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CHARACTERIZATION OF THE NASAL BACTERIAL MICROFLORA OF HOUSEHOLD DOGS

MARGARET ZEMANEK
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Reviewed and approved* by the following:

Bhushan Jayarao
Professor of Veterinary and Biomedical Sciences
Resident Director, Penn State Animal Diagnostic Laboratory
Thesis Supervisor

Lester Griel Jr.
Professor Emeritus of Veterinary Sciences
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

In this study, the bacterial microflora in the nasal cavity of healthy dogs and their resistance to antimicrobials was determined. Identification of bacterial isolates was done using MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometer) and the results compared to 16S rRNA sequence analysis. A total of 203 isolates were recovered from the nasal passages of 63 dogs. The 203 isolates belonged to 58 bacterial species. The predominant genera were *Streptococcus* and *Staphylococcus*, followed by *Corynebacterium*, *Rothia*, and *Carnobacterium*. The species most commonly isolated were *Streptococcus pluranimalium* and *Staphylococcus pseudointermedius*, followed by *Rothia nasimurium*, *Carnobacterium inhibens*, and *Staphylococcus epidermidis*. Many of the other bacterial species were infrequently isolated from nasal passages, accounting for one or two dogs in the study. MALDI-TOF identified certain groups of bacteria, specifically non-spore-forming, catalase-positive, gram-positive cocci, but was less reliable in identifying non-spore-forming, catalase-negative, gram-positive rods. This study showed that MALDI-TOF can be used for identifying "clinically relevant" bacteria, but many times failed to identify less important species. These findings indicate the need for improvement and expansion of the Bruker Biotyper database comprising of bacteria isolated from nasal cavities of healthy canines.

A total of 177 isolates were examined for their resistance to antimicrobials. The antimicrobials used for assessing antimicrobial sensitivity of gram-positive bacteria were amoxicillin-clavulanate, ampicillin, cefoxitin, cefpodoxime, cephalothin, chloramphenicol, clindamycin, enrofloxacin, erythromycin, imipenem, penicillin, tetracycline, and trimethoprim/sulfamethoxazole, and *Staphylococcus* species were also examined for their

resistance to oxacillin. Gram-negative bacteria were tested with amikacin, amoxicillin-clavulanate, ampicillin, cefoxitin, cefpodoxime, cephalothin, gentamicin, chloramphenicol, enrofloxacin, imipenem, tetracycline, and trimethoprim/sulfamethoxazole. Of the two most prevalent genera, Staphylococci had high rates of resistance, especially to ampicillin and penicillin, whereas Streptococcal isolates were pan susceptible to all antimicrobials tested. Spore-forming, gram-positive rods were mostly susceptible to the majority of antimicrobials tested with the exception of *Bacillus mobilis*, which was resistant to eight of the antimicrobials. Non-spore-forming, catalase-positive, Gram-positive rods were all susceptible to the tested antimicrobials except for *Rothia nasimurium*, which was resistant to clindamycin, and *Corynebacterium flavescens*, which showed resistance to clindamycin, enrofloxacin, erythromycin, and imipenem. Non-spore-forming, catalase-negative, Gram-positive rods were mostly susceptible, although *Carnobacterium maltaromaticum* showed resistance to cefpodoxime, and clindamycin. Large levels of resistance were observed in non-spore-forming, catalase-positive Gram-positive cocci. Except for *Staphylococcus schleiferi,* all Staphylococcal isolates demonstrated some form of resistance, of which *Staphylococcus pseudintermedius* isolates which showed resistance to ampicillin, amoxicillin-clavulanate, cefpodoxime, chloramphenicol, clindamycin, enrofloxacin, erythromycin, oxacillin, penicillin, tetracycline, and trimethoprim/sulfamethoxazole. Non-spore-forming, catalase-negative, Gram-positive cocci were susceptible to all antimicrobials tested. Most of the gram-negative rods were also susceptible to the antimicrobials tested, although resistance was seen in *Acinetobacter modestus, Aeromonas hydrophila, Pseudomonas koreensis, Pantoea agglomerans, Pantoea vagans* and *Stenotrophomonas rhizophila.* In general, common and/or pathogenic species often were

resistant to many different antimicrobials, whereas species less prevalent or not as commonly pathogenic showed more susceptibility to antimicrobials.

In conclusion, the findings of the study suggest that there is considerable diversity in the bacterial microflora of healthy dogs that were available for this study. Resistance to antimicrobials, in particular beta-lactams and cephalosporins was observed with isolates belonging to the genus Staphylococci. These findings of the study can be used to develop a more comprehensive descriptive and or analytic epidemiologic study and inform veterinarians in clinical practice on the diversity of nasal bacterial microflora and aid in selection of antimicrobials for treating bacterial infections related to the nasal passages and upper respiratory tract of dogs.

TABLE OF CONTENTS

LIST OF TABLES

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INTRODUCTION

The relationship between dogs and humans can be traced back to an estimated 15,000 years ago as gray wolves began to be domesticated. Dogs have lived in close contact with humans ever since, providing companionship and serving vital roles in the work force or assisting those with disabilities (Ostrander et al., 2017). Currently, pet ownership rates are on the rise throughout the United States with dogs as the most popular pet. A total of around 70,00,000 pet dogs are owned by an estimated 36.5% of American households (AVMA, 2012) However, as humans and dogs continue to share close environments, there is an increased risk for the transfer of pathogens between humans and their pets.

The nasal passages of these pet dogs can harbor a wide array of microbiota that are capable of causing disease in both the host and those in contact (Tress et al., 2017). Studies have shown that dog ownership plays an important role in the acquired microbiome of owners (Misic et al., 2015).

In addition to infection due to cohabitation, the anatomical connection of the mouth and nose allows bacteria of the nasal passage to infect humans and other animals through dog bites. Dog bites commonly occur throughout the United States, resulting in potentially serious injuries and infections. It is estimated that over 4.5 million people are bitten by dogs each year in the United States, resulting in emergency room visits, hospitalizations, and even death (CDC, 2018). Therefore, a better understanding of the canine nasal passage microbiome may help both veterinarians and physicians in treating dog bite wounds.

Few studies have been conducted to determine the microbial community in canine nasal passages. *Moraxellaceae*, especially *Moraxella* spp. was shown to be the most prevalent bacteria present in healthy canine nasal passages according to next-generation sequencing of 16S rRNA genes (Tress et al., 2017). However, attempts to culture *Moraxella* spp. from the nasal passage were not successful. Other studies have shown that aerobic bacteria including *Staphylococci*, *Streptococci*, *Acinetobacter*, and *Enterococci* dominated the nasal passages of healthy canines when culture-based methods were used (Abramson et al., 1976; Balish et al., 1977; Abramson et al., 1980). Studies have also isolated bacteria in canine nasal cavities that are common inhabitants. of soil and water, suggesting that dogs could acquire bacteria naturally through the environment. (Tress et al., 2017).

Nasal disease of canines can also affect the microbial populations found in their nasal passages. Rhinitis characterized by inflammation and damage to the mucous membrane of the nose is the most common upper respiratory tract disorder in canines. It often occurs alongside sinusitis, an inflammation of the lining of the sinuses. Together, they may damage the filtration function, leading to nasal passage deterioration. This leads to an increased risk in microorganism and dust exposure to the lungs (Kuehn, 2019). Diseased dogs were shown to have a decrease in *Moraxellaceae*, with an associated increase in *Pasteurellaceae* species (Tress et al., 2017)

Some of the bacterial populations in canine nasal passages may be zoonotic and serve as a serious public health concern to humans. Dogs are an important reservoir for zoonotic infections and can transmit infectious agents to humans through a variety of mechanisms including bites, aerosols, and direct contact (Ghasemzadeh and Namazi, 2015).

In a clinical setting, antimicrobials are often used empirically to treat bacterial infections. In recent years, there is evidence to suggest that veterinarians are prescribing increasing amounts

of antimicrobials, many of these antimicrobials are also used extensively in human medicine. There is also evidence to suggest that bacteria isolated from healthy and sick dogs and cats have developed resistance to many of the commonly used antimicrobials (Lloyd, 2007). Limited studies have been done on the development of antimicrobial resistance in companion animals compared to the extensive attention given to food animals. Pets, such as dogs, may play an important role as reservoirs for antimicrobial resistant bacteria and may pose a large threat in transmitting zoonotic pathogens resistant to a wide range of antimicrobials (Guardabassi et al., 2004). Therefore, a better understanding of the microbiome of canines and their susceptibility to a wide array of antimicrobials is necessary for veterinarians and physicians alike.

The aim of this study was to characterize the bacterial microflora of the nasal passages of household dogs and examine the isolates for their resistance to antimicrobials.

The objectives of the study are:

1. To characterize the bacterial diversity of the canine nasal cavities
2. To compare the efficacy of MALDI-TOF biotyper using 16S rRNA sequencing technique as a reference method
3. To identify the antimicrobial resistance of bacteria isolated from canine nasal passages.

BACKGROUND AND REVIEW OF LITERATURE

Companion animals including canines acquire bacteria through a variety of mechanisms and may play an important role in microbial transfer to their owners (Misic et al., 2015). The nasal passages of healthy canines can harbor a wide array of microbiota that have the potential to cause disease in the host and other animals in contact (Tress et al., 2017).

The olfactory perception of canines is thought to be approximately 10,000 times more acute than that of humans (Walker et al., 2006). Olfaction is used by canines to find food and mating partners in the wild, as well as for predator avoidance. Domesticated breeds are indispensable to humans for detecting illicit substances such as drugs and explosives (Gazit and Terkel, 2003). Active sniffing is used by canines to exploit their keen sense of smell. Active sniffing, as opposed to breathing, delivers an estimated 2.5 times more air to the olfactory recess (Rygg et al., 2017). This tendency of dogs to sniff at a variety of locations can increase their exposure to different bacteria and may affect their sense of olfaction (Isaiah et al., 2017).

The nasal passages of canines are a complex anatomical structure that serves multiple functions. The nasal conchae is branched to allow for a large surface area for the transfer of heat, moisture, and odorants (Craven et al., 2007) The nasal cavity contains four types of epithelium: squamous, respiratory, olfactory, and transitional. An important component of the respiratory epithelium is the motile cilia that projects from the surface (Mygind et al.,1982). The rich vasculature of the nose is in the lamina propria just under the respiratory epithelium. These vessels have extensive constricting and dilating capabilities (Negus and Straatsma, 1960). In

addition, the head and neck contain an elaborate system of lymphatics involving over 300 nodes and their intermediate channels. They are bound by aponeuroses along with muscles, nerves, and vessels of the head and neck. The lymph nodes of the neck receive direct drainage from the nasal cavity (Koroulakis and Agarwal, 2018). This lymphatic network can play an important role in bacterial pathogen dissemination (Lynskey et al., 2015). Once colonized in the lymph nodes, certain bacteria can escape into the bloodstream, leading to septic shock and death (Gonzalez et al., 2015).

The noses, ears, and throats of animals are clinically connected to one another. It is now understood that these organs are associated within the fields of anatomy, physiology, and pathology. Anatomically, there is a continuation of the inner ear, eustachian tube, nasopharynx, oropharynx, and larynx. Therefore, the spread of infections and malignancies are commonly seen through this cavity. The physiology of these closely related regions helps to explain the hearing or balance deficits that result from complications within these cavities (Yalamanchili, 2009).

The skull of the domestic dog (*Canis familiaris*) varies more in shape and size than any other mammal. The shape of the skull can be classified into three categories. Dogs with long and narrow skulls are considered dolichocephalic, while those with short and wide skulls are brachiocephalic while mesocephalic skulls fall between the two categories (Evans, 1993).

Although bacterial involvement in the pathophysiology of canine nasal disease is unclear, differences in bacterial communities of healthy and diseased canines have been observed. A decrease in the abundance of *Moraxellaceae*, has been shown to be associated with an increase in *Pasteurellaceae* species in diseased canines (Tress et al., 2017). Cultural methods showed that bacteria isolated from the upper respiratory tract of dogs showing respiratory signs

were frequently *Staphylococcus intermedius*, *E. coli*, α-hemolyzing *Streptococcus*, and *Pasteurella multocida* (Schultz et al., 2006). However, these most likely originated from the normal bacterial flora and are not recognized as primary pathogens. Excluding *E. coli*, these bacteria were also found in the nasal passages of healthy canines, but at a lower prevalence. *Staphylococcus* and *Pasteurella* species were present in most of the dogs, whereas *Streptococcus* was found in very few of the canines (Tress et al., 2017).

In addition, dogs with nasal neoplasia may be susceptible to certain secondary pathogens due to a decreased mucosal defense mechanism. Bacteria from the *Neisseriaceae* family were more commonly found in canines with nasal neoplasia (Cohn and Reinero, 2007) These affected canine were also observed to have *Moraxella spp*. colonizing the nasal passages, however, they accounted for a much lower proportion of the total taxa compared to healthy individuals. Lastly, dogs affected with nasal neoplasia had a significantly higher amount of *Haemophilus parainfluenza* and *Pasteurella multocida* (Tress et al., 2017).

Isaiah et al. (2017) also used sequencing-based methods to determine the nasal microbiota of working dogs. The study found that there appears to be no significant difference in the microbiome in nasal samples based on age, breed, or sex. However, oral samples from the same study indicated a significant difference in bacterial communities based on age and breed with no significant difference based on sex. Nasal bacterial communities have been shown to differ based on geographic location. Predominant phyla of the nasal cavity in this study were Proteobacteria and Bacteroidetes, along with Firmicutes, Tenericutes, and GN02 occurring at a lower abundance. In addition, the bacterial communities found in the nasal cavities of this study were also detected in soil and plants likely due to host interaction with the environment (Isaiah et al., 2017).

Small animal practices commonly encounter nasal diseases in canines. Rhinitis is one of the most common upper respiratory tract disorders, that is characterized by inflammation and damage to the mucous membranes of the nose. Sinusitis, an inflammation of the lining of the sinuses is commonly associated with rhinitis. Consequently, there may be a loss of filtration function as a result of nasal passage deterioration. This could result in the lungs being at a greater risk of exposure to microorganisms and dust (Kuehn, 2019).

Nasal diseases are most often found in doliocephalic and mesocephalic canines (Lefebvre et al., 2005). These diseases can be due to fungal, viral, or bacterial pathogens. In addition, nasal disease can also result from dental disease, neoplasia, foreign bodies, or allergies. Secondary bacterial infections can result from any nasal disease that disrupts protective mechanisms in the nasal passage (Cohn, 2014). Relationships may exist between the age or sex of canines and the incidence of nasal disease. Nasal neoplasia and periodontal disease is more likely to be seen in older dogs (Avner et al., 2008; Meler et al., 2008). Fungal rhinitis is most often observed in young to middle-aged adult dogs (Sharman and Mansfield, 2012).

.The primary risk factor for zoonotic diseases is living in close contact with pets. Those most at risk of becoming infected with zoonotic bacteria are immunocompromised individuals, the elderly, neonates, and pregnant women (Glaser et al., 1994; Mani and Maguire, 2009; Stull et al., 2015). In addition, those that work in prolonged contact with animals such as veterinary professionals, farmers, animal handlers, and researchers may also be at an increased risk of contracting an infection (Mani and Maguire, 2009). Zoonotic diseases can be transmitted to humans through saliva, aerosols, contaminated urine and feces, and by direct contact with a dog. The anatomy of the nose and mouth allows nasal infections to spread to dogs' mouths.

Therefore, dog bites can serve as an important source of zoonotic infections (Ghasemzadeh and Namazi 2015).

Staphylococcus intermedius is a common inhabitant in the anterior part of the nasal cavity of many animals including dogs, pigeons, and horses. It has also been isolated from healthy gingiva of dogs (Hoekstra and Paulton, 2002). Although it is not a common zoonotic pathogen in humans, it has been isolated from dog bite wounds and infection with S. intermedius could result in cellulitis of the affected tissue (Talan et al.,1989; Tanner et al., 2000). *Methicillin-resistant Staphylococcus aureus* (MRSA) is a major public health concern. Pets have been shown to be potential reservoirs of MRSA and may increase the risk of MRSA carriage in those working closely with animals, specifically veterinarians (Moodley et al., 2008). In addition, humans cases of *Methicillin-resistant Staphylococcus pseudintermedius* (MRSP) have been linked to pets (Paul et al., 2011).

Canibacter oris, an anaerobic, gram-positive bacteria, has also been shown to be a potential zoonotic pathogen. This species has been isolated from infections that were the result of dog bites (Aravena-Román et al., 2014).

Acinetobacter species are aerobic, gram-negative bacteria that have been isolated from healthy companion animals at numerous sites on the body including the rectum and mouth (Turton et al., 2010; Pailhoriès et al., 2015). However, these species have been isolated from various infections and have been recognized as a nosocomial pathogen in humans. Recently, it has been suggested that this bacteria is emerging as a significant nosocomial pathogen in dogs as well (Turton et al., 2010; Mitchell et al., 2018).

Streptococcus canis is a common pathogen of dogs that results in cutaneous, respiratory, genital, and urinary infections (Lamm et al., 2010). In addition, they have been linked to severe

or fatal diseases of cats and in cows with mastitis (Hassan et al., 2005; Pesavento et al., 2007). *Streptococcus canis* can also cause infections in humans including soft tissue infection, bacteremia, urinary infection, bone infection, and pneumonia. This species has also cause death in humans due to sepsis (Galpérine et al., 2007). *S. pluranimalium* is an emerging animal *Streptococcal* species with a zoonotic potential. Literature on the pathogenic significance of this bacteria in humans is still limited, however, cases of human infections with *S. pluranimalium* are increasing and have recently been linked to cause human brain abscesses (Maher et al., 2018).

Corynebacterium species has been linked to infections in dogs and humans. A non-toxigenic gene bearing *Corynebacterium ulceran*s has been isolated from the wound of a human, along with a nasal sample from the pet dog (Fuursted et al., 2015). In addition, *C. ulcerans* was the causative agent of a serious respiratory tract infection in an elderly woman. The same bacteria was also isolated from the patient's dog (Monaco et al., 2017).

Neisseria. weaveri can cause skin and soft tissue infections resulting from dog bites. In addition, it has the potential to cause severe septicemia in humans (Shinha, 2018). *Neisseria weaveri* has also been shown to cause lower respiratory tract infections in humans (Panagea et al., 2002). *Neisseria animaloris* and *N. zoodegmatis* are clinically important and have commonly been isolated from dog bites (Heydecke et al., 2013).

Moraxella species are gram-negative diplococci that are a commensal of dogs and cats. Occasionally it can be isolated from clinical samples in humans. *Moraxella canis* has been isolated from an ulcerated metastatic lymph node from a terminal human patient (Vaneechoutte et al., 2000). Another human patient had polyarticular septic arthritis that was associated with a predisposing malignancy. The septic arthritis was the result of *M. canis* infection due to immunosuppression (Ottaviani et al., 2009).

MATERIALS AND METHODS

Survey and Sample Collection

The animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC No. PROTO201700128) at Penn State University. Volunteers were solicited from the community through word-of-mouth publicity, outreach through university listservs, and advertising through a local dog training group. All together 63 dogs were available for the study. Study participants completed a questionnaire that sought information about dog's demographic history, and lifestyle factors. The questionnaire comprised of basic questions on the dogs' prior respiratory illness status, vaccination history, and use of antimicrobials. Owners also provided crucial information pertaining to the pets' interactions with animals and exposure to different geographical areas. The frequency of visits to places such as the dog park, vet office, and boarding were all obtained.

Nasal swabs were collected from study dogs using the procedure described by Hanson, Tripp, and Harvey (Hanson et al. 2016). The dog was restrained and where needed a muzzle was applied, following which a sterile swab was gently inserted as deep as possible in a twisting motion upward and toward the midline. The swab was placed in a test tube containing sterile Brain Heart Infusion Broth (BD Diagnostics, Sparks, MD) and refrigerated for later testing.

Bacteria Isolation

The nasal swabs in the sterile media were vortexed for approximately 1 min. A sterile inoculating loop was inserted into the media and a loopful of it was spread on both Tryptic Soy Agar (TSA) plates with 5% sheep blood (Remel Inc., Lenexa, KS) and Spectra MRSA (Remel, Inc.) plates. The plates were then incubated at 37°C for 24-48 h. The bacterial growth was observed on both TSA and Spectra MRSA plates and 4-8 individual colonies were selected for subculturing. A TSA plate with 5% sheep blood was separated into quadrants and a loopful of each colony was streaked onto each quadrant. The plates were then incubated at 37°C for 24-48 h. A loopful of bacterial growth was taken from each quadrant and plated onto an individual TSA plate and incubated again at 37°C for 24-48 h.

Bruker MALDI-TOF MS Identification System

Bacterial isolates were grown on TSA plates with 5% sheep blood (Remel, Inc) and incubated for 48 h. at 37°C. The tube extraction method was done as described by Rodrıguez-Sanchez et al. (2014) and Savage et al. (2017). A single large colony or several smaller colonies were selected and added to an Eppendorf tube containing 300 µl of sterile water. After vortexing, 900 µl of ethanol was added to the tube to inactivate the bacteria. The mixture was vortexed then centrifuged for 2 min. at 13,000 rpm. A pipette was used to remove the ethanol. The tube was then left out at room temperature to evaporate any remaining ethanol. 50 µl of 70% formic acid was added. The mixture was then vortexed and allowed to stand for 5 min. 50µl of 100% acetonitrile was added to the mixture and then centrifuged for 2 min. 1µl was taken from the supernatant and pipetted onto a steer target and allowed to dry. 1µl of the matrix

solution, consisting of a-cyano-4-hydroxy-cinnamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid was added to the dried spot. A bacterial test standard (BTS) was added 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), operated in the linear mode and equipped with a 337-nm nitrogen laser using FlexControl 3.3 software (Bruker Daltonics). The mass spectra were collected within a 2000 to 20,000 m/z mass range. MALDI-TOF Biotyper 2.0 software was used to analyze Spectra (Bruker Daltonics). A peak list generated for each sample was used in matching a reference library by the integrated pattern-matching algorithm. Results were based on a log score and ranged up to 3.0. Only scores greater than 2.0 gave probable species identification. Identifications with scores lower than 2.0 were not used.

16S rRNA Sequencing

The protocol described by Savage et al. (2017) was used to perform 16S rRNA sequencing. Briefly, bacterial isolates were grown on TSA plates with 5% sheep blood (Remel, Inc) and incubated for 24-48 h. at 37°C. DNA extraction was performed using the boiled prep method. One large colony or several smaller colonies were placed into a 1.7 µl Eppendorf tube. The tube was centrifuged at 5000 rpm for 5 min. to harvest the bacteria. The supernatant was decanted and 200 µl of nuclease free water was added to the tube. The mixture was then placed in a water bath of 100°C for approximately 30 min. Each tube was vortexed to break cell membranes and then centrifuged at 6000 rpm for 5 min. to precipitate the cell debris. The supernatant was then collected and stored at -20°C

PCR was then performed in 50 µl reactions on PTC-200 DNA Engine Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA). The 16S rRNA primers used in this study were described by Relman et al. (1992). Sequences of the forward and reverse primers used were p8FPL-p806R (834 bp product) F 5' GCG GAT CCG CGG CCG CTG CAG AGT TTG ATC CTG GCT CAG 3',R5' GCG GAT CCG CGG CCG CGG ACT ACC AGG GTA TCT AAT 3' and p515FPL-p13B (904 bp product) F 5' GCG GAT CCT CTA GAC TGC AGT GCC AGC AGC CGC GGT AA 3',R5' CGG GAT CCC AGG CCC GGG AAC GTA TTC AC 3'. Each reaction included 22.1µl of water, 4µl of each primer pair at 0.4 µM, 0.5 µl of DNTPs at 0.1 µM, 0.4 µl of Taq Polymerase (Promega, Madison, WI) at 2 U per reaction, 4 µl of MgCl2 at 2 mM, 5 µl of 1 x Taq Polymerase Buffer (100 mM Tris HCl; 500 mM KCl, 15mM MgCl2, 0.01% gelatin), and 10 µl of DNA template.

The thermocycling conditions used were: 94°C for 2 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; then, 72°C for 10 min and 4°C holding. For visualization, PCR products were ran on 2% agarose gel for 45 min at 180V using PCR markers for molecular weight standards (Promega). Positive results were purified with QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). After purification, the products were sent to Penn State Genomics Core facility (University Park, PA) following preparation instructions.

The PCR products (834 and 904-bp) were sequenced bidirectionally and identified using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/).

Antimicrobial Sensitivity Testing

Antimicrobial sensitivity was conducted according to the standard protocol from the Clinical and Laboratory Standards Institute (CLSI 2018). The antibiotics disks used for assessing antimicrobial sensitivity of gram-positive bacteria were: Imipenem (10 µg/mL), Cephalothin (30 µg/mL), Cefoxitin (30 µg/mL), Cefpodoxime (10 µg/mL), Ampicillin (10 µg/mL), Trimethoprim/Sulfamethoxazole (1.25+23.75 µg/mL), Clindamycin (2 µg/mL), Erythromycin (15 µg/mL), Penicillin (1 µg/mL), Amoxicillin-clavulanate (20+10 µg/mL), Chloramphenicol (30 µg/mL), Enrofloxacin (5 µg/mL), and Tetracycline (30 µg/mL) (Remel, Inc.). In addition, Oxacillin (5 µg/mL) (Remel, Inc.) was also added for *Staphylococcus* species. The antibiotic disks used for gram-negative bacteria were: Amikacin (30 µg/mL), Gentamicin (10 µg/mL), Imipenem (10 µg/mL), Cephalothin (30 µg/mL), Cefoxitin (30 µg/mL), Cefpodoxime (10 µg/mL), Ampicillin (10 µg/mL), Trimethoprim/Sulfamethoxazole (1.25+23.75 µg/mL), Amoxicillin-clavulanate(20+10 µg/mL), Chloramphenicol (30 µg/mL), Enrofloxacin (5 µg/mL), and Tetracycline (30 µg/mL) (Remel, Inc.).

Bacteria were grown on TSA plates with 5% sheep blood for 24-48 h. Bacteria was then added to 2 ml of 0.85% saline solution to create a 0.5 McFarland concentration. The inoculum was spread evenly onto 150 mm Mueller-Hinton Agar plates (Remel, Inc.) using sterile cotton swabs and allowed to dry for 5 min. Disks saturated with each antibiotic were applied using Sensi-Disc dispensers. The plates were then inverted and incubated for 18-24 hours. The diameter of the zone of inhibition was measured to the nearest millimeter using a ruler. Measured values were compared to standard values from CLSI. The cut off values for the most closely related species with standard values were used for bacterial species not given a standard

value. These values were then used to classify bacteria into one of three categories: Sensitive (S), Intermediate (I), or Resistant (R).

RESULTS

This study included 62 dogs from Centre County and one from Virginia; the 63 dogs comprised of 33 females and 30 males. The average age of the dogs was 6.8 years old (Table 1). It was noted that 4.8% of the dogs at the time of sampling had minor respiratory signs of illness. The study comprised of 24 purebred breeds and 13 mixed breeds. Golden retrievers were the most common breed that was surveyed (11%). It was noted that 6.3% of the dogs had received antimicrobials within the past one month during the time of sample collection.

The study sought to identify the sampled dogs' exposure to high population areas where there may be greater risks of bacterial transmission. Table 2 summarizes the frequency of the dogs' visits to potential high-risk areas including boarding facilities, training classes, dog day care, groomers, dog parks, veterinary practices, and their interactions with non-household dogs. It was observed that 35% of the dogs had traveled out of Pennsylvania, mostly to nearby states, while 90% of the dogs in the study lived in households with other pets, most frequently other dogs.

Table 1 Distribution of breed, sex and age of dogs in the study

Breed	**Sex**	**Age (years)**
Australian cattle dog	M (2), F(1)	1, 11, 14
Australian shepherd	M (3)	3, 6, 6,
Basset hound	F (2)	3.5, 5.5
Border collie	M (2), F (2)	4, 4, 5, 9.5
Dogue de bordeaux	M (2), F (1)	2.5, 2.5, 7
English bulldog	F (1)	9
Flat-coated retriever	F (2)	0.3, 5
French bulldog	M (1)	1
German shorthaired pointer	M (2)	4.5 ,12
Golden retriever	M (1), F (6)	0.8, 3, 4, 6, 8, 10, 10
Great pyrenees	M (1)	10
Greyhound	M (1)	10
Jack russell	F (1)	4
Keeshond	M (1)	11
Labrador retriever	M (3), F (1)	8, 10, 13, -
Llewellin setter	F (1)	5
Mix	M (5), F (8)	2, 4, 5, 5, 5, 5, 7, 7, 8, 10, 10, 11, 18
Newfoundland	M (1)	10
Pitbull	F (1)	4
Pug	M (1)	4
Rat terrier	M (1)	9
Sheltie	F (2)	4, 8
Siberian husky	M (1), F (3)	1.5, 2.5, 5, 8
Weimaraner	M (1), F (1)	2.5, 7
Welsh springer spaniel	M (1)	2
Total	**M (30), F (33)**	

Table 2: Frequency of sampled dogs' exposure to high risk areas

	Never	Rarely	Regularly	Frequently
Boarding facilities	55 (87%)	1 (2%)	0 (0%)	7 (11%)
Training classes	42 (66%)	4 (6%)	6 (10%)	11 (17%)
Day care	63 (100%)	0 (0%)	1 (0%)	0 (0%)
Groomer	51 (81%)	9 (14%)	3 (5%)	0 (0%)
Dog park	55 (87%)	8 (13%)	0%	0%
Veterinary Practice	16 (25%)	43 (68%)	4 (6%)	0%
Interactions with non-household dogs	15 (24%)	20 (32%)	11 (17%)	17 (27%)

Proportion of dogs (n=63) sampled visiting boarding facilities, training classes, day care, grooming facilities, dog parks, veterinary offices, and interacting with non-household pets. Options were broken down by never, rarely (1-2 stays in the past 6 months), regularly (3-5 stays in the past 6 months), or frequently (more than 5 stays in the past six months).

Bacterial microflora isolated from nasal passages of dogs.

A total of 203 isolates were recovered from the nasal passages of 63 dogs. These 203 isolates belonged to 58 bacterial species. The bacteria were categorized into 5 groups: 1) gram-positive, catalase-positive species, 2) gram-positive, catalase-negative species, 3) gram-negative non-spore forming species, 4) regular non-spore forming, gram-positive rods, and 5) and irregular non-spore forming, gram-positive rods. *Streptococcus pluranimalium* was most commonly isolated, followed by *Staphylococcus pseudointermedius. Rothia nasimurium, Carnobacterium inhibens*, and *Staphylococcus epidermidis* were also found in a number of the sampled dogs. The species were identified using MALDI-TOF and results were compared to the 16S rRNA gene sequencing technique, which served as the reference method (Tables 3-8).

Table 3: Species identification of Gram-positive spore-forming rods isolated from nasal cavity of dogs.

Identified by 16S sequencing [a]			Identified by MALDI-TOF [a]		
Species	**No. of Isolates**	**% ID**	**Species**	**No. of Isolates**	**MALDI - TOF score**
Bacillus mobilis[b]	1	99.1	*Bacillus cereus*	1	2.31
Bacillus megaterium	1	97.6	*Bacillus megaterium*	1	2.33
Bacillus simplex	2	99.0 - 99.7	*Bacillus simplex*	1	2.40
Bacillus toyonensis[b]	1	99.7	-	-	-
Paenibacillus cookii	1	94.5	*Paenibacillus cookii*	1	1.93

[a] As compared to 16S sequencing 64 and 50% of isolates identified by MALDI-TOF to the genus and species level, respectively
[b] Not present in the Bruker Biotyper 2.0 database

Six Gram-positive spore-forming rods belonging to five different species were isolated and speciated (Table 3). On comparison to the 16S sequencing method, MALDI-TOF correctly identified four of the six isolated to the genus level (64%) and three were identified to the species level (50%). Two of the five species, *Bacillus mobilis* and *Bacillus toyonensis* were not present in the Bruker biotyperdatabase. Instead, *Bacillus mobilis* was misidentified as *Bacillus cereus*, while the *Bacillus toyonensis* isolate gave no reliable identification through MALDI-TOF.

Table 4: Species identification of non-spore-forming, catalase-positive Gram-positive rods isolated from nasal cavity of dogs

Identified by 16S sequencing [a]			Identified by MALDI-TOF [a]		
Species	**No. of Isolates**	**% ID**	**Species**	**No. of Isolates**	**MALDI-TOF score**
Corynebacterium amycolatum[c]	1	98.3	-	-	-
Corynebacterium efficiens[c]	1	93.7	-	-	-
Corynebacterium epidermidicanis[b]	1	96.9	-	-	-
Corynebacterium flavescens	1	98.8	*Corynebacterium striatum*	1	1.88
Corynebacterium lactis[b]	1	97.9	-	-	-
Corynebacterium mastitidis	1	99.0	*Corynebacterium mastitidis*	1	1.87
Corynebacterium mustelae[b]	1	99.5	-	-	-
Corynebacterium auriscanis	1	98.3	*Corynebacterium auriscanis*	1	1.83
Rothia nasimurium	11	98.6 - 99.9	*Rothia nasimurium*	7	2.09 - 2.40

[a]As compared to 16S sequencing 53 and 47% of isolates identified by MALDI -TOF to the genus and species level, respectively
[b] Not present in the Bruker Biotyper 2.0 database
[c] Present in the Bruker Biotyper 2.0 database, but no reliable identification

Nineteen non-spore-forming, catalase-positive Gram-positive rods belonging to nine different species were isolated and speciated (Table 4). On comparison to the 16S sequencing method, MALDI-TOF correctly identified 10 of the 19 isolated to the genus level (53%) and nine were identified to the species level (47%). Three of the five species, *Corynebacterium epidermidicanis, Corynebacterium lactis,* and *Corynebacterium mustelae* were not present in the database. Instead, all three isolates received no reliable identification through MALDI-TOF. On

the other hand, *Corynebacterium efficiens* was present in the database, however, could not be identified using the MALDI-TOF system.

Table 5: Species identification of non-spore-forming, catalase-negative, Gram-positive rods isolated from nasal cavity of dogs.

Identified by 16S sequencing [a]			**Identified by MALDI-TOF** [a]		
Species	**No. of Isolates**	**% ID**	**Species**	**No. of Isolates**	**MALDI-TOF score**
Canibacter oris[b]	4	96.6 - 97.6	-	-	-
Carnobacterium inhibens[b]	12	94.8 -99.9	-	-	-
Carnobacterium maltaromaticum[c]	2	99.3 - 99.9	-	-	-
Carnobacterium viridans[b]	2	99.6 -99.7	-	-	-

[a]As compared to 16S sequencing 0 and 0% of isolates identified by MALDI-TOF to the genus and species level, respectively
[b] Not present in the Bruker Biotyper 2.0 database
[c] Present in the Bruker Biotyper 2.0 database, but no reliable identification

20 non-spore-forming, catalase-negative, gram-positive rods belonging to four different species were isolated (Table 5). On comparison to the 16S sequencing method, all isolates were unidentified by MALDI-TOF. Three of the five species, *Canibacter oris, Carnobacterium inhibens* and *Carnobacterium viridans* were not present in the database. Of the four species, only *Carnobacterium maltaromaticum* was present in the database. However, MALDI-TOF was still unable to identify the isolate.

Table 6: Species identification of non-spore-forming, catalase-positive, gram-positive cocci isolated from nasal cavity of dogs.

Identified by 16S sequencing [a]			Identified by MALDI -TOF[a]		
Species	**No. of Isolates**	**% ID**	**Species**	**No. of Isolates**	**MALDI-TOF Score**
Arthrobacter luteolus	4	95.8 -100	*Arthrobacter gandavensis*	4	2.39 – 2.47
Arthrobacter woluwensis	3	99.3 - 99.7	*Arthrobacter woluwensis*	3	2.15 – 2.21
Micrococcus luteus	2	98.9 - 100	*Micrococcus luteus*	1	2.22
Staphylococcus aureus	7	99.1 - 99.8	*Staphylococcus aureus*	7	2.37 – 2.51
Staphylococcus epidermidis	6	99.5 - 100	*Staphylococcus epidermidis*	5	1.94 – 2.25
			Staphylococcus pseudintermedius	1	2.21
Staphylococcus hominis	1	99.4	*Staphylococcus hominis*	1	2.43
Staphylococcus pseudintermedius	42	92.7 - 100	*Staphylococcus pseudintermedius*	35	1.71 – 2.41
			Staphylococcus intermedius	7	1.98 – 2.13
Staphylococcus schleiferi	2	99.3 - 99.8	*Staphylococcus schleiferi*	2	2.26 – 2.39
Staphylococcus sciuri	5	95.8 - 99.9	*Staphylococcus sciuri*	3	1.75 – 1.80
Staphylococcus warneri	1	99.8	*Staphylococcus warneri*	1	2.14
Staphylococcus vitulinus	1	99.5	*Staphylococcus vitulinus*	1	1.75
Planococcus psychotoleratus[b]	1	99.5	*Streptococcus hyovaginalis*	1	1.81

[a]As compared to 16S sequencing 95 and 79% of isolates identified by MALDI-TOF to the genus and species level, respectively

[b] Not present in the Bruker biotyper 2.0 database

75 non-spore-forming, catalase-positive, gram-positive cocci belonging to 12 different species were isolated (Table 6). On comparison to the 16S sequencing method, MALDI-TOF correctly identified 71 of the 75 isolated to the genus level (95%) and 59 were identified to the species level (79%). One of the 17 species, *Planococcus psychotoleratus* was not present in the database. Instead, *Planococcus psychotoleratus* was misidentified as *Streptococcus hyovaginalis*. *Arthrobacter luteolus* was incorrectly identified as *Arthrobacter gandavensis* by MALDI-TOF. In addition, MALDI-TOF misidentified one isolate of *Staphylococcus epidermidis* as *Staphylococcus pseudintermedius*. *Staphylococcus pseudintermedius* was misidentified as *Staphylococcus intermedius* in seven of the isolates.

Table 7: Species identification of non-spore-forming, catalase-negative, gram-positive cocci isolated from nasal cavity of dogs.

Identified by 16S sequencing [a]			**Identified by MALDI-TOF** [a]		
Species	**No. of Isolates**	**% ID**	**Species**	**No. of Isolates**	**MALDI-TOF Score**
Streptococcus canis	4	97.4 - 99.9	*Streptococcus canis*	4	2.14 – 2.35
Streptococcus minor	2	99.4	*Streptococcus minor*	1	1.80
Streptococcus oralis[c]	1	99.7	-	-	-
Streptococcus ovis[c]	1	89.9	-	-	-
Streptococcus pluranimalium	36	94.3 -99.8	*Streptococcus pluranimalium*	8	1.74 – 1.95
			Streptococcus hyovaginalis	6	1.76 – 2.30
			Streptococcus thoraltensis	5	1.82 – 2.00
			Neisseria animaloris	1	2.27

[g] As compared to 16S sequencing 55 and 30% of isolates identified by MALDI-TOF to the genus and species level, respectively
[b] Not present in the Bruker Biotyper 2.0 database
[c] Present in the Bruker Biotyper 2.0 database, but no reliable identification

A total of 44 non-spore-forming, catalase-negative, gram-positive cocci belonging to five different species were speciated using MALDI-TOF (Table 7). On comparison to the 16S sequencing method, MALDI-TOF correctly identified 24 of the 44 isolated to the genus level (55%) and 13 were identified to the species level (30%). All species were present in the database, however *Streptococcus oralis* and *Streptococcus ovis* were not given a reliable identification by MALDI-TOF. In addition, *Streptococcus pluranimalium* was misidentified 12 times as either *Streptococcus hyovaginalis, Streptococcus thoraltensis,* or *Neisseria animaloris.* [8 *S. pluranimalium* isolates were correctly identified by MALDI-TOF although, due to its low MALDI-TOF score (> 2.0), the identification is classified as not reliable.]

Table 8: Species identification of gram-negative rods isolated from nasal cavity of dogs.

Identified by 16S sequencing [a]			Identified by MALDI-TOF [a]		
Species	**No. of Isolates**	**% ID**	**Species**	**No. of Isolates**	**MALDI - TOF Score**
Acinetobacter lwoffii	3	99.4 -100	*Acinetobacter lwoffii*	1	2.30
Acinetobacter modestus[b]	1	99.3	-	-	-
Aeromonas hydrophila	1	100	*Aeromonas salmonicida ssp masoucida*	1	2.30
Moraxella canis	2	96.9 - 98.8	*Moraxella canis* *Moraxella_sg_Branhamella ovis*	1 1	2.22 2.27
Moraxella cuniculi[b]	5	94.4 - 96.3	-	-	-
Neisseria animaloris	1	99.5	*Neisseria animaloris*	1	2.25
Neisseria canis[c]	1	99.2	-	-	-
Neisseria weaveri	2	99.7 - 100	*Neisseria weaveri*	2	2.42 - 2.43
Neisseria zoodegmatis[c]	1	98.9	-	-	-
Pantoea agglomerans[c]	1	99.7	-	-	-
Pantoea vagans[b]	2	93.4 - 99.7	-	-	-
Pseudomonas koreensis	1	99.8	*Pseudomonas koreensis*	1	2.44
Psychrobacter maritimus[b]	2	97.5 - 99.7	-	-	-
Psychrobacter psychrophilus[b]	6	98.9 - 100	-	-	-
Riemerella columbina	2	92.3 - 99.7	*Riemerella columbina*	1	2.37
Stenotrophomonas rhizophila	1	96.7	*Stenotrophomonas acidaminiphila*	1	1.71
Suttonella ornithocola[b]	2	94.3	-	-	-

[a] As compared to 16S sequencing 29 and 21% of isolates identified by MALDI-TOF to the genus and species level, respectively

[b] Not present in the Bruker Biotyper 2.0 database

[c] Present in the Bruker Biotyper 2.0 database, but no reliable identification

34 Gram-negative rods belonging to 17 different species were speciated using MALDI-TOF and 16S rRNA gene sequencing (Table 8). On comparison to the 16S sequencing method,

MALDI-TOF correctly identified 10 of the 34 isolates to the genus level (29%) and seven were identified to the species level (21%). Neither *Acinetobacter modestus, Moraxella cuniculi, Pantoea vagans, Psychrobacter maritimus, Psychrobacter psychrophilus,* or *Suttonella ornithocola* were present in the database. None of these isolates were identified by MALDI-TOF. *Aeromonas hydrophila* and *Stenotrophomonas rhizophila* were misidentified as *Aeromonas salmonicida* and *Stenotrophomonas acidaminiphila,* respectively. One of the two *Moraxella canis* isolates was incorrectly identified as *Moraxella_sg_Branhamella ovis.*

Antimicrobial Resistance

Of the 203 isolates identified, 177 isolates were examined for their resistance to antimicrobials. The 177 isolates were representative from each of the five bacterial groups identified in this study.

Table 9: Antimicrobial resistance patterns of spore-forming, gram-positive rods isolated from nasal cavity of dogs.

Organism	No. of dogs	No. of Isolates	Resistance Patterns *
Bacillus mobilis	1 (1.6%)	1	1- AM, AMC, FOX, CPD, CF, E, P, SXT
Bacillus megaterium	1 (1.6%)	1	1-CC
Bacillus simplex	2 (3.2%)	2	1 -sensitive 1-E
Paenibacillus cookie	1 (1.6%)	1	1-TE, C, CC

amikacin (AK), amoxicillin-clavulanate(AMC), ampicillin (AM), cephalothin (CF), cefpodoxime (CPD), cefoxitin (FOX), gentamicin (GM), chloramphenicol (C), clindamycin (CC), enrofloxacin (ENO), erythromycin (E), imipenem (IMP), oxacillin (OX), penicillin (P), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT)
*Underlined antimicrobial signifies an intermediate resistance to the antimicrobial.

Antimicrobial susceptibility testing was performed on five isolates that were spore-forming, gram-positive rods belonging to four different species (Table 9). A single isolate of *Bacillus mobilis* was observed to be resistant to five antimicrobials including [trimethoprim/sulfamethoxazole and four beta-lactams (ampicillin, penicillin and two cephalosporins: cepfodoxime and cephalothin).] This isolate also showed intermediate resistance to amoxicillin-clavulanate, cefoxitin and erythromycin. One isolate of *Bacillus megaterium* was resistant to clindamycin. It was observed that *Paenibacillus cookii* was resistant to tetracycline and showed intermediate resistance to chloramphenicol and clindamycin. Of the two isolates of *Bacillus simplex* isolated from nasal swabs from dogs, one was sensitive to all the antimicrobials tested, while the other showed intermediate resistance to erythromycin.

Table 10: Antimicrobial resistance patterns of non-spore-forming, catalase-positive Gram-positive rods isolated from nasal cavity of dogs.

Organism	No. of dogs	No. of Isolates	Resistance Patterns
Corynebacterium amycolatum	1 (1.6%)	1	1-sensitive
Corynebacterium epidermidicanis	1 (1.6%)	1	1-sensitive
Corynebacterium flavescens	1 (1.6%)	2	1-C 1- ENO, E, IPM
Corynebacterium lactis	1 (1.6%)	1	1-sensitive
Corynebacterium mastitidis	1 (1.6%)	1	1-sensitive
Corynebacterium mustelae	1 (1.6%)	1	1-sensitive
Rothia nasimurium	7 (11.1%)	10	7-sensitve 1-<u>CC</u> 1-CC 1-<u>E</u>

amikacin (AK), amoxicillin-clavulanate(AMC), ampicillin (AM), cephalothin (CF), cefpodoxime (CPD), cefoxitin (FOX), gentamicin (GM), chloramphenicol (C), clindamycin (CC), enrofloxacin (ENO), erythromycin (E), imipenem (IMP), oxacillin (OX), penicillin (P), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT)
*Underlined antimicrobial signifies an intermediate resistance to the antimicrobial.

A total of 17 isolates that were non-spore forming catalase positive and Gram-positive rods comprised of seven different species (Table 10). A total of five *Corynebacterium* species

were examined for their resistance to antimicrobials. *Corynebacterium amycolatum*, *C. epidermidicanis*, *C. lactis*, *C. mastitidis*, and *C. mustelae* were sensitive to all the antimicrobials tested in this study. Of the two isolates of *C. flavescens*, one isolate showed intermediate resistance to chloramphenicol, while the other isolate showed resistance to enrofloxacin, erythromycin, and imipenem. In this class of bacteria, *Rothia nasimurium* was isolated from seven dogs, of the ten isolates examined, seven were sensitive to all antimicrobials. Two isolates showed intermediate resistance to clindamycin, while the other isolates showed intermediate resistance to erythromycin.

Table 11: Antimicrobial resistance patterns of non-spore-forming, catalase-negative, Gram-positive rods isolated from nasal cavity of dogs.

Species	No. of dogs	No. of Isolates	Resistance Patterns*
Canibacter oris	4 (6.3%)	4	3- sensitive 1- E
Carnobacterium inhibens	4 (6.3%)	11	10 - sensitive 1- CPD
Carnobacterium maltaromaticum	1 (1.6%)	2	1-sensitive 1- CC, CPD
Carnobacterium viridans	2 (3.2%	2	1-sensitive 1- TE

amikacin (AK), amoxicillin-clavulanate(AMC), ampicillin (AM), cephalothin (CF), cefpodoxime (CPD), cefoxitin (FOX), gentamicin (GM), chloramphenicol (C), clindamycin (CC), enrofloxacin (ENO), erythromycin (E), imipenem (IMP), oxacillin (OX), penicillin (P), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT)
*Underlined antimicrobial signifies an intermediate resistance to the antimicrobial.

A total of 18 isolates that were non-spore forming, catalase negative gram-positive rods belonged four different species (Table 11). *Canibacter oris* was isolated from four dogs., Three isolates were sensitive to all the antimicrobials tested, while one isolate was resistant to erythromycin. A total of 11 isolates of *Carnobacterium inhibens* were recovered from four dogs. of the 11 isolates, 10 were sensitive all 16 antimicrobials, while one isolate showed intermediate

resistance to cefpodoxime. Two isolates of *Carnobacterium maltaromaticum* were recovered from one dog. One isolate was sensitive to all 16 antimicrobials while the other showed intermediate resistance to cefpodoxime and clindamycin. Two isolates of *Carnobacterium viridans* were recovered from two dogs, of which one isolate was sensitive to all 16 antimicrobials, while the other showed intermediate resistance to tetracycline.

Table 12: Antimicrobial resistance patterns of non-spore-forming, catalase-positive Gram-positive cocci isolated from nasal cavity of dogs

Species	No. of dogs	No. of Isolates	Resistance Pattern*
Arthrobacter luteolus	1 (1.6%)	4	2 - sensitive 1 - CPD 1 - AMC, CPD
Arthrobacter woluwensis	1 (1.6%)	3	1 - C, CC, CPD, FOX
Micrococcus luteus	2 (3.2%)	2	1 - CC, E, 1 - C
Staphylococcus aureus	4 (6.3%)	7	7 - AM, P
Staphylococcus epidermidis	5 (7.9%)	6	1 - CC, E, P 1 – E, ENO, P 1 - CC, P 1 – AM 2 - CC, E
Staphylococcus pseudintermedius	14 (22.2%)	34	9 – sensitive 3 - AM, CPD, ENO, P, SXT 3 - AM, ENO, P, SXT 1 - AM, CC, CPD, E, ENO, OX, P, SXT, TE 3 - AM, CC, E, ENO, P, SXT, TE 1 - AM, C, CC, E, P 6 - AM, P 2 - TE 1 – CC, CPD, E, ENO, OX, P, SXT, TE 1 – P 2 - AM, P, SXT 1 - AM, P, TE
Staphylococcus schleiferi	2 (3.2%)	2	2 - sensitive
Staphylococcus sciuri	3 (4.8%)	4	1 – sensitive 1 - CPD 1 - CC 1 – P
Staphylococcus warneri	1 (1.6%)	1	1 - AM, CC, E, P
Staphylococcus vitulinus	1 (1.6%)	1	1 - TE

amikacin (AK), amoxicillin-clavulanate(AMC), ampicillin (AM), cephalothin (CF), cefpodoxime (CPD), cefoxitin (FOX), gentamicin (GM), chloramphenicol (C), clindamycin (CC), enrofloxacin (ENO), erythromycin (E), imipenem (IMP), oxacillin (OX), penicillin (P), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT)

*Underlined antimicrobial signifies an intermediate resistance to the antimicrobial.

A total of 64 isolates that were non-spore forming, catalase-positive, Gram-positive cocci belonged to 10 different species (Table 12). Four isolates of *Arthrobacter luteolus* were recovered from one dog. Two isolates were sensitive to all 16 antimicrobials. One isolate was resistant to amoxicillin-clavulanate and had intermediate resistance to cefpodoxime and one isolate was resistant to cefpodoxime. *Arthrobacter woluwensis* was recovered from one dog in which all three isolates were resistant to cefoxitin and cefpodoxime and had intermediate resistance to chloramphenicol and clindamycin. Two isolates of *Micrococcus luteus* were recovered from 2 dogs in which one was resistant to clindamycin and erythromycin, while the other was resistant to just chloramphenicol. Seven isolates of *Staphylococcus aureus* were recovered from four dogs with all isolates resistant to ampicillin and penicillin. Six isolates of *Staphylococcus epidermidis* from 5 dogs had varying resistance. One was resistant to ampicillin and another to enrofloxacin. Three were resistant to erythromycin and two of these were also resistant to penicillin. The third was resistant to clindamycin. Another isolate was also resistant to clindamycin and another had intermediate resistance. *Staphylococcus pseudintermedius* was isolated from 14 dogs with a total of 34 isolates. This species also had varying amounts of resistance with 9 isolates being completely sensitive to all antimicrobials. Two isolates of *Staphylococcus schleiferi* recovered from two dogs were both sensitive to all antimicrobials. Four isolates of *Staphylococcus sciuri* were recovered from 3 dogs. One isolate was sensitive to all tested antimicrobials, one had intermediate resistance to cefpodoxime and another to clindamycin. The fourth isolate was resistant to penicillin. *Staphylococcus warneri* isolated from one dog was resistant to ampicillin, clindamycin, erythromycin, and penicillin. The one isolate of *Staphylococcus vitulinus* was resistant to tetracycline.

Table 13: Antimicrobial resistance patterns of non-spore-forming, catalase-negative, Gram-positive cocci isolated from nasal cavity of dogs

Organism	No. of dogs	No. of Isolates	Resistance Pattern*
Streptococcus canis	3 (4.8%)	4	4 - sensitive
Streptococcus minor	1 (1.6%)	2	2 - sensitive
Streptococcus oralis	1 (1.6%)	1	1 - sensitive
Streptococcus ovis	1 (1.6%)	1	1 - sensitive
Streptococcus pluranimalium	20 (31.7%)	36	36 - sensitive

Amikacin (AK), Amoxicillin-clavulanate(AMC), Ampicillin (AM), Cephalothin (CF), Cefpodoxime (CPD), Cefoxitin (FOX), Gentamicin (GM), Chloramphenicol (C), Clindamycin (CC), Enrofloxacin (ENO), Erythromycin (E), Imipenem (IMP), Oxacillin (OX), Penicillin (P), Tetracycline (TE), Trimethoprim/Sulfamethoxazole (SXT)

* Underlined antimicrobial signifies an intermediate resistance to the antimicrobial.

A total of 44 isolates that were non-spore forming, catalase negative gram-positive cocci belonged to five different species (Table 13). All Streptococcus species were susceptible to the antimicrobials tested.

Table 14: Antimicrobial resistance patterns of Gram-negative rods isolated from nasal cavity of dogs

Organism	No. of dogs	No. of Isolates	Resistance Pattern*
Acinetobacter lwoffii	2 (3.2%)	2	2 - sensitive
Acinetobacter modestus	1 (1.6%)	1	1 - CF
Aeromonas hydrophila	1 (1.6%)	1	1 - AM, CF, FOX
Moraxella canis	2 (3.2%)	2	2 - sensitive
Moraxella cuniculi	2 (3.2%)	4	4 - sensitive
Neisseria animaloris	1 (1.6%)	1	1 - sensitive
Neisseria canis	1 (1.6%)	1	1 - sensitive
Neisseria weaveri	2 (3.2%)	2	2 - sensitive
Neisseria zoodegmatis	1 (1.6%)	1	1 - sensitive
Pantoea agglomerans	1 (1.6%)	1	1 - CPD
Pantoea vagans	1 (1.6%)	1	1 – C, CPD
Pseudomonas koreensis	1 (1.6%)	1	1 - AM, AMC, C, CF, CPD, FOX
Psychrobacter maritimus	3 (4.8%)	4	4 - sensitive
Psychrobacter psychrophilus	2 (3.2%)	2	2 - sensitive
Riemerella columbina	2 (3.2%)	2	2 - sensitive
Stenotrophomonas rhizophila	1 (1.6%)	1	1 - CF, CPD, FOX
Suttonella ornithocola	2 (3.2%)	2	2 - sensitive

amikacin (AK), amoxicillin-clavulanate(AMC), ampicillin (AM), cephalothin (CF), cefpodoxime (CPD), cefoxitin (FOX), gentamicin (GM), chloramphenicol (C), clindamycin (CC), enrofloxacin (ENO), erythromycin (E), imipenem (IMP), oxacillin (OX), penicillin (P), tetracycline (TE), trimethoprim/sulfamethoxazole (SXT)
*Underlined antimicrobial signifies an intermediate resistance to the antimicrobial.

A total of 29 isolates that were Gram-negative rods belonged to 17 different species (Table 14). *Acinetobacter modestus* isolated from one dog was resistant to cephalothin. A single *Aeromonas hydrophila* was recovered from one dog that was resistant to ampicillin, cefoxitin, and Cephalothin. *Pantoea agglomerans isolate* was determined to be resistant to cefpodoxime. An isolate of *Pantoea vagans* recovered was resistant to cefpodoxime and chloramphenicol. Resistance to ampicillin, amoxicillin-clavulanate, cefoxitin, cephalothin, cefpodoxime, and

chloramphenicol was observed in one isolate of *Pseudomonas koreensis*. The isolate of *Stenotrophomonas rhizophila* was resistant to cefoxitin, cephalothin, and cefpodoxime.

DISCUSSION

Evaluation of MALDI-TOF for identification of bacterial microflora from nasal passages of dogs.

One of the goals of this study was to evaluate the accuracy of species identification by Bruker MALDI-TOF MS biotyper system for identifying as compared to the 16 S rRNA sequencing technique which was used as a reference method, as 16S rRNA sequencing is recognized as the "gold standard" for bacterial identification by taxonomists with over 100,000 16S rRNA sequences available in public databases. MALDI-TOF is becoming a more commonly used method for rapid and accurate identification of bacterial species. The Bruker biotyper 2.0 database includes over 3000 unique entries that aid in the identifying and bacterial species (El-Bouri et al., 2012). This technique has been successfully used to identify bacterial from animals, humans and the environment pathogens (Pavlovic et al., 2015).

Although studies have been limited on evaluating the efficacy of MALDI-TOF for routine identification of veterinary bacteria, there is evidence to suggest that MALDI-TOF is a reliable alternative method for bacterial species identification isolated from animals (Pavlovic et al., 2015), In identifying bacteria isolated from veterinary clinical specimens, 95.2% were correctly identified at the genus level and 90.1% at the species level. This study concluded that MALDI-TOF was an appropriate technique for classification and identification of veterinary bacterial isolates (Pavlovic et al. 2015). A study conducted by Randall et al. (2015) used

MALDI-TOF to identify isolates submitted to a veterinary diagnostic laboratory which showed that MALDI-TOF successfully identified 100% of 620 isolates to the genus level and 95.3% to the species level. This study determined MALDI-TOF to be a rapid and reliable method to identify bacteria isolated in a veterinary diagnostic laboratory (Randall et al., 2015). Savage et al. (2017) in their study found MALDI-TOF to be more reliable in species identification of bacteria isolated from bovine mastitis and bulk tank milk samples than two other widely used bacterial identification systems in veterinary laboratories: Sensititre Automated Reading and Incubation System 2x System (ARIS) and API (API). In their study, 100 and 96.9% of catalase-negative, Gram-positive cocci isolates were identified to the genus and species level, respectively. For catalase-positive, Gram-positive cocci isolates MALDI-TOF correctly identified 100% and 97.7% of the isolates both to the genus and species level. MALDI-TOF identified 97.9% of Gram-negative isolates to the genus level and 95.8% to the species level. In general, the study concluded that MALDI-TOF was a time-efficient and cost effective method for identifying bacteria in this setting (Savage et al., 2017).

Studies have clearly shown the efficacy of MALDI-TOF; however, there can be some potential limitations on solely relying on MALDI-TOF for bacterial species identification. In general, the Bruker Biotyper 2.0 database includes more clinically relevant bacteria that are involved in disease and infection and may be unable to identify less relevant bacteria. In doing so, MALDI-TOF may underestimate the bacterial diversity in studies by only identifying clinically important species.

This study focused on evaluating the accuracy of the MALDI-TOF system using 16S rRNA gene sequence analysis as the reference method. Overall, MALDI-TOF accurately

identified 83% of all bacteria isolated to the genus level and 62% to the species level. However, some bacteria groups were better identified than others. MALDI-TOF correctly identified 60% of the gram-positive spore-forming rod isolates to the genus level and 46% identified to the species level. For non-spore-forming, catalase-positive Gram-positive rods isolated from the canine nasal passage, 53 and 47% of isolates were identified by MALDI-TOF to the genus and species level, respectively. Non-spore-forming, catalase-negative, gram-positive cocci had 55 and 30% of isolates identified by MALDI-TOF to the genus and species level, respectively. Notably, non-spore-forming, catalase-positive, gram-positive cocci were most commonly accurately identified by MALDI-TOF. 95 and 79% of their isolates were identified by MALDI-TOF to the genus and species level, respectively. On the other hand, MALDI-TOF was less reliable in identifying non-spore-forming, catalase-negative, gram-positive rods. None of the 20 isolates in this group were able to be given any reliable identification by MALDI-TOF. Similarly, MALDI-TOF was less reliable in identifying gram-negative rods as only 29% of the isolates were identified to the genus level and 21% were identified to the species level.

MALDI-TOF was successful in identifying certain groups of bacteria, specifically non-spore-forming, catalase-positive, gram-positive cocci. However, other grouping such as non-spore-forming, catalase-negative, gram-positive rods were unable to be given any reliable identification. The canine nasal passages often harbored very unique bacterial species that often were not present in the Bruker Biotyper 2.0 database. Unlike previous studies evaluating the efficacy of MALDI-TOF, this present study sought to characterize the diversity of canine nasal cavities, instead of identifying only clinically important species. This may explain why this present study was less effective at obtaining bacterial identifications than previous studies and why species such as *Staphylococcus* were more commonly identified by MALDI-TOF than

species such as *Acinetobacter*. *Staphylococcus* species are often clinically relevant, whereas *Acinetobacter species* are only now becoming emerging pathogens. Therefore, data on certain *Acinetobacter* species were not present in the database.

In addition, some species that were present in the database were still unable to be identified. Isolates may have different MALDI-TOF profiles depending on their origin that could affect MALDI's accuracy (Randall et al., 2015). The Bruker Biotyper 2.0 database mostly includes reference spectra derived from human isolates. Therefore, if small differences exist between human and animal isolates of the same bacterial species, MALDI-TOF results may be affected. Also, the number of main spectra (MSPs) the database provides may also affect MALDI's accuracy in identifying species. Some bacteria that were misidentified by MALDI-TOF, such as *Streptococcus pluranimalium*, were commonly identified as species including *Streptococcus hyovaginalis* and *Streptococcus thoraltensis*. In the database, both *S. hyovaginalis* and *S. thoraltensis* have two biotyper MSPs, whereas *S. pluranimalium* only has one MSP. This may explain why *S. pluranimalium* was more readily identified as *S. hyovaginalis* and *S. thoraltensis*. In conclusion, MALDI-TOF is successful in identifying more common, clinically relevant samples and ineffective at times in identifying unique or clinically irrelevant bacteria, or those that are emerging as pathogens. Although the database is continuously supplemented extensively, efforts should be taken to include a broader, more unique range of bacteria.

Bacterial Diversity of Canine Nasal Passages

This study was able to demonstrate the presence of a highly unique and diverse microbiome in canine nasal cavities. Some of the species isolated were similar to ones characterized in previously reported studies (Abramson et al., 1976; Balish et al., 1977; Abramson et al., 1980; Isaiah et al., 2017; Tress et al., 2017), Although a few bacterial species detected have never or only rarely been associated with the canine nasal cavity. The genera most commonly found in the nasal cavities were *Streptococcus* and *Staphylococcus* both isolated from 26 different dogs, followed by *Corynebacterium* from eight dogs, *Rothia* from seven dogs, and *Carnobacterium* from six different dogs. Of the 58 different bacterial species isolated from 63 dogs, *Streptococcus pluranimalium* and *Staphylococcus pseudointermedius* were the most prevalent, followed by *Rothia nasimurium, Carnobacterium inhibens*, and *Staphylococcus epidermidis*. Many of the other bacterial species found were unique and only isolated from 1 or 2 of the dogs sampled.

Other studies on bacterial diversity in canine respiratory tract have resulted in observations that are different from those in this study. Tress et al. (2017) used next-generation sequencing methods to study the bacterial diversity of the respiratory tract in 47 dogs from Germany. Tress observed a dominance of *Moraxellaceae*, especially *Moraxella* spp., with other bacterial families existing at much lower levels. In their study, *Staphylococcus* and *Streptococcus* species were not as significant and were detected less frequently. (Isaiah et al., 2017) observed a more diverse bacterial community in the respiratory tract of 81 dogs from The United States in which the predominant genera were *Moraxella, Mycoplasma, Prevotella, Helcoccus, Cardiobacterium*. The discrepancy of the results from different studies show that the

diversity of bacterial communities in canine respiratory tract is influenced by several factors. As highlighted by Tress et al. (2017), the nasal microbiome in healthy dogs is influenced by interaction between host factors like breed, age and sex and environmental background. Therefore, it is most likely that dogs sampled from canine communites in different geographic locations will harbor different microbial diversities in their respiratory tracts. Furthermore, the difference between this present study and those in the studies by (Tress et al., 2017) and (Isaiah et al., 2017) may be explained by the fact that this study focused on the diversity of culturable bacteria while the next-generation sequencing techniques used by Tress et al. (2017) and Isaiah et al. (2017) allow the detection of all bacteria including those that cannot be detected with culture methods.

The results of this study are more in agreement with other studies that used culture-based methods to analyze the microbiome of canine nasal passages. The high prevalence of *Staphylococci* and *Streptococci* species found in this present study are in agreement with the previous studies done by Balish et al. (1977) and the studies by Abramson et al. (1976 and 1980).

Studies have also been done to determine the bacterial diversity of human nasal cavities. Hilty et al. (2010) used sequencing-based methods to determine that there was an abundance of bacteria belonging to the genera of *Corynebacterium, Staphylococcus, Streptococcus, Veillonella, Neisseria,* and *Moraxella* in human nasal samples (Hilty et al., 2010). Besides *Veillonella* spp. these genera were all isolated from the nasal cavity at varying abundances. The study by Hilty et al. (1980) and this present study show the similarities that exist between the bacteria harbored in both the human and canine nasal passages, most likely due to their close contact with each other.

Streptococcal organisms were the most predominant bacteria isolated in this study. A total of 36 isolates were recovered from 26 different dogs (41%). *Streptococcus pluranimalium,* was the most commonly isolated species of the canine nasal passage in this study, and is considered an emerging pathogen (Kalhoro, 2015). This bacteria has been known to cause septicemia, endocarditis and salpingitis in broilers (Hedegaard et al., 2009), as well as reproductive disturbances in sheep and cattle (Foster et al., 2008; Foster et al., 2010). *Streptococcus pluranimalium* is commonly isolated from a variety of animals from varying locations, including mastitic milk, vagina and cervix of cattle, lungs and lesions of birds, tonsil of goats and cats (Devriese et al., 1999) and humans (Dhotre et al., 2014). This species has been isolated from a dog presented for evaluation of respiratory syndrome using 16S rRNA sequencing technique (Maher et al., 2018). In an experimental study, the virulence of *S. pluranimalium* was determined by experimentally inoculating mice. It was shown that *Streptococcus pluranimalium was* capable of crossing the blood brain barrier to cause brain damage (Kalhoro, 2015). It is also speculated that this organism can cause canine infectious respiratory disease (Kalhoro, 2015). Human infections with cases of *S. pluranimalium* are increasing and have been linked to human brain abscesses (Maher et al., 2018). Another frequently isolated pathogen, *Streptococcus canis*, has been shown to cause cutaneous, respiratory, genital, and urinary infections (Lamm et al., 2010). This organism was isolated from three dogs in this study. *Streptococcus canis* has been shown to cause infections in humans including soft tissue infection, bacteremia, urinary infection, bone infection, and pneumonia (Galpérine et al., 2007).

Staphylococcus pseudintermedius (n=42 isolates), was one of the predominant species, isolated from 26 different dogs in this study. *Staphylococcus pseudintermedius* can act as an opportunistic pathogen and can cause infections of the skin and ear, as well as infections of other body tissues and cavities in dogs (Griffeth et al., 2008; Weese and van Duijkeren, 2010). *S. pseudintermedius* is commonly found as part of the healthy skin, nares, mouth, pharynx, and anus of healthy dogs (Cox et al., 1988; Talan et al., 1989; Rubin and Chirino-Trejo, 2011). Humans have been shown to carry the same strain of *Staphylococcus pseudintermedius* as their pets when suffering from soft tissue infections (Guardabassi et al., 2004). This bacterial species has been determined to be the causative agent in sino-nasal infections in humans and has been responsible for chronic rhinosinusitis that is resistant to aggressive medical treatment (Kuan et al., 2016). Most concerningly, *S. pseudointermedius* has shown resistance to many of the conventional oral antimicrobial agents to treat human and animal infections (Perreten et al., 2010). *Methicillin-resistant Staphylococcus pseudointermedius* (MRSP) was isolated in this study from two of the dogs. *Staphylococcus pseudintermedius*, along with *Staphylococcus aureus*, isolated from four dogs can pose a significant risk for transfer of antimicrobial resistance. Pets can act as reservoirs for *Methicillin-resistant Staphylococcus aureus* (MRSA) and have shown to increase the risk of MRSA carriage in humans in close contact with animals (Moodley et al., 2008). Cases of MRSP in humans have been linked back to their pets (Paul et al., 2011). In this study, *Staphylococcus epidermidis*, was isolated from four of the dogs. This common commensal in dog may act as a reservoir for methicillin resistance for *Staphylococcus aureus* (Barbier et al., 2010; Smyth et al., 2011). This present study also included important β-hemolysin– producing *Staphylococcus* species, specifically *Staphylococcus pseudintermedius* and *Staphylococcus schleiferi*. These β-hemolysin– producing *Staphylococcus* species can

induce synergistic hemolysis when combined with nonhemolytic isolates of catalase-positive, Gram-positive cocci, including *Rothia nasimurium*. *Rothia nasimurium* was one of the more prevalent species isolated, recovered from seven of the dogs tested. *Rothia nasimurium* has been infrequently reported as a as a commensal organism in dogs, with an unknown role as a contributor to polymicrobial infections in dogs (Bemis et al., 2014).

Canibacter oris was isolated from five dogs that participated in the study. This bacteria has been isolated from a human with an infected dog bite wound (Aravena-Román et al., 2014). *Neisseria* species have also been isolated from infections in humans (Heydecke et al., 2013; Shinha, 2018). Five of the dogs in the study harbored this genus. *Neisseria weaveri was* isolated from two of the dogs. Shinha (2018), reported that *N. weaveri* can cause skin and soft tissue infections resulting from dog bites and if untreated can result in severe septicemia in humans (Shinha 2018). *Neisseria animaloris* and *N. zoodegmatis* were each isolated from one dog. These species have both been commonly isolated from dog bite infections and are considered clinically important (Heydecke et al., 2013). *Moraxella* species, isolated from five of the dogs, is a known common commensal of canines. In this study, *Moraxella canis* was isolated from two of the dogs and has been shown to cause clinical disease in humans. *Moraxella canis* was isolated from an ulcerated metastatic lymph node from a terminal human patient (Vaneechoutte et al., 2000). Another patient developed polyarticular septic arthritis that was associated with a predisposing malignancy. The septic arthritis was the result of *M. canis* infection due to immunosuppression. It has been the first septic arthritis case due to *M. canis* (Ottaviani et al., 2009) *Acinetobacter* species were isolated from three of the dogs in the study. These bacteria, although isolated from healthy animals, have been recognized as a nosocomial pathogen in humans and have been isolated from various infection sites. It has now recently been proposed

to be emerging as a significant nosocomial pathogen in dogs as well (Turton et al., 2010; Mitchell et al., 2018).

Antimicrobial Resistance

This study demonstrates the presence of a rich community of bacterial inhabitants of the nose cavities of dogs, some of which are pathogenic to both dogs and humans. The bacteria species isolated from nasal passages of dogs were tested for their susceptibility to the major groups of antimicrobials. Antimicrobial Resistance (AMR) has become one of the biggest threats to global public health. The increase in multidrug resistant (MDR) bacteria has resulted in therapeutic failures and constitutes a hindrance to a successful management of bacterial infectious diseases in both animals and humans. Most of the attention had focused on misuse of antimicrobial in food-production animals as a major cause of AMR. However, in a society where pet animals, including dogs, have become closer to humans (Guardabassi et al., 2004), it is sensible to believe that bacteria can easily pass from animals to humans and vice-versa. Due to the ability of bacteria to pass antimicrobial resistance to other bacteria through horizontal gene transfer (Lerminiaux and Cameron, 2018), it is very likely that AMR patterns will be shared between bacterial communities in humans and pet animals.. Therefore, a better understanding of the level of resistance in canine nasal bacteria will help veterinarians and physicians alike to estimate the burden of AMR and guide them in setting therapeutic strategies.

In this study, the bacteria species tested for AMR had varying levels of resistance to the antimicrobials tested. Many of the bacterial isolates in this study showed high levels of resistance with 23 isolates from eight species being multi-resistant (resistant to three or more

antimicrobials). The species that were found to have the most multi-resistant isolates were *Bacillus mobilis, Corynebacterium flavescens, Staphylococcus epidermidis, Staphylococcus pseudintermedius, Staphylococcus warneri, Aeromonas hydrophila, Pseudomonas koreensis,* and *Stenotrophomonas rhizophila.* In general, bacteria that were less frequently isolated were either susceptible or showed resistance to one or two antimicrobials. *Staphylococcal* species were resistant to several antimicrobials. Conversely, *Streptococcus pluranimalium,* that was just as commonly isolated as *Staphylococcal* spp. showed either pan susceptibility or resistant to one or two antimicrobials.

Some of these *Staphylococcus* spp. may have high rates of AMR because they commonly cause disease in animals and humans. Bacteria that are known to cause infections such as *Staphylococcus* spp. will more exposed to antimicrobials and therefore have a higher risk of developing antimicrobial resistance due to selection pressure (Zhang et al., 2015).

Staphylococcus pseudintermedius had the highest level of resistance, with several isolates resistant to the majority of the antimicrobials tested. Some *Staphylococcus pseudintermedius* isolates were able to be grown on the SPECTRA plates, suggestive of resistance to methicillin. *Staphylococcus epidermidis* also had high levels of resistance, with all six isolates tested having some form of resistance, particularly to clindamycin, erythromycin, and penicillin. In addition, all seven isolates of *Staphylococcus aureus* tested were resistant to both ampicillin and penicillin.

Staphylococcus pseudintermedius is the most prevalent inhabitant of dog mucosa and skin, as well as the major causative agent of canine skin and ear infections (Griffeth et al., 2008; Bannoehr, 2012). This prevalence may also help explain the high levels of resistance seen in this species, including multidrug resistance and methicillin resistance. Each year there is an increasing number of infections globally due to methicillin-resistant *S. pseudintermedius*

(MRSP) (Hanselman et al., 2008). These infections become extremely difficult to treat due to their multidrug resistance (Frank and Loeffler, 2012; Ventrella et al., 2017). In addition, humans are capable of developing infections due to MRSP, suggesting a risk of zoonotic transmission (Grandolfo 2018).

Even non-virulent strains of *Staphylococcus pseudintermedius* can cause problems as they can pass their AMR determinants carried on mobile gene elements to other pathogenic bacteria. Some of the dogs with resistant *Staphylococcus pseudintermedius* in their nasal cavities also harbored other species with similar antimicrobial resistant profiles. Although none of the *Staphylococcus aureus* isolates in this study were methicillin resistant, they may be susceptible to gaining methicillin resistance genes through horizontal transfer from the MRSP also colonizing their nasal cavity. Another resistant species in this study, *Staphylococcus epidermidis,* may also play a role in transferring AMR determinants. Studies have found that a persistent co-carriage of resistant *Staphylococcus epidermidis* may act as a source of antimicrobial resistance genes for *Staphylococcus pseudintermedius* and *Staphylococcus aureus* (Gómez-Sanz et al., 2013). In this study, several dogs had co-carriage of *Staphylococcus epidermidis* with other bacteria in the nasal cavities with very similar antimicrobial resistance profiles. This indicates the potential transmission of AMR determinants on *Staphylococcus epidermidis* to previously susceptible bacteria.

In this study, *Streptococcus* species, especially *Streptococcus pluranimalium*, were of similar prevalence to *Staphylococcus* species. However, unlike *Staphylococcus* species, these isolates were completely susceptible to all antimicrobials tested. This AMR study included *Streptococcus canis, Streptococcus minor, Streptococcus oralis, Streptococcus ovis,* and *Streptococcus pluranimalium.* None of the 44 isolates tested developed any sign of resistance.

This may be due to the fact that many of these species are infrequently associated with disease as compared to *Staphylococcus* species.

Similarly, *Streptococcus* species, *Acinetobacter* species have commonly been isolated from dogs at numerous sites on the body (Turton et al., 2010; Pailhoriès et al., 2015). However, because it is not as commonly associated with disease as *Staphylococcus* species, based on this observation it can be speculated that the level of resistance observed in this study were low. Studies have suggested that *Streptococcus pluranimalium* is emerging as a pathogen in dogs (Mitchell et al., 2018), and therefore, perhaps antimicrobial resistant strains of *S. pluranimalium* strains could emerge in the near future.

In conclusion, this study demonstrates the presence of a highly diverse population of bacteria colonizing the nasal passages of household dogs. Staphylococci and streptococci species dominated the nasal cavities of the sampled dogs, with other genera isolated much less frequently. Notably, the nasal cavity harbors potentially pathogenic species that are capable of being transmitted to humans. Some of the species, specifically those often involved in clinical disease, had high levels of resistance to antimicrobials. Staphylococci species had highest levels of resistance, especially to beta-lactams and cephalosporins. On the other hand, all streptococci species isolated and tested in this study were susceptible to all antimicrobials tested. The results of this study can be used to help veterinarians develop a more effective antimicrobial treatment plan for infections of the nasal passage and upper respiratory tracts of dogs. In addition, these results can help aid further studies in developing a more extensive descriptive and epidemiologic study to better understand the bacterial species inhabiting canine nasal passages.

Appendix A

Questionnaire

Survey #

Please take a few minutes to fill out this questionnaire about your dog.

Veterinary Clinic_____ Owner's Name_____
Your Zip Code _____ Pet's Name _____

General Patient Information

Breed_____ Age_____ Sex_____ Spayed/Neutered Yes No

Has your dog had some form of respiratory illness within the last 6 months?

☐ Yes,
☐ No

If yes, briefly explain the signs? _____

Has your dog received the Canine Influenza Vaccine?

☐ Yes,
☐ No

If yes, when was it administered? _____

Has your dog taken an antibiotic within the past month?

☐ Yes,
☐ No

Did you acquire your dog within the last 6 months?

☐ Yes,
☐ No

If yes, how did you acquire your dog?

☐ Dog breeder ☐ Animal shelter ☐ Pet store ☐ Other _____

Interactions with Other Animals

In the last 6 months, how often has you dog stayed at a boarding facility/kennel? ☐ Never ☐ 1-2 stays in the past six months ☐ 3-5 stays in the past six months ☐ More than 5 stays in the past six months	In the last 6 months, how often has your dog gone to a training class? ☐ Never ☐ Rarely (at most 1-2 times/month) ☐ Regularly (at least 1 time/week) ☐ Frequently (more than 2 times/week)
In the last 6 months, has your dog gone to doggie day care? ☐ Never ☐ Rarely (at most 1-2 times/month) ☐ Regularly (at least 1 time/week) ☐ Frequently (more than 2 times/week)	In the last 6 months, how often has your dog gone to the groomer? ☐ Never ☐ Rarely (at most 1-2 times/month) ☐ Regularly (at least 1 time/week) ☐ Frequently (more than 2 times/week)
In the last 6 months, how often has your dog gone to a dog park? ☐ Never ☐ Rarely (at most 1-2 times/month) ☐ Regularly (at least 1 time/week) ☐ Frequently (more than 2 times/week)	In the last 6 months, how often has your dog interacted with dogs outside of the household? ☐ Never ☐ Rarely (at most 1-2 times/month) ☐ Regularly (at least 1 time/week) ☐ Frequently (more than 2 times/week)
In the last 6 months, how often has your dog gone to a vet clinic? ☐ Never ☐ Rarely (at most 1-2 times/month) ☐ Regularly (at least 1 time/week) ☐ Frequently (more than 2 times/week)	In the last 6 months, has your dog traveled out of state? ☐ Yes ☐ No If yes, where? _____
Are there any other pets at home? ☐ Yes ☐ No If yes, what kind(s)?_____	Has your dog had contact with livestock in the last 6 months? ☐ Yes ☐ No If yes, what kind(s)? _____

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ACADEMIC VITA

Margaret Zemanek

| 202 W. Wayne Ave Easton, PA | 610-390-2187 | mcz5057@psu.edu |

Education:

B.S. Veterinary and Biomedical Sciences — Spring 2019
Pennsylvania State University - Schreyer Honors College

Animal Experience

Mt. Nittany Veterinary Hospital - State College, PA — 2018
Veterinary assistant

All Creatures Veterinary Care - Centre Hall, PA — 2017 - 2018
Veterinary assistant

Centre Wildlife Care - Port Matilda, PA — 2015 - 2019
Animal Care Supervisor
Help rehabilitate injured and orphaned wildlife

Lehigh Valley Zoo - Allentown, PA — 2016 - 2017
Docent
Provided animal care and gave educational presentations

Large Animal Veterinary Practice in the Tropics, Belize — 2017
Participated in a pre-vet program working with large animals

Warren County Animal Hospital and Easton Animal Hospital — 2015 - 2016
Observational Learning

Research Experience:

Animal Diagnostic Lab - State College, PA — 2017- 2018
Research on the Canine Influenza virus in Pennsylvania

Dr. Bhushan Jayarao's Lab – State College, PA — 2018 - 2019
Cultured bacteria from nasal swabs
Identified bacteria using MALDI-TOF and 16S rRNA sequencing
Antimicrobial resistance testing

Lehigh University - Bethlehem, PA — 2017
Morphological research on snakes and their feeding behavior

Penn State Langkilde Lab - State College, PA 2016 - 2017
Research on Fence lizards

Teaching Experience
Penn State Learning Assistant
Penn State Chemistry Department- General Chemistry I 2016
Penn State Mathematics Department- Calculus and Biology I 2017