Identification of bacterial species in mastitic cattle using the 16S rRNA gene

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Allegheny College
Department of Biology

Identification of bacterial species in mastitic cattle via PCR amplification of a 600 base pair region of the 16S rRNA gene

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

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Pledge

Approval by the Senior Thesis Committee:

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Abstract

Mastitis is one of the most prominent diseases within the dairy industry today, worldwide it is estimated to cost the dairy industry $2 billion in losses annually. Mastitis is inflammation of the bovine mammary tissue caused by the infection of various pathogens; these infections cause numerous problems for the cow and the farmer. Many methods of treatment exist for mastitis with antibiotic therapies being the most common method of treatment, but because antibiotics have been used so heavily many bacterial strains are currently demonstrating resistance. To avoid unnecessary use of antibiotics proper identification of the causative agent is crucial. While microbiological culture is the traditional diagnostic method PCR amplification has shown identify causative agents more accurately. The purpose of this study was to identify mastitis pathogens via PCR amplification of the 16S rRNA gene in raw milk samples of mastitic cattle from one farm in Western Pennsylvania. Of the collected samples one included 11 colonies that were successfully sequenced after PCR amplification. Multiple *Staphylococcus* species were identified as well as *Bacillus, Psychrobacter, Corynebacterium,* and *Brevibacterium*. Thus even though not all samples were successfully sequenced PCR was able to identify multiple species in just one sample that culture methods would be likely to miss. These results suggest that PCR amplification could be a more complete and accurate tool in mastitis diagnoses.

Introduction

Mastitis has been a prevalent disease in cattle since the 1940's. Today this disease is one of the biggest challenges the dairy business faces. In the United States, and worldwide, mastitis causes such a significant economic loss that it is the most costly disease in the dairy industry. Mastitis has been

reported to cost up to $200 per cow per year and worldwide it has been estimated to cause annual losses of $2 billion (Halasa et al., 2007). However, concerns stemming from this disease are not only economic. In regards to the health of the animal, mastitis not only causes a decrease in milk production and quality, but also can ultimately mean the loss of the animal. Humans later consuming infected milk are ingesting milk of poor quality and possibly antibiotic residues from treatment. Many treatments and management programs exist for mastitis but this complex disease still remains one of the most problematic for farmers, veterinarians, and researchers all over the world (Bradley, 2002).

Mastitis is inflammation of the bovine mammary tissue caused by pathogen infection. What makes the disease so complex is the diversity of the causative infectious agents. Bacteria, fungi, viruses, yeasts, and algae have all been found responsible for causing mastitis; in one study 137 different species of organisms were identified as causative agents (Watts, 1988). Another complicating factor is that mastitis can be either subclinical or clinical, in which clinical cases demonstrate symptoms and subclinical cases are asymptomatic. It is estimated that 90-95% of all cases are subclinical while the remaining 5-10% are clinical (Bradley 2002). This is a great issue because a majority of the subclinical cases go without treatment and therefore usually endure longer and end up causing more damage.

Bacterial pathogens causing mastitis can be divided into two groups: contagious and environmental. Contagious pathogens are those that can be spread from cow to cow. Most often these are spread through milking machines and related procedures. Some of the most common contagious bacterial agents include *Streptococcus agalactiae*, *Staphylococcus aureus*, and various mycomplasmas. Environmental pathogens are those obtained from growth locations in the environment; these can be found all over the farm but are most commonly found in the cow bedding. Choice of bedding varies from farm to farm. Common bedding used includes sawdust, wood shavings, sand, straw, and shredded paper. Each material boasts different properties and so creates different environments to host different

bacteria in. This is one reason why the diversity of all infectious pathogens is so great. Common environmental agents include *Streptococcus uberis, S. dysgalactiae,* coagulase-negative *Staphylococci,* and coliforms (*Escherichia coli, Klebsiella pnuemoniae*) (Paulrud, 2005).

When foreign bacteria enter the teat and eventually the mammary gland of the cow an inflammatory response is initiated, thus causing the inflammation of the udder (Harmon, 1994). To prevent foreign substances from entering the mammary gland the cow's first line of defense is a smooth muscle sphincter (Figure 1.). Cells lining the canal between the sphincter muscles produce keratin, which possesses bacteriostatic properties. Trauma to the teat end can result from many things including anything during the milking procedure such as incorrect use of udder washes and cleaning compounds, poor condition or misuse of milking equipment, and over milking, to any sort of damage accrued from the environment; thus the cows first line of defense being damaged renders the udder much more vulnerable to invasion of foreign substances (Jones, 1984). If bacteria are able to cross into the teat sinus they are now able to multiply and spread throughout the lumen of the mammary glands and eventually can enter the blood stream. Now that the infectious agents are inside the mammary tissue the second line of defense, the inflammatory response, is triggered. In response to the presence and multiplication of the foreign bacteria and any toxins they secrete polymorphonuclear leukocytes, other phagocytes, and immune system agents flood to the site of infection (Harmon, 1994). Not only can the bacteria and their

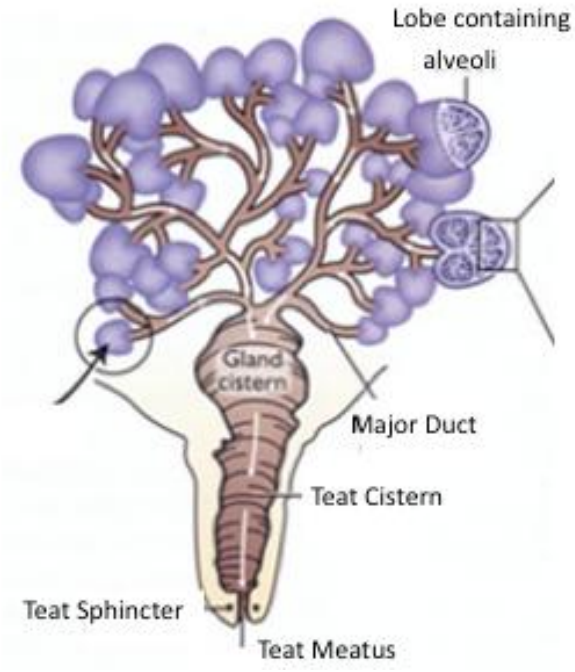

Figure 1. Basic anatomy of the bovine teat and mammary tissue (Digitális Tankonyvtár, 2011).

secretions cause damage but the increased volume of somatic cells in the lumen of alveoli can damage secretory cells as well, sometimes resulting in permanent damage (Jones, 1984).

Many treatments exist for mastitis. Contagious mastitis has been controlled well with good management practices, such as clean and careful milking procedures, whereas environmental mastitis is much harder to control from a management standpoint (Bradley, 2002). If the infection is caught early much of the infection can be removed from the infected quarter of the utter by stripping (emptying of milk) as often as every two hours. Oxytocin and aspirin are common drugs used during this treatment to help empty the utter (Paulrud, 2005). Beside oxytocin and aspirin, antibiotics are the next line of treatment. Rigorous programs of antibiotic treatment are very often successful, but, one great problem with antibiotic treatments is that select infectious bacteria have become or are becoming resistant to these antibiotics (Getahun et al., 2008). Antibiotics are most effective against contagious pathogens while there has been a lot of recent resistance developed by environmental pathogens such as coagulase-negative *Staphylococci*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, and *E.coli* (Getahun et al. 2008, Gill et al., 2006, Tanji et al. 2008).

Antibiotic resistance in bovine mastitis is becoming a major problem all over the world. Myllys et al. (1998) assessed the increase of antibiotic resistance in Finland over a seven year period. The proportion of *S. aureus* strains resistant to at least one antibacterial drug increased from 36.9% in 1988 to 63.6% in 1995. During those same years resistance of coagulase-negative Staphylococci to at least one antibacterial increased from 26.6% to 49.7%. Three years later another study in Finland found that *S. aureus* isolated from 52.1% of mastitic samples and coagulase-negative Stapylococci isolated from 32% of samples were resistant to the most commonly used antibiotic for mastitis: penicillin (Pitkala et al., 2004). More recently in 2007 resistance patterns were assessed in Selalle, Ethiopia. Isolates of *S. aureus* exhibited moderate to extremely high resistance to four commonly used antibiotics: penicillin

(45.3%), ampicillin (53.4%), streptomycin (63.4%), and polymixin B (97.7%). Not only did *S. aureus* isolates display high levels of resistance to commonly used antibiotics but *S. epidermidis* did as well: ampicillin (70.8%), oxytetracycline (83.3%), and polymixin B (95.2%). *Enterococci, S. agalactiae,* and *S. uberis* also exhibited moderate to very high resistance rates in these studies (Pitkala et al., 2004, Getahun et al., 2007). With resistance rates to commonly used antibiotics in mastitis treatment rising the importance of accurate identification of the infectious agent is crucial to proper treatment and avoiding use of unnecessary antibiotics.

Traditional diagnostic methods include Somatic Cell Counts (SCC), the California Mastitis Test, and microbiological cultures. An SCC is used to predict an intramammary infection (IMI); greater than 200,000 cells per milliliter is indicative of IMI (Harmon, 2001). SCC requires the sample to be sent to a laboratory, but to avoid any wait period the California Mastitis Test can be used cow-side to detect infection via the degree of detergent and somatic cell interaction(that will form a visible gel) and a pH indicator, although this method does not give an actual SCC (Schalm & Noorlander, 1957). To determine what causative agents are present traditional microbiological methods can be easily followed in a veterinary clinic according to the National Mastitis Council procedures (Farnsworth, 1993, Houghtby et al., 1993, Painter & Schnepper, 1965**)**. The most traditional culture method uses a quad agar plate that includes one quadrant of a rich media (usually a blood agar) as a positive control, a *Staphylococcus* selective media, a *Streptococcus* selective media, and a Coliform selective media. Diagnoses are made by growth on one of the selective quadrants with concurrent growth on the positive control quadrant (Houghtby et al., 1993). Although selective media is used to identify organisms at the genus and species level it has been found that this is not always a reliable diagnostic method.

In many cases it has been shown that microbiological methods can yield false-negative and incorrect diagnoses; it is estimated that up to half of milk samples submitted for culturing fail to be

diagnosed correctly (Bexiga 2010). In a study done by Bexiga et al. (2010) mastitis pathogens were identified via PCR in 47% of no growth, or culture negative, samples. The most common species identified in the culture negative samples were *E. coli, S. uberis, Arcanobacterium pyogenes,* and *S. dysgalactiae*; many of these which traditional culture methods have the ability to identify. In a more recent study by Keane et al. (2013) 30% of cultured samples did not identify any pathogen. Of these culture negative samples 79% yielded identification of a mastitis pathogen via PCR.

The purpose of this study was to generate a list of bacterial species identified from mastitic cattle on one farm in western Pennsylvania via PCR amplification of a region of the 16S rRNA gene. The 16S rRNA gene was chosen in an effort to identify as many species as possible because of its variability among species but its conservation within species (Riffon et al., 2001). This study aimed to assess if the 16S rRNA gene is suitable for bacterial species identification in mastitic bovine milk. Furthermore the purpose is also to look at similarities and differences of bacterial species identified in each cow of the one farm. It is expected that a very broad variety of species will be identified and that a portion of the species identified will include species not commonly identified via traditional culturing techniques. If PCR is able to identify many pathogenic bacterial species, especially those not normally identified in traditional culturing methods, it supports the move from microbiological culturing as the standard diagnostic method to PCR amplification of the 16SrRNA gene as the new standard method, providing farmers with a more accurate diagnosis and the ability for veterinarians to administer proper treatments.

Materials and Methods

Subjects & Sample Collection

Raw milk samples from the ten cows with the highest somatic cell count in a heard of Swiss and Holstein cattle were obtained from Breeze Ridge Farms (Zelienople, PA) (Table 1). Somatic Cell Counts (SCCs) of 200,000 cell/ml or greater are considered intra-mammary infections (IMI) and thus the cow is considered mastitic. Cows differed in age, lactation number, and were either pregnant or not (Table 1.). Samples were collected by the Pennsylvania Dairy Heard Improvement Association (DHIA) directly from the teat of a nonspecific quarter of the utter in Everseal sterile10 cc flip top sample tubes. After samples were collected they were preserved by freezing at -20° C until they were picked up and transported to Allegheny College on dry ice, and finally stored at -20° C.

Table 1. Milk Samples from Breeze Ridge Farm and Somatic Cell Counts: Each cow was allotted a number for sample identification. Somatic Cell Counts are measured and reported to the farmer as a linear score, anything above two is considered an IMI. The SCC linear scores were converted into cells/ml according to Western Canadian Dairy Heard Improvement Services (Agromedia).

Cow	Sample Number	SCC Linear Score	SCC (cells/ml)	Lactation Number	Pregnant	Age
20307	1	6.9	1,493,000	2	N	3
20328	2	6.1	857,000	1	N	3
20186	3	6.0	800,000	4	N	6
Paula	4	6.0	800,000	2	Y	3
Gretta	5	5.8	696,000	6	N	7
Juliett	6	5.7	650,000	2	Y	3
108Alis	7	5.2	459,000	3	Y	4
Satin	8	5.1	400,000	4	Y	5
Dora	9	4.8	348,000	3	N	4
Fran	10	4.7	325,000	2	N	3

Raw Milk Culture

Raw milk samples were cultured on plates made with 5% sheep's blood agar media (EMD Biosciences, San Diego, CA), which is a rich media and will help to grow fastidious organisms. Each sample was vortexed and diluted to a 10^{-1} concentration in a 1.5 ml microcentrifuge tube using sterile

water. A volume of 0.1 ml of the 10^{-1} dilution was spread on one blood agar plate and then repeated on a second, separate plate. One spread plate was put in an incubator at 35°C with 5% CO_2 for 48 hours while the other spread plate was put in an incubator at 37° for 48 hours. After 48 hours samples were removed from the incubators and placed in a 4°C refrigerator for short-term storage. The number of colonies on each plate was counted. These totals were then used to calculate the number of colony forming units (CFUs) by multiplying the number of colonies counted by the dilution factor and then dividing by the volume plated. The number of CFUs was determined towards assessing if there was significant difference in the amount of growth attributable to the varying incubation conditions. To assess whether there was significantly more growth on plates incubated in a regular incubator compared to those same samples incubated in a CO_2 incubator a paired T-test was run via IBM SPSS 19 (http://www.01.ibm.com/software/analytics/spss). The amount of growth between the two groups was considered statistically significant if $p<0.05$.

DNA extraction

Colonies were selected for DNA extraction based on morphological differences. A diverse range of colony morphologies was selected in hopes of obtaining as many unique bacterial species as possible. Six different morphological factors were taken into consideration during colony selection according to the American Society for Microbiology Microbe Library: pigmentation, form, elevation, margin, texture, and opacity (Figure 2) (American Society for Microbiology, 2013). Each colony was picked from the agar using a sterile pipette tip and swished in 50 µl of Molecular Biology Grade water in a 0.2 ml PCR tube. The PCR tubes where then placed in the thermal cycler at 98° for 5 minutes to burst open the cells and release the DNA. Extracts were then stored at 4°C. Selected colonies will be referred to as "sample number.colony number". For example, the first colony selected from sample five was referred to as "5.1".

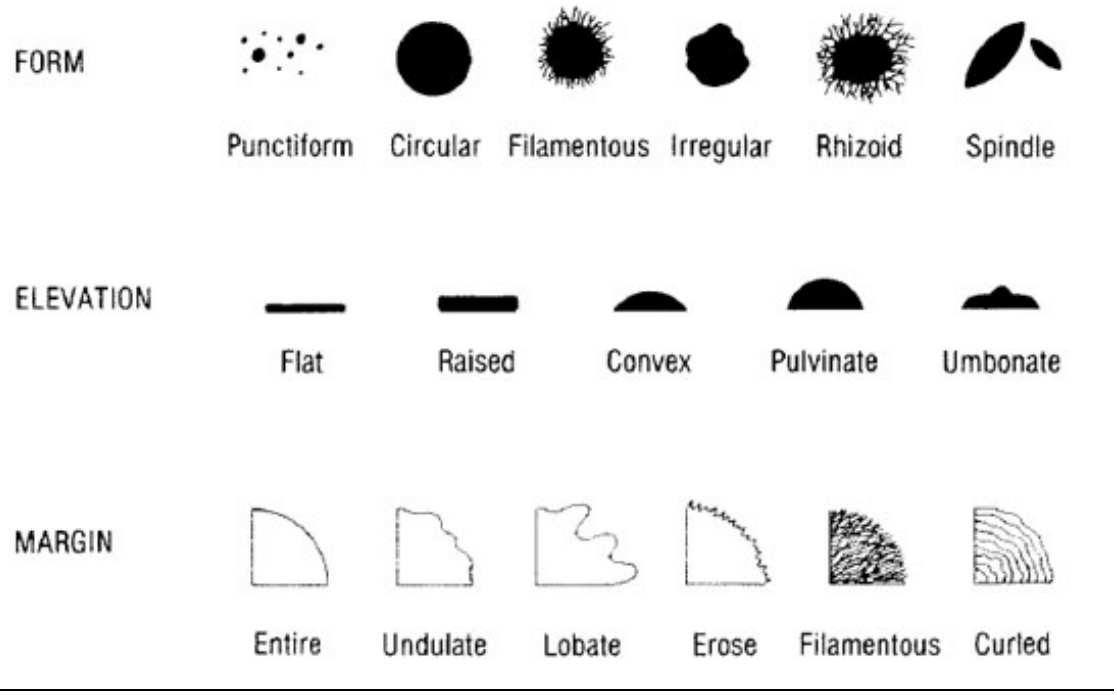

Figure 2. Morphological characteristics for colony selection: Pictured above are the morphologies used to identify form elevation, and margin. Pigmentation was either a uniform pigment or diffusing pigment. Texture was mucoid, rough, or smooth. Opacity was translucent or opaque (American Society for Microbiology, 2013).

PCR Amplification

A 600 base pair region of the 16s gene rRNA gene was amplified through Polymerase Chain Reaction (PCR) using universal primers. The previously designed universal forward primer used was 5'- GATTAGATACCCTGGTAGTCCAC -3' and the previously designed universal reverse primer used was 5'-CCCGGGAACGTATTCACCG-3' (Byrne et al. 2009). Primers were diluted from a stock concentration of 100 μM to 2 μM using Molecular Biology Grade water. Each PCR was set up in a 25 μl reaction that contained 2.5 μl of each primer (2uM), 5 μl of extracted DNA, 2.5 μl of DNAase/RNAase free water, and 12.5 μl of *Taq* 2X Master Mix that contained *Taq* DNA polymerase, dNTPs, $MgCl_2$, KCl and stabilizers (New England BioLabs, Ipswitch, Massachusetts). A negative

control was set up for each set of PCRs in which the 5 µl of extracted DNA was replaced with 5 µl of Molecular Biology Grade water. Thermal cycler conditions were set to: 95°C for 2 minutes for initial denaturing, 40 cycles of denaturing at 95°C for 15s, annealing at 50°C for 30s, and extension at 72°C for 30, followed by a final extension of 72°C for 5 minutes. Thermal cycler conditions were previously published by Bryne et al. 2009 and modified by lowering the annealing temperature (published at 58°C). The success of each PCR was determined using 1% agarose gel electrophoresis and a low mass ladder (Invitrogen, Carlsbad, California). PCR was deemed successful if a single, bright, 600 bp band was displayed on the gel. The concentration and size of the PCR products was determined via comparison to the low mass ladder.

DNA Sequencing

PCR products were purified and sent to MWG Eurofins Operon (Lousiville, Kentucky) for DNA sequencing. The amount of PCR product sent to Eurofins MWG Operon was calculated to meet their specified parameters (http://www.operon.com/services/dna-sequencing/sample-prep.aspx) using the previously determined size and concentration of the PCR products. To purify the product and remove any excess primers and unincorporated nucleotides 2 µl of ExoSAP-IT (Affymetrix, Santa Clara, California) was added per every 5 µl of PCR product being sent for DNA sequencing. After products were purified and prepared they were shipped overnight to MWG Eurofins Operon.

Editing of DNA Sequences and Identification of Bacterial Species

The DNA sequences were returned from MWG Eurofins Operon (Lousiville, Kentucky) as chromatograms and were viewed and edited using FinchTV software (http://www.geospiza.com/Products/finchtv.shtml). The sequences were then entered into the Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov) which returned the most similar bacterial sequences found from the nucleotide database Genbank. All sequences that were returned with

95% similarity or greater to the newly collected sequence were used to reconstruct a phylogenetic tree for identification of the bacterial species. Multiple Sequence Comparison by Log-Expectation (MUSCLE) was used to align the sequences and the program MEGA 5 (Tamura et al., 2011) was used to build the phylogenetic trees via Maximum Likelihood. Both Maximum Likelihood phylogenetic trees were built using the Kimura 2-parameter model via nearest neighbor with a bootstrap of 500. The Kimura-2-parameter model was chose because it utilizes the transitional and transversional substitution rates while also correcting for multiple hits and still assumes that each nucleotide frequency is the same and that rates of substitution do not vary among sites (Kimura et al., 1980). The phylogenetic trees were then analyzed to determine which bacterial species identifies most closely to the sample sequenced.

Results

Raw Milk Culture

All twenty plates exhibited successful growth with multiple colonies and very diverse morphologies. Although the amount of growth on each plate looked very similar at first glance after counting colonies and calculating colony forming units (CFUs), a paired samples T-test indicated that there was significantly more growth on the plates that were incubated in 35°C with 5% CO_2 compared to 37°C with 11.4% more growth on the plates incubated in 35°C with 5% CO_2 (T_{20}=4.173, P=0.002) (Table 2).

Table 2. Bacterial Growth: More growth was exhibited on plates incubated in CO_2 (T_{20}=4.173, P=0.002).

Plate	CFUs Regular Incubator	CFUs CO_2 Incubator
1	462000	483000
2	326000	334000
3	144000	79000
4	165000	185000
5	357000	386000
6	40000	60000
7	92000	106000
8	17000	25000
9	18000	64000
10	2000	26000

Although more CFUs were counted on plate three there was only growth on less than half of the plate because the sample was not spread across the whole plate by accident. The sample was not included in the T-test.

DNA Extraction and PCR Amplification

Nineteen colonies were selected for DNA extraction from two different plates that were both incubated in 35°C with 5% CO_2 for 48 hours (Table 3). Eleven colonies from plate 5 and eight colonies from plate 3 were selected and DNA was extracted (Table 3). Eleven of the nineteen colonies were successful in PCR amplification as they each exhibited one single bright band on the 1% agarose gel and were all around the 600 bp region of the low mass ladder.

Table 3. Colonies Selected for Extraction: Nineteen Colonies were selected from two different plates. Each plate was incubated in 35°C with 5% CO_2 for 48 hours . The nineteen colonies were selected because they exhibited the following diverse morphologies

Sample Number	Colony Number	pigmentation	form	elevation	margin	texture	opacity	PCR Success
5	1	diffusing	circular	umbonate	entire	mucoid	translucent	Y
	2	uniform	circular	convex	entire	mucoid	opaque	Y
	3	uniform	circular	raised	entire	smooth	opaque	Y
	4	diffusing	punctiform	convex	entire	mucoid	translucent	Y
	5	diffusing	circular	convex	undulate	mucoid	translucent	Y
	6	diffusing	irregular	umbonate	lobate	smooth	opaque	Y
	7	uniform	irregular	pulvinate	entire	mucoid	translucent	Y
	8	uniform	circular	pulvinate	entire	mucoid	opaque	Y
	9	diffusing	circular	pulvinate	entire	mucoid	translucent	Y
	10	uniform	punctiform	convex	entire	mucoid	opaque	Y
	11	diffusing	circular	pulvinate	entire	mucoid	translucent	Y
3	12	uniform	filamentous	raised	filamentous	rough	opaque	N
	13	diffusing	circular	flat	erose	mucoid	opaque	N
	14	diffusing	circular	flat	entire	smooth	transparent	N
	15	diffusing	circular	Raised	Entire	mucoid	translucent	N
	16	diffusing	filamentous	raised	filamentous	rough	opaque	N
	17	uniform	circular	umbonate	undulate	rough	opaque	N
	18	uniform	irregular	raised	undulate	mucoid	translucent	N
	19	uniform	irregular	flat	undulate	rough	opaque	N

BLAST and Phylogenetic Analysis: Staphylococcus Species

Of colonies sequenced, six of them were identified as being *Staphylococcus* species as indicated by high levels of sequence similarity (Figure 3). When then trimmed and edited sequences were entered into BLAST all of them returned multiple Staphylococcus species with very high levels of sequence similarity (≧99%) to *Staphylococcus* species in the Genbank database (Table 4). The Maximum Likelihood tree revealed that the sequence amplified for sample 5.1 is equally similar to that of *S. saprophyticus, S. succinus CPO, S. xylosus,* and *S. cohnii*. Samples 5.2 and 5.3 appear to identify most closely with *S. succinus PK-1*. Sample 5.4 appears to be most closely related to three different species, two of the species including two different strains: *S. arueus Y19, S. aureus Y.22, S. haemolyticus, S.*

epidermidis D0061, and *S. epidermidis CO181*. Sample 5.5 identifies most closely to *S. equorum.* Sample 5.10 does not show a close relationship in the phylogenetic tree with neither the other samples collected nor the sequences collected from the Genbank database.

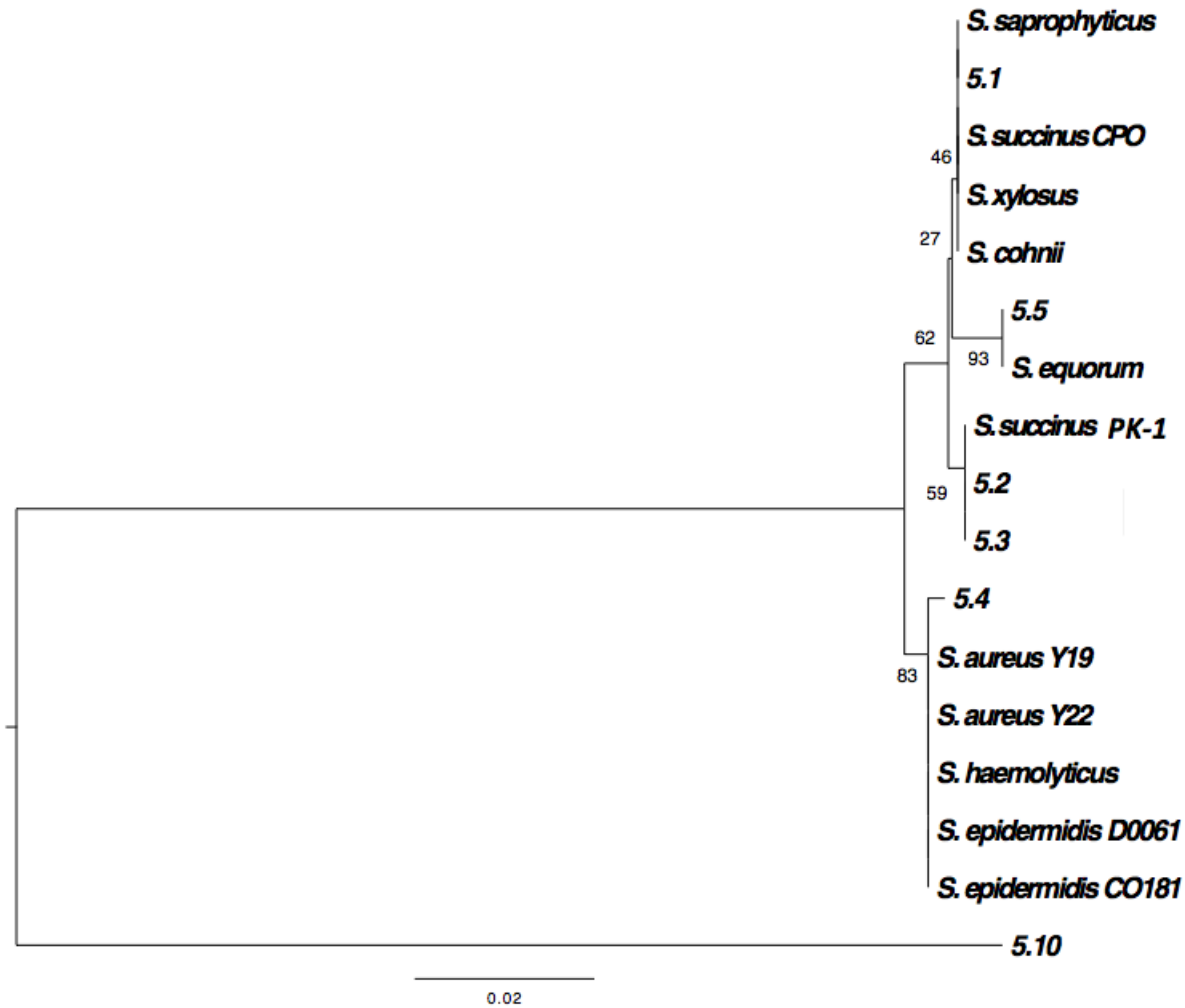

Figure 3. Phylogenetic analysis of Staphylococcus Identified Species: The sequences labeled as only numbers are the colonies sequenced in this study (sample number.colony number) and the sequences with species names are those that identified with ≧99% sequence similarity, downloaded from Genbank. Values by the nodes represent the bootstrap values.

Table 4. BLAST Results: BLAST results included more than one species for each sequence entered in the search. Each species BLAST returned from the Genbank database had very high identities, all greater than or equal to 96% similar identity with very few gaps.

Sample	Blast Species Returned	% Identity	Gaps	Accession number
5.1	*S. xylosus*	99%	1	HG941664.1
	S. saprophyticus	99%	1	KF923830.1
	S. succinus CPO	99%	1	KF923830.1
	S. cohnii	99%	1	KC456514.1
5.2	*S. succinus PK-1*	99%	1	KF254621.1
	S.xylosus	99%	1	HG941664.1
	S. saprophyticus	99%	1	KF923830.1
	S. cohnii	99%	1	KC456514.1
5.3	*S. succinus PK-1*	99%	2	KF254621.1
	S. xylosus	99%	2	HG941664.1
	S. saprophyticus	99%	2	KF923830.1
	S. cohnii	99%	2	KC456514.1
5.4	*S. haemolyticus*	99%	0	HG941667.1
	S. areus Y19	99%	0	KF923961.1
	S. aureus Y22	99%	0	KF923962.1
	S. epidermidis D0061	99%	0	KF575156.1
	S. epidermidis CO181	99%	0	KF575160.1
5.5	*S. equorum*	99%	4	HG941668.1
	S. haemolyticus	99%	4	KF318857.1
5.6	*B. mycoides*	99%	2	KC415246.1
	B. cereus SVK1	99%	2	KF612021.1
	B. thurnigiensis	99%	3	KF921618.1
	B. anthracis	99%	3	KF512667.1
5.7	*B. mycoides*	100%	0	KC415246.1
	B. cereus CPO4	99%	1	KF921620.1
	B. thuringiensis	99%	1	KF921618.1
	B. anthracis	99%	1	HG424433.1
5.8	*P. pulmonis*	99%	1	KF819801.1
	P. faecalis	99%	1	KF806878.1
5.9	*C. xerosis*	99%	1	KF410882.1
5.10	*S. xylosus*	99%	0	HG941664.1
	S. saprophyticus	99%	0	KF923830.1
	S. succinus PK-1	99%	0	KF254621.1
	S. cohnii	99%	0	KC456514.1
5.11	*B.pityocampae*	99%	4	JX876856.1
	B.yomogidense	97%	4	AB609748.1
	B. daeguense	97%	4	NR_109070.1
	B. salitolerans	96%	7	GU117109.1
	B. album	96%	7	NR_044126.1
	B. ravenspurgense	96%	9	NR_044398.1

BLAST and Phylogenetic Analysis: Non-Staphylococcus Species

Of the sequences returned from MWG Eurofins Operon five identified as a non-Staphylococcus bacterial species. After trimming, editing, and entering into BLAST samples 5.6 and 5.7 returned multiple *Bacillus* species that all had a 99% similar identity (Table 4). When a phylogenetic tree was constructed using the Kimura 2-parameter model via nearest neighbor with a bootstrap value of 500 the two samples identified equally with all *Bacillus* species (Figure 4). Sample 5.6 and 5.7 could be four possible *Bacillus* species (one including two different strains): *B. thuringiensis, B. anthracis, B. cereus CPO4, B. cereus SVK1, or B. mycoides*. When entered into BLAST sample 5.8 returned two *Psychrobacter* species that had 99% similarity: *P. pulmonis* and *P. faecalis;* both of which identified equally in the phylogenetic tree. When sample 5.9 was entered into BLAST it identified 100% with *Corynebacterium xerosis* and no other species were returned. When the sequences of both sample 5.9 and *C. xerosis* were aligned and then included in the phylogenetic tree the identification agreed with that of BLAST. The BLAST results of sample 5.11 included six *Brevibacterium* species ranging from 99%-96% similar identities: *B. pityocampae* (99%), *B. yomogindense* (97%), *B. daeguense* (97%), *B. salitolerans* (96%), *B. album* (96%), and *B. ravenspurgense* (96%). After construction of the phylogenetic tree sample 13 most closely identifies with *B. pityocampae*.

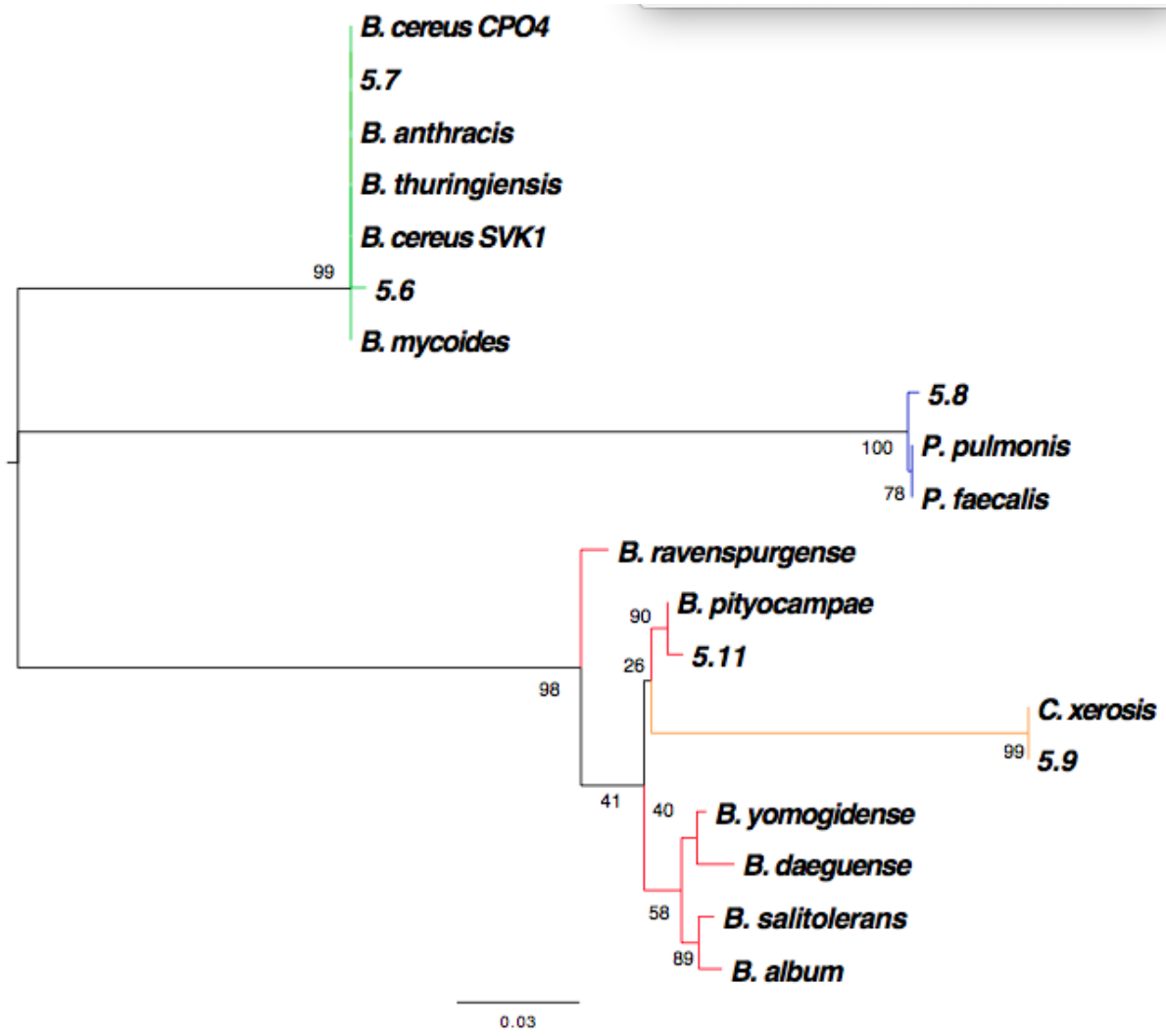

Figure 4. Phylogenetic analysis of non-Staphylococcus Identified Species: The sequences labeled as only numbers are the colonies sequenced in this study (sample number.colony number) and the sequences with species names are those that identified with high similarity downloaded from Genbank. The green clade indicates all Bacillus species, blue indicates *Psychrobacter* species, Red indicates *Brevibacterium* species, and Orange indicates *Corynebacterium* species. Values by the nodes represent the bootstrap values.

Discussion

Raw Milk Culture Conditions and Growth Trends

Significantly more growth was observed on plates incubated in 35°C with 5% CO_2 compared to 37°C (T_{10}=4.173, P=0.002) (Table 2) most likely because the samples came from an anaerobic environment – the inside of the cows udder (Table 2). Standard culture procedures of mastitis samples by veterinarians most often only include an incubator without a CO_2 source. Not only does the use of selective agar plates prevent a complete assay but it's possible the incubator conditions do as well. Even though in traditional culturing methods most split-agar plates include a non-selective media it is only used as a positive control (Painter & Schnepper, 1965). If a significant amount of unrecognizable growth is observed further culturing techniques will be applied, but these can only be applied if the colonies were observed when samples were tested in standard conditions (Farnsworth et al., 1993). Incubation in only a regular incubator can lead to a false negative (no growth) diagnosis as not all bacterial species possibly causing mastitis can grow in these conditions. It has been found that 30% of milk samples submitted for diagnosis via bacterial culture yield false negative results due to the presence of species that will not grow under standard mastitis culture conditions (Taponen et al., 2009).

Although an extensive and comprehensive list of species was not generated via DNA sequencing, good growth and variety of diverse colony morphologies was observed on the blood-agar plates as expected (Table 3). Although many different morphologies were observed different colony morphology cannot always be assumed to mean different bacterial species. In a study that isolated *S. aureus* from mastitic bovine milk only 83% of the cultures exhibited similar colony morphologies (Sutra et al., 1990). This is a likely explanation for the amount of species that identified with *Staphylococcus* but also had different colony morphologies (Table 4 and Figure 3). It has also been suggested that morphologies can differ within a species depending on other microorganisms present because of

different extracellular toxins, proteins, and other signaling molecules neighboring species can produce (Shapiro, 1998). In contrast even though many similar colony morphologies were observed as well these cannot all be assumed to be the same species. For example, both species in the Staphylococcus genus and the Streptococcus genus exhibit very similar colony morphologies as both can be circular, entire, mucoid, opaque or translucent, and similar colorings (Foresman et al., 1997). Colonies 2 and 8 exhibited very similar morphologies; both were uniform, circular, entire, opaque, and only differed in elevation: colony 2 was convex and colony 8 was pulvinate but the difference was not extremely clear (Table 3). Each of these colonies identified in two different genera: *Staphylococcus* and *Psychrobacter* (Table 4, Figure 3, and Figure 4). Although morphologically a colony may appear similar to or different from another it is often necessary to assess the colonies further to properly identify the organisms.

PCR amplification and Sequence Identification

During the past thirty years over 100 bacterial species have been identified in mastitic cattle using various culturing and molecular techniques. Watts (1988) identified 137 different species of bacteria from both clinical and subclinical cases via culturing methods and more recently Bhatt el al. 2012 identified 56 different species of bacteria in just subclinical cases using metagenomic analysis. Here, the 16S rRNA gene from 11 bacterial colonies present in one sample of milk taken from a cow infected with mastitis were successfully sequenced resulting in as few as eight or as many as 11 different species (Figure 3. and Figure 4.). The low number of identified species was due to trouble obtaining successful, non-contaminated, PCR products. The use of universal primers allowed for the 16s rRNA gene of any bacterial species present in the PCR – whether it be from my samples, the equipment, or simply bacteria in the air – to be amplified. Because the first step in the PCR thermal cycler conditions is almost identical to the DNA extraction conditions DNA from bacterial contaminants would likely be extracted in the beginning of PCR. Although species specific primers would most likely have

eliminated a great deal of the contamination issues only the species specific to the chosen primers would have been identified; this would not have allowed for possible identification of unexpected or unknown bacterial species. One other potential issue that would not have allowed for identification is that if the sequence is not on Genbank database due to either the species not being previously identified or the sequence not being uploaded to the database.

The identification of many *Staphylococcus* species was expected as this genus has been responsible for causing the most mastitis cases over the years. In one study of clinical mastitis cases a *Staphylococcus* species was isolated from 57% of the samples and specifically *S. aureus* was isolated from 44.7% of the samples (Waage 1999). Even though standard quad-agar plates include a quadrant selective specifically for *Staphylococcus* species, and the microbiological identification of *S. aureus* is relatively simple, just identifying the species has shown to be insufficient with regards to successful treatment. Attempts to cure *S. aureus* infections via antibiotic infusion typically have less than a 50% cure rate (Riffon et al., 2001). It has been shown that treatment of this species is so difficult because different strains of *S. aureus* respond differently to antibiotic therapies depending on clonal complexes, toxins secreted, and much more that is not clearly understood (Monecke et al., 2007). To type the *S. aureus* strains accurately molecular methods are required. A similar argument can be made for the many other *Staphylococcus* species that are potentially causing an infection. Although the quad agar plate indicates a *Staphylococcus* infection the exact species cannot always be indentified under traditional microbiological conditions, thus molecular analysis is essential to the successful identification and ultimately treatment of a cow infected by a *Staphylococcus* species (Zdanowicz et al., 2004). It is possible though that because only three of the six samples were identified to species level that species-specific primers or a universal primer other than region of 16S rRNA targeted(as it appears here to not be variable enough between species) would be necessary for more accurate identification.

Even with the small sample number, sequenced samples were identified that would normally not be identified with standard culture conditions. Sample 5.8 was identified a species of the *Psychrobacter* genus and showed a close relationship to *P. pulmonis* and *P faecalis* (Figure 4 and Table 4). Both of these species are considered fastidious strains and require rich agar for growth (Alves et al., 2004). Although as a rich media the positive control quadrant on a diagnostic plate is sufficient for growth of *Psychrobacter* species the remaining three quadrants are not. Because you must have growth outside of the positive control quadrant as well to make a diagnosis members of this genus are usually not identified under standard culture conditions. Sample 5.11 was identified as being most closely related to *Brevibacterium pityocampae* (Figure 4 and Table 4). This is especially interesting because *B. pityocampae* is a recently identified and proposed species of the genus *Brevibacterium* (Kati et al. 2010). Other *Brevibacterium* that have previously been identified in mastitis cases include *B. stationis* and *B. linens;* these species were only reported as being identified in relation to bovine mastitis cases via DNA sequencing methods (Chiang et al., 2008, Watts et al., 2001). No mastitis culturing studies were found that included the identification of *Brevibacterium* and thus if an infection by this organism is to be correctly diagnosed DNA sequencing methods, such as PCR, are required.

Sample 5.9 identified most closely with *Corynebacterium xerosis* (Figure 4 and Table 4) . *C. xerosis* is another species that is not routinely diagnosed in standard culturing conditions. *C. xerosis* is a gram-positive species and thus will not appear on either the gram-negative, *Staphylococcus* specific, or *Streptococcus* specific quadrants (Colman et al., 1992). This species has been identified in mastitis via culture before, but with methods veterinarians do not perform routinely. A screening test for *C. xerosis* would include the detection of pyrazinamidase activity and cystinase activity as well as an Elek test, but none of these were reported as diagnostic options on the DHIA's microbiology website (Colman et al., 1992).

The bacterial species identified in this study were only selected from a sample from one cow but the list of species identified still support the idea that molecular methods are necessary for accurate diagnoses and treatments. DNA sequencing is important in identifying less common bacteria, fastidious bacteria, and even accurate identification of some of the most common species (Viguier et al., 2009). In one study that compared culture and PCR results, culturing techniques identified pathogens in 77% of the samples whereas PCR identified pathogens in 89% of the samples using species-specific primers for 11 species. Furthermore 53 samples that were negative for *Staphylococcus* species in cultures were positive for the species in PCR (Koskinen et al., 2010). PCR is a key step in advancing diagnostic techniques for mastitis.

Although the use of species specific primers in PCR eliminates many contamination issues and allows pathogen identification directly from milk in as little as four hours, current PCR kits only include primers designed for only eleven of the major mastitis-associated pathogens, thus very much limiting species detection (Viguir et al., 2009). To utilize molecular identification to detect as much as possible as efficiently as possible more research needs to be done. The variety of species-specific primers in the mastitis PCR kits should be expanded upon for more complete results and to avoid the same problem as standard culturing techniques: false negatives. Although including a greater variety of primers would increase the identification of PCR more molecular methods should be assessed for diagnosing mastitis. One method that is similar to basic PCR is PCR-single strand conformation polymorphism (SSCP) which targets a 400 bp fragment of the bacterial 16s rRNA gene (Schweiger & Tebbe, 1998). In 8 of 11 cases, specific diagnoses were made from culture results but when the same samples were assessed with SSCP not only were all samples diagnosed but culture diagnoses of only six samples agreed with SSCP results. In some samples multiple bands were detected with different intensities suggesting differing

amounts of the bacterial species to be present. This is a reminder that just because an organism is identified it does not always mean it is a causative agent.

Research in mastitis with Real-time PCR has recently been increasing because of its capability to quantify as well as qualify (Koskinen et al., 2010, Mahmmod et al., 2013, Taponen et al., 2009, Viguier et al., 2009). A measure of 100,000 cells/ml was reported to be a baseline for diagnosis of infection by a bacterial organism (Taponen et al., 2009). Although a common baseline exists species-specific baselines were not found to be reported in the literature (Koskinen et al., 2010, Taponen et al., 2009, Viguier et al., 2009). The lack of species-specific baseline infection numbers is most likely due to the difficulty of accurately identifying these values. Because of the complexity of the udder environment and the presence of so many bacterial organisms at the same trying to quantify just one *in vivo* is just as difficult as building an accurate *in vitro* model for quantification. The quantification of the amount of a species necessary to cause an infection has proved difficult, but is one of the most important areas of research that should be continued – especially for the successful use of Real-Time PCR as a diagnostic tool.

Much more research is needed to determine the amount of a single species that is needed to actually cause an infection as well as if certain bacteria are dangerous alone or in conjunction with another species. It has been suggested that *A.pyogenes* and *Peptoniphilus* species are only dangerous when present together In one study both species were detected concurrently in samples from multiple cows reported as clinically severe mastitis cases (Schwaiger et al., 2012). Another factor that must be taken into consideration is if bacteria being identified are actually alive and functioning as dangerous pathogens. One method has been proposed that is said to be not only quicker but more sensitive than PCR because it can discriminate between dead and living organisms: Real-time Nucleic Acid Sequence

Based Amplification (NASBA). Real-time NASBA has only been applied in mastitis research with *Bacillus species*, but it displayed promising results (Gore et al., 2003).

Although this study resulted in identifying pathogens of only one cow, PCR still identified organisms that would not be identified in traditional culture techniques. Thus, PCR has the ability to correctly identify many more pathogens than microbiological culturing methods. Not only can PCR identify more pathogens, it can identify more pathogens faster. In this study identification was not always as specific as hoped, as bacteria were not always identified to a specific species, and so it is likely that a gene with more variability within species is necessary. It is possible that assessment of a larger region of or the complete 16S rRNA gene may improve upon the specificity of identification. The continued study and development of modern molecular methods for bacterial pathogen identification in milk is crucial to improving accurate diagnoses. Future studies should not only assess identification methods but quantification methods as well. In order to accurately diagnose an infection the amount of bacteria present, not simply the presence, should be taken into consideration. Therefore future studies should focus on quantifying bacterial infections. Also, future studies should focus on any trends where multiple bacteria are present. It is possible that in some cases a bacterium may be present in a large amount but may not be harmful unless in conjunction with another species. Ultimately if diagnoses become extremely accurate, especially down to the strain level for *Staphylococcus* species, proper treatment can be administered so cure rates will rise and resistance rates will decrease with the use of unnecessary antibiotics being avoided.

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Appendix

Appendix 1: Sequences collected from sample 5 (Gretta): The sequences collected from the samples 5.1-5.11 are included in fasta format. The sample names are consistent with table 3 of the results and table 1 of the methods.

>5.1
------ATGAGTGCTAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTC
AAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGAAAACTCTA
GAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAGGTTGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT
TATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTA
AACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTA
GTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAG
CATGCTACGGTGAATAC—

>5.2
----------TGCTAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCA
GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAAT
TCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGAAAACTC
TAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGG
TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAGGTTGACT
GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGC
TAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTG
TAGTCTGCAACTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATC
AACATGCTACGGTGAATACGTTCCCGGGA--

>5.3
-AGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCA
CTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCCTTC
CCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAG
TTGCCATCATTAAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTA
CACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCAT
GCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGAC
TACATGAAGCTGGAATCGCTAGTAATCGTAGATCAACATGCTACGGTGAA

ACGTTTCCCGGGAGA

>5.4
TAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCA
CTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAAATCTTGACATCCTTTGACAACTCTAGAGATAGAGCCTTC
CCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAG
TTGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGG
AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTA
CACACGTGCTACAATGGACAATACAAAGGGCAGCGAAACCGCGAGGTCAA
GCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGAC
TACATGAAGCTGGAATCGCTAGTAATCGTAGATCAACATGCTACGGTGAA
TAG-TTCCCGGGA

>5.5
GAGTGC-AGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCAT
TAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGATAGA
GCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAA
ACTTAGTTGCCAGCATTTAGTTGGGCACTCTAGGTTGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTT
GGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGA
GGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAA
CTCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTAC
GGTGAA-CGTTTTCCCGGGA

>5.6
TGAGTGCTAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCA
TTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA
CGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAG
GGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT
CTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTG
GGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAG
GTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAAC
TCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG
GTGAAACGTTTCCCGGGA

>5.7
GAGTGCTAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCAT

TAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGG
GCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATC
TTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAA
CCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGG
GCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGG
TGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACT
CGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGG
TGAATAC

>5.8
GTCTACTAGTCGTTGGGTCCTTGAGGACTTAGTGACGCAGCTAACGCAAT
AAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACG
CGAAGAACCTTACCTGGTCTTGACATATCTAGAATCCTGCAGAGATGCGG
GAGTGCCTTCGGGAATTAGAATACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCT
TAGTTACCAGCGGTTTGGCCGGGAACTCTAAGGATACTGCCAGTGACAAA
CTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGACCAGG
GCTACACACGTGCTACAATGGTAGGTACAGAGGGCAGCTACACAGCGATG
TGATGCGAATCTCAAAAAGCCTATCGTAGTCCAGATTGGAGTCTGCAACT
CGACTCCATGAAGTAGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGG
TGAAT

>5.9
TCTTCCACGACTTCTGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGG
GAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTG
GGCTTGACATATACGGGACCGGGCCAGAGATGGTCCTTCCCTTGTGGCTC
GTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTCTTATGTTGCCAGCACGTAAT
GGTGGGGACTCGTGAGAAACTGCCGGGGTCAACTCGGAGGAAGGTGGGGA
TGACGTCAAATCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACA
ATGGTCGGTACAGTGGGTTGCGATGCCGTGAGGTGGAGCTAATCCCTGAA
AGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGG
AGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTTCCCGG

>5.10
TGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTC
CGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGA
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
CTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCCTTCCCC
TTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTG

CCATCATTAAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACAC
ACGTGCTACAATGGACAATACAAAGGGCANCTAAACCGCGAGGTCATGCA
AATCCCATAAAGTTGTTCTCAGTTCCGA

>5.11
GGTGTGGGGTCATTCCACGGATTCCGCGCCGTAGCTAACGCATTAAGTGC
CCCGCCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGG
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CGAATCCCAGAAAGCCGGTCTCAGTTCGGATCGTAGTCTGCAACTCGACT
ACGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAA
TACGTTTCCC

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