 ° C.

2. Bruker MALDI-TOF MS Identification System

Staphylococcus pseudintermedius isolates were reconfirmed using Bruker MALDI-TOF-MS Identification System. Isolates were grown on TSA plates with 5% sheep blood (Remel, Inc) and incubated for 48 hours at 37 ° C. Tube extraction, as described by Savage et al. (2017), was followed. To an Eppendorf tube, several small colonies were added into 300 µl of sterile water and vortexed. 900 µl of ethanol was added to the tube, and then the mixture was vortexed and centrifuged for 2 minutes at 13,000 rpm. The ethanol was decanted, and the remaining ethanol was evaporated under the hood at room temperature. Once dried, 50 µl of 70% formic acid was added to the tube, vortexed, and let rest for 5 minutes. Then, 50 µl of 100% acetonitrile was added to the mixture and centrifuged for 2 minutes at 13,000 rpm. 1 µl of supernatant was pipetted onto a steel target plate and dried. 1 µl of matrix solution, consisting of a-cyano-4-

hydroxy-cinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, was added on top of the dried spot. A bacterial test standard (BTS) was included to the steel plate for calibration.

The MALDI-TOF MS was performed in a Bruker Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The machine was operated using the same settings as Zemanek (2019).

3. Whole Genome Sequencing

3.1. Library Preparation

Staphylococcus pseudintermedius isolates were grown overnight at 37 ° C. Then an isolated colony was centrifuged, pelleted, washed, and used for DNA extraction using the QIAGEN Genomic-tip 100/G kit (Qiagen Germantown, MD) following the manufacturer's instructions for gram-positive bacterial cell lysate. Next-generation sequencing libraries were prepared with a Nextera DNA Flex library kit (Illumina, San Diego, CA) following the manufacturer's instructions. The pooled libraries were sequenced on an Illumina MiniSeq using a 300-cycle reagent kit. The sequencing ran for approximately 23 hours and generated 150 bp paired reads.

3.2. Sequence Data Processing and Genome Assembly

FastQC (Andrews, 2010) was used for quality assessment of the Illumina reads. Quality improvement and adapter trimming was conducted using BBDuk v38.96 (Bushnell et al., 2017)

with the parameters trimq=20 (average quality), qtrim=rl (trim both ends), minlength = 50. SPAdes v3.15.4 (Bankevich et al., 2012) was used to create de novo assemblies of all the Illumina reads with careful command. Quast (Gurevich et al., 2013) was used to evaluate genome assemblies. Pan-genome analysis was completed through Roary 3.7.0 (Page et al., 2015) using GFF3 files produced by Prokka 1.12 (Seemann, 2014) from isolate assemblies and annotation files of the isolates. A phylogenetic tree was created using iTOL (Letunic and Bork, 2021).

3.3. Genome Characterization

Virulence-associated genes of the assembled genomes were determined using a custom database of *S. pseudintermedius* virulence genes. NCBI blast v.2.11.0 (Camacho et al., 2009) was used to find high similarity (>90%) of virulence genes between the assembled genomes and the custom database. Antimicrobial resistance genes were determined using a locally installed version of ResFinder v4.0 (Bortolaia et al., 2020) from the Center for Genomic Epidemiology (CGE)'s database (https://bitbucket.org/genomicepidemiology) using *S. aureus* as a model. Multilocus sequence types were determined using a locally installed version of MLST pipeline also from CGE. Phages within the bacterial genome were identified using PHASTER (Arndt et al., 2016; Zhou et al., 2011) by uploading FASTA files.

4. Determination of Biofilm Formation

The ability of *S. pseudintermedius* to create biofilms was determined following the method described by Kwasny and Opperman (1999) and Singh et al. (2017) with modifications.

Isolates were cultured on TSA with 5% blood agar (Remel, Inc.) and transferred into Muller Hinton Broth at a MacFarland Standard of 0.5 (~1.5 x 108 CFU/mL). Isolates were tested in triplicate on a 96-well plate. 10 µl of the bacterial suspension was mixed with 190 µl Tryptic soy broth containing 1% glucose and incubated for 24 hours at 37 ° C. Then, the bacterial supernatant was aspirated, and the well was washed with phosphate buffer saline (PBS, pH 7.2). The plate was fixed in an incubator at 60 ° C for 2 hours. The wells were dyed using crystal violet and rinsed with distilled water. The plate was dried for 30 minutes before reading in an ELISA plate reader at the PSU Animal Diagnostic Lab. The biofilm was dissolved using 33% glacial acetic acid for 30 minutes, and the biofilm production was quantified by measuring absorbance at 595nm with a 96-well plate reader.

RESULTS

A total of 18 *S. pseudintermedius* isolates were obtained from a previously conducted study by Zemanek in 2019 (https://honors.libraries.psu.edu/catalog/6100mcz5057) in which the nasal bacterial flora of healthy dogs in Centre County was examined. The isolates were re-grown from stock cultures maintained by the Bacteriology Section of the Penn State Animal Diagnostic Laboratory. *Staphylococcus pseudintermedius* isolates were reidentified using Bruker MALDI-TOF-MS Identification System. All isolates had a MALDI score > greater than 2.0 and were correctly identified to the species level as *S. pseudintermedius.*

1. Genome Characteristics

A summary of the genome, including contigs, size, GC contents, N50 values, and number of genes is included in Table 2. The size of the assembled genome ranged from 2,460,547 bp to 2,714,597 bp. Average assembled genome size was 2,566,825 bp. All isolates were assembled with less than 50 contigs. The G-C contents ranged from 37.07% to 37.64%. The N50 values ranged from 95,656 bp to 272,791 bp. The number of predicted protein-coding sequences (CDSs) ranged from 2284 to 2615. The average CDS was 2,418.11 genes. All isolates had 2 to 6 rRNAs and 31 to 59 tRNA loci. All isolates had 1 tmRNA.

Multilocus sequence types could not be identified for all isolates; however, the closest sequence type was determined for isolates lacking a sequence type (Table 3). Sequence types were identified for 7 isolates, including which belonged to 5 different sequence types: MLST188, MLST241 (n=2), MLST527, MLST764, MLST1296 (n=2). The closest sequence type was provided for the remaining 11 isolates.

A phylogenetic tree of *S. pseudintermedius* isolates was created to visualize genetic relatedness of isolates (Fig 1).

Table 2. Genomic characteristics of *S. pseudintermedius* isolates from the nasal passages of healthy dogs in Centre County.

Isolate No.	# Contigs	Total length (bp)	GC (%)	N50 (bp)	Total number of genes (CDS)
1	31	2568997	37.48	168228	2516
2	32	2589848	37.47	212574	2533
3	48	2621398	37.41	96608	2531
4	49	2598111	37.41	114347	2520
5	43	2666298	37.13	133247	2611
6	41	2714597	37.07	136173	2681
7	26	2582145	37.31	272791	2491
8	44	2460547	37.54	126797	2341
9	37	2589700	37.49	160967	2537
10	42	2547843	37.42	95781	2447
11	44	2547593	37.42	95656	2449
12	36	2496741	37.59	143077	2393
13	32	2608227	37.38	227094	2505
14	34	2611105	37.38	227095	2504
15	37	2465657	37.55	143304	2349
16	39	2464546	37.56	126798	2351
17	25	2519216	37.64	202674	2428
18	30	2550278	37.51	239904	2454

Table 3. Multilocus sequence types (MLST) of *S. pseudintermedius* isolates from the nasal passages of healthy dogs in Centre County.

Isolate No.	MLST	Closest Sequence Type
4	188	-
10, 11	241	-
3	527	-
7	764	-
13, 14	1296	-
8, 15, 16	-	689
1	-	1477
18	-	1916
17	-	172, 1168
9	-	850, 1200, 1724
5, 6	-	113, 116, 179, 2331, 2555
12	-	87, 537, 546, 1055, 1359, 1383, 1558, 1858, 2360, 2417, 2550
2	-	22, 23, 204, 215, 471, 481, 536, 634, 1215, 1225, 1396, 1465, 1466, 1564, 1630, 1933, 2035, 2138, 2160, 2335

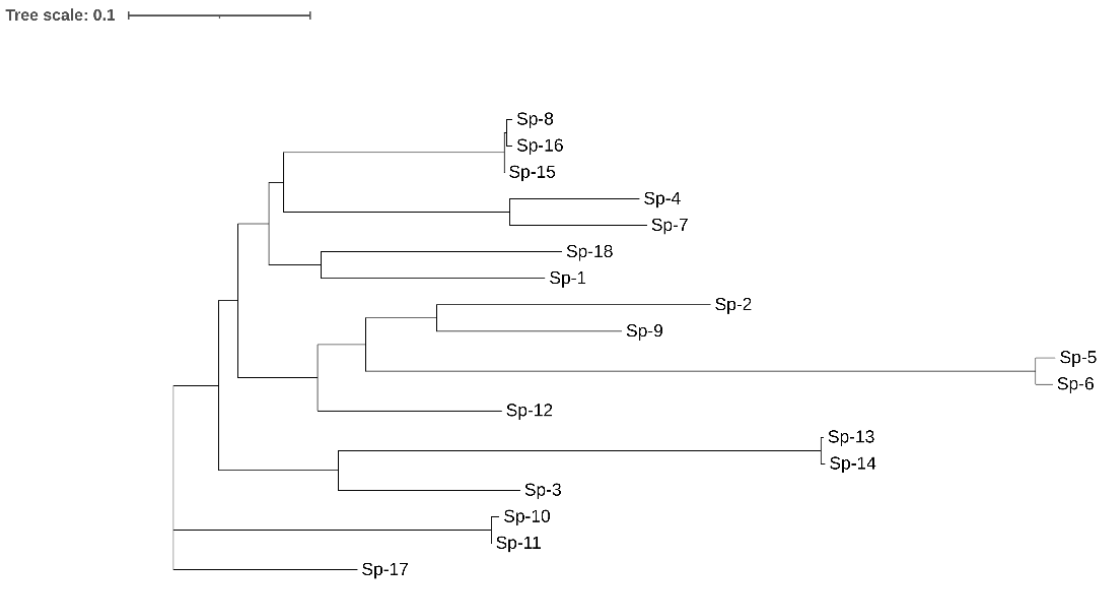

Figure 1. Phylogenetic tree of *Staphylococcus pseudeintermedius* isolates.

*Sp: *Staphylococcus pseudintermedius*

2. Virulence Genes and Accessory/Regulatory Genes

Fifty-one genes encoding virulence factors and other genes were identified in the genome of the *S. pseudintermedius* isolates (Table 4). All isolates contained seven genes associated with enzymatic virulence factors. These include proteases (*clpX*), coagulases (*coa*), serine proteases (*htrA*), triacylglycerol lipase (*lip*), and thermo-nuclease (*nucA, nucB, nucC*). Twelve isolates (67%) contained the *nanB* gene, responsible for sialidase production.

Six genes associated with toxin production were identified. Toxins include hemolysins, leukocidins, enterotoxins, and exfoliative toxins. All isolates contained *hlb*, encoding beta-hemolysin production, *LukF-I* and *LukS-I*, encoding leukocidin production, *sei*, encoding enterotoxin production, and *siet* and *speta*, encoding exfoliative toxin production (Table 4).

Twenty-three genes related to bacterial adhesion were identified. The most diversity in virulence genes was seen with adhesions, specifically *S. pseudintermedius* surface protein genes: *spsA, spsB, spsC, spsD, spsE, spsF, spsG, spsH, spsI, spsJ, spsK, spsL, spsM, spsN, spsO, spsP, spsQ,* and *spsR.* All isolates contained *spsA-E, spsG-N*, and *spsR* genes. Ten isolates (56%) contained *spsO*, five isolates (28%) contained *spsF*, and three isolates (17%) contained *spsP* and *spsQ*. These genes are responsible for the adherence of bacterial cells to extracellular matrix proteins of the host. All isolates contained elastin-binding protein gene (*ebpS*), which is another cell wall associated protein related to adherence and potentially biofilm production. Intracellular adhesion protein, encoded by *icaA, icaB, icaC, icaD* genes, was detected in 17 (94%) isolates and is also related to biofilm production (Table 4).

All isolates contained accessory genes and other regulatory genes expected in the genome. These include accessory gene regulars (*agrA, agrB, agrC, agrD*), *Staphylococcus aureus* exoprotein expression regulator (*saeR, saeS*), repressor of toxins (*roT*), and

Staphylococcal accessory gene regulator (*sarA, sarR*). In addition, all isolates contained autolysis related locus genes (*arlR, arlS, lytR, lytS*) and Staphylococcal respiratory response protein gene (*srrA*) (Table 4).

Table 4. *Staphylococcus pseudintermedius* genes of nasal isolates from healthy dogs in Centre County identified with whole genome sequencing.

Gene group	**Protein/Function**	**Genes**	***S. pseudintermedius* isolates (n=18) (%)**
Enzymes	Protease	*clpX*	18 (100)
	Coagulase	*coa*	18 (100)
	Serine protease	*htrA*	18 (100)
	Triacylglycerol lipase	*lip*	18 (100)
	Thermo-nuclease	*nucA, nucB, nucC*	18 (100)
	Sialidase	*nanB*	12 (67)
Toxins	Hemolysins	*hlb*	18 (100)
	Leukocidins	*LukF-I; LukS-I*	18 (100)
	Enterotoxin	*sei*	18 (100)
	Exfoliative toxins	*siet; speta*	18 (100)
Adhesions	Elastin-binding protein	*ebpS*	18 (100)
	Intracellular adhesion proteins	*icaA, icaB, icaC, icaD*	17 (94)
	S. pseudintermedius surface proteins	*spsA, spsB, spsC, spsD, spsE*	18 (100)
		spsF	5 (28)
		spsG, spsH, spsI, spsJ, spsK, spsL, spsM, spsN	18 (100)
		spsO	10 (56)

		spsP, spsQ	3 (17)
		spsR	18 (100)
Accessory, regulatory, and repressor genes	Accessory gene regulator	*agrA, agrB, agrC, agrD*	18 (100)
	S. aureus exoprotein expression regulator	*saeR, saeS*	18 (100)
	Repressor of toxins	*roT*	18 (100)
	Staphylococcal accessory gene regulator	*sarA, sarR*	18 (100)
Other genes	Autolysis related locus	*arlR, arlS, lytR, lytS*	18 (100)
	Staphylococcal respiratory response protein	*srrA*	18 (100)

3. Antimicrobial Resistance Genes

Staphylococcus pseudintermedius isolates encoded for antimicrobial resistance for aminoglycosides, beta-lactams, chloramphenicol, trimethoprim, macrolides/lincosamides, quaternary ammonium compound, and tetracycline (Table 5). Aminoglycoside resistance genes identified were *ant(6)-Ia, aph(3")-III,* and *aac(6')-aph(2").* Beta-lactam resistance belonged to *blaZ* and *mecA* types of resistance to beta-lactams. Chloramphenicol (*cat(pC221)*), trimethoprim (*dfrG*), and erythromycin and clindamycin (*erm(B)*) resistance genes were identified in sequenced isolates. In addition, resistance genes for tetracycline (*tet(M)*) and quaternary ammonium compound by *qacF* and *qacG* were identified.

The most frequent resistance was against beta-lactams and produced by the *blaZ* gene (78%). Resistance to beta-lactams due to the *mecA* gene was also detected in 22% of isolates. Resistance due to aminoglycoside genes *ant(6)-Ia/aph(3')-IIIa* and *aac(6')-aph(2")* (28% and 22%, respectively), trimethoprim gene *dfrG* (33%), and erythromycin gene *erm(B)* (28%) was also common. Resistance was less frequently driven by tetracycline gene *tet(M)* (22%), chloramphenicol gene *cat(pC221)* (11%), and quaternary ammonium compound genes *qacF* and *qacG* (11% and 6%, respectively) (Table 5).

The antimicrobial resistance profiles studied by Zemanek (2019) and antimicrobial resistance gene determinants of this study are listed in Table 6.

Fifteen isolates had a resistance gene correlating with at least one antimicrobial class. Four isolates contained only the *blaZ* gene, and one isolates contained only the *qacG* gene. The remaining 10 isolates were resistant to more than one drug class and contained a combination of *ant(6)-Ia/aph(3')-IIIa, aac(6')-aph(2"), blaZ, mecA, cat(pC221), dfrG, erm(B), qacF, and tet(M)* genes. Three isolates did not encode for resistance genes.

Table 5. Antimicrobial resistance genes identified in *S. pseudintermedius* nasal isolates from healthy dogs in Centre County.

Antimicrobial Class	**Antimicrobial Resistance Gene**	***S. pseudintermedius* isolates (n=18) (%)**
Beta-lactam resistance	*blaZ*	14 (78)
	mecA	4 (22)
Trimethoprim resistance	*dfrG*	6 (33)
Macrolide/lincosamide resistance	*erm(B)*	5 (28)
Aminoglycoside resistance	*ant(6)-Ia*	5 (28)
	aph(3")-III	5 (28)
	aac(6')-aph(2")	4 (22)
Tetracycline resistance	*tet(M)*	4 (22)
Chloramphenicol resistance	*cat(pC221)*	2 (11)
Disinfectant resistance	*qacF*	2 (11)
	qacG	1 (6)

Table 6. Antimicrobial resistance profiles and antimicrobial resistance gene determinants of *S. pseudintermedius* nasal isolates from healthy dogs in Centre County.

Isolates	Antimicrobial [a]	Antimicrobial resistance gene determinants [b]
1, 12, 18	Sensitive	-
17	Sensitive	*qacG*
7	TE	*blaZ, tet(M)*
15	P	*blaZ*
4, 8, 16	AM, P	*blaZ*
2	AM, P, SXT	*blaZ, dfrG*
3	AM, P, TE	*blaZ, tet(M)*
6	AM, P, SXT, ENO	*blaZ, mecA, dfrG, aac(6')-aph(2')*
5	AM, P, CPD, SXT, ENO	*blaZ, mecA, dfrG, aac(6')-aph(2')*
9	AM, P, SXT, E, CC	*blaZ, dfrG, erm(B), ant(6)-Ia, aph(3')-III*
10,11	AM, P, E, C, CC	*blaZ, erm(B), cat(pC221), ant(6)-Ia, aph(3')-III*
13	AM, P, SXT, E, TE, CC, ENO	*blaZ, mecA, dfrG, erm(B), tet(M), qacF, ant(6)-Ia, aph(3')-III, aac(6')-aph(2'')*
14	P, OX, CPD, SXT, E, TE, CC, ENO	*blaZ, mecA, dfrG, erm(B), tet(M), qacF, ant(6)-Ia, aph(3')-III, aac(6')-aph(2'')*

[a] Ampicillin (AM), Cefpodoxime (CPD), Chloramphenicol (C), Clindamycin (CC), Enrofloxacin (ENO), Erythromycin (E), Oxacillin (OX), Penicillin (P), Trimethoprim-sulfamethoxazole (SXT), Tetracycline (TE)
[b] Genes described in text.

4. Prophages

Of the 18 total isolates, 16 intact phages from several bacterial taxa were detected using PHASTER. The number of phages per isolate ranged from 1-3. The most common phage (n = 13 isolates) was Staphylococcal phage 187. In addition, a variety of other Staphylococcal phages were detected including vB_SpsS_QT1, PT1028, 2638A, SPbetalike, SA7, phiRS7, 37, StauST398, and B236. In 2 isolates, one lactococcal phage each, bIL286 and bIL311, was detected. In addition, a *Lactobacillus* phage, Lj965, was detected in one isolate. Streptococcal

phage 315.4 was detected in one isolate. *Enterococcus* phage EFC_1 was detected in one isolate. Interestingly, a Pseudomonal phage JD024 was detected in 2 isolates.

Phage characteristics are described in Table 7. The phage length varied greatly from 14.4 Kb to 127.4 Kb, but the average length was 43.2Kb and the median length was 41.4 Kb. The total proteins ranged from 22 to 156 proteins, with an average of 62 proteins. GC content ranged from 30.54% - 64.24%.

Table 7. Characteristics of prophage regions identified by PHASTER. Prophage regions with a score greater than 90 are intact, a score between 70-90 are questionable, and a score less than 70 are incomplete.

Most Common Phage	Length (Kb)	Score	Total Proteins	Phage + Hypothetical Protein %	Region Position	GC %	Submitted	NCBI Reference Sequence
PHAGE_Staphy_187	39.4	142	77	100	117-39548	34.25	(Kwan, *et al.*, 2005)	NC_007047
PHAGE_Staphy_2638A	41.1	150	57	100	52-41216	36.92	(Kwan, *et al.*, 2005)	NC_007051
PHAGE_Staphy_vB_SpsS_QT1	42.8	150	60	100	61-42927	36.88	(Zeman, *et al.*, 2019)	NC_048192
PHAGE_Staphy_SA7	34.7	147	53	100	1-34730	34.13	(Kim, 2017) Unpublished	NC_048658
PHAGE_Lactoc_bIL286	41.7	150	61	100	1-41756	35.32	(Chopin, *et al.*, 2001)	NC_002667
PHAGE_Staphy_SPbeta_like	127.4	150	156	100	240-127668	30.54	(Kornyenko *et al.*, 2015) Unpublished	NC_029119
PHAGE_Staphy_phiRS7	43.2	150	62	100	1-43246	34.26	(McDole Somera *et al.*, 2013) Unpublished	NC_022914
PHAGE_Lactob_Lj965	40	150	46	100	72-40085	35.18	(Ventura, *et al.*, 2004)	NC_005355
PHAGE_Staphy_37	43.4	150	70	100	22-43478	35.14	(Kwan, *et al.*, 2005)	NC_007055
PHAGE_Strept_315.4	41.7	150	64	100	1-41780	38.58	(Beres, *et al.*, 2002)	NC_004587
PHAGE_Lactoc_bIL311	14.4	150	22	100	1-14490	34.26	(Chopin, *et al.*, 2001)	NC_002670
PHAGE_Staphy_StauST398_2	45.3	140	62	100	85-45425	33.35	(Van der Mee-Marquet *et. al.*, 2012) Unpublished	NC_021323
PHAGE_Staphy_PT1028	15.5	150	22	100	7-15599	31.42	(Kwan, *et al.*, 2005)	NC_007045
PHAGE_Pseudo_JD024	37.1	147	58	100	168-37295	64.24	(Cui, 2014) Unpublished	NC_024330
PHAGE_Staphy_B236	43	150	67	100	33-43067	35.59	(Botka, *et al.*, 2015)	NC_028915
PHAGE_Entero_EFC_1	40	150	59	100	84-40103	35.08	(Yoon and Chang, 2014) Unpublished	NC_025453

5. Biofilm Formation

Isolates were tested for their ability to form biofilms, and classified as a strong, moderate, or weak biofilm formers based on optical density cut-offs that were standardized from *S. pseudintermedius* ATCC 49051. Of the 18 isolates, one isolate was identified as a non-biofilm former with an optical density (OD) of <0.383. Eight isolates were determined to be weak biofilm formers with ODs that ranged from 0.393 to 0.766, while nine isolates were identified as moderate biofilm formers with ODs ranging from 0.766 to 1.532. None of the isolates were identified as strong biofilm producers (Table 8).

Table 8. Biofilm strength of *S. pseudintermedius* isolates collected from the nasal passages of healthy dogs in Centre County.

Biofilm strength	***Optical density***	***S. pseudintermedius* isolates (n=18) (%)**
None	<0.383	1 (6%)
Weak	0.393 to 0.766	8 (44%)
Moderate	0.766 to 1.532	9 (50%)
Strong	>1.532	0 (0)

DISCUSSION

Genome-based sciences have been used extensively in recent years to expand knowledge related to the epidemiology of pathogens, such as *S. pseudintermedius*. *Staphylococcus pseudintermedius* is an opportunistic skin pathogen, primarily of dogs, which has been rapidly emerging in terms of its geographic spread and development of antimicrobial resistance. In this study, a genomic analysis of *S. pseudintermedius* isolates obtained from the nasal passages of healthy dogs in Centre County, Pennsylvania was performed. Whole genome sequencing was used to examine general genome characteristics, virulence genes, antimicrobial resistance genes, and prophages. In addition, isolates were categorized based on their ability to produce biofilms based on optical density measurements of the biofilm. This study was conducted to better understand genome aspects of *S. pseudintermedius* isolates and to connect how biofilm formation, an important virulence factor, is associated with these genes.

1. Genome Characteristics

The *S. pseudintermedius* genome was measured based on its size, G-C content, and predicted protein coding sequences (CDSs). The size of the assembled genome ranged from 2,460,547 bp to 2,714,597 bp, the C-G contents ranged from 37.07% to 37.67%, and the CDS ranged from 2284 to 2615. This is supported by other genome assemblies reported in the literature, such as by Zakour et al. (2011), Francino et al. (2021), and Fàbregas et al. (2023) that sequenced isolates from dogs with skin infections or healthy dogs. Similarly, greater amounts of tRNA's (31-59) compared to rRNAs (2-6) were found, which is also supported by these studies.

However, Fàbregas et al. (2023) reports the amount of tRNAs up to 19, which is greater than in this study.

Identified sequence types included MLST188, MLST241, MLST527, MLST764, MLST1296, and remaining sequence types were unknown to the CGE database. MLST188 has been identified in a previous study conducted in Spain from the nasal passage of healthy stray dogs (Gómez-Sanz et al., 2013). The most prevalent sequence type in the United States, MLST68, was not detected in this study because it is usually isolated from the skin and not the nasal passages (Zukancic et al., 2020). Common MLST's were identified in four of the isolates, and common predicted MLST were identified in five isolates. The variation in MLST of the isolates represents diversity within this collection.

2. Virulence Genes

The pathogenesis of *S. pseudintermedius* is reliant upon a variety of virulence factors encoded by genes in the core genome or located on mobile genetic elements. Eight genes encoding exoenzymes were detected in this study, including *clpX*, *coa*, *htrA*, *lip*, *nucA*, *nucB*, *nucC*, and *nanB*. These genes encode proteases that scavenge nutrients from the environment or the host, or aid in the evasion of host immunity. *ClpX* encodes a Clpx ATPase, which is expressed during heat stress to cleave specific substrates and control the expression of other virulence factors (Frees et al., 2003). *Coa* encodes for a coagulase enzyme responsible for disruption of the coagulase cascade and complement pathways (Sewid et al., 2018). *HtrA* encodes high temperature requirement A proteins that are envelope-associated head shock serine proteases (Song et al., 2022). *Lip* encodes triacylglycerol lipase, which breaks down sebum and

encourages colonization of the skin (Kmieciak and Szewczyk, 2018). Three nuc genes, *nucA*, *nucB*, and *nucC*, encode nucleases that degrade DNA traps to evade neutrophils (Bünsow et al., 2021). *NanB* encodes sialidase, which frees carbon and regulates the presence of sialic acid on cell surfaces (Rynhoud et al., 2021). All isolates contained *clpX*, *coa*, *htrA*, *lip*, and *nuc* genes, and variation was seen with the *nanB* gene, present in 12 isolates. This pattern has been commonly seen in sequenced isolates (McCathy, et al., 2015). Variation of the *nanB* gene may provide some isolates with greater ability to colonize the host and cause infection.

The identified toxin genes including *hlb*, *LukF-I*, *LukS-I*, *sei*, *siet*, and *speta* were found in all isolates. The Luk-I leukocidin, encoded by both *LukF-I* and *LukS-I*, is a bicomponent toxin analogous to Panton-Valentine leukocidin (PVL) of *S. aureus*. Both genes are required to produce a functioning exotoxin, which is composed of an S subunit that recognizes specific receptors and an F subunit that creates pores in canine leukocytes (Spaan et al., 2017). *LukF-I* and *LukS-I* are frequently found in *S. pseudintermedius* isolates and play a major role in hindering host immunity (Abouelhair et al., 2018). Interestingly, some *S. pseudintermedius* isolates have been reported to encode *PVL* as well (Wang et al., 2022), but that was not seen in this study.

Staphylococcal enterotoxin (SE) I is a putative enterotoxin related to food-borne illness encoded by *sei*, but enterotoxins are not as well characterized in *S. pseudintermedius* as an *S. aureus*. At least 17 genes encoding staphylococcal enterotoxins have been detected to varying frequencies (Gharsa et al., 2013; Phumthanakorn et al., 2018). Phumthanakorn (2018) found that *S. pseudintermedius* isolates from humans carried a more diverse set of SE genes compared to canine isolates, highlighting a greater potential for pathogenicity in isolates from humans in this manner. Many SE genes are located on mobile genetic elements, such as bacteriophages and

pathogenicity islands; however, *sei* is a non-mobile gene on a genomic island (Phumthanakorn et al., 2018).

Exfoliative toxin SIET is encoded by *siet* and understood to cause erythema, exfoliation and crusting when injected into healthy dogs. However, the role of SIET in pathogenesis is not completely understood because *siet* is frequently seen in *S. pseudintermedius* isolates regardless of collection from infected or healthy dogs and it contains little similarity in amino acid sequence to other known exfoliative toxins (Banovic et al., 2017). SPETA is an exfoliative toxin of *S. pseudintermedius,* encoded by *speta*, that is similar to SHETA of *S. hyicus*. However, the role of SPETA in pathogenesis has not been completely characterized (Banovic et al., 2017). In this study, *siet* and *speta* were detected in all isolates, and its frequent detection is agreeable with other studies (Bergot et al., 2018). This warrants further investigation of their function. Other exfoliative toxins, encoded by *expA* and *expB,* digest desmoglein 1, inhibiting cell-cell adhesion of healthy skin (Iyori et al., 2010). These genes are observed much less frequently than *siet* in *S. pseudintermedius* isolates (Gómez-Sanz et al., 2013) and were not detected in this study.

Eighteen cell wall-associated (CWA) proteins, *spsA-R,* are classified into the *S. pseudintermedius* surface protein family. Although the function of every protein is not completely understood, these proteins are responsible for adhesion of bacterial cells to extracellular matrix molecules. Particularly, *spsD*, *spsL,* and *spsO* have been shown to play a major role in *S. pseudintermedius* infection by adhering to fibrinogen, fibronectin, cytokeratin 10, or other unknown receptors (Bannoehr et al., 2011; Pietrocola et al., 2015). In this study, variation of frequency was detected in *spsF* and *spsO,* which were detected in five and 10 isolates, respectively. Other studies, such as by McCarthy et al. (2015), have also detected variation in the frequency of *spsO*, *spsF*, *spsQ,* and *spsP*. Of note, *spsQ* and *spsP* are orthologs to

the *spa* gene in *S. aureus* that encodes *S. aureus* staphylococcal protein A. In dogs, SpsQ enables bacteria to evade complement and phagocytosis by binding the Fc region of canine IgG and therefore may play a major role in infection. Both genes are located adjacent to each other on the *oriC* environ within the genome, so they were detected with the same frequency in 3 isolates (Zukancic et al., 2020). In other studies, *spsQ* was found much less frequently compared to other CWA proteins, which agrees with the findings of this study (Zukancic et al., 2020); however, other studies have reported *spsQ* in most isolates (Rynhoud et al., 2021).

Two-component systems are essential for bacteria to respond to environmental changes through the expression of genes. The *agr* group, including *agrABCD*, encodes the two-component accessory gene regulator system, which influences the action of over 70 genes via quorum sensing mechanisms. These genes are related to biofilm formation, secretion of virulence factors, or expression of surface adhesions (Jenul and Horswill, 2018; Little et al., 2019). Another two-component regulatory system includes the *lytS* and *lytR* genes, which moderate the rate of autolysis and alterations of the cell wall in *S. aureus* (Brunskill and Bayles, 1995). Similarly, in the autolysis related locus, *arlS* and *arlR* regulate many cellular functions, such as adhesion, biofilm production, secretion of virulence factors, and control of other regulatory systems (Crosby et al., 2020). Finally, *saeR* and *saeS* encode the *S. aureus* exoprotein expression two-component system that aids the production of exoproteins (Jenul and Horswill, 2018). The staphylococcal respiratory response system, encoded by *srrA* and *srrB*, regulates virulence factors in low oxygen conditions, especially the *ica* operon (Jenul and Horswill, 2018). Lastly, repressor of toxins, encoded by *roT*, is a regulatory protein that increases or decreases the expression of many toxins (Jenul and Horswill, 2018). These genes have been greatly studied in

S. aureus and were present in all the studied isolates, which points towards their importance related to gene expression in *S. pseudintermedius*.

Overall, the isolates encoded a similar range of virulence genes, which are essential for the survival or stress tolerance of bacteria. Differences in virulence genes represent the variation of virulence factors in the genome of *S. pseudintermedius*.

3. Antimicrobial Resistance

Staphylococcus pseudintermedius obtained from the canine nasal passage contained high levels of antimicrobial resistance (Zemanek, 2019). Isolates of this study were phenotypically resistant to beta lactams (penicillin, ampicillin, oxacillin, cefpodoxime), macrolides (erythromycin), lincosamides (clindamycin), fluoroquinolones (enrofloxacin), chloramphenicol, trimethoprim, and tetracycline, as previously conducted by Zemanek (2019). Four isolates were phenotypically pan-resistant to all antimicrobials tested. All isolates were susceptible to imipenem, cephalothin, cefoxitin, and amoxicillin-clavulanate. Most isolates were resistant to penicillin (72%) and ampicillin (61%). Several isolates were also resistant to trimethoprim (33%), tetracycline (28%), erythromycin (28%), clindamycin (28%), and enrofloxacin (22%). Few isolates were resistant to cefpodoxime (11%) and oxacillin (6%) (Zemanek, 2019).

In this study, it was observed that resistance genotypes matched resistance phenotypes in all but 1 isolate; however, the data is limited because phenotypic resistance to aminoglycosides and quaternary ammonium compounds was not studied. Of the isolates used in this study, many were phenotypically resistant to beta-lactams, particularly penicillin (n=13) and ampicillin (n= 11). All isolates that were resistant to penicillin contained the *blaZ* gene and 4 contained the

mecA gene. In contrast, one isolate that was phenotypically resistant to only tetracycline contained both the *blaZ* and *tet(M)* genes. The presence of *blaZ* in penicillin-susceptible isolates has been reported by Tyson et al. (2018), and a lack of phenotypic expression could be explained by a disruption in the gene. Similarly, Wegener et al. (2018) reports the presence of *aac(6')-aph(2")* in several aminoglycoside-susceptible isolates. Additionally, enrofloxacin resistance was seen in four isolates; resistance to fluoroquinolones is related to the *gyrA* and *grlA* genes (Kizerwetter-Świda et al., 2016), which were not detected in this study. Interestingly, all isolates resistant to enrofloxacin were also resistant to trimethoprim. For all other antimicrobial classes (trimethoprim, erythromycin, clindamycin, tetracycline, chloramphenicol), the phenotypic resistance was observed along with its respective gene (*dfrG, erm(B), tet(M), cat(*p_{C221}*)*). Several other studies report a strong correlation between resistance genotypes and phenotypes, particularly of erythromycin, chloramphenicol, tetracycline, and trimethoprim, indicating that whole-genome sequencing may be used to identify antimicrobial resistance in *S. pseudintermedius* (Rynhoud et al., 2021; Tyson et al., 2018; Wegener et al., 2018).

The most common antimicrobial resistance gene was the *blaZ* gene, which was detected in 14 of the isolates, resulting in resistance to penicillin. Many other studies have also reported a predominance of *blaZ* in isolates (Bergot et al., 2018; McCarthy et al., 2015; Tyson et al., 2021). There has been a significant increase of resistance towards penicillin over time due to the increasing prevalence of the *blaZ* gene (Moodley et al., 2014), indicating that it is the most widespread and predominant antimicrobial resistance gene of *S. pseudintermedius*. Otherwise, patterns of resistance are often associated with sequence type, where some lineages encode diverse patterns of many resistance genes (ST71), some are diverse but have few resistance genes (ST258) and others have conserved resistance gene patterns (ST45) (Wegener et al., 2018).

Seven isolates in this study were multidrug resistant (MDR) and contained at least 4 and at most 9 antimicrobial resistance genes. Resistance to aminoglycosides (*ant(6)-Ia*, *aph(3")-III*, *aac(6')-aph(2")*, methicillin (*mecA*), erythromycin/clindamycin (*erm(B)*), and chloramphenicol (*cat(*p_{C221}*)*) were unique to MDR isolates in this study. Genes found in non-MDR isolates, *tet(M)*, *dfrG*, and *blaZ*, were also found in some MDR isolates. A trend between resistance genes was that all isolates encoding *ant(6')-Ia* also contained the *erm(B)* gene. Previous studies have reported associations between aminoglycoside and erythromycin resistance in *S. pseudintermedius*, which could be due to the presence of Tn5405-like elements that encode these respective genes in close proximity (Kadlec and Schwarz, 2012). Multidrug resistance is identified in both MRSP and MSSP strains to high frequencies (Bardiau et al., 2013; Lee and Yang, 2020), indicating the need for surveillance on the acquisition of antimicrobial resistance by *S. pseudintermedius.*

Four isolates in this study were MRSP and contained the *mecA* gene. Two of the MRSP isolates contained 9 resistance genes conferring resistance to 6 antimicrobial classes: aminoglycosides, beta-lactams, trimethoprim, erythromycin, quaternary ammonium, and tetracycline. It is frequently recorded that MRSP isolates have greater numbers of antimicrobial resistance genes than MSSP (Lee and Yang, 2020), supporting the risk methicillin-resistance poses by limiting treatment options. MRSP is more often found on dogs with skin infections, further complicating treatment, and it is occasionally found on healthy dogs as well (Ference et al., 2019). Detection of MRSP in the nasal passageways of healthy dogs indicates the need to further characterize *S. pseudintermedius* due to the high number of resistance genes it harbors and potential for the movement of mobile genetic elements.

4. Biofilms

Biofilm formation is an important virulence factor that improves nutrient absorption and waste removal, promotes evasion of host immunity, and increases tolerance to antimicrobials, which enables bacteria to colonize a host more efficiently. *S. pseudintermedius* and other coagulase positive staphylococci are known to produce biofilms (Wang et al., 2022), and in this study 8 isolates created weak biofilms and 9 isolates created moderate biofilms. One isolate was a non-biofilm former.

Biofilm formation has been associated with genotypic factors, such as the presence of the *ica* operon that encodes polysaccharide intracellular adhesion molecule (PIA). Expression of both *icaA* and *icaD* promotes PIA activity in *S. epidermidis* facilitating the creation of biofilms. In the present study, isolate 18 lacked the *ica* operon, but was classified as a weak biofilm former, indicating the ability to produce biofilms without PIA. A similar finding was seen in a study conducted by Casagrande et al. (2015) in 4 isolates. Conversely, isolate four contained the entire *ica* operon and was classified as a non-biofilm former. Non-biofilm formers have been reported to be *icaA*/*icaD* positive in other studies examining *S. pseudintermedius* and *S. aureus* (Casagrande et al., 2015; Little et al., 2019). From this data, the complete role of the *ica* operon and other adhesion molecules in the formation of biofilms is unclear. Future studies can be directed at other genotypic factors that may promote biofilm production in the absence of the *ica* operon or inhibit biofilm production when the *ica* operon is present.

5. Prophages

A prophage is the genetic material of a bacteriophage that has been integrated into the bacterial accessory genome. A total of 16 different intact phages were detected, and phages were of a variety of bacterial origin, such as staphylococcal, streptococcal, lactococcal, lactobacillus, enterococcal, and pseudomonal phages. Many phages of *S. aureus* and other coagulase negative Staphylococci are of the *Siphoviridae* family, which are temperate phages that may increase the virulence of infected bacteria through lysogenic conversion (Moodley et al., 2019). For example, Staphy_B236 contains the *eta* gene that encodes exfoliative toxins responsible for epidermal splitting (Botka et al., 2015). Even though some phages do not appear to contain virulence factors or antimicrobial genes, such as with Staphy_187, it encodes many hypothetical proteins with unknown functions (Moodley et al., 2019).

Detection of phages of a variety of bacterial species represents the diversity of genes that can be introduced into the bacterial genome. Phages can encode interesting virulence factors, such as Strept_315.4, which has been detected in highly virulent strains of Group A Streptococcus. It encodes a homologue to streptococcal pyrogenic exotoxins and a protein similar to neurotoxins produced by venomous snakes (Beres et al., 2002).

6. Public Health Significance

Staphylococcus pseudintermedius is a known etiologic agent for pyoderma in dogs and can be found in the nasal passages of dogs; however, it is a pathogen also of human health significance. There have been several documented cases of human infection, including skin and soft tissue infections, prosthetic joint infections, and sinonasal infections (Ference et al., 2019;

Somayaji et al., 2016). While it is often unclear if zoonotic transmission occurred between pet owners and their dogs, people who own dogs are more likely to become infected with *S. pseudintermedius* compared to people who do not own dogs (Ference et al., 2019; Guardabassi et al., 2004). In addition, numerous MDR resistant strains, such as to penicillin, trimethoprim, clindamycin, and erythromycin, have been isolated from human infections (Ference et al., 2019). Similarly, MRSP has been isolated from human infections (Somayaji et al., 2016). From the perspective of treatment, the wide array of antimicrobial resistance patterns of MRSP poses a threat to both animals and humans. Furthermore, diagnostics of *S. pseudintermedius* utilizing routine lab tests is challenging and is often misreported as *S. aureus* in humans, resulting in incomplete epidemiological data. *Staphylococcus pseudintermedius* in addition to encoding for biofilm genes also encodes many virulence factors, an array of antimicrobial resistance genes, and prophages from many bacterial species. Consequently, these described characteristics that influence its ability to cause opportunistic infection make *S. pseudintermedius* a pathogen of both veterinary and human health concern.

CONCLUSION

Staphylococcus pseudintermedius is a common inhabitant of the normal canine skin and cause of opportunistic infection. An increasing global spread and acquisition of antimicrobial resistance, particularly to methicillin, has been observed over the years. In addition, *S. pseudintermedius* encodes similar virulence factors to *S. aureus* and has been reported to cause infection in humans. An increased understanding of the genomic factors influencing opportunistic infection and of the spread of antimicrobial resistance provides valuable epidemiological information for public health officials, veterinarians, and doctors.

In this study, a diverse set of *S. pseudintermedius* isolates from nasal swabs of healthy dogs in Centre County, Pennsylvania was sequenced and analyzed. The objectives of this study were to characterize isolates based on MLST, examine virulence factors and antimicrobial genes, identify prophages, and study biofilm formation of *S. pseudintermedius* isolates.

Five MLSTs were identified in seven isolates, including MLST188, MLST241, MLST527, MLST764, MLST1296, and remaining sequence types were unknown. Similar ranges of virulence factors, including exoenzymes, toxins, adhesions, and regulatory systems, were observed in the isolates. On the contrary, the isolates displayed wide ranges of antimicrobial resistance genes that correlated with phenotypic resistance. Most isolates were resistant to penicillin and contained the *blaZ* gene, as commonly reported in other studies. In addition, four methicillin resistant isolates were detected and encoded the *mecA* gene. Lastly, prophages of several bacterial species were identified in the study, potentially encoding genes that confer virulent actions.

In terms of biofilms, most isolates were either a weak or strong biofilm former, supporting the role of *S. pseudintermedius* in surgery site infections. Using the results of this

study, it is unclear if the presence of the *ica* operon is the sole determinant of biofilm formation. All but one isolate encoded the *ica* operon; however, the isolate without these genes was able to produce biofilms. Meanwhile, the one isolate that did not produce biofilms encoded the *ica* operon. There are possibly other genomic factors that account for biofilm formation other than the *ica* operon that cannot be resolved in this study.

The results of this study illustrate the genomic characteristics observed in *S. pseudintermedius*, such as virulence genes, antimicrobial resistance genes, and prophages, which may influence its ability cause opportunistic infection in dogs.

Appendix A

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