ABSTRACT

The Distribution and Resistance to Antibiotics of *Staphylococcus* Organisms Among the Equine Population of Central Texas

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Staphylococcus species are common microbes capable of causing infection in human and nonhuman populations. Microbial resistance to antibiotics has become a threat to antisepsis and public health. There is limited knowledge of *Staphylococcus* organisms specific to equine populations and less of their resistance to antibiotics within these populations. To survey the *Staphylococcus* species in the equine population of the Central Texas region, a total of 65 horses from three farms were swabbed within the nasal cavity, behind the ear, and at the left girth. Swab sites were chosen for accessibility and high moisture levels that could support bacterial growth. The farms varied in level of human contact. Interactions between species may increase the diversity of microbes shared. Biochemical tests were used to characterize strains, which were then identified using the Advanced Bacterial Identification Software (ABIS) and the Siemens Microscan Autoscan System. 25 *Staphylococcus* species were isolated between the three farms. The most common isolates were *Staphylococcus cohnii cohnii, Staphylococcus xylosus, and Staphylococcus haemolyticus.* An increase in level of human contact and potential for treatment between Farms One and Three correlated with an increase in microbial resistance to antibiotics, specifically penicillin, oxacillin, trimethoprim-sulfmethoxazole, and ciprofloxacin.

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THE DISTRIBUTION AND RESISTANCE TO ANTIBIOTICS OF *STAPHYLOCOCCUS* ORGANISMS AMONG THE EQUINE POPULATION OF CENTRAL TEXAS

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By

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All philosophy is based on two things only: curiosity and poor eyesight... The trouble is, we want to know more than we can see. Again, if we could really see things as they are, we would really know something, but we see things other than as they are. So true philosophers spend a lifetime not believing what they do see, and theorizing on what they don't see.

– Bernard Le Bovier de Fontenelle

CHAPTER ONE

Introduction

Bacteria are ubiquitous organisms. Specific flora have adapted to various host organisms, and interactions between different host species can facilitate transfer of the adapted strains from one host to another. Of particular interest are bacteria in the *Staphylococcus* genus, many of which are normal flora of humans and other mammals. The *Staphylococcus* genus was named for its morphology, from the Greek "staphyle" ("bunch") and "kokkos" ("berry"); together, these translate as a "bunch of berries," a description of the individual shape and collective pattern staphylococci form under a microscope (29). Basic hygiene practices such as handwashing can decrease the exchange and spread of microbes.

Staphylococcal species are commonly grouped as either coagulase-negative staphylococci (CoNS) or coagulase-positive staphylococci (CoPS). Both groups are capable of causing infection. CoNS are typically opportunistic pathogens and are frequently associated with medical device infections.

Despite fewer *Staphylococcus* species being coagulase-positive, there is a stronger research interest in CoPS because they can be primary pathogens. The CoPS of primary concern are *Staphylococcus aureus* and *Staphylococcus*

pseudointermedius. In one retrospective study of CoPS implicated in horse necropsies, *Staphylococcus aureus* and *Staphylococcus pseudointermedius* were isolated from 60 out of 3,457 total necropsies (1.7%). Necropsies were performed over a ten-year period on horses that died or were euthanized. The results were startling: 54 of the 60 isolates (90%) were strongly associated with the death of the animal (16).

The primary purpose of this study is to identify the common staphylococcal flora of the equine population in the Central Texas region. A secondary interest of this study seeks to analyze the degree of microbial resistance to antibiotics when the level of human contact varies between the equine populations. By surveying the distribution of species and the antibiotic resistance profile of this population, this study is additionally evaluating potential methicillin-resistant *Staphylococcus aureus* carriage in the equine population of Central Texas.

Staphylococcus organisms are seemingly ubiquitous in themselves. The possibility of hosting staphylococci is no surprise, but the region-specific distribution of the species deserves investigation. Many staphylococci other than *Staphylococcus aureus* have been commonly isolated from horses, such as *Staphylococcus equorum* (initially isolated from horses, as its species name implies). The primary purpose of this study is to identify the common staphylococcal flora on the equine population of Central Texas.

The most common strains of *Staphylococcus* species seem to resist all beta-lactam antibiotics. One study shows that nearly 80 percent of CoNS isolates showed resistance to oxacillin (methicillin) in human hospitals across the United States, Canada, Latin America, Europe, and the Western Pacific Region (10). Furthermore, resistance of *Staphylococcus* species to methicillin has been genetically linked to the mecA gene (2, 33). Microbial resistance to antibiotics has become a potential threat to public health. It is suspected that many bacteria developed antibiotic resistance due to the genetically selective pressure of microbial evolution in human hospital settings (8, 12).

Given that MRSA infections can cause quickly spreading diseases in confined human populations, it is possible that a similar scenario involving other *Staphylococcus* species can occur in animal populations as well. The antibiotic resistance of *Staphylococcus aureus* as a primary pathogen and of other *Staphylococcus* organisms as opportunistic pathogens is alarming because treatment options are potentially limited by this adaptation.

A secondary purpose of this study is to examine the resistance to antibiotics within the Central Texas equine population. Contact between humans and horses varied between farms. This study examines the potential correlation between level of human contact (including likelihood of receiving treatment) and degree of antibiotic resistance. It is likely that degree of human contact is not the only factor pertaining to antibiotic resistance. It is quite possible that this resistance is simply specific to the staphylococcal

species, and interaction between the horses allows for the spread of these resistant microbial strains. This is expected especially for strains that are most commonly isolated from animal species.

Staphylococcus aureus was first named for its golden appearance, in contrast to the white-colored *Staphylococcus albus*, now known as *Staphylococcus epidermidis* (27). Since this initial discovery, many other staphylococcal species have been discovered and named; however, certain strains of *Staphylococcus aureus* have acquired resistance to methicillin, a penicillin-based antibiotic. Many *Staphylococcus* organisms carry the mecA gene, which codes for beta-lactamase. Beta-lactamase is an enzyme that catabolizes the beta-lactam ring of many antibiotics, such as many penicillin-based drugs. The mecA gene is a common mechanism of resistance among *Staphylococcus* organisms. Methicillin-resistant *Staphylococcus aureus* (MRSA) organisms pose a serious threat in hospital environments and areas of close confinement.

Many MRSA strains specifically adapted either to humans or to animals can transfer between species and remain on their new host asymptomatically as opportunistic pathogens. MRSA strains have been known, however, to act as primary pathogens, and have been found on many different host animals, including domesticated ruminants, pigs, horses, dogs, and cats (27). MRSA can be found in many raw dairy products, meats, and cheeses. *Staphylococcus aureus* is typically considered a human pathogen,

but animal-adapted strains have been identified. In one study, human infection was observed only four hours after contact with a MRSA-colonized foal (27). Due to the endemic and potential pathogenic nature of MRSA, another secondary purpose of this study is to investigate the potential distribution of methicillin-resistant *Staphylococcus aureus* in the equine population of the Central Texas region.

Information on the distribution and degree of antibiotic resistance of *Staphylococcus* organisms in the Central Texas region is limited. Examining the distribution of *Staphylococcus* species can potentially provide insight into the migration patterns of bacteria, as well as inform and caution the public on the potential microbes that are common flora to this region. This study compares the farm to species identification, the level of human contact (by farm) to resistance of six different antibiotics, and species identification to resistance of those same antibiotics. Information from this study on antibiotic resistance can assist scientists in better understanding the truly ubiquitous presence of microbial resistance to various beta-lactam drugs. This study provides a basis from which the equine bacterial flora of Central Texas can be compared to other regions across the United States and, potentially, other countries across the world.

CHAPTER TWO

Methods and Materials

Procedure

The study was conducted at three Central Texas farms between August 2012 and May 2014. Horses were swabbed behind the left ear, within the nostril, and at the left girth. Swab sites were chosen for accessibility and high moisture levels that could support bacterial growth.

Swabs were immediately transported to a microbiology laboratory in the Baylor University Sciences Building, inoculated onto Mannitol Salt Agar (MSA), and incubated for 48 hours at 35 degrees Celsius. After incubation, growth, mannitol fermentation, and physical observations were noted. Small, circular, entire, and convex colonies from each plate were selected for isolation on Tryptic Soy Agar (TSA). Isolates were gram-stained, and their morphology was observed under the microscope.

Biochemical testing included determination of catalase production, coagulase production, urease production, nitrate reduction, arginine dihydrolase production, oxidase production, and fermentation of sugars (glucose, xylose, sucrose, and lactose). Oxygen requirements were determined by growth patterns in fluid thioglycollate broth. Hemolysis reaction was observed on blood agar plates. All of these characteristics were used to better

describe and group colonies based on their similarities and differences. Antibiotic susceptibility testing was performed using the Kirby-Bauer method. Group representative colonies from the first farm were chosen and identified using the Siemens Microscan autoscan system. The remaining colonies from the first farm were classified based on their similarities to the identified colonies. Cultures from the second and third farms were identified using the Advanced Bacterial Identification System (ABIS).

Farm Selection

Three farms were used in this study. The farms were selected for use in this study based on level of human interaction, which includes the possibility for exchange of microbes with varying numbers of humans, as well as the frequency of and access to treatment. Degree of human interaction was determined by how frequently the horses were ridden and groomed. The first farm was privately owned, and the horses had minimal human contact. A local riding association owned the second farm, and the horses had a moderate level of human contact, characterized by periodic grooming and riding. The third farm, a riding stable, had a high level of human contact; horses were housed in stalls, and were groomed and ridden multiple times per week.

Permission Forms and Horse Information

Owners or farm managers granted permission to use their horses in this study and provided physical information on each horse, including predominant breed, hair color(s), sex, age, and weight.

Mannitol Salt Agar (MSA)

MSA contains the following ingredients per liter: 5.0 grams of enzymatic digest of casein, 5.0 grams of enzymatic digest of animal tissue, 1.0 gram of beef extract, 10.0 grams of d-mannitol, 75.0 grams of sodium chloride, 0.025 grams of phenol red, and 15.0 grams of agar.

With a high concentration of sodium chloride (salt; NaCl), MSA is selective for gram-positive halophiles, such as *Staphylococcus* organisms. Some *Streptococcus* species will not grow, nor will most gram-negative bacteria.

In addition, some bacteria are able to ferment mannitol (a sugar). The fermentation of mannitol will produce an acidic byproduct, and the phenol red pH indicator in the agar will change from red to yellow. If the bacteria cannot ferment mannitol, the agar will remain red.

Bacterial colonies were observed and grouped based on physical characteristics visible to the naked eye (4). Colony size (in millimeters), elevation, margins, and color were noted, with a brief description of each. An approximate percentage of fermenter colonies per plate was recorded, as was

the approximate number of colonies growing on each MSA plate. Colonies that were small, circular, entire, and convex were selected for further study and transferred to the tryptic soy agar.

Tryptic Soy Agar (TSA)

TSA contains the following ingredients per liter: 15.0 grams of pancreatic digest of casein, 5.0 grams of peptic digest of soybean meal, 5.0 grams of sodium chloride, and 15.0 grams of agar. This agar is considered a general growth medium for microorganisms in lab settings. TSA was used to maintain cultures on library plates.

Catalase

Catalase is an enzyme that facilitates the cellular detoxification and neutralization of the effects of hydrogen peroxide (H_2O_2), whose oxidizing ability is damaging to cells. Testing for the catalase enzyme assists in differentiating *Streptococcus* from *Staphylococcus* species (32). In this study, a variation of the slide (drop) method is used. Aliquots of hydrogen peroxide are applied to a Plexiglass grid. Using a wooden applicator stick, a minute amount of the organism is transferred into a discrete hydrogen peroxide droplet. If the hydrogen peroxide begins to form bubbles (effervescence), the test is positive (*Staphylococcus*). If effervescence does not occur, the test is negative (*Streptococcus*).

Gram Stain

Gram staining assists in the classification and differentiation of organisms in the domain Bacteria based on the composition of their cell wall. *Staphylococcus* organisms are gram-positive, and have a cell wall primarily composed of a thick layer of peptidoglycan, exterior to the cell membrane. Bacteria whose cell wall is primarily composed of a small peptidoglycan layer sandwiched between two membranes will stain pink, a negative test. The Gram Stain method was first used in 1884 by Hans Christian Gram to identify pneumococci (a gram-positive organism), and typhoid bacilli (a gram-negative organism) (38). Gram's method has four steps: crystal violet primary stain, iodine mordant, ethanol rinse, and safranin secondary stain. The primary and secondary stains are positively charged, and bind to the negatively charged structures of the cell wall. The iodine operates as a mordant, which intensifies the color by binding to the crystal violet cation. The alcohol reacts with the outermost lipid layer of the outer membrane, causing the gram-negative cell walls to leak, removing the purple coloring from gram-negative bacteria. Finally, the safranin stain is applied. Gram-positive cells stain purple, and gram-negative cells stain pink.

Coagulase

The enzyme coagulase is produced by pathogenic *Staphylococcus* species, including *Staphylococcus aureus*, and differentiates *Staphylococcus*

aureus from other staphylococcal species. As the bacteria multiply in the plasma, they secrete staphylocoagulase, which binds to prothrombin, causing the formation of a fibrin clot (24). Coagulase, as its name suggests, plays a role in the coagulation and clotting of blood. In this study, the tube coagulase test is used, and clumping within 24 hours indicates a coagulase-positive organism. In vivo, this keeps infections confined as a boil or sty by producing clots in localized blood vessels.

Urease

The urease enzyme hydrolyzes urea to carbon dioxide and ammonia. As urea is broken down to ammonia (a positive test), pH increases and the phenol red pH indicator changes from yellow-orange to pink (5).

Nitrate and Nitrite Reduction

Testing for the reduction of nitrate (NO_3^+) to nitrite (NO_2^-) can be a useful tool in characterizing and identifying bacterial species. The reduction of nitrate is catalyzed by nitrate reductase (7). Following inoculation of the nitrate broth and a minimum of 48 hours of incubation, reagents A (α-naphthylamine) and B (sulfanilic acid) are added to the nitrate broth; if nitrites are present (implying that nitrates have been reduced), the reagents will acidify and change from a colorless solution to a red, rust-colored solution, indicating a positive test.

Fermentation of Glucose, Xylose, Sucrose, and Lactose

Fermentation is a catabolic process in which a carbohydrate is broken down by a microbe to form an organic acid. The pH indicator, phenol red, changes the carbohydrate broth from red to a bright, lemon-yellow color when acids are produced. There are a variety of carbohydrate substrates, and bacteria are typically capable of breaking down some carbohydrates but not others. Fermentation of glucose, xylose, sucrose, and lactose are four simple tests that are used to characterize and identify bacteria in this study.

Arginine Dihydrolase

Arginine is an amino acid that many bacteria catalyze for energy production, and the carbon present in arginine can be utilized as a building block for other organic substances. Bacteria that have this ability possess arginine dihydrolase. To determine arginine dihydrolase activity, bacteria are transferred to a broth containing glucose, arginine, and a pH indicator, brom cresol purple, which is purple under basic conditions, but turns yellow when its environment drops below a pH of 5.2. The yellow color indicates a positive test for arginine dihydrolase. This test is useful for differentiating between staphylococcal species.

Oxidase

Oxidase is an enzyme used by some bacteria in the final stage of respiration known as the electron transport chain. The colorless reagent is an electron donor that turns purple when exposed to a fresh (18 to 24 hours), oxidase-positive bacterial culture. In 1928, this biochemical test was first used to differentiate *Neisseria gonorrhoeae* (oxidase-positive) from *Staphylococcus* and *Streptococcus* organisms (both are oxidase-negative) (36). The test has since been adapted to provide information for identification purposes.

Fluid Thioglycollate

Sodium thioglycollate prevents diffusion of oxygen in the medium. After autoclaving, oxygen will diffuse into the surface, and no oxygen will be available at the bottom of the test tube. This is considered a redox potential and will cause the bacteria to migrate within the test tube to the areas that best support their oxygen requirements (13). This permits the classification of bacteria as aerobic, facultative, or anaerobic. Additionally, motility can be observed as movement away from the stab line. For example, if the microbe is an obligate aerobe, growth will be present along the top four to five millimeters of the inoculation stab, and along the surface; if the microbe is facultative and motile, growth would be present throughout the medium, and a stab line would be obscured in the medium. A stab line would not be

obscured if the inoculate is facultative and nonmotile because the bacteria will grow in both aerobic and anaerobic areas but will not separate from the stab line. Obligate anaerobes grow only in the bottom of the tube.

Hemolysis

Testing for hemolysis is medically beneficial because some bacteria possess hemolysins that can digest the contents of erythrocytes (specifically hemoglobin) to produce energy, which can cause damage to many other cells, in addition to red blood cells. The effects of hemolysins on a blood agar are visible to the naked eye, and are classified into three categories: beta hemolysis, alpha hemolysis, and gamma hemolysis. Beta hemolysis occurs when red blood cells are completely lysed and the agar around the colony is clear. Alpha hemolysis reflects incomplete hemolysis at the colony. It is degraded to a green metabolite, and the agar will still be opaque. Gamma hemolysis shows no visible difference in the agar, despite the growth of bacteria.

Antibiotic Sensitivities

A microbe's sensitivity or resistance to any given antibiotic can be tested using the Kirby-Bauer method. This procedure is particularly useful in identifying antibiotics useful for treatment of both human and nonhuman patients (19). Mueller-Hinton agar is considered the standard medium, and

contains the following ingredients per liter: 2.0 grams of infused beef, 17.5 grams of acid digest of casamino, 1.5 grams of starch, and 17.0 grams of agar. Isolates are first transferred from a freshly incubated TSA culture (between 18 and 24 hours old) to a nutrient broth. Mueller-Hinton plates are then streaked with the broth using a three-way streak pattern to completely coat the agar surface. One inoculum is transferred to each nutrient broth and then to each Mueller-Hinton plate.

Six-millimeter paper discs containing specific antibiotics are then stamped onto the surface of the agar. Plates are then incubated for 24 hours. Zones of inhibition are measured and compared to standard tables to determine microbial susceptibility or resistance to that antibiotic. This study tested microbial susceptibility to oxacillin, penicillin, trimethoprim-sulfmethoxazole, ciprofloxacin, clindamycin, and gentamicin.

Advanced Bacterial Identification System (ABIS)

ABIS is an online database useful for identifying bacteria using biochemical test results. Due to its vast expanse of information, the database is divided into groups based on bacterial strain, morphology, and gram stain. Once the initial bacterial grouping has been selected, biochemical test results can be entered to identify the species. Because there are many test results that can characterize a species, it is not necessary to have results for every test to receive an accurate identification. Once test results have been entered,

there are two options: a normal mode of identification, and a forced mode of identification. In this study, the normal mode of identification was utilized for better accuracy of strain taxon-- forced identification is typically only utilized when the number of biochemical tests or results is significantly limited.

Identification results include: the top, most-likely taxa; a percentage of correct test results (out of the total number of tests entered) based on the species' biochemical descriptions in the ABIS database; the expected test result, if the results entered do not match the expected database descriptions; and a list of suggested tests which might assist in discriminating between the top, most-likely taxa. ABIS results were used in this study to decide which tests would best help differentiate between similar identifications. Additionally, ABIS is the primary database used in this study to distinguish and identify species.

Siemens Microscan Autoscan System

This system is used to identify species and measure microbial resistance to antibiotics. A broth is inoculated with one organism and poured into a panel, which is then sealed. Test materials are injected into the panel, and panels are incubated overnight and read by the autoscan system the next day. The autoscan is useful for identifying species, characterizing isolates by their test results, and measuring antibiotic resistance by the minimum

inhibitory concentrations (MICs) of each antibiotic. This system was only used to characterize strains from Farm One.

CHAPTER THREE

Results

A total of 65 horses were sampled in this study. 34 horses were swabbed at Farm One, ten horses were swabbed at Farm Two, and 21 horses were swabbed at Farm Three. In total, 184 viable isolates qualified as *Staphylococcus* based on culture characteristics, growth on MSA, catalase production, and presence of gram-positive cocci. Isolates were then analyzed further. Farm One had 62 isolates, 21 isolates came from Farm Two, and 101 isolates came from Farm Three. Isolates were analyzed with two primary comparisons in mind:

(1) Compare the identified species from each farm,

(2) Compare antibiotic resistance patterns of isolates from each farm.

Additionally, to ensure that the antibiotic resistance was a characteristic of the farm and not of the species themselves, a third, secondary comparison was analyzed:

(3) Compare identified species with their levels of antibiotic resistance.

Distribution of Species

On Farm One, five different *Staphylococcus* species were identified (Figure 1). Out of 62 total isolates, 30 isolates were *Staphylococcus xylosus* (48.39%), sixteen were *Staphylococcus cohnii cohnii* (25.81%), eight were *Staphylococcus sciuri* (12.90%), four were *Staphylococcus cohnii urea* (6.45%), and four were *Staphylococcus capitis ureo* (6.45%).

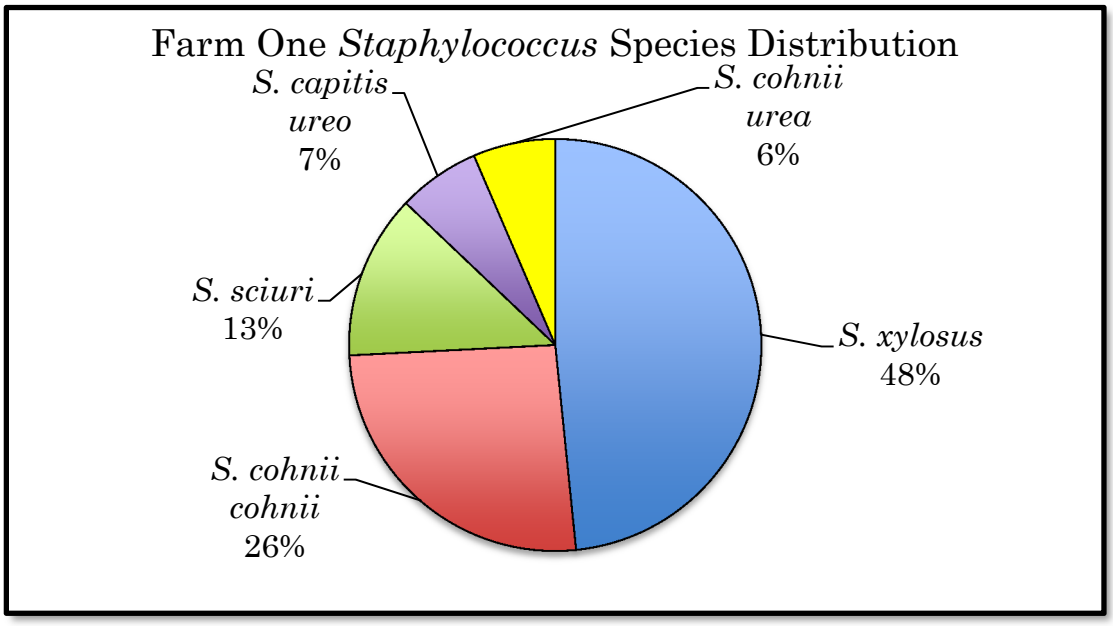

Figure 1. Equine bacteria were isolated from the ear, nostril, and girth. Farm One had a low level of human contact. Out of 62 total isolates, five species were isolated from Farm One: *S. xylosus, S. cohnii cohnii, S. sciuri, S. capitis ureo,* and *S. cohnii urea.*

Microbial prevalence in a community is more apparent if the species' presence is analyzed as the number of carriers of that microbe in said community. A total of 34 horses were sampled from Farm One. All species were present on more than one horse (Figure 2). *Staphylococcus xylosus* was

isolated from seventeen of the 34 horses (present on 50% of the equine population); *Staphylococcus cohnii cohnii* was isolated from twelve horses (35.3%); *Staphylococcus sciuri* was isolated from eight horses (23.5%); and *Staphylococcus capitis-ureo* and *Staphylococcus urea* were respectively isolated from four different horses (11.8%).

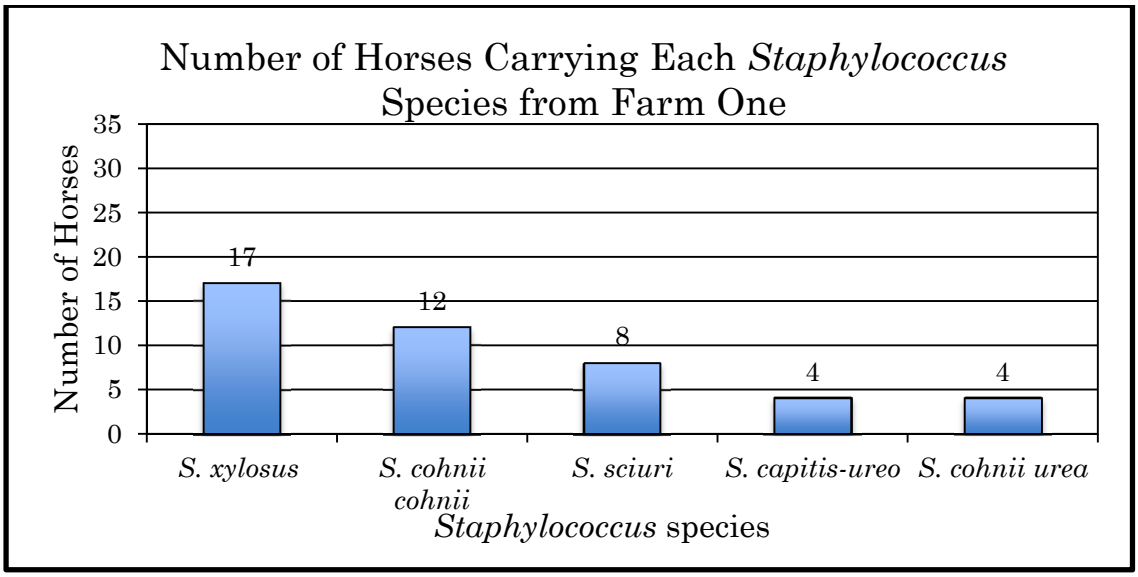

Figure 2. Isolates were analyzed as the number of horses carrying each species. Five species were identified from 34 horses at Farm One: *S. xylosus*, *S. cohnii cohnii, S. sciuri, S. capitis-ureo,* and *S. cohnii urea.*

From Farm Two, a total of ten different species were identified (Figure 3). Out of 21 isolates, the five most isolated species were *Staphylococcus kloosii* (four isolates, 19.05%), *Staphylococcus capitis capitis* (three isolates, 14.29%), *Staphylococcus carnosus* (three isolates, 14.29%), *Staphylococcus cohnii cohnii* (three isolates, 14.29%), and *Staphylococcus sciuri* (two isolates, 9.52%). Five other species collectively represent about 29 percent of the isolates from Farm Two, including *Staphylococcus*

haemolyticus, Staphylococcus lentus, Staphylococcus microti, Staphylococcus gallinarum, and *Staphylococcus simiae.*

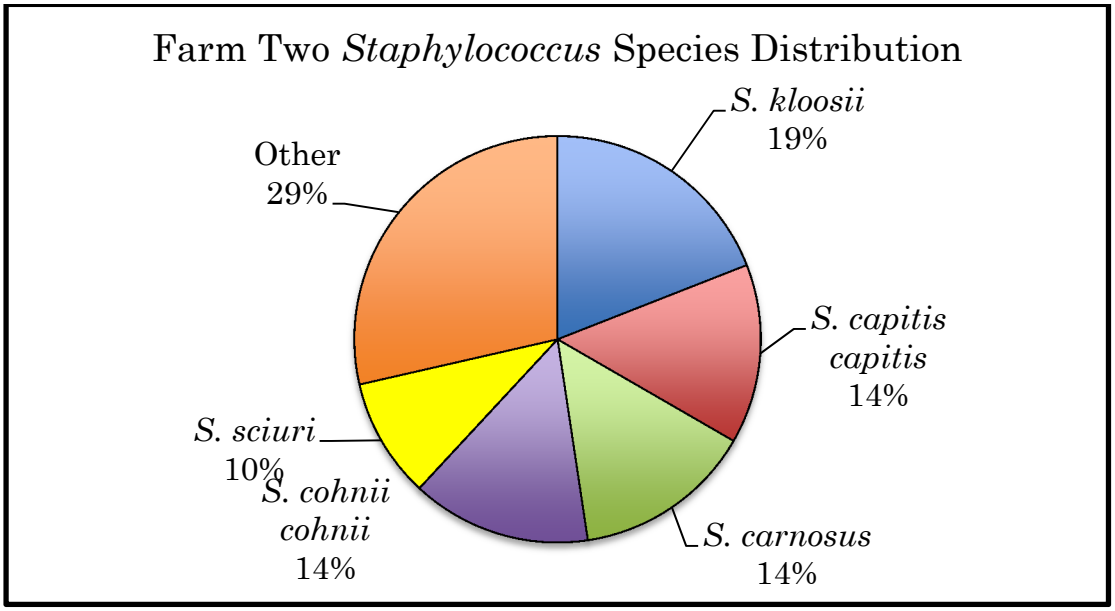

Figure 3. Equine bacteria were isolated from the ear, nostril, and girth. Farm Two had more human contact than Farm One, but less human contact than Farm Three. Out of twenty-one isolates, ten species were isolated from Farm Two: *S. kloosii, S. capitis capitis, S. carnosus, S. cohnii cohnii,* and *S. sciuri.* Other species include: *S. haemolyticus, S. lentus, S. microti, S. gallinarum,* and *S. simiae.*

Ten horses were sampled from Farm Two. Some species were present on more than one horse (Figure 4). *Staphylococcus cohnii cohnii* was isolated from three horses (present on 30% of the equine at Farm Two); *Staphylococcus capitis capitis, Staphylococcus carnosus, Staphylococcus kloosii,* and *Staphylococcus simiae* were each isolated from two horses (20%); *Staphylococcus gallinarum, Staphylococcus haemolyticus, Staphylococcus lentus, Staphylococcus microti,* and *Staphylococcus sciuri* were each isolated from one horse (10%).

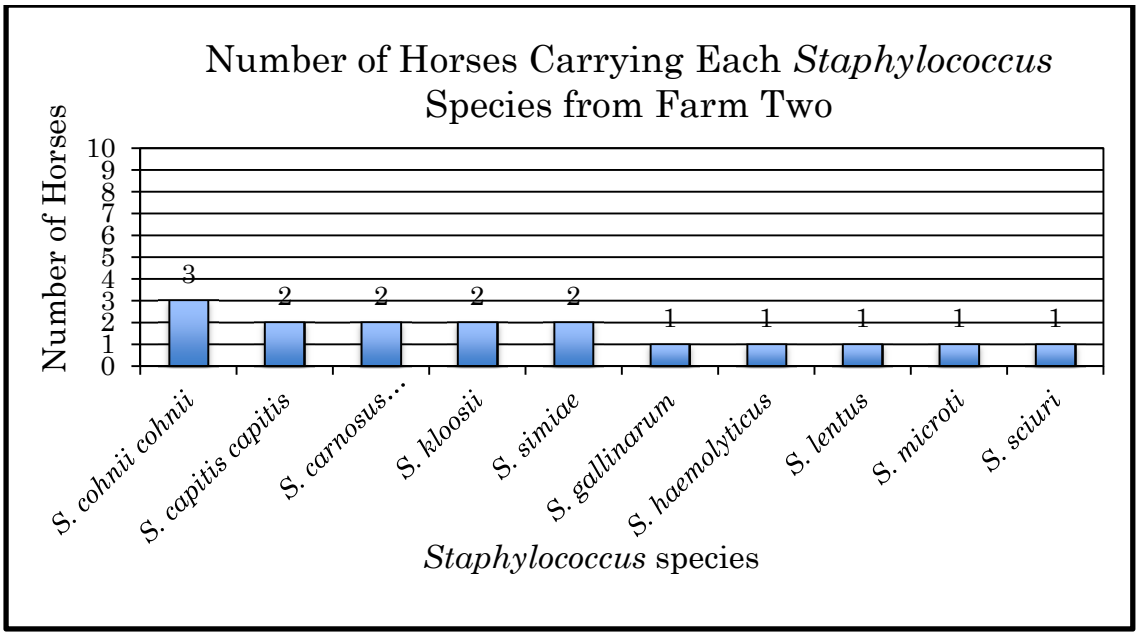

Figure 4. Isolates were analyzed as the number of horses carrying each species. Ten species were isolated from ten horses at Farm Two: *S. cohnii cohnii, S. capitis capitis, S. carnosus, S. kloosii, S. simiae, S. gallinarum, S. haemolyticus, S. lentus, S. microti,* and *S. sciuri.*

From Farm Three, 101 isolated strains and eighteen different species were identified (Figure 5). The five most prevalent isolates were *Staphylococcus haemolyticus* (31 isolates, 30.69%)*, Staphylococcus capitis capitis* (21 isolates, 20.79%), *Staphylococcus kloosii* (ten isolates, 9.90%)*, Staphylococcus cohnii cohnii* (ten isolates, 9.90%)*,* and *Staphylococcus simiae* (seven isolates, 6.93%). Thirteen other species collectively represent twenty-one percent of the isolates from Farm Three, including*: Staphylococcus warneri, Staphylococcus carnosus, Staphylococcus arlettae, Staphylococcus condimenti, Staphylococcus caprae, Staphylococcus equorum equorum, Staphylococcus gallinarum, Staphylococcus homini novobiosepticus,*

Staphylococcus muscae, Staphylococcus vitulinus, and four species that could not be definitively identified.

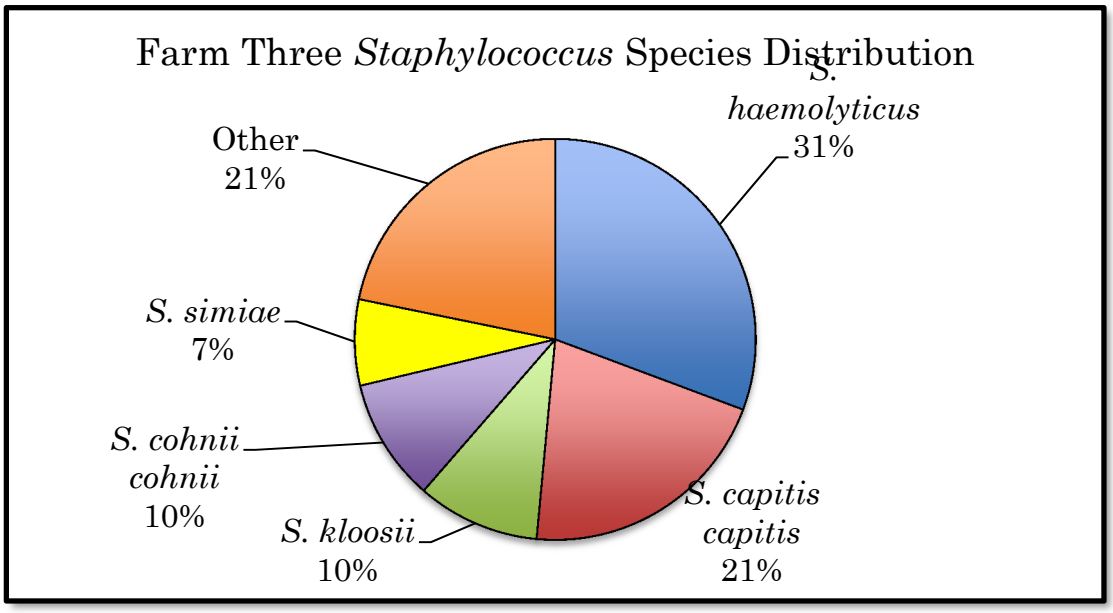

Figure 5. Equine bacteria were isolated from the ear, nostril, and girth. Farm Three had a high level of human contact. Eighteen species were isolated from Farm Three: *S. haemolyticus, S. capitis capitis, S. kloosii, S. cohnii cohnii,* and *S. simiae.* Other species include: *S. warneri, S. carnosus, S. arlettae, S. condimenti, S. caprae, S. equorum equorum, S. gallinarum, S. homini novobiosepticus, S. muscae,* and *S. vitulinus,* and three other species that could not be identified definitively.

Twenty-one horses were sampled from Farm Three. Some species were present on more than one horse (Figure 6). *Staphylococcus haemolyticus* was isolated from twelve of the twenty-one total horses (57.1%); *Staphylococcus capitis capitis* was isolated from nine horses (42.9%); *Staphylococcus kloosii* and *Staphylococcus simiae* were each isolated from six horses (28.6%); *Staphylococcus cohnii cohnii* was isolated from five horses (23.8%)*;* *Staphylococcus warneri* was isolated from three horses (14.3%)*;* and

Staphylococcus carnosus and *Staphylococcus condimenti* were each isolated from two horses (9.5%). Other species were isolated from only one horse (from 4.8% of the population): *Staphylococcus arlettae, Staphylococcus caprae, Staphylococcus equorum equorum, Staphylococcus gallinarum, Staphylococcus hominis novobiosepticus, Staphylococcus muscae,* and *Staphylococcus vitulinus.*

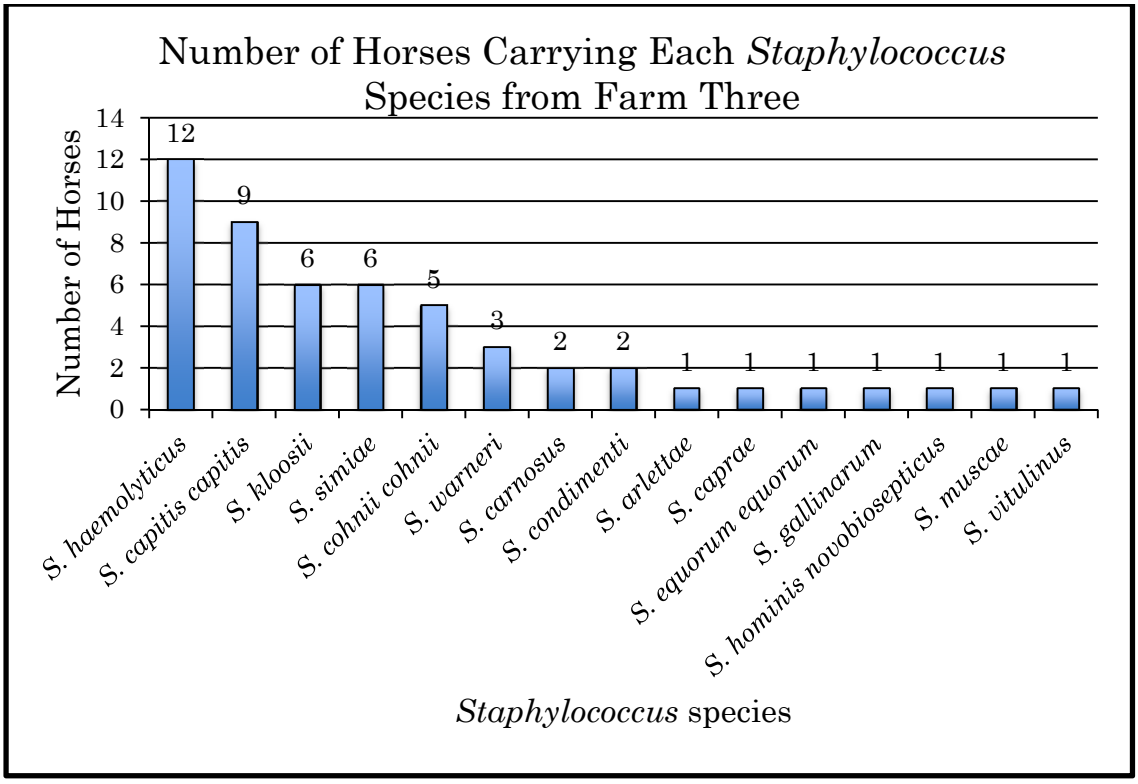

Figure 6. Isolates were analyzed as the number of horses carrying each species. Eighteen species were isolated from 21 horses at Farm Three: *S. haemolyticus*, *S. capitis capitis, S. kloosii, S. simiae, S. cohnii cohnii, S. warneri, S. carnosus,* and *S. condimenti.* Other species include: *S. arlettae, S. caprae, S. equorum equorum, S. gallinarum, S. hominis novobiosepticus, S. muscae,* and *S. vitulinus.* Four species could not be definitively identified.

Many species were isolated from more than one farm site. Twenty-five different species were isolated between all three farms. Three species were isolated only from Farm One: *Staphylococcus xylosus, Staphylococcus capitis ureo,* and *Staphylococcus cohnii urea.* Similarly, two species were isolated only from Farm Two: *Staphylococcus lentus* and *Staphylococcus microti.* Eleven species were isolated only on Farm Three: *Staphylococcus warneri*, *Staphylococcus arlettae, Staphylococcus caprae, Staphylococcus equorum equorum, Staphylococcus homini novobiosepticus, Staphylococcus muscae, Staphylococcus vitulinus,* and four species that could not be definitively identified.

One species was isolated from only Farms One and Two: *Staphylococcus sciuri.* There were no common isolates between only Farms One and Three. Six species were isolated only from Farms Two and Three: *Staphylococcus carnosus, Staphylococcus gallinarum, Staphylococcus kloosii, Staphylococcus capitis capitis, Staphylococcus simiae,* and *Staphylococcus haemolyticus.* One species was isolated from Farms One, Two, and Three: *Staphylococcus cohnii cohnii.*

Sixty-five horses were sampled in this study. For a general distribution of *Staphylococcus* species in the equine population of Central Texas, it is useful to analyze the total number of horses carrying each species across all three farms (Figure 7). *Staphylococcus cohnii cohnii* was found on twenty of the 65 horses sampled (present in 30.8% of horses in Central Texas);

Staphylococcus xylosus was found on seventeen horses (26.2%); *Staphylococcus haemolyticus* was isolated from thirteen horses (20.0%); *Staphylococcus capitis capitis* was isolated from eleven horses (16.9%); *Staphylococcus sciuri* was isolated from nine horses (13.8%); and *Staphylococcus simiae* and *Staphylococcus kloosii* were each isolated from eight different horses (12.3%, respectively). All other species were isolated fewer than five times (present on less than 8% of Central Texas equine).

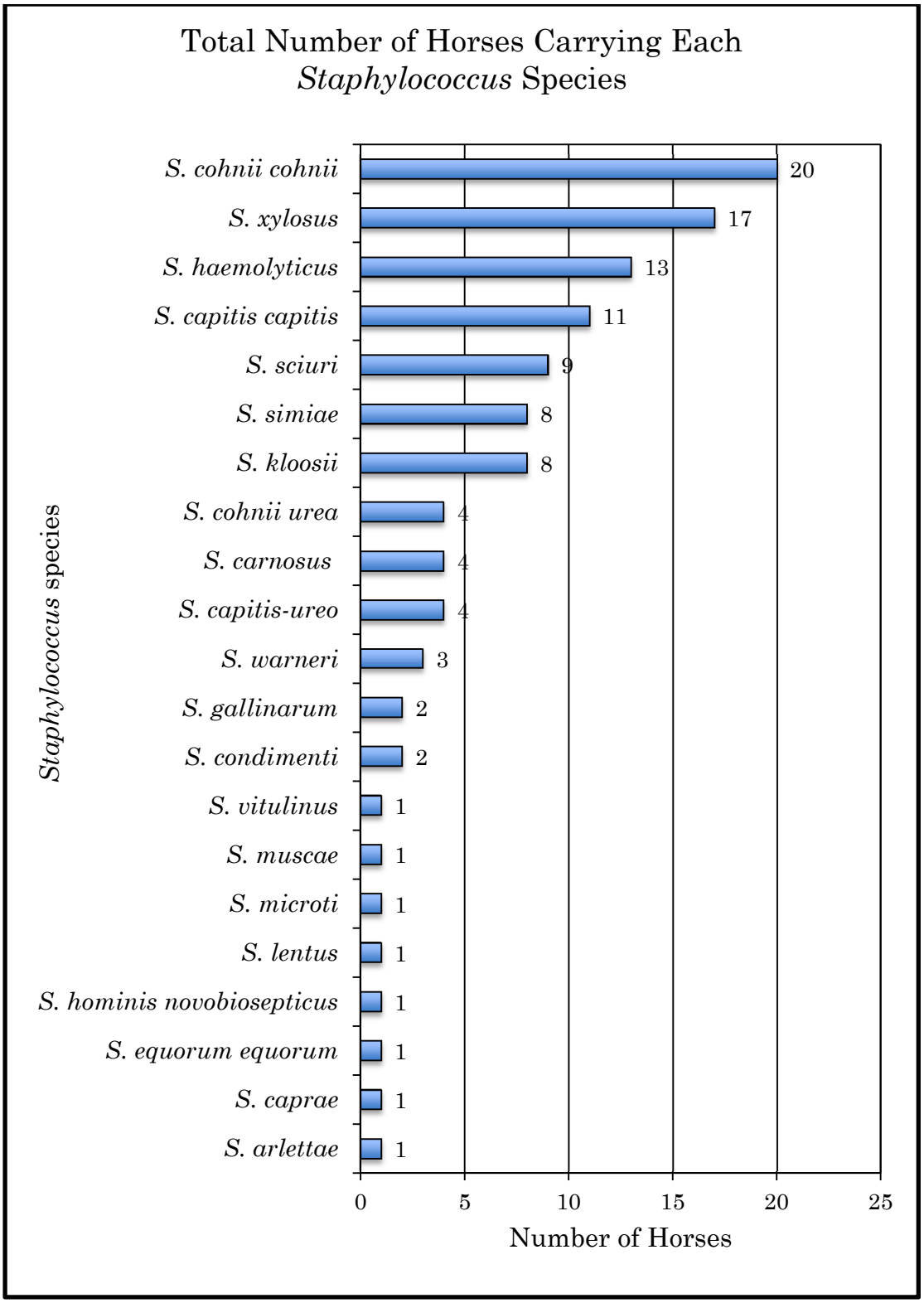

Figure 7. This study examined the distribution of *Staphylococcus* species in the equine population of Central Texas by analyzing the total number of horses from all three farms carrying each identified species.

Microbial Resistance to Antibiotics

Microbial resistance to six antibiotics was tested: oxacillin, penicillin, trimethoprim-sulfmethoxazole, ciprofloxacin, clindamycin, and gentamicin. All strains were sensitive to clindamycin and gentamicin. One isolate showed resistance to ciprofloxacin. Resistance to penicillin, oxacillin, and trimethoprim-sulfmethoxazole was observed in four common patterns (Figure 8). Strains were either (1) sensitive to all six antibiotics, (2) resistant only to penicillin, (3) resistant to penicillin and oxacillin, or (4) resistant to penicillin and trimethoprim-sulfmethoxazole.

Forty-six out of the 62 strains (74.19%) of Farm One were sensitive to all six antibiotics (Figure 8). Five isolates (8.06%) were resistant only to penicillin, and eleven isolates (17.74%) showed resistance to both penicillin and oxacillin. All 21 isolated strains from Farm Two were susceptible to all six tested antibiotics. On Farm Three, only eight of the 101 isolates (7.92%) were sensitive to all tested antibiotics. 28 isolates (27.72%) were resistant only to penicillin and 55 isolates were resistant to both penicillin and oxacillin (54.46%). Seven isolates were resistant to penicillin and trimethoprim-sulfmethoxazole (6.93%). One isolate was resistant solely to trimethoprim-sulfmethoxazole (0.99%), one isolate was resistant to penicillin, oxacillin, and trimethoprim-sulfmethoxazole (0.99%), and one isolate was resistant to penicillin, oxacillin, and ciprofloxacin (0.99%).

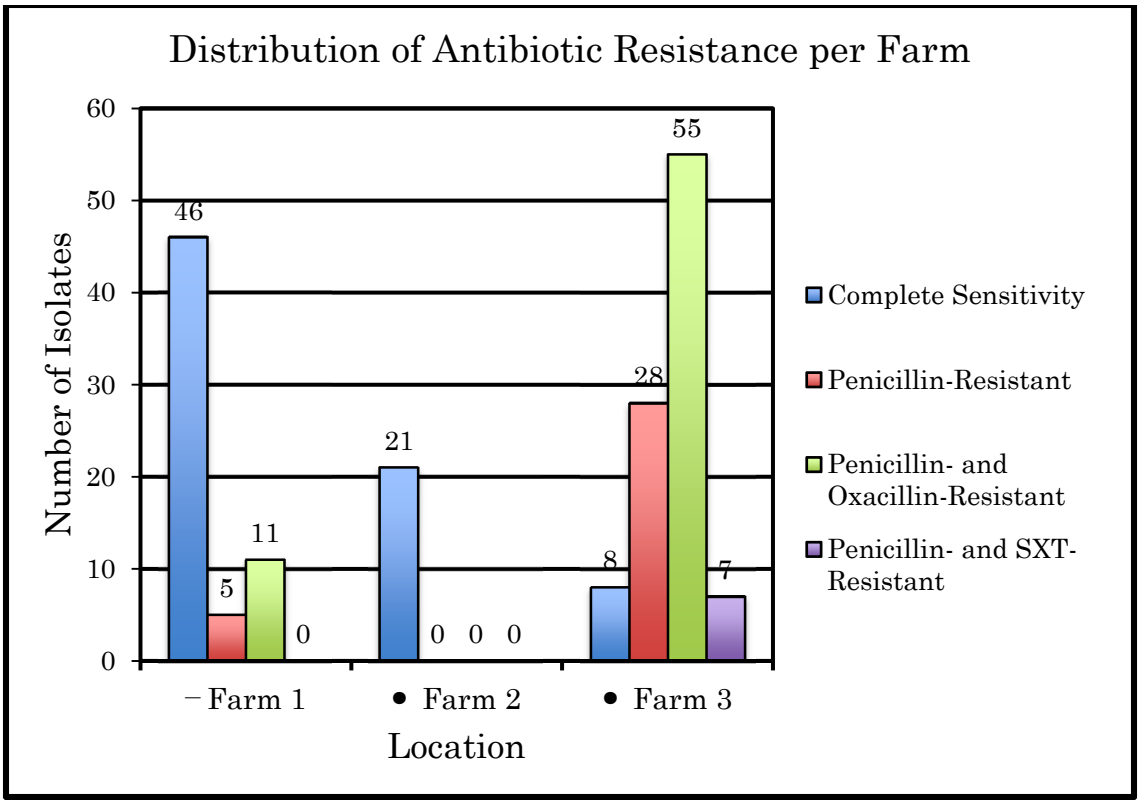

Figure 8. Resistance to antibiotics was measured by the number of isolates within each resistance pattern from three separate farms. Most isolates from Farm One were sensitive to all tested antibiotics. Some isolates showed resistance to oxacillin and penicillin. No trimethoprim-sulfmethoxazole-resistant bacteria or ciprofloxacin-resistant bacteria were isolated. From Farm Two, isolates showed complete sensitivity. From Farm Three, most isolates showed resistance to both penicillin and oxacillin. Complete sensitivity, resistance to trimethoprim-sulfmethoxazole, and resistance to ciprofloxacin were also observed.

Many species naturally possess resistance to various antibiotics. Twenty-one different species were identified between Farms One, Two, and Three. It is likely that among these species, some are naturally predisposed to antibiotic resistance. If the species are commonly resistant, it cannot be assumed that antibiotic resistance is due to high levels of human contact;

therefore, this study is additionally examining the degree of antibiotic resistance per species isolated (Figure 9).

None of the various species had at least one isolate in every observed pattern of resistance. Two species had isolates in at least four commonly observed resistance patterns. Of 24 *Staphylococcus capitis capitis* isolates, three were completely sensitive, four were penicillin-resistant, twelve were resistant to penicillin and oxacillin, and three were resistant to penicillin and trimethoprim-sulfmethoxazole. Additionally, one *Staphylococcus capitis capitis* isolate was resistant to penicillin, oxacillin, and trimethoprim-sulfmethoxazole, and another was resistant to penicillin, oxacillin, and ciprofloxacin. Of 33 *Staphylococcus haemolyticus* isolates, five were completely sensitive, five were resistant to only penicillin, twenty-one were resistant to penicillin and oxacillin, and two isolates were resistant to penicillin and trimethoprim-sulfmethoxazole.

Four identified species had at least one isolate that followed three of the seven total resistance patterns. *Staphylococcus cohnii cohnii* was isolated 29 times; four isolates were completely sensitive, thirteen were resistant to only penicillin, and twelve were resistant to penicillin and oxacillin. *Staphylococcus kloosii* was isolated fourteen times; four isolates were completely sensitive, four were resistant to only penicillin, and six were resistant to penicillin and oxacillin. Of nine isolates of *Staphylococcus simiae,* four were completely susceptible while one was resistant only to penicillin

and four were resistant to both penicillin and oxacillin. *Staphylococcus carnosus* was isolated only five times; three isolates were completely sensitive to antibiotics, one was resistant to only penicillin, and one was resistant to penicillin and trimethoprim-sulfmethoxazole.

Four species had isolates that followed at least two different resistance patterns. *Staphylococcus xylosus* was isolated 30 times; 29 isolates were completely susceptible to antibiotics and one isolate was resistant only to penicillin. *Staphylococcus warneri* was isolated three times; one isolate was resistant to only penicillin and two isolates were resistant to both penicillin and oxacillin. *Staphylococcus gallinarum* was isolated only twice; one isolate was completely sensitive, and the other was resistant only to penicillin. *Staphylococcus condimenti* was also isolated twice; one isolate was completely sensitive while the other was resistant to both penicillin and oxacillin.

Isolates of the remaining fourteen species followed only one resistance pattern. All nine isolates of *Staphylococcus sciuri* were sensitive to all antibiotics, as were the four total isolates of *Staphylococcus cohnii urea* and four total isolates of *Staphylococcus capitis ureo.* Unfortunately, the remaining eleven species were only isolated once, thus making their resistance pattern grouping less significant. Isolated species that were completely sensitive to the tested antibiotics include *Staphylococcus vitulinus, Staphylococcus microti, and Staphylococcus lentus. Staphylococcus equorum equorum* was the only isolated penicillin-resistant species, and

Staphylococcus arlettae, Staphylococcus muscae, Staphylococcus caprae, and *Staphylococcus homini novobiosepticus* were resistant to both penicillin and oxacillin.

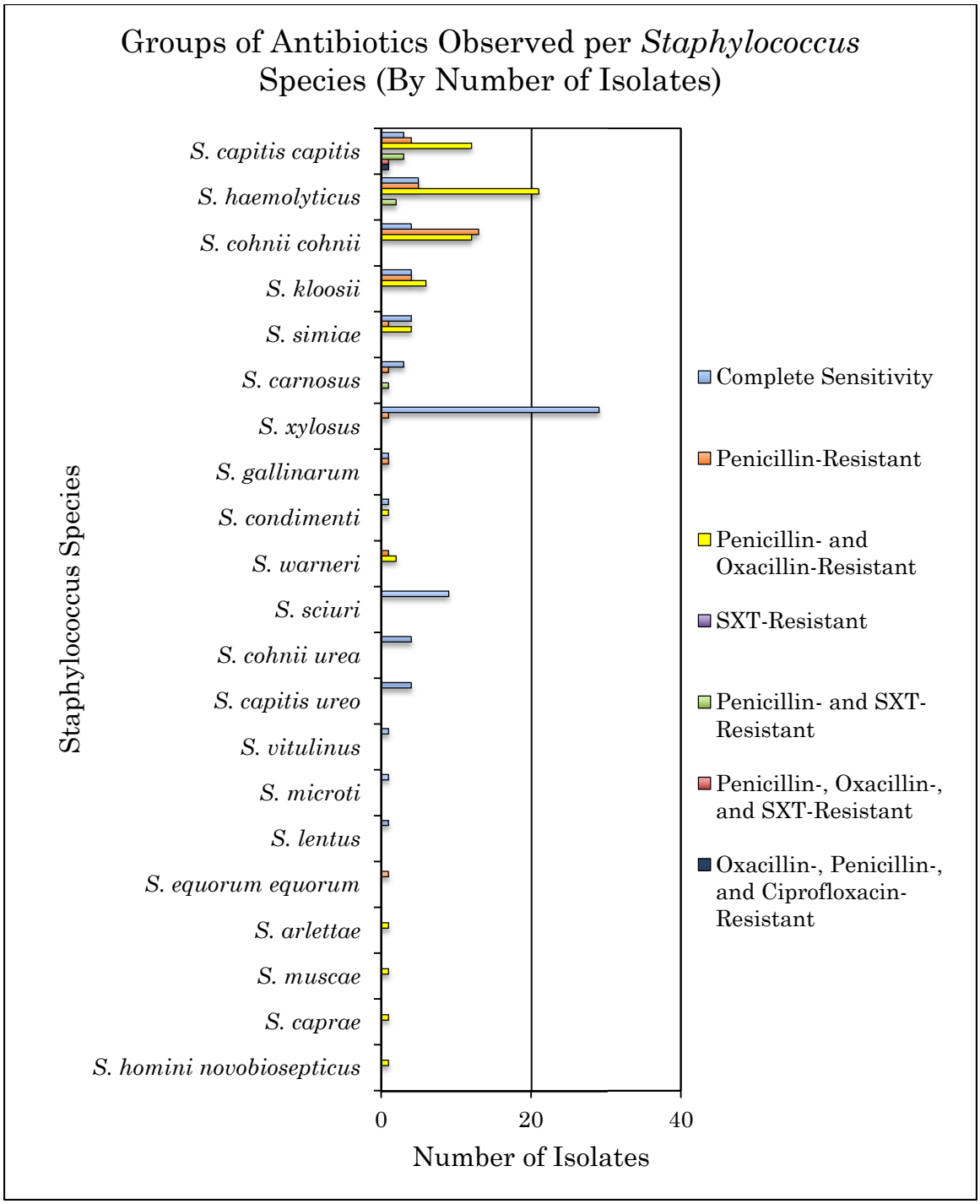

Figure 9. Twenty-one species were identified, and seven total patterns of antibiotic resistance were observed. One species had at least one isolate in six of the seven resistance patterns, and one species had at least one isolate in four of the resistance patterns. Five species had isolates that followed only three of the patterns; four species had isolates that followed only two of the patterns; isolates from eleven identified species followed only one pattern of resistance.

CHAPTER FOUR

Discussion and Conclusions

The primary purpose of this study was to identify the different *Staphylococcus* flora of the equine of the Central Texas region. Significantly fewer species were found on Farm One than on Farms Two and Three. This is most likely because Farm One is a closed herd, with established contact between small mammals and a limited number of humans. On the contrary, Farm One had fewer species than Farm Two despite having more isolates. *Staphylococcus xylosus* (48%)*, Staphylococcus cohnii cohnii* (26%)*,* and *Staphylococcus sciuri* (13%) are the primary species isolated from Farm One. These three species alone comprise 87 percent of the isolated population.

In contrast, *Staphylococcus kloosii* (19%)*, Staphylococcus capitis capitis* (14%)*, Staphylococcus carnosus* (14%)*, Staphylococcus cohnii cohnii* (14%)*, and Staphylococcus sciuri* (10%) are the five primary species isolated from Farm Two and they contribute to 71 percent of the isolates. *Staphylococcus kloosii*, the most isolates species from Farm Two, contributed only to nineteen percent of the population, unlike *Staphylococcus xylosus,* which contributed to nearly half of the isolated population of Farm One (48%). *Staphylococcus sciuri* was only isolated from Farms One and Two and is probably indicative of exposure to small mammals in a pastoral setting.

Farm Two is a known open herd, with a variety of microbial sources, such as new horses and humans, being introduced to the community.

Eighty percent of Farm Three's isolated population consists of five primary species, including *Staphylococcus haemolyticus* (32%), *Staphylococcus capitis capitis* (21%), *Staphylococcus kloosii* (10%), *Staphylococcus cohnii cohnii* (10%), and *Staphylococcus simiae* (7%). Farm Three had close contact between the stalls, and close contact between humans. Many bacterial sources were constant in the community while many bacterial sources were introduced into the community from a variety of other locations, increasing the range of potential transfer from multiple other humans and horses not present in this community. Contact between horses of neighboring stalls could facilitate the rapid spread of a bacterium throughout the community.

The spread of bacterial strains is easily detected when analyzing the number of horses that colonize each particular species. The three species isolated from the most equine are *Staphylococcus cohnii cohnii* (twenty horses), *Staphylococcus xylosus* (seventeen horses), and *Staphylococcus haemolyticus* (thirteen horses). *Staphylococcus capitis capitis* was isolated from eleven horses, and all other species were isolated from fewer than ten hosts, with eight species isolated from only one host.

Staphylococcus cohnii cohnii is the only species that was isolated from all three farms. It was isolated from twenty horses overall and is clearly

present within the Central Texas region. *Staphylococcus cohnii cohnii* is most likely a commensal of horses. As a coagulase-negative *Staphylococcus* (CoNS) organism, an infection typically would occur only if the hosts were immunocompromised. Understanding a microbe's distribution across a population and its prevalence in a habitat is valuable when evaluating the impact of that microbe in the community.

Staphylococcus xylosus is the most prevalent species isolated from Farm One, and it was not found in either of the other farms. Despite its absence on Farms Two and Three, it was still isolated more times than all other species, excluding *Staphylococcus cohnii cohnii*. It is expected to find *Staphylococcus xylosus* among equine because *Staphylococcus xylosus* is a commonly isolated skin commensal of equine, as well as humans and other mammals, and even birds (11). *Staphylococcus xylosus* is a common inhabitant of pastures; it is surprising then that *Staphylococcus xylosus* was not isolated from either Farms Two or Three. Because of its typically ubiquitous nature in humans and other mammals, the isolation of *Staphylococcus xylosus* is not evidence of human contact. It could, however, be indicative of a less confined, more pastoral habitat due to its particular niche in soils in biofilm formation (11, 30), therefore suggesting that the tight confinement of stables inhibits the proliferation of *Staphylococcus xylosus*.

Staphylococcus haemolyticus was isolated twice from Farm Two and 31 times from Farm Three, making it the most frequently isolated species of

Farm Three. This organism has likely been introduced from an outside source and has adapted to the equine hosts at Farm Three, spreading to nearly 60 percent of the horses in this community. Methicillin resistance is characteristic among *Staphylococcus haemolyticus* organisms. In one study, 87 percent of *Staphylococcus haemolyticus* implicated in various infections were methicillin-resistant, with 75 percent of isolates being multiresistant and 55 percent possessing the mecA gene typically associated with methicillin resistance (2). In this study, 28 of 33 isolates (84.8%) showed resistance to penicillin, and 21 of the 33 isolates (63.64%) additionally showed resistance to oxacillin, confirming that methicillin resistance is a likely common characteristic of *Staphylococcus haemolyticus*.

A secondary focus of this study was to identify potential methicillin-resistant *Staphylococcus aureus* (MRSA) in the equine population of Central Texas. Most studies involving MRSA, especially in veterinary medicine, are published in response to an outbreak, or sudden increase in cases in some population of close confinement, such as a hospital. No MRSA strains were isolated in this study. The results of this study are useful because, at this time, no published MRSA surveys have been conducted that were not provoked by an outbreak, much less any that specifically involve the specific population of Central Texas equine. This study would be useful in comparison with other similar populations worldwide. Because MRSA is typically a human pathogen, its presence in equine populations would be unexpected,

unless MRSA bacteria were asymptomatically carried on a horse after zoonotic transfer from an infected human.

Finally, this study sought to analyze the degree of microbial resistance to antibiotics when the level of human contact varied between the communities of the equine population of Central Texas. Antibiotic resistance was tested for oxacillin, penicillin, trimethoprim-sulfmethoxazole, ciprofloxacin, clindamycin, and gentamicin. These antibiotics were tested because of their frequent use in combatting *Staphylococcus*-associated infections in equine. Susceptibility to clindamycin and gentamicin was invariably universal among isolates from all three farms. These drugs would, therefore, be beneficial in controlling and treating staphylococcal infections. Complete susceptibility to all six tested antibiotics was observed in 75 of 184 isolates (40.76%). Many isolates, however, showed resistance to antibiotics, namely penicillin and oxacillin. Overall, 108 of 184 isolates (58.70%) were sensitive to penicillin, and 68 isolates (36.96%) were resistant to oxacillin and to penicillin. Nine isolates showed resistance to trimethoprim-sulfmethoxazole-- one isolate (0.54%) was resistant to only trimethoprim-sulfmethoxazole, seven isolates (3.80%) were resistant to trimethoprim-sulfmethoxazole and penicillin, and one isolate (0.54%) was resistant to penicillin, oxacillin, and trimethoprim-sulfmethoxazole. Finally, one isolate (0.54%) was resistant to penicillin, oxacillin, and ciprofloxacin.

Another similar study shows that nearly 80 percent of coagulase-negative *Staphylococcus* organisms showed resistance to oxacillin in hospitals across the United States, Canada, Latin America, Europe, and the Western Pacific Region (10). It is expected that a nonhuman microbial population would be more susceptible to antibiotics than a human population because these populations have less exposure to the drugs. As speculated, 36.96 percent of equine isolates in this study (as opposed to 80 percent of isolates in the international study) were resistant to oxacillin. In all, nearly two-thirds of equine isolates (59.24%) showed some degree of resistance to antibiotics.

An hypothesis of this study was that an increase in human contact, through grooming and riding, would lead to an increase in antibiotic resistance. A stark difference was apparent between Farms One and Three. On Farm One, over two-thirds of isolates (74.19%) demonstrated complete sensitivity to all tested antibiotics. On Farm Three, only a little over seven percent of isolates showed complete sensitivity, meaning that over 90 percent of isolates showed some degree of resistance to antibiotics. This drastic increase in resistance from Farm One to Farm Three could be directly due to human contact or indirectly, through close confinement within a stable and "sharing" of microbial resistance genes. Farm Two did not fit this trend, in part because it was an open herd with new horses and their "history" of microbial variations.

An additional study interest was added to ensure that microbial resistance or susceptibility was not characteristic of a given species. The only species in this study that was isolated a significant number of times and had 100 percent identical antibiotic profiles among its isolates is *Staphylococcus sciuri*, which was isolated from both Farms Two and Three. All nine strains of *Staphylococcus sciuri* were completely susceptible to all tested antibiotics. Other studies' data contradict this, however. A study similar to this one, which surveyed nasal isolates from 42 healthy horses from four farms, identified 100 percent of their *Staphylococcus sciuri* isolates as methicillin-resistant (23). A comparison of the studies implies that *Staphylococcus sciuri* can be either resistant or susceptible to penicillin drugs. The resistance could be the result of degree of human contact; or, the resistance could be simply due to location—the methicillin-resistant *Staphylococcus sciuri* results were studied in Poland.

Future Study Considerations

Many schematics are available to identify species, and also to determine antibiotic susceptibility. Using a more formal schematic would be beneficial, especially in reducing the potential for MRSA identification earlier on in the study. Specifically, a phylogenetic schematic would be useful to determine what test results are expected for a given species, and which results are common between species due to their evolutionary ancestry. A

schematic of this type would also potentially explain how species have developed their resistance and the mechanisms used to resist the antibiotics, like MRSA's production of beta-lactamase, which breaks down the beta-lactam ring in methicillin.

The Advanced Biochemical Identification System (ABIS) could not definitively identify some isolates based on biochemical test results used for characterization. Limiting ABIS identifications by adding more test results is possible, but no tests significantly changed the outcome identification provided by the ABIS algorithm.

In Farm Two, significantly fewer isolates were observed. The balanced distribution of the five most prevalent species across the equine population could easily be affected by the addition of one new isolate. Considering the most isolated species from Farm Two was isolated merely four times, the data from Farm Two as percentages is not as reliable as desired. Despite this, the plain identification of Farm Two isolates is data that will not change, regardless of an increased number of isolates.

Because a full survey was not completed of the microbial profile of each horse, it would not be accurate for this study to analyze the species' frequencies of growth between the ear, nose, and girth. Ideally, a study would isolate every skin-dwelling microbe to get a full profile per horse, and profiling the total equine population. Accepting more than two isolates from each swabbed site would increase either the number of different species or

would increase the frequency of an already-isolated species. Given that some bacteria were isolated only once, any additional isolates of that species would clearly and dramatically improve the frequency of that species.

It is not possible for this study to determine definitively why some *Staphylococcus* organisms are more commonly isolated or more resistant to antibiotics. The closed herd of Farm One with an established equine population, consistent small mammal exposure, and limited human contact has a balance of commensal organisms limited to five prevalent staphylococcal species. Farm Two is an open herd in that horses are added periodically and bring their own microbial mix to the group. In addition, a variety of different humans have periodic contact with the horses and horses may be mixed in various paddocks or pastures. Microbial populations from Farm Two blend *Staphylococcus* species associated with pastoral settings and human contact. Farm Three has a relatively constant number of stabled horses. Human "contact" includes Waco riders, riders from other cities and states, and the organisms that riders may carry from contact with horses from their "home" area. Findings suggest that the variety of organisms and antibiotic resistance is correlated with movement of horses (within a herd or between herds), presence of small mammals, and human contact.

Oxacillin is a useful replacement drug when testing for methicillin resistance. Unfortunately, however, many studies have proven that oxacillin resistance does not necessarily imply methicillin resistance, so oxacillin is not

the end-all when antibiotic resistance is concerned. Many studies recommend testing both oxacillin and cefoxitin susceptibility when evaluating methicillin resistance. There is often discrepancy between the two results, but if the strain shows resistance to either antibiotic, resistance to methicillin is presumed (20). While the mecA gene is the most frequent association with methicillin resistance, its absence does not necessarily indicate methicillin susceptibility (2). Genetic testing is a common method for determining susceptibility because of the mecA gene, which codes for beta-lactamase, the antibiotic-resistance mechanism of *Staphylococcus* organisms. While using the Kirby-Bauer method can still determine antibiotic susceptibility in most organisms, some organisms cannot be properly characterized using this method. For this reason, genetic testing through Polymerase Chain Reaction would be beneficial in addition to the Kirby-Bauer method.

Bacteria are undeniably ubiquitous organisms, whether in human populations, in horse populations, or otherwise. The proliferation of bacterial species is heightened in habitats of close confinement, and the degree of antibiotic resistance is significantly increased in nonhuman populations when levels of interaction and contact with humans increase, as demonstrated in this study. The identification of these correlations and the survey of microbial organisms and their potential resistance to antibiotics gives rise to a potential for further equine population studies of the distribution and resistance to antibiotics of *Staphylococcus* organisms.

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