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Assessment of Antimicrobial Resistance in Pathogens Responsible for Causing Bovine Mastitis in Kentucky

Erica Denise West
Eastern Kentucky University

ASSESSMENT OF ANTIMICROBIAL RESISTANCE IN PATHOGENS RESPONSIBLE FOR CAUSING BOVINE MASTITIS IN KENTUCKY

By

Erica D. West

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Chair, Advisory Committee

Member, Advisory Committee

Member, Advisory Committee

Member, Advisory Committee

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By

Erica D. West

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DEDICATION

This thesis is dedicated to my husband
Nathan Phillips,
for his unwavering support.

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ABSTRACT

Bovine mastitis is most significant disease seen in dairy farms worldwide, resulting in the largest profit loss of any other disease affecting dairy cows. The aim of this thesis was to determine the predominant species responsible for bovine mastitis in a subset of ten Kentucky dairy herds, and to assess the presence of antibiotic resistance in these pathogens. In this study, 308 milk samples were obtained from cow's selected based on their recent somatic cell count. Samples positive for growth were identified using the gram stain and various biochemical tests. After identification, resistance to 11 antimicrobial agents was assessed using the Kirby-Bauer test. *Staphylococcus aureus* was found to be the most common species causing bovine mastitis, which was identified in 13% of milk samples. Coagulase negative Staphylococci (11%) and streptococci species (10%) were also found to be major causes of mastitis in Kentucky. Only one isolate of *Streptococcus agalactiae* was identified, indicating that this species is not prevalent in this state. *S. aureus* isolates were highly susceptible to all antibiotics used in the laboratory, with the only minor resistance seen in penicillin (7%), ampicillin (5%), oxacillin (2%), and cephalothin (2%). Coagulase negative Staphylococci species showed their highest resistance to oxacillin (31%), pirlimycin (23%), tetracycline (17%), and ampicillin (14%). Streptococci species were the least susceptible group of all the major pathogens identified, with many of these species resistance to kanamycin (69%), tetracycline (59%), and oxacillin (50%). Overall, the major pathogens recovered in this study were largely susceptible to cephalosporins, indicating that this group of antibiotics may be effective in the treatment of Kentucky's common bovine mastitis infections.

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CHAPTER 1

Introduction – Literature Review

Mastitis is the most common disease seen in dairy cattle worldwide, making it a significant problem in terms of cow health and agricultural productivity. The pathogens responsible for causing mastitis in cattle range from a number of gram positive bacterial species, including members of the genera *Staphylococcus* and *Streptococcus*, as well as gram negative bacteria, such as the species *Escherichia coli*, which are associated with the intestinal tract of mammals (Barkema et al. 2009, Guler et al., 2005, Nam et al., 2009). Correct identification of these pathogens to the species level is important to ensure proper treatment due to the variability in each pathogen's susceptibility to antibiotic treatment (Pitkala et al., 2008). This disease is the primary reason that antibiotics are used in dairy cows (Barkema et al. 2009; De Oliveira et al., 2000; Guler et al., 2005; Kalmus et al., 2011). As a result of this reliance on antibiotics, the level of resistance in these pathogens should be monitored. Inappropriate use of antibiotics to treat bovine mastitis can lead to an increase of resistance in these pathogens (Gianneechini et al., 2002).

Common Causes of Bovine Mastitis:

There are over 135 different microorganisms that have been found to cause bovine mastitis, but the major pathogens responsible are the Staphylococci, Streptococci, and Gram negative rods (De Oliveira et al., 2000). Mastitis pathogens are normally classified as either contagious or environmental based on their method of infection and

spread through the herd. Contagious pathogens are those that are transmitted from an infected cow to a susceptible cow, which often occurs during milking (Harmon, 1996). These infections are seen to increase in the absence of post milking teat disinfection (Barkema et al., 2009; Harmon, 1996; Neave et al., 1969). In contrast, some cases of mastitis result from pathogens found in the cow's immediate environment. These infections are seen to increase in the absence of pre-milking teat disinfection (Verkamp, 2005). *Streptococcus agalactiae, Staphylococcus aureus,* and *Mycoplasma* species are the major contagious pathogens responsible for bovine mastitis (Barkema et al., 2009; Harmon, 1996). These organisms gain entrance into the mammary gland through the teat canal, with the exception of some mycoplasmal infections that may originate in other sites and spread systemically (NMC – "A practical look", n.d.). Environmental pathogens thought to spread in a contagious manner include *Streptococcus dysgalactiae*, *Streptococcus canis*, *Streptococcus uberis*, and *Klebsiella pneumoniae* (Barkema et al., 2009).

Staphylococcus species are one of the major groups of bacteria that cause bovine mastitis. This genus is separated into two groups based on the species' ability to coagulate (clump) rabbit plasma, which is considered an important phenotypic determinant (Guler et al., 2005; NMC 1999; Taponen and Pyorala, 2008). These two groups are commonly referred to as coagulase positive *Staphylococcus* species (CPS), which most notably includes *S. aureus*, and coagulase negative *Staphylococcus* species (CNS). It has been speculated that the clumping ability of the coagulase protein could result in the formation of a fibrin layer surrounding staphylococcal abscesses, which

could in turn localize the infection preventing phagocytosis (Medical Microbiology 6th edition pg. 214, 2009).

Staphylococcus aureus is one of the most common causes of contagious mastitis on dairy farms (Barkema et al., 2009; Juhasz-Kaszanyitzky et al., 2007; Middleton, n.d.; Olde Riekerink et al., 2008; Wilson et al., 1997). Many phenotypically and genotypically different strains of *S. aureus* exist, but there is little information about the distribution of the strains existing within herds and geographic locations (Guler et al., 2005). *S. aureus* is known to produce chronic subclinical infections, accompanied by periods of mild clinical symptoms (Taponen and Pyorala, 2008). Infections from this species have also occasionally produced severe clinical symptoms, such as gangrene (NMC – "A practical look", n.d.).

S. aureus infections occur when the teat skin or canal are colonized during the milking process. These infections result in increased somatic cell counts and decreased milk production, and are more damaging to the milk tissues than *S. agalactiae* infections (NMC – "A practical look", n.d.). After entry into the mammary gland, *S. aureus* will form pockets of infections within the milk ducts and eventually form abscesses. Due to the damage from infection, these abscesses become walled off when scar tissue is formed. This wall formation has been implicated as a possible reason it is so difficult to treat *S. aureus* infections with antibiotics (NMC – "A practical look", n.d.). Tissue damage from infections with this species can be minimized if animals are treated during the early stages of infection.

S. agalactiae is also considered a major contagious mastitis pathogen, but is much more easily controlled than *S. aureus*. This species generally responds well to β-lactam

antibiotic therapy (NMC – "A practical look", n.d.), and due to the implementation of mastitis control practices developed in the 1960s, it has been largely eradicated in the UK and other parts of Europe (Kalmus et al., 2011; Zadoks and Fitzpatrick, 2009). Even so, *S. agalactiae* remains prevalent in countries such as Brazil (Duarte et al., 2004), Germany (Tenhagen et al., 2006), and Uruguay (Ginannechini et al., 2002).

S. agalactiae is an obligate parasite of the bovine mammary gland (Keefe, 1997). Once this species enters the mammary gland, it infects the cisterns and ducts and produces an inflammatory response. This results in high somatic cell counts, much higher than what is seen in *S. aureus* infections, and a decrease in milk production (NMC – "A practical look", n.d.). Whenever the bulk tank somatic cell count is 1,000,000 cells/ml or higher, this species is suspected to be the cause of infection (NMC – "A practical look", n.d.).

In humans, *S. agalactiae* is a common cause of neonatal septicemia, and is known to exist as part of the normal flora in the throat, genitourinary tract, and rectum of humans. Even though the majority of human infections are acquired from other human sources, there is always some risk of infection to those who come in direct contact with infected cows or raw milk (Keefe, 1997). Interestingly, Wagner and Dunney found that a great deal of homology exists between strains isolated from septicemic infants and mastitic cows (Wagner and Dunny, 1985). Rarely, it has even been seen that an individual animal or bulk tank sample tested positive for *S. agalactiae* due to the presence of a human strain of this species (Barkema et al., 2009).

Coagulase negative staphylococci (CNS) are considered opportunistic pathogens, and are found as part of the normal micro flora on the cow. These species are known to

predominately cause minor infections normally characterized by a slight decrease in milk production and increased somatic cell counts (Luthje and Schwartz, 2006). It is also common for these species to cause co-infections with other microorganisms (Taponen and Pyorala, 2008). CNS are generally more resistant to antibiotics in laboratory susceptibility testing when compared to *S. aureus*, but they respond better to antibiotic treatment within the cow (Taponen and Pyorala, 2008). In routine diagnostics, this group of staphylococci is not normally identified to the species level, as the absence of the coagulase protein is sufficient for their identification (Pyorala and Taponen, 2008).

Environmental streptococci are significant causes of both clinical and subclinical cases of bovine mastitis around the world (Nam et al., 2009; Wang et al., 1999), but are known to cause higher rates of clinical cases than contagious pathogens. These species are commonly found in the soil, bedding, and on the skin of cows (NMC, 1999). *Streptococcus uberis* and *S. dysgalactiae* are the most common environmental streptococcal species recovered from dairy farms, with *S. uberis* being the more prevalent of the two (Nam et al., 2009; Wang et al., 1999). *S. uberis* is especially found in older cows during dry periods, and is a major cause of clinical mastitis during early lactation (Wang et al., 1999). *S. dysgalactiae* is also a common cause of infections during the dry period and early lactation (Wang et al., 1999). Kalmus et al. (2011) reported *S. uberis* as the most prevalent species recovered from bovine milk samples during a two year study. Other common species of environmental streptococci include *S. bovis*, *S. canis*, *S. equinus*, and *S. equi* subspecies *zooepidemicus* (Nam et al., 2009).

Other major environmental pathogens consist of gram-negative enteric rods, such as *E. coli* and *Klebsiella*, and *Enterobacter* species. Cows can become infected with

these species if they come in contact with contaminated bedding, water, soil, or plant material (NMC, 1999). Infections with coliform bacteria are more likely during the first two weeks of the dry of period, and the two weeks immediately prior to calving (NMC, 1999). These infections are normally short, lasting less than a month, and are not likely to become chronic. Infections with these bacteria account for approximately 40% of the clinical cases within herds that are well managed (NMC, 1999).

Detection and Control of Mastitis:

Leukocytes and white blood cells travel to the udder during the early stages of infection (Harmon, 1999). This response results in an increase in the total amount of cells that can be detected in the milk. The number of cells within milk can be measured and is known as the somatic cell count (SCC). An infection is indicated when an individual cow's SCC increases above 200,000 cells/ml (Harmon, 1999). SCCs vary greatly depending on what type of microorganism is present in the mammary gland, and the degree of immune response elicited by its presence.

The normal proportion of somatic cells within the milk of uninfected cows has been reported to be 80% macrophages, 16% lymphocytes, 3% polymorphonuclear leukocytes, and 2% epithelial cells (Sharma et al., 2011). This proportion changes dramatically during inflammation of the udder, in which over 90% of the cells present within the milk are neutrophils (Harmon, 2001; Leitner et al., 2008; Sharma et al., 2011). Polymorphonuclear leukocytes flood into the mammary gland during early infection and function to engulf and digest the invading microorganisms. These leukocytes also release

substances to attract more leukocytes to the area in order to continue the process of eliminating the infection (Harmon, 2001).

It is possible that the proportional differences of somatic cells found within infected milk could be used to help detect what pathogen is causing the infection. One study in particular (Leitner et al., 2008) looked at the leukocyte populations of quarters infected with *S. aureus*, *E. coli*, and *S. dysgalactiae*. This study found uninfected quarters to contain more epithelial cells than polymorphonuclear cells. Leukocytes made up 56% of the cells in uninfected quarters (Leitner et al., 2008). Neutrophils were the main cell type identified in quarters with acute infections of either *E. coli* or *S. aureus*, as well as in chronic *S. dysgalactiae* infected quarters. Cow chronically infected with *S. aureus* or CNS showed a higher proportion of polymorphonuclear leukocytes than what was seen in the other infections, but remained similar to the distribution seen in healthy cows (Leitner et al., 2008). $CD4^+$ and $CD8^+$ T cells were also seen to increase significantly in acute *E. coli* and *S. aureus* infections, and in chronic *S. aureus* infections (Leitner et al., 2008).

Common laboratory methods used to measure the SCC of the entire herd include the Coulter Milk Cell Counter, which uses the current of an electric field to count cells, and the Fossomatic, where cells are stained using a florescent dye (Sharma et al., 2011). Routine SCC testing is a crucial part of maintaining the health of the herd. Dairy producers participating in the Dairy Herd Improvement Association (DHIA) are able to receive monthly SCC records by sampling during the same time milk yields are recorded. The milk samples must be collected correctly to ensure that the fat particles within the milk are evenly dispersed, as somatic cells are known to attach to butterfat particles

(McAllister and Witherspoon, 2013). Milk samples are drawn by either placing a sampling device on the apparatus used to measure milk yield, or all four quarters are sampled after the milking equipment has been put on for at least 2 – 3 minutes. Each quarter sample is then mixed together thoroughly, and a single sample is obtained from the mix (McAllister and Witherspoon, 2013). All Kentucky DHI milk samples are sent to the Mid-South Dairy Records laboratory in Springfield, Missouri for testing (McAllister and Witherspoon, 2013).

A cow-side SCC test known as the California Mastitis Test (CMT) can also be used in between DHI testing dates, or to identify potentially infected quarters for microbiological culturing. This simple test is performed by adding milk from each quarter to four corresponding wells on a plastic paddle. An equal amount of reagent is then added to each well. This reagent acts as a detergent with a pH indicator, bromcresol (Ruegg and Reinemann, 2002), meaning it will disrupt the cell wall of somatic cells present in the milk causing the cells to release their contents. The DNA released from the cells' nuclei will string together forming a gel, which is indicative of an increased somatic cell count (Ruegg and Reinemann, 2002).

Routine monitoring of SCCs is especially beneficial for the detection of contagious mastitis outbreaks, which are indicated by bulk tank SCCs above 300,000 cells/ml (NMC, 1999). Even so, it is still common for herds to have significant problems with individual infections, without necessarily increasing the bulk tank SCC (NMC, 1999). Infections caused by environmental pathogens such as *E. coli*, *S. uberis*, and *S. dysgalactiae* are known to cause clinical mastitis. The overall prevalence of environmental infections at a given time can be low (NMC, 1999). In this case, the bulk

tank SCC would not be an effective method for monitoring udder health due to clinical mastitis. Environmental infections are also known to be short in duration, and many occur during the dry period and calving (NMC, 1999).

Another important reason to monitor SCC within the herd is due to the national regulations in place. In the United States, dairy producers must keep the bulk tank SCC of their herd below 750,000 cells/ml in order to sell their milk as Grade A (USDA, 2011). If national regulations are not met, the dairy producer could have their license suspended (USDA, 2011). Also, if a producer wishes to export their milk to the European Union, Canada, Australia, or New Zealand, all of these countries enforce a limit of 400,000 cells/ml (USDA, 2011). There has recently been support to lower the limit in the United States to 400,000 cells/ml, but the National Conference on Interstate Milk Shipments (NCIMS) has yet to vote in favor of this limit (USDA, 2011). Thus, it is extremely important to lower the bulk tank SCC as much as possible. This is achieved through good control practices and by removing cows with chronic infections from the herd (Harmon, 1999).

Standard control practices for the treatment and prevention of mastitis have been in place since the late 1960s. Results from the Neave et al. (1969) study led to the development of a five-point mastitis control plan that would function to control the spread and duration of contagious infections within a herd. This plan sought to ensure 1) proper milking procedures and equipment, 2) application of a post-milking teat disinfectant, 3) dry cow therapy antibiotic treatment of infected cows, 4) proper treatment and recording of all clinical mastitis infections, and 5) culling of any chronically infected cows (Middleton, n.d.; Neave et al., 1969). Results of this plan showed a significant

reduction of infections caused by *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Streptococcus dysgalactiae* (Neave et al., 1969). However, it was not as effective in controlling infections resulting from environmental pathogens, such as *Streptococcus uberis* (Neave et al., 1969). Thus, a ten-point mastitis control plan was later developed by the NMC in 2001, in order to also decrease the prevalence of infections resulting from environmental pathogens (Middleton, n.d.; Veerkamp, 2005).

Intramammary infusion of antibiotics is the most common method available for treating bovine mastitis (Barkema et al., 2009; De Oliveira et al., 2000; Guler et al., 2005; Kalmus et al., 2011). This treatment is also commonly used at the beginning of the dry off period as a prophylactic in order to prevent and eliminate any existing infections (USDA, 2008). The method is performed by using an antibiotic tube with a plastic cannula attached to the end, and inserting the cannula partially or fully into the teat canal. The antibiotics are then completely infused into the teat cistern, after which the teat is pinched off and the antibiotics are massaged upward into the mammary gland. The most common antibiotics reported by the USDA (2008) used for bovine mastitis are cephalosporin (53.2%), β-lactam (19.7%), and lincosamide (19.4%).

Knowing the antimicrobial susceptibilities of common mastitis pathogens can help aid veterinarians in their choice of an effective antibiotic treatment for an individual infection (De Oliveira, 2000; Nunes et al., 2007; Pitkala et al., 2008). Studies have reported the *in vitro* antimicrobial susceptibility of *S. aureus* and coagulase negative *Staphylococcus* species (CNS) isolated from mammary glands in cattle (Nunes et al., 2007). Information on the susceptibility traits is essential for antimicrobial resistance monitoring and could help to accurately define specific breakpoints for mastitis

pathogens. The majority of breakpoints for staphylococci testing is based on human data and does not take into account the specificity of the udder environment (Nunes et al., 2007).

The production of β-lactamase is the most commonly found method of resistance in staphylococcal species (Taponen and Pyorala, 2008). Chances of a successful cure through antibiotic treatment vary greatly depending on which species is causing the infection. *S. aureus* tends to respond poorly to antibiotic therapy, while CNS species generally respond well. Antibiotic cure rates for *S. aureus* range greatly due to many factors, such as lactation number, duration of infection, somatic cell count prior to treatment, and the particular susceptibility profile of the isolate (Taponen and Pyorala, 2008). Antibiotics such as pirlimycin have been shown to be effective in the treatment against *S. aureus* as a result of their chemical nature, which allows them to penetrate mammary tissues (Guler et al., 2005).

Due to the severity of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in humans, and the use of cloxacillin to treat bovine mastitis, it is important to monitor the antibiotic resistance patterns of *S. aureus* within the dairy industry (Barkema et al., 2009). Although rare, the transmission of MRSA from animal sources to humans has been reported in dogs, pigs, horses, and recently in cows (Barkema et al., 2009; Juhasz-Kaszanyitzky et al., 2007). It is unknown whether transmission occurred from cow to human or vice versa, but the same strain was found in several cows as well as a human carrier who worked in close contact with the herd (Juhasz-Kaszanyitzky et al., 2007).

Methicillin resistance is much more commonly reported in CNS species than in *S. aureus*. Resistant CNS species have been found to carry the *mecA* gene, which is the gene responsible for conferring methicillin resistance (Taponen and Pyorala, 2008). CNS species which carry the *mecA* gene could possibly be a source of methicillin resistance through a mechanism known as horizontal gene transfer. Co-infections with CNS and *S. aureus* are common in bovine mastitis infections. If this mechanism were to occur during a co-infection with *S.aureus* and a CNS species containing the *mecA* gene, it is possible that the *S. aureus* strain could pick up this gene, resulting in the acquisition of methicillin resistance. Horizontal gene transfer has also been implicated as the possible method by which *S. aureus* originally obtained the *mecA* gene when it was first described in humans (Brody et al., 2008).

Antimicrobial susceptibility studies of environmental streptococcal species have shown high levels of resistance to tetracycline (Kalmus et al., 2011; Gianneechini et al., 2002; Nam et al., 2009). In one study, *S. dygalactiae* was found to be resistant to tetracycline, while other streptococcal species and *Enterococci* were found to be susceptible (Gianneechini et al., 2002). These species have been reported to show resistance to oxacillin, but susceptibility in other β-lactam antibiotics (Nam et al., 2009). Overall, these streptococcal species seem to show high levels of susceptibility to cepthalothin and penicillin (Kalmus et al., 2011; Nam et al., 2009; Gianneechini et al., 2002).

The Viridans group of streptococci have been reported as becoming increasingly more resistant to numerous antimicrobial agents (Nam et al., 2009) and should be monitored. These bacteria are also considered a possible source of antibiotic resistant

genes, due to the possibility of transfer of genes conferring resistance to other pathogenic species (Nam et al., 2009). Unfortunately, there is limited information on the susceptibility and resistance patterns of the more uncommon species that represent this group of organisms (Nam et al., 2009).

A great deal of attention has also been paid to gram-negative bacteria due to extensive antibiotic resistance in some species that poses a threat to public health (Lockhart et al., 2007). In one study, 70% of all gram-negative bacteria isolates from mastitis had resistance to more than three different antimicrobial agents (Nam et al., 2009). Over 90% of *Pseudomonas* species showed resistance to almost all antimicrobials (Nam et al., 2009).

Purpose of Research:

Antibiotic resistance in bacterial organisms causing both human and animal diseases is becoming increasingly problematic. Due to this reliance on antibiotic therapy, it is important to monitor the resistance and susceptibility patterns of the pathogens responsible. This study sought to identify the species responsible for causing bovine mastitis in Kentucky, and to assess the antibiotic resistance found in these microorganisms. The conclusions of this study aim to further the knowledge of dairy scientists and veterinarians in order to assist in the effective control and treatment of these infections.

CHAPTER 2

Materials and Methods

Collection of Milk Samples:

IACUC approval was received on March 17, 2011 prior to the start of this project, to allow the use of dairy cows for milk collection. The IACUC protocol number for this study is 03-2011. Upon approval, recommendations for farms to contact were made by Dr. Jeffrey Bewley, at the Department of Animal and Food Sciences, University of Kentucky. Any herds within approximately 150 miles of Richmond, KY, with SCCs higher than 250,000 cells/mL, were the primary target for this study. Each farm was contacted by phone to obtain permission for the sample collection visit, and farmers were provided with the results of all milk sample culturing. Individual cows from each herd were selected based on their latest Dairy Herd Improvement (DHI) SCC results. An average of 30 samples per farm were collected from cows with highest SCC scores. Farmers were also able to request the culturing of other cows within the herd at the time of sampling, and on occasion, previously selected cows were unable to be sampled from since they were sold prior to the sampling date. Cows with SCCs below 250,000 cells/mL were only sampled in herds with less than 20 cows above this threshold.

Before obtaining each sample from a selected cow, the first few streams of milk (forestrip) were discarded and the teats were brushed off and pre-dipped with the provided teat dip. Each quarter was then wiped clean using a paper towel, and subsequently disinfected with 70% alcohol wipes. Disinfecting continued until the wipes remained clean, upon which a period of 30 seconds was allowed for the teat to dry.

Quarters were tested using the California Mastitis Test (CMT), a cow side indicator of somatic cell count, in order to identify possible infected quarters. A four-well plastic paddle was used to collect two squirts of milk from each quarter, and an equal volume of CMT reagent was added to each well. The paddle was gently swirled for 5 – 10 seconds in order to agitate the milk/reagent mixture, and any trace of gelling within 20 seconds was noted as a positive reaction (NMC 1999). Milk was collected from each positive quarter by holding a collection tube at a 45° angle to prevent contamination. Approximately 4 mL was collected from each quarter sampled and each were immediately labeled and stored on ice (NMC 1999).

Identification of Mastitis Pathogens:

The milk samples were brought to the microbiology lab the same day as collection. Each sample was vortexed and 0.1 ml was plated once each on Trypticase Soy Agar supplemented with 5% sheep's blood (BAP) and MacConkey agar (MAC). Plates were inverted and incubated at 37ºC for 24 hours, after which they were checked for growth and purity. The colony color on both MAC and BAP was noted, and the presence or absence of hemolysis for each unique colony was recorded. Plates that had growth of more than two morphologically different colonies were labeled as contaminated (NMC, 1999), and no attempt was made to identify the possible pathogens. Distinct colonies from plates positive for the growth were subcultured on BAP and frozen down to -80ºC in a 10% serum-sorbitol solution. Isolates were analyzed first by using the Gram's stain and the catalase test. When performing the catalase test, colonies were carefully collected, making sure not to dig into the agar, and were placed on a coverslip

with a drop of hydrogen peroxide. This was repeated twice for each isolate to confirm positive reactions, due to the ability of blood in BAP to react since it also contains the catalase enzyme. Results of these two tests test determined what further analyses were necessary (Figure 1).

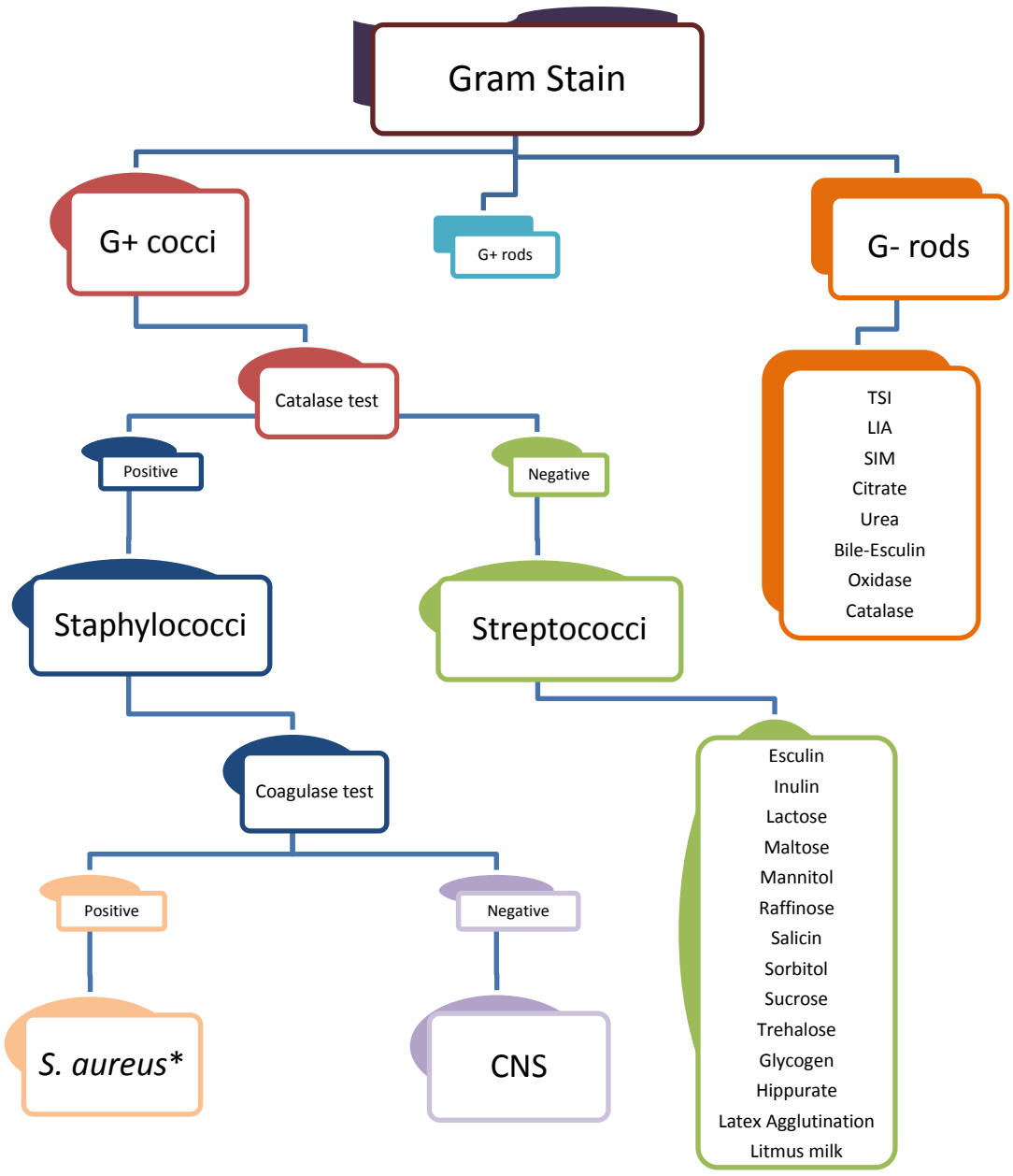

Figure 1: Flow chart for the identification of bovine mastitis pathogens isolated from milk samples (Fortin et. al 2003, National Mastitis Council 1999, Odierno et. al 2006, personal communication with Dr. Erol). *S. *aureus* was identified when a coagulase positive *Staphylococcus* spp. tested positive for the fermentation of Maltose, Mannitol, and Trehalose, but *S. lutrae* and *S. delphini* can also test positive for these sugars (Foster et al, 1997), (personal communication Dr. Erdal Erol).

Several tests were used for the species level identification of coagulase positive *Staphylococcus* species (Table 1), *Streptococcus* species (Table 2), and gram-negative rods (Table 3) present in milk samples. Coagulase negative staphylococci were not identified to the species level, as they are considered as minor pathogens and the absence of the coagulase enzyme is sufficient for identification (NMC, 1999; Pyorala and Taponen, 2008). Gram positive rods were only gram stained and observed on BAP, since these species are rarely a cause of infection it was unnecessary to identify them (NMC, 1999; personal communication Dr. Bob Harmon).

Table 1: Media and tests used to differentiate coagulase positive staphylococci (Foster et al, 1997), (personal communication Dr. Erdal Erol). v=variable; w=weak reaction; (-) = more than 90% of species are negative

Species	Maltose	Mannitol	Trehalose
S. aureus	+	+	+
S. schleiferi ss. coagulans	-	+	v
S. lutrae	+	v	+
S. intermedius	w	v	+
S. hyicus ss. hyicus	-	-	+
S. delphini	+	+	(-)

Table 2: Reactions for tests used to identify *Streptococcus* species present in bovine milk samples (NMC 1999), (Fortin et al. 2003), (Odierno et al. 2006) (personal communication Dr. Edal Erol). A=acid (pink); R=reduction (white); C=curd; v=variable

	S.agalactiae B-hemolytic	*S.agalactiae* non hemolytic	*S. dysgalactiae*	*S. uberis*	*S.bovis*	*E. feacalis*	*Entercoccus* spp.
Lancefield group	B	B	C	no group	D	D	D
Litmus milk	A/C	R/C	A/R	A/Rv/C	A/R/C	A/R/C	A/R/C
Esculin	-	-	-	+	+	+	+
Inulin	-	-	-	+	+	-	+
Lactose		+		+	+	+v	
Maltose		+		+	+	+	
Mannitol	-	-	-	+	+ or -	+	+
Raffinose	-	-	-	+ or -	+	-	+
Salicin	-	+	-	+	+	+	+
Sorbitol	-	-	+ or -	+	-	+	+
Sucrose	+	+	+	+	+	+	+
Trehalose	+	+ or -	+	+	*	+	+
Glycogen			+ or -	-	+		
Hippurate	+	+	-	+	-	-	-

Table 3: Media and tests used to differentiate Gram-negative rods (NMC 1999).

Secondary Media and Tests for Gram-negative rods	
TSI	Fermentation of lactose, sucrose, and glucose; production of gas and hydrogen sulfide
LIA	Tests for the presence of the enzymes lysine decarboxylase and lysine deaminase
Urea	Ability to hydrolyze urea into ammonia and carbon dioxide
Simmons Citrate	To determine if citrate can be used as sole carbon source
SIM	To determine sulfur production; indole production; molitity
Bile-Esculin	Ability to hydrolyze esculin in the presence of bile
Oxidase	Production of the enzyme cytochrome oxidase
Catalase	Production of the enzyme catalase

Determination of Antibiotic Resistance:

The antibiotic resistance of all isolated *Staphylococcus*, *Streptococcus*, and Gram-negative species was determined using the Kirby-Bauer test. Each isolate was prepared in a bacterial suspension of sterile saline with turbidity equal to a 0.5 McFarland

standard. Muller-Hinton agar was used for *Staphylococcus* and Gram negative species, while Muller-Hinton agar supplemented with 5% sheep's blood (Hardy Diagnostics) was used for *Streptococcus* species. A bacterial lawn was inoculated on its respective agar plate using sterile swabs dipped into the bacterial suspension. Antibiotic agents used for routine testing in veterinary microbiology laboratories (Table 4) were chosen and placed 4 cm apart on each Mueller-Hinton agar. Plates were inverted and incubated for 18 – 24 hours, after which zones of inhibition for each agent were recorded in millimeters. Susceptibility or resistance was determined according to the interpretive standards set by the Clinical Laboratory Standards Institute (NCCLS, 2004) for bacteria isolated from animals.

Table 4: The antimicrobial agents used for Kirby-Bauer susceptibility testing of species recovered from bovine milk samples.

Antimicrobial agent	Disk Content
Ampicillin (AMP)	10 µg
Cefazolin (CZ)	30 µg
Ceftiofur (XNL)	30 µg
Cephalothin (CF)	30 µg
Erythromycin (E)	15 µg
Kanamycin (K)	30 µg
Oxacillin (OX)	1 µg
Penicillin (P)	10 units
Penicillin-novobiocin (P10/NB)	10 units/ 30 µg
Pirlimycin (PRL)	2 µg
Tetracycline (TE)	30 µg

CHAPTER 3

Results

Identification of Mastitis Pathogens:

A total of 308 milk samples were collected from 198 Kentucky dairy cows (Tables 5, 6, 7). Quarters that resulted in the growth of two organisms were isolated and counted as two samples, but recorded as a single quarter (Table 5). There were also duplicates of quarter samples (see appendix) when the farmer provided frozen samples that had been taken prior to sample collection. Duplicates were also counted as separate samples, but recorded as a single quarter. Due to contamination, 7 samples were not included in cultural analysis. Prevalence of mastitis in all milk samples was 128/308. *Staphylococcus aureus* was the major bacteria identified in milk samples, while both coagulase negative staphylococcal species (CNS) and streptococcal species were also main causes of infection. *Staphylococcus aureus* and coagulase negative staphylococci (CNS) accounted for 76/128 positive samples (Table 5). *S. aureus* was the predominant contagious agent (41/128) recovered, and *Streptococcus uberis* was the major environmental pathogen (9/128).

Determination of Antibiotic Resistance:

A total of 116 isolates were tested against 11 antibiotic agents. Gram positive rods and yeast species recovered from milk samples were not analyzed. *S. aureus* isolates were highly susceptible to all antibiotics used in this study (Table 8), and both CNS (Table 9) and streptococci species (Table 10) were highly susceptible to

cephalosporins. Gram negative rods were also susceptible to cephalosporins, as well as kanamycin (Table 11). CNS had the highest resistance to β-lactam antibiotics and pirlimycin (Table 9), while streptococcal species were resistant to oxacillin, kanamycin, and tetracycline (Table 10).

It appears that *S. aureus* is the primary cause of mastitis in this subset of Kentucky dairy herds, which was found in approximately 32% of the positive samples identified in this study. The level of resistance found for this species in the laboratory does not appear to be high.

Table 5: Total number of cows sampled from 10 dairy herds in Kentucky, and the average of individual cows and quarters sampled for each farm.

Farm Number	Cows Sampled	Quarters Sampled	Positive Quarters	Negative Quarters
1	24	30	15	15
2	27	31	18	13
3	8	10	3	7
4	17	27	9	19
5	21	36	15	21
6	25	42	10	32
7	19	28	19	9
8	11	21	11	10
9	27	41	12	29
10	19	27	15	12
Total	198	293	127	167
Average	20	29	13	17

Note: Contaminated quarters were included in the positive column to show that growth had occurred.

Table 6: The distribution of the number of cow quarters sampled per cow from 10 Kentucky dairy herds.

Farm Number	Cows Sampled from 1 Quarter	Cows Sampled from 2 Quarters	Cows Sampled from 3 Quarters	Cows Sampled from 4 Quarters
1	19	4	1	-
2	23	4	-	-
3	6	2	-	-
4	11	2	4	-
5	13	3	3	2
6	17	2	3	3
7	10	9	-	-
8	4	4	3	-
9	15	10	2	-
10	11	8	-	-
Total	129	48	16	5

Table 7: Total number of milk samples collected from 10 Kentucky dairy herds, and the percent of each pathogen present.

Species	n	%
Staphylococcus aureus	41	13
Staphylococcus delphini	1	0
CNS	35	11
Streptococcus uberis	9	3
Streptococcus dysgalactiae	7	2
Streptococcus agalactiae	1	0
Enterococcus spp.	2	1
Group A Streptococci	3	1
Group B Streptococci	1	0
Group C Streptococci	2	1
Other Streptococci spp.	7	2
Klebsiella spp.	4	1
E. coli (non motile)	1	0
Enterobacter spp.	1	0
Citrobacter spp.	1	0
G + rods	4	1
Yeasts	7	2
Non pathogenic organism	1	0
Positive Samples	128	42
Negative Samples	173	56
Contaminated	7	2
Analyzed samples	308	100

Table 8: Antibiotic susceptibility/resistance of *S. aureus*, and *S. delphini* recovered from Kentucky dairy cow milk samples.

Antibiotic	*S. aureus, S. delphini*					
	Susceptible		Intermediate		Resistant	
	n	%	n	%	n	%
Ampicillin (AMP)	40	95	0	0	2	5
Cefazolin (CZ)	42	100	0	0	0	0
Ceftiofur (XNL)	41	98	1	2	0	0
Cephalothin (CF)	41	98	0	0	1	2
Erythromycin (E)	41	98	1	2	0	0
Kanamycin (K)	42	100	0	0	0	0
Oxacillin (OX)	41	98	0	0	1	2
Penicillin (P)	39	93	0	0	3	7
Penicillin-novobiocin (P10/NB)	42	100	0	0	0	0
Pirlimycin (PRL)	42	100	0	0	0	0
Tetracycline (TE)	42	100	0	0	0	0

Table 9: Antibiotic susceptibility/resistance of coagulase negative staphylococci recovered from Kentucky dairy cow milk samples.

Antibiotic	Coagulase Negative Staphlyococci					
	Susceptible		Intermediate		Resistant	
	n	%	n	%	n	%
Ampicillin (AMP)	29	83	1	3	5	14
Cefazolin (CZ)	34	97	0	0	1	3
Ceftiofur (XNL)	34	97	0	0	1	3
Cephalothin (CF)	34	97	0	0	1	3
Erythromycin (E)	30	86	1	3	4	11
Kanamycin (K)	35	100	0	0	0	0
Oxacillin (OX)	24	69	0	0	11	31
Penicillin (P)	28	80	0	0	7	20
Penicillin-novobiocin (P10/NB)	30	86	2	6	3	9
Pirlimycin (PRL)	27	77	0	0	8	23
Tetracycline (TE)	27	77	2	6	6	17

Table 10: Antibiotic susceptibility/resistance of *Streptococcus* species recovered from Kentucky dairy cow milk samples.

Antibiotic	*Streptococcus* species					
	Susceptible		Intermediate		Resistant	
	n	%	n	%	n	%
Ampicillin (AMP)	31	97	0	0	1	3
Cefazolin (CZ)	32	100.00	0	0	0	0
Ceftiofur (XNL)	32	100.00	0	0	0	0
Cephalothin (CF)	30	94	0	0	2	6
Erythromycin (E)	28	88	1	3	3	9
Kanamycin (K)	4	13	6	19	22	69
Oxacillin (OX)	16	50	0	0	16	50
Penicillin (P)	30	94	0	0	2	6
Penicillin-novobiocin (P10/NB)	31	97	0	0	1	3
Pirlimycin (PRL)	26	81	0	0	6	19
Tetracycline (TE)	13	41	0	0	19	59

Table 11: Antibiotic susceptibility/resistance of Gram-negative rod species recovered from Kentucky dairy herd milk samples.

Antibiotic	Gram-negative rods					
	Susceptible		Intermediate		Resistant	
	n	%	n	%	n	%
Ampicillin (AMP)	1	14	0	0	6	86
Cefazolin (CZ)	6	86	0	0	1	14
Ceftiofur (XNL)	7	100	0	0	0	0
Cephalothin (CF)	6	86	0	0	1	14
Erythromycin (E)	2	29	0	0	5	71
Kanamycin (K)	7	100	0	0	0	0
Oxacillin (OX)	0	0	1	14	6	86
Penicillin (P)	0	0	0	0	7	100
Penicillin-novobiocin (P10/NB)	0	0	2	29	5	71
Pirlimycin (PRL)	0	0	0	0	7	100
Tetracycline (TE)	4	57	0	0	3	43

CHAPTER 4

Discussion

Identification of Mastitis Pathogens:

Staphylococcus aureus is a coagulase positive staphylococcal species known to be a major cause of bovine mastitis (Barkema et al., 2009; Juhasz-Kaszanyitzky et al., 2007; Middleton, n.d; Olde Riekerink et al., 2008; Wilson et al., 1997). In order to positively identify this species, presence of the coagulase enzyme is an important phenotypic determinant (Guler et al., 2005; NMC, 1999; Taponen and Pyorala, 2008), but it should be noted that several other coagulase positive staphylococcal species exist (Bannoehr et al., 2007; Devriese et al., 2005; Foster et al., 1997; Sasaki et al., 2007; Varoldo et al., 1988). Results from this study suggest that the major pathogen responsible for bovine mastitis in a coalition of Kentucky dairy cows is *Staphylococcus aureus*, which was recovered in approximately 13% of all bovine milk samples obtained. Coagulase negative staphylococcal species (CNS) and streptococcal species were also major sources of infection, representing 11% and 10% of isolates recovered in this study respectively. Previous publications have also reported *S. aureus* as the most common cause of bovine mastitis (Juhasz-Kaszanyitzky et al., 2007; Olde Riekerink et al., 2008).

S. delphini was the only other coagulase positive staphylococcal species identified in this study, based on the isolate's inability to ferment trehalose. Greater than 90% of strains within this species will be positive for acid production on trehalose, but it is possible for some strains to produce a negative result (Foster et al., 2003). Considering

the fact that each of these species are able to ferment maltose, mannitol, and trehalose, it is possible that this study misidentified *S. intermedius*, *S. delphini, or S. lutrae* as *S. aureus*. Previous studies have noted that coagulase positive staphylococcal species are commonly misidentified as *S. aureus* or *S. intermedius* (Bannoehr et al., 2007; Devriese et al., 2005; Sasaki et al., 2007). *S. delphini*, however, has not been commonly identified since it was first described as a novel species in 1988 (Bannoehr et al., 2007; Varoldo et al., 1988). The only other case of this species being documented from bovine origin occurred in Norway (Bjorland, 2007). It is possible that this species is more prevalent than the dairy industry realizes, due to the fact that the methods suggested for the identification of coagulase positive staphylococci in the National Mastitis Handbook (NMC, 1999) are not specific enough to positively identify these species. In order to confidently identify these organisms correctly, molecular methods or comprehensive phenotypic testing is required (Devriese et al., 2005).

Coagulase negative staphylococcal species were the second highest group identified in this study, representing approximately 11% of all milk samples recovered. Classification of these organisms to the species level was unnecessary, as they are considered minor pathogens that only cause mild infections (Taponen et al., 2006). Pyorala and Taponen (Pyorala and Taponen, 2008) stated that this perspective may need to be reassessed, as several studies have found CNS species to be the most common causative mastitis species (Pitkala et al., 2004; Tenhagen et al., 2006; Wilson et al., 1997). This data indicates that CNS species are now more prevalent than *S.aureus*, *S. agalactiae*, and other streptococcal species in some areas, depending on geographic location. This shift of prevalence could have resulted from a decrease in contagious

infections due to the better control practices in place. Still, the number of CNS species recovered from clinical cases of mastitis remains low (Olde Riekerink et al., 2007; Pyorala and Taponen, 2008).

Streptococcus agalactiae is known to be one of the major contagious pathogens causing bovine mastitis (Barkema et al., 2009; Keefe, G. P., 1997; Zoldoks and Fitzpatrick, 2009). However, results from my study suggest that this species is not a significant pathogen in Kentucky. After the introduction of standard control practices in the 1960s, *S.agalactiae* infections have become more sporadic (Zoldoks and Fitzpatrick, 2009), as they are susceptible to penicillin therapy causing them to be easily eradicated in a closed herd (Keefe, 1997). Even so, intramammary infections due to this species are still common. For example, a 2004 study in Brazil found 60% of their herds positive for *S. agalactiae* (Duarte et al. 2004). In 2006, a study in Germany reported 28.7% of herds samples were positive for *S. agalactiae* (Tenhagen et al., 2006).

The identification of streptococcal species recovered in this study was based on several publications (Facklam 2002; Fortin et al., 2003; NMC 1999), and personal communication with Dr. Erdal Erol. When species level or group identification could not be made, isolates were classified as other streptococcal species. It is possible that some of these isolates were *Enterococcus* species, *Lactococcus* species, or *S. uberis* (Fortin et al., 2003). *Entercococci* species belong to the Lancefield group G (NMC, 1999), which was not found in any of the seven isolates classified as other streptococcal species. Even so, the latex agglutination test alone is not sufficient for identification (Facklam, 2002). Use of API 20 STREP test is recommended in order to identify these isolates (Fortin et. al., 2003).

All non-*agalactiae* streptococcal species identified in this study represented approximately 10% of milk samples recovered, making them the third most prevalent group recovered. Nine of these isolates were identified as *S. uberis* (3%), seven as *S. dysgalactiae* (2%), and two as *Enterococcus* species (1%). This group represented a higher overall percentage of the milk cultures when compared to what was found in Germany (Tenhagen et al., 2006) and the United States (Wilson et al. 1997). In contrast, a 2011 study from Estonia found a much greater overall prevalence of streptococci species, reporting that *S. uberis* was identified in 18.4% of milk samples recovered (Kalmus et al., 2011).

Antibiotic Resistance and Susceptibility:

S. aureus and *S. delphini* species identified were overall found to be highly susceptible to the antibiotics used in this study. This finding was inconsistent with what previous publications have shown. De Oliveira et al. (2000) found that a significant number of their isolates contained the enzyme β-lactamase, which renders lactam antibiotics ineffective. It was also found in Portugal that 66.7% of their *S. aureus* isolates also contained β-lactamase and were resistant to penicillin (Nunes et al., 2007). *S.aureus* species analyzed by Guler et al. (2005) showed higher resistance to penicillin, ampicillin, and tetracycline, when compared to my results, and only 29.8% of their strains were susceptible to all antibiotics. Kalmus et al. (2011) found approximately 60% of their isolates resistant to penicillin and ampicillin.

CNS species showed some resistance to oxacillin (31%), pirlimycin (23%), and penicillin (20%). Results for this study showed increased resistance to oxacillin,

penicillin/novobiocin, and pirlimycin for CNS when compared to what was found in Germany (Luthje, P. and S. Schwartz, 2006). Similar to the level of oxacillin resistance observed, Nunes et al. in Portugal (2007) reported that 77.4% of their *S. epidermidis* isolates were positive for β-lactamase, and 29% were resistant to oxacillin. Most CNS isolates from this study were susceptible to ampicillin (83%), which is comparable to what was reported in Germany (Luthje, P and S. Schwartz, 2006). Kanamycin and the cephalosporins showed 100% and 97% susceptibility respectively, and had the greatest bactericidal effect of all agents used. This indicates that these antibiotics could effectively be used to treat CNS infections.

Streptococcal species identified in this study showed their highest resistance to kanamycin (69 %), tetracycline (59%), and oxacillin (50%). When compared to earlier studies in Uruguay (Gianneechini et al., 2002) and Estonia (Kalmus et al., 2011), these species represented an increased resistance to tetracycline. Oxacillin showed the smallest bactericidal effect of all β- lactams antibiotics used on these isolates, which agreed with Nam et al. (2009). Streptococcal isolates were also overall very susceptible to cephalothin and penicillin, agreeing with studies done in Korea (Nam et al., 2009), Uruguay (Gianneechini et al., 2002), and Estonia (Kalmus et al., 2011).

For the nine confirmed isolates of *S. uberis*, six (67%) were resistant to kanamycin and oxacillin, while three (33%) were resistant to tetracycline. Five (71%) isolates of *S. dysgalactiae* were resistant to kanamycin, while all seven (100%) were resistant to tetracycline. This increased resistance to tetracycline seen with *S. dysgalactiae* when compared to *S. uberis* was also found in Uruguay (Gianneechini et al., 2002). Of the other streptococcal species unable to be grouped or identified to the

species level, 71% were resistant to kanamycin and tetracycline, while 57% were resistant to oxacillin.

The only isolate of *S. agalactiae* found in this study was 100% susceptible to all antibiotics, which is in contrast to what was reported by Nam et al. (2009) and Kalmus et al. (2011). Considering only one isolate was identified, this result could easily change if more isolates of this species were obtained from a larger study. It is also possible that *S. agalactiae* is not prevalent in Kentucky's DHIA farms due to the control measures in practice.

Gram negative rods were only recovered in approximately 2% of milk samples. Even so, these cases present an increasing issue of highly resistant strains against which antibiotic treatment is problematic. At least one of these isolates were resistant to 9 out of 11 (82%) of the antimicrobials they were tested against, which is in agreement with resistance patterns reported by Nam et al. (2009). Overall, they were highly resistant to β-lactam antibiotics, erythromycin, and pirlimycin. However, all gram negative isolates were 100% susceptible to ceftiofur and kanamycin, suggesting that these two agents would be effective in treating infections caused by this group of bacteria.

Conclusion:

It is not often cost effective for the farmer to culture every cow when an infection has been indicated. Results from studies like this can be beneficial to farmers and veterinarians even when culturing is not completed by providing resistance and/or susceptibility information about common pathogens in their geographical area.

Varying degrees of antibiotic resistance patterns were observed in the species identified during this study. Overall, gram-negative rods and CNS showed the highest resistance to β-lactam antibiotics, while *S. aureus* and *Streptococcus* species showed the highest susceptibility. Both *S. aureus* and CNS identified in this study were highly susceptible to kanamycin (100%) and cephalosporins (> 97%). *Streptococcus* species were highly susceptible to ampicillin, penicillin, penicillin-novobiocin, and erythromycin. *S. aureus* and CNS were 100% susceptible to kanamycin, while 69% of *Streptococcus* species were resistant.

The majority of bovine mastitis infections in the United States have been reportedly treated with cephaloporin, β-lactam, and lincosamide antibiotics (USDA, 2008). Results from this study indicated that cephalosporins would be the most effective agents to use for antibiotic therapy in the cows from which bacteria were cultured. Even so, the susceptibility patterns observed in the laboratory can vary greatly from the cure rates observed in the udder environment. There are several factors that contribute to this discrepancy. One is that the standards set for determining antimicrobial susceptibility or resistance in common mastitis pathogens are based on human reports, and do not take into account the specific udder environment (Nunes et al., 2007). Virulence factors, such as the formation of biofilms, slime layers, and capsules, are also undoubtedly a major contributor, which enable microorganisms to evade death resulting from both innate and extrinsic microbicidal elements. It is also known that chronic infections of *S. aureus* can cause the bacteria to become walled off, effectively separating them from leukocytes and antibiotics (NMC – "A practical look", n.d.). A previous study (Hoe and Ruegg, 2005)

even attempted to determine a relationship between results of in vitro susceptibility testing and the cure rate of cows with clinical mastitis, but no relationship was found.

If this study were to be continued, the collection of milk samples from a greater proportion of herds across the state would be recommended in order to better represent the prevalence of pathogens in the state of Kentucky. The sampling strategy might also be changed to a stratified random sampling, and each cow selected could be sampled from all quarters instead of relying on the CMT to identify potentially infected quarters.

Results of the CMT have also been stated to be subjective, thus a more efficient method of rapidly estimating infections in cows would be useful in future studies. Recently, another such method has been developed by Dairy Quality Inc., which utilizes a device attached to an iPhone. Once this device is attached to the iPhone, a sample of milk can be placed into the device for analyses. Within a few seconds the application determines the SCC, and also suggests the most probable pathogen causing infection or whether a clinical infection is present (Dairy Quality Inc., 2012). The testing device claims to actually count the cells, so it is possible that causative pathogens are suggested due to the proportion of leukocytes present within the milk samples. An interesting direction this study could take would be to compare results between the use of this iPhone device to results found using the CMT, cell counts by flow cytometry, and either conventional microbiological methods or next generation sequencing for species identification.

It would also be advised to increase the sensitivity of the identification protocol for streptococcal species, through use of the API 20 STREP test. As an alternative to microbiological diagnostics, next generation sequencing could be used as a fast and

effective method to identify species isolated from milk samples. Another recommendation would be to determine what antibiotic resistance genes are present in the species identified by using primers specific for known resistance genes and PCR amplification.

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APPENDIX

Table 12: Reactions of species present in milk samples from Farm 1 (Mercer County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
Royal RR	S. agalactiae	132	grey β-hemolytic	N/G	G+ cocci	-	N/A	+	+	+
Danica RR	Enterococcus spp.	348	grey	N/G	G+ cocci	-	N/A	+	+	+
Missy LR	S. aureus	650	grey /white	N/G	G+ cocci	+	+	+	+	+
Ruby RR	S. aureus	528	grey/white	N/G	G+ cocci	+	+	+	+	+
Melanie LF	S. aureus	492	yellow	N/G	G+ cocci	+	+	+	+	+
Deedra RR	S. aureus	38	grey β-hemolytic	N/G	G+ cocci	+	+	+	+	+
Missy LF	S. delphini	650	white β-hemolytic	N/G	G+ cocci	+	+	-	+	+
Style RR	CNS	115	beige	N/G	G+ cocci	+	-	N/A	N/A	N/A
Legacy RF	CNS	460	yellow	N/G	G+ cocci	+	-	N/A	N/A	N/A
Dayna RR	CNS	57	white	N/G	G+ cocci	+	-	N/A	N/A	N/A
Royal RR	CNS	132	white	N/G	G+ cocci	+	-	N/A	N/A	N/A
791 LR	CNS	54	white	N/G	G+ cocci	+	-	N/A	N/A	N/A
Classic LR	CNS		white	N/G	G+ cocci	+	-	N/A	N/A	N/A

Table 13: Reactions for *Streptococcus* species present in milk samples from Farm 1 (Mercer County, KY).

Sample Name	Species ID	Esculin	Inulin	Lactose	Raffinose	Salicin	Sorbitol	Sucrose	Glycogen	Hippurate	Litmus milk	Latex Agg
Royal RR	S. agalactiae	-	-	+	-	-	-	+	-	+	A/C	Group B
Danica RR	Enterococcus spp.	+	+	+	+	+	+	+	-	-	A/R/C	Group D

Table 14: Kirby-Bauer test results for species identified in milk samples from Farm 1 (Mercer County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
Royal RR	S. agalactiae	S	S	S	S	S	S	S	S	S	S	S
Danica RR	Enterococcus spp.	R	S	S	S	R	R	R	R	R	R	S
Missy LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
Ruby RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
Melanie LF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
Deedra RR	S. aureus	R	S	I	S	I	S	R	R	S	S	S
Missy LF	S. delphini	S	S	S	S	S	S	S	S	S	S	S
Style RR	CNS	S	S	S	S	S	S	S	S	S	S	S
Legacy RF	CNS	S	S	S	S	S	S	S	S	S	S	S
Dayna RR	CNS	S	S	S	S	S	R	R	S	S	S	R
Royal RR	CNS	S	S	S	S	S	S	S	S	S	S	S
791 LR	CNS	S	S	S	S	S	S	S	S	S	S	S
Classic LR	CNS	S	S	S	S	S	S	S	S	S	S	S

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 15: Reactions of species present in milk samples from Farm 2 (Taylor County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
121LF	Streptococcus spp.	857	grey	N/G	G+ cocci	-	N/A	+	+	-
1025RF	Group A Strep	492	grey	N/G	G+ cocci	-	N/A	N/A	N/A	N/A
901LF	Group C Strep	1838	grey	N/G	G+ cocci	-	N/A	+	+	+
1088RF	S. aureus	919	grey/white	N/G	G+ cocci	+	+	+	+	+
10RF	S. aureus	606	grey/white	N/G	G+ cocci	+	+	+	+	+
1052RR	S. aureus	919	grey/white	N/G	G+ cocci	+	+	+	+	+
1108LR	S. aureus	152	yellow	N/G	G+ cocci	+	+	+	+	+
1043RF	S. aureus	746	β-hemo grey/white	N/G	G+ cocci	+	+	+	+	+
1064RR	S. aureus	141	white/yellow	N/G	G+ cocci	+	+	+	+	+
1111LR	S. aureus	696	white	N/G	G+ cocci	+	+	+	+	+
1111RR	S. aureus	696	white/yellow	N/G	G+ cocci	+	+	+	+	+
1088RR	S. aureus	919	grey / white	N/G	G+ cocci	+	+	+	+	+
08RR	S. aureus	528	grey / white	N/G	G+ cocci	+	+	+	+	+

Table 15 (continued):

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
1150RR	CNS	303	grey / white	N/G	G+ cocci	+	-	N/A	N/A	N/A
147RR	CNS	325	gold	N/G	G+ cocci	+	-	N/A	N/A	N/A
939LF	Gram + rod	1393	transparent	N/G	G+ rod	-	N/A	N/A	N/A	N/A
864RR	Gram + rod	746	mucoid grey	N/G	G+ rod	+	N/A	N/A	N/A	N/A
1025RF	E. coli	492	mucoid grey	pink	G- rod	+	N/A	N/A	N/A	N/A

Table 16: Reactions for *Streptococcus* and Gram-negative rod species present in milk samples from Farm 2 (Taylor County, KY).

Sample Name	Species ID	Esculin	Inulin	Lactose	Raffinose	Salicin	Sorbitol	Sucrose	Glycogen	Hippurate	Litmus milk	Latex Agg
121LF[a]	Streptococcus spp.	+	-	+	-	-	+	+	+	+	A/R/C	No rxn
901LF[b]	Group C Streptococcus	-	+	+	-	-	+	-	-	-	A/R	Group C
1025RF	Group A Streptococcus											Group A
		Sulfur	Indole	Motility	Urea	Bile	LIA	Cit	TSI	H_2S		
1025RF	E. coli	-	-	-	-	+	K/A	-	A/A	-		

Notes: 121 LF[a] – Negative results on mannitol, inulin, and salicin rendered this species unidentifiable. Use of the API 20 STREP test is recommended. 901 LF[b] – The Lancefield group C latex agglutination reaction alone is not sufficient in order to identify *S. dysgalactiae*. This isolate was positive for inulin and mannitol, which rendered this species unidentifiable. Use of the API 20 STREP test is recommended.

Table 17: Kirby-Bauer test results for species identified in milk samples from Farm 2 (Taylor County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
121LF	Streptococcus spp.	S	S	S	S	S	R	S	S	S	S	R
1025RF sm	Group A Strep	S	S	S	S	S	R	R	S	S	S	S
901LF	Group C Strep	S	S	S	S	S	R	R	S	S	R	S
1088RF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
10RF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1052RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1108LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1043RF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1064RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1111LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1111RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1088RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
08RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1150RR	CNS	S	S	S	S	S	S	S	S	S	S	S
147RR	CNS	S	S	S	S	S	S	S	S	S	S	S
1025RF big	E. coli	S	S	S	S	S	S	R	R	R	R	S

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 18: Reactions of species present in milk samples from Farm 3 (Adair County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
436 LR	S. aureus	283	grey/white β-hemo	N/G	G+ cocci	+	+	+	+	+
263 RF	CNS	283	grey/white	N/G	G+ cocci	+	-	N/A	N/A	N/A
436 LF	Enterobacter spp.	283	beige carpet	Pink	G- rod	weak +	N/A	N/A	N/A	N/A

Table 19: Reactions of Gram-negative rod species present in milk samples from Farm 3 (Adair County, KY).

Sample Name	Species ID	Sulfur	Indole	Motility	Urea	Bile	LIA	Cit	TSI	H_2S	Malonate
436 LF	Enterobacter spp.	-	-	-	+	+	K/K	+	A/Ag	-	+

Table 20: Kirby-Bauer test results for species identified in milk samples from Farm 3 (Adair County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
486 LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
263 RF	CNS	R	S	S	S	R	S	R	R	S	R	R
436 LF	Enterobacter spp.	R	S	S	S	R	S	I	R	R	R	S

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 21: Reactions of species present in milk samples from Farm 4 (Adair County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
986 LR	S. uberis	325	white α-hemo	N/G	G+ cocci	-	N/A	+	+	+
1087 LR	S. dysgalactiae	696	grey α-hemo	N/G	G+ cocci	-	N/A	+	+	-
1160 LR	S. aureus	283	gold/white β-hemo	N/G	G+ cocci	+	+	+	+	+
1039 LR	S. aureus	650	gold/white β-hemo	N/G	G+ cocci	+	+	+	+	+
986 LF	S. aureus	325	grey/white β-hemo	N/G	G+ cocci	+	+	+	+	+
893 RR	S. aureus	3940	grey/white β-hemo	N/G	G+ cocci	+	+	+	+	+
893 LF	S. aureus	3940	grey/white β-hemo	N/G	G+ cocci	+	+	+	+	+
961 LR	Bacillus spp.		mucosal white filamentous	N/G	G+ rod	+	N/A	N/A	N/A	N/A

Table 22: Reactions of *Streptococcus* species present in milk samples from Farm 4 (Adair County, KY).

Sample Name	Species ID	Esculin	Inulin	Lactose	Raffinose	Salicin	Sorbitol	Sucrose	Glycogen	Hippurate	Litmus milk	Latex Agg
986 LR	S. uberis	+	+	+	+	+	-	+	-	+	A	No group
1087 LR	S. dysgalactiae	-	-	+	-	-	-	-	+	-	A/R	Group C

Table 23: Kirby-Bauer test results for species identified in milk samples from Farm 4 (Adair County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
986 LR	S. uberis	S	S	S	S	R	R	R	S	S	R	R
1087 LR	S. dysgalactiae	S	S	S	S	S	I	S	S	S	S	R
893 RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1160 LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
893 LF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
986 LF	S. aureus	R	S	S	R	S	S	S	R	S	S	S
1039 LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 24: Reactions of species present in milk samples from Farm 5 (Green County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
607 LF small	S. dysgalactiae	8445	grey α-hemo	N/G	G+ cocci	-	N/A	+	+	-
607 LF big	Streptococcus spp.	8445	white	N/G	G+ cocci	-	N/A	+	+	+
252 LF tiny	S. uberis	400	transparent	N/G	G+ cocci	-	N/A	+	+	+
252 LF big	Group A Strep	400	transparent β-hemo	N/G	G+ cocci	-	N/A	-	-	-
642 LR	Streptococcus spp.	264	white	N/G	G+ cocci	-	N/A	+	+	+
118	S. aureus	650	grey/white β-hemo	N/G	G+ cocci	+	+	+	+	+
387 BS RF	S. aureus	1970	grey/white	N/G	G+ cocci	+	+	+	+	+
369 BS LR	S. aureus	1838	grey/white	N/G	G+ cocci	+	+	+	+	+
473 LF	CNS	528	white	N/G	G+ cocci	+	N/A	N/A	N/A	N/A
638 RF	CNS	9701	yellow β-hemo	N/G	G+ cocci	+	N/A	N/A	N/A	N/A
647 RR Moya	CNS	800	white	N/G	G+ cocci	+	N/A	N/A	N/A	N/A
387 BS RR	K. pneumoniae	1970	white raised β-hemo	pink raised	G- rod	+	N/A	N/A	N/A	N/A

Table 24 (continued):

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
470 RR	Yeast	800	transparent	N/G	G+	+	N/A	N/A	N/A	N/A
470 LF	Yeast	800	transparent	N/G	G+	+	N/A	N/A	N/A	N/A
470 LR	Yeast	800	transparent	N/G	G+	+	N/A	N/A	N/A	N/A
465 LR	Yeast	76	white	N/G	G+	+	N/A	N/A	N/A	N/A
470 RF	Yeast	800	transparent	N/G	G+	+	N/A	N/A	N/A	N/A

Table 25: Reactions of *Streptococcus* and Gram-negative rod species present in milk samples from Farm 5 (Green County, KY).

Sample Name	Species ID	Esculin	Inulin	Lactose	Raffinose	Salicin	Sorbitol	Sucrose	Glycogen	Hippurate	Litmus milk	Latex Agg
607 LF small	S. dysgalactiae	-	-	+	-	-	-	+	+	-	A/R	Group C
607 LF big[a]	Streptococcus spp.	+	-	+	-	-	-	+	-	+	R/C	no rxn
252 LF tiny	S. uberis	+	+	+	-	+	+	+	-	+	A/R	no rxn
252 LF big	Group A Strep	-	-	-	-	-	-	-	-	-	Alk	Group A
642 LR[b]	Streptococcus spp.	+	-	+	-	+	+	+	-	+	A/R/C	no rxn
		Sulfur	Indole	Motility	Urea	Bile	LIA	Cit	TSI	H_2S		
387 BS RR	K. pneumonia	-	-	-	+	+	K/K	+	A/Ag	-		

Notes: 607 LF big[a]; 642 LR[b] – Due to the negative inulin results, it is possible that these isolates could be *S. uberis, Enterococcus*, or *Lactococcus* species. All three of these species can have a negative result on inulin. Enterococci species belong to the Lancefield group D, but this test alone is not sufficient for identification.

Table 26: Kirby-Bauer test results for species identified in milk samples from Farm 5 (Green County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
607 LF	S. dysgalactiae	S	S	S	S	S	R	S	S	S	S	R
607 LF	Streptococcus spp.	S	S	S	S	S	R	S	S	S	S	R
252 LF	S. uberis	S	S	S	S	S	I	R	S	S	S	S
252 LF	Group A Strep	I	S	S	R	S	S	R	R	S	R	S
642 LR	Streptococcus spp.	S	S	S	S	S	R	R	S	S	S	R
118	S. aureus	S	S	S	S	S	S	S	S	S	S	S
387 BS RF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
369 BS LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
473 LF	CNS	R	R	R	R	S	S	R	R	S	R	R
638 RF	CNS	S	S	S	S	S	S	R	R	R	R	S
647 RR Moya	CNS	S	S	S	S	R	S	R	R	R	R	S
387 BS RR	K. pneumoniae	R	S	S	S	S	S	R	R	R	R	S

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 27: Reactions of species present in milk samples from Farm 6 (Taylor County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
531 LF	S. aureus	429	yellow	N/G	G+ cocci	+	+	+	+	+
258 LF	S. aureus	373	yellow	N/G	G+ cocci	+	+	+	+	+
6138 RF	S. aureus	373	grey/white	N/G	G+ cocci	+	+	+	+	+
531 LR	CNS	429	yellow/white	N/G	G+ cocci	+	-	N/A	N/A	N/A
60 RR	CNS	200	yellow	N/G	G+ cocci	+	-	N/A	N/A	N/A
242 RR	CNS	3676	white	N/G	G+ cocci	+	-	N/A	N/A	N/A
236 RR	Yeast	1838	white	N/G	yeast	+	N/A	N/A	N/A	N/A
201 RF	Non-pathogenic organism (not bacteria)	246	white	N/G	unknown	+	N/A	N/A	N/A	N/A

Table 28: Kirby-Bauer test results for species identified in milk samples from Farm 6 (Taylor County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
531 LF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
258 LF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
6138 RF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
531 LR	CNS	S	S	S	S	S	S	S	R	S	S	S
60 RR	CNS	S	S	S	S	S	S	S	S	S	S	S
242 RR	CNS	S	S	S	S	S	S	R	S	S	S	S

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 29: Reactions of species present in milk samples from Farm 7 (Washington County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
670 LF	E. faecalis	1715	white	N/G	G+ cocci	-	N/A	+	+	+
501 RF	S. uberis	1600	grey	N/G	G+ cocci	-	N/A	+	+	+
634 RR	S. uberis	5572	grey	N/G	G+ cocci	-	N/A	+	+	+
388 RF	S. uberis	5199	grey	N/G	G+ cocci	-	N/A	+	+	+
634 RF	S. uberis	5572	grey	N/G	G+ cocci	-	N/A	+	+	+
634 RF	Streptococcus spp.	5572	white	N/G	G+ cocci	-	N/A	+	+	+
885 LR	S. aureus	1131	golden	N/G	G+ cocci	+	+	+	+	+
436 LF	S. aureus	985	golden β-hemo	N/G	G+ cocci	+	+	+	+	+
855LF	S. aureus		golden	N/G	G+ cocci	+	+	+	+	+
1822 LR	S. aureus	492	golden β-hemo	N/G	G+ cocci	+	+	+	+	+
646 LR	S. aureus	1300	golden	N/G	G+ cocci	+	+	+	+	+

Table 29 (continued):

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
810 LF	CNS	1056	grey	N/G	G+ cocci	+	-	N/A	N/A	N/A
685 LR	CNS	6400	grey	N/G	G+ cocci	+	-	N/A	N/A	N/A
1801 RR	CNS	115	golden	N/G	G+ cocci	+	-	N/A	N/A	N/A
855 RR	CNS		golden weak β-hemo	N/G	G+ cocci	+	-	N/A	N/A	N/A
685 RR	CNS	6400	grey	N/G	G+ cocci	+	-	N/A	N/A	N/A
715 RR	CNS	174	grey	N/G	G+ cocci	+	-	N/A	N/A	N/A
478 LF	CNS	1838	golden	N/G	G+ cocci	+	-	N/A	N/A	N/A
478 LF	CNS	1838	grey	N/G	G+ cocci	+	-	N/A	N/A	N/A
670 LF	K. oxytoca	1715	mucoid cream	mucoid pink	G- rods	+	-	N/A	N/A	N/A
685 LR	K. pneumoniae	6400	mucoid cream	mucoid pink	G- rods	+	-	N/A	N/A	N/A
493 RF	G+ rod	1838	wet β-hemo	N/G	G+ rods	+	N/A	N/A	N/A	N/A

Table 30: Reactions of *Streptococcus* and Gram-negative rod species present in milk samples from Farm 7 (Washington County, KY).

Sample Name	Species ID	Esculin	Inulin	Lactose	Raffinose	Salicin	Sorbitol	Sucrose	Glycogen	Hippurate	Litmus milk	Latex Agg
670 LF	E. faecalis	+	-	+	-	+	+	+	-	-	A/R/C	Group D
501 RF	S. uberis	+	+	+	+	+	+	+	-	+	A/C	No group
634 RR	S. uberis	+	+	+	+	+	+	+	-	+	A/C	No group
388 RF	S. uberis	+	+	+	+	+	+	+	-	+	A/C	No group
634 RF	S. uberis	+	+	+	+	+	+	+	-	+	A/C	No group
634 RF[a]	Streptococcus spp.	-	+	+	+	+	+	+	-	-	A/C	No group
		Sulfur	Indole	Motility	Urea	Bile	LIA	Cit	TSI	H_2S		
670 LF	K. oxytoca	-	+	-	+	+	K/K	+	A/Ag	-		
685 LR	K. pneumonia	-	-	-	+	+	K/K	+	A/Ag	-		

Note: 634 RF[a]**:** Due to the negative results on esculin and hippurate, as well as the absence of a latex agglutination reaction, this species could not be identified. Further testing is necessary. Use of API 20 STREP test is recommended.

Table 31: Kirby-Bauer test results for species identified in milk samples from Farm 7 (Washington County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
670 LF	E. faecalis	S	S	S	R	I	I	R	S	S	R	R
501 RF	S. uberis	S	S	S	S	S	I	R	S	S	S	S
634 RR	S. uberis	S	S	S	S	S	I	R	S	S	S	R
388 RF	S. uberis	S	S	S	S	S	R	S	S	S	S	S
634 RF	Streptococcus spp.	S	S	S	S	S	S	R	S	S	R	R
885 LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
436 LF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
855 LF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
1822 LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
646 LR	S. aureus	S	S	S	S	S	S	S	R	S	S	S
810 LF	CNS	S	S	S	S	S	S	S	S	S	S	S
685 LR	CNS	R	S	S	S	R	S	R	R	I	R	R
1801 RR	CNS	S	S	S	S	S	S	S	S	S	S	S
855 RR	CNS	S	S	S	S	S	S	S	S	S	S	S
685 RR	CNS	R	S	S	S	R	S	R	R	I	R	R
715 RR	CNS	S	S	S	S	S	S	R	S	S	S	R
478 LF	CNS large golden	S	S	S	S	S	S	S	S	S	S	I
478 LF	CNS tiny grey	S	S	S	S	S	S	S	S	S	S	S
493 RF	G+ rod	S	S	S	S	S	S	S	S	S	S	S
670 LF	K. oxytoca	R	S	S	S	R	S	R	R	I	R	R
685 LR	K. pneumoniae	R	S	S	S	R	S	R	R	I	R	R

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 32: Reactions of species present in milk samples from Farm 8 (Henry County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
Rae LR	S. uberis	132	grey	N/G	G+ cocci	-	N/A	+	+	+
Rae RR	S. uberis	132	white	N/G	G+ cocci	-	N/A	+	+	+
40 LR	Streptococcus spp.	650	α-hemo grey	N/G	G+ cocci	-	N/A	+	+	+
Star LF	S. uberis	1970	white	N/G	G+ cocci	-	N/A	+	+	+
118 LR	S. dysgalactiae	919	α-hemo, grey round	N/G	G+ cocci	-	N/A	+	+	-
118 RR	Group C Strep	919	grey flat	N/G	G+ cocci	-	N/A	+	+	-
58 RR	S. dysgalactiae	800	α-hemo, grey round	N/G	G+ cocci	-	N/A	+	+	-
54 LF	S. aureus	746	β-hemo, grey/white	N/G	G+ cocci	+	+	+	+	+
Rae LR	CNS	132	yellow	N/G	G+ cocci	+	-	N/A	N/A	N/A
58 LF	CNS	800	beige	N/G	G+ cocci	+	-	N/A	N/A	N/A

Table 33: Reactions of *Streptococcus* species present in milk samples from Farm 8 (Henry County, KY).

Sample Name	Species ID	Esculin	Inulin	Lactose	Raffinose	Salicin	Sorbitol	Sucrose	Glycogen	Hippurate	Litmus milk	Latex Agg
Rae LR	S. uberis	+	+	+	-	+	+	+	-	+	A/C	no group
Rae RR	S. uberis	+	+	+	+	+	+	+	-	+	A/C	no group
40 LR[a]	Streptococcus spp.	+	-	+	-	+	+	+	-	+	A/C	no group
Star LF	S. uberis	+	+	+	+	+	+	+	-	+	A/C	no group
118 LR	S. dysgalactiae	-	-	+	-	-	-	+	+	-	A/R	Group C
118 RR[b]	Group C Strep	+	-	+	-	-	-	+	-	-	A/R	Group C
58 RR	S. dysgalactiae	-	-	+	-	-	-	+	+	-	A/R	Group C

Notes: 40 LR[a] – Due to the negative inulin result, it is possible that this isolate could be an *Enterococcus* or *Lactococcus* species or *S. uberis*. All three of these species can have a negative result on inulin. Entercococci species belong to the Lancefield group G, but this test alone is not sufficient for identification. 118 RR[b] – Due to the positive esculin result, this species could not be identified. Further testing is necessary. Use of API 20 STREP test is recommended.

Table 34: Kirby-Bauer test results for species identified in milk samples from Farm 8 (Henry County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
Rae LR	S. uberis	I	S	S	S	S	R	R	S	S	S	S
Rae RR	S. uberis	S	S	S	S	S	R	S	S	S	S	S
118 LR	S. dysgalactiae	S	S	S	S	S	R	S	S	S	S	R
118 RR	Group C Strep	S	S	S	S	S	R	S	S	S	S	R
40 LR	Streptococcus spp.	S	S	S	S	S	R	R	S	S	S	S
58 RR	S. dysgalactiae	S	S	S	S	S	R	S	S	S	S	R
Star LF	S. uberis	S	S	S	S	S	R	S	S	S	S	S
Rae LR	CNS	S	S	S	S	S	S	S	S	S	S	R
58 LF	CNS	S	S	S	S	S	S	S	S	S	S	S
54 LF	S. aureus	S	S	S	S	S	S	S	S	S	S	S

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 35: Reactions of species present in milk samples from Farm 9 (Lincoln County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
R38 RR	Group B Strep	1393	grey	N/G	G+ cocci	-	N/A	+	+	+
W146 RR	Streptococci spp.	650	grey	N/G	G+ cocci	-	N/A	+	+	+
O94 LF	Streptococci spp.	492	white	N/G	G+ cocci	-	N/A	+	+	+
Y45 RF	S. dysgalactiae	400	grey α-hemo	N/G	G+ cocci	-	N/A	+	+	-
O94 LF	Group A Strep	492	white	N/G	G+ cocci	-	N/A	+	+	+
R21 LR	S. aureus	1131	grey/white	N/G	G+ cocci	+	+	+	+	+
O80 LF	S. aureus	200	yellow	N/G	G+ cocci	+	+	+	+	+
Y74 RR	CNS	100	gold	N/G	G+ cocci	+	-	N/A	N/A	N/A
R38 LF	CNS	1393	gold	N/G	G+ cocci	+	-	N/A	N/A	N/A
R25 RF	CNS	81	grey mucoid	N/G	G+ cocci	+	-	N/A	N/A	N/A
R6 RR	CNS	62	grey	N/G	G+ cocci	+	-	N/A	N/A	N/A
Y32 LR	CNS	100	yellow	N/G	G+ cocci	+	-	N/A	N/A	N/A
R21 LR	Citrobacter spp.	1131	grey mucoid	pink mucoid	G- rods	+		N/A	N/A	N/A

Table 36: Reactions of *Streptococcus* and Gram-negative rod species present in milk samples from Farm 9 (Lincoln County, KY).

Sample Name	Species ID	Esculin	Inulin	Lactose	Raffinose	Salicin	Sorbitol	Sucrose	Glycogen	Hippurate	Litmus milk	Latex Agg
R38 RR[a]	Group B Strep	+	+	+	-	+	+	+	+	+	purple	Group B
W146 RR[b]	Streptococcus spp.	+	-	+	-	+	+	+	-	+	A/C	No group
O94 LF[c]	Streptococcus spp.	+	-	+	-	+	+	+	-	+	A/C	No group
Y45 RF	S. dysgalactiae	-	-	+	-	-	-	+	+	-	A/R	Group C
O94 LF	Group A Strep	+	+	+	-	+	+	+	-	-	A/C	Group A
		Sulfur	Indole	Motility	Urea	Bile	LIA	Cit	TSI	H_2S	Malonate	
R21 LR	Citrobacter spp.	-	-	+	+		K/A	+	K/Ag	-	+	

Notes: R38 RR[a] – This species was not identified as *S. agalactiae* due to the positive results for mannitol, esculin, inulin, and sorbitol. Even so, the national mastitis council states that *S. agalactiae* can be identified based on the positive Lancefield group B agglutination reaction. This species was also non hemolytic. W146 RR[b]; O94 LF[c] – These isolates were negative for inulin, indicating that they could possibly be an *Enterococcus* species, *Lactococcus* species, or *S. uberis*. All three of these species can have a negative result on inulin. Entercococci species belong to the Lancefield group G, but this test alone is not sufficient for identification. The API 20 Strep test is recommended.

Table 37: Kirby-Bauer test results for species identified in milk samples from Farm 9 (Lincoln County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
R38 RR	Group B Strep	I	S	S	S	S	R	S	S	S	S	R
W146 RR	Streptococcus spp.	S	S	S	S	S	R	R	S	S	S	R
Y45 RF	S. dysgalactiae	S	S	S	S	S	R	S	S	S	S	R
O94 LF	Streptococcus spp.	S	S	S	S	S	S	S	S	S	S	S
O94 LF	Group A Strep	S	S	S	S	R	R	R	S	S	S	R
R21 LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
O80 LF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
Y74 RR	CNS	S	S	S	S	S	S	R	S	S	S	S
R38 LF	CNS	S	S	S	S	S	S	S	S	S	S	S
R25 RF	CNS	S	S	S	S	S	S	S	S	S	S	S
R6 RR	CNS	S	S	S	S	S	S	S	S	S	S	S
Y32 LR	CNS	S	S	S	S	S	S	R	S	S	S	S
R21 LR	Citrobacter spp.	R	R	S	R	R	S	R	R	R	R	S

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant

Table 38: Reactions of species present in milk samples from Farm 10 (Oldham County, KY).

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehalose	Maltose	Mannitol
953 LF	S. uberis	1056	grey	N/G	G+ cocci	-	N/A	+	+	+
956 LR	S. dysgalactiae	1213	grey α-hemo	N/G	G+ cocci	-	N/A	+	+	-
871 RR frozen	S. dysgalactiae	283	grey α-hemo	N/G	G+ cocci	-	N/A	+	+	-
914 RR	S. aureus	2111	grey/white	N/G	G+ cocci	+	+	+	+	+
912 RF	S. aureus	606	grey/white β-hemo	N/G	G+ cocci	+	+	+	+	+
903 RR	S. aureus	1970	grey/white β-hemo	N/G	G+ cocci	+	+	+	+	+
958 LR	S. aureus	2263	gold	N/G	G+ cocci	+	+	+	+	+
874 RR	S. aureus	4851	gold	N/G	G+ cocci	+	+	+	+	+
958 RR	S. aureus	2263	yellow	N/G	G+ cocci	+	+	+	+	+
914 RF	S. aureus	2111	grey/white β-hemo	N/G	G+ cocci	+	+	+	+	+

Table 38 (continued):

Sample Name	Species ID	SCC	BAP colonies	MAC colonies	Gram stain	Catalase	Coagulase	Trehaose	Maltose	Mannitol
855 RF	CNS	528	gold β-hemo	N/G	G+ cocci	+	-	N/A	N/A	N/A
956 LR	CNS	1213	gold	N/G	G+ cocci	+	-	N/A	N/A	N/A
958 LR	CNS	2263	white	N/G	G+ cocci	+	-	N/A	N/A	N/A
958 RR	CNS	2263	white	N/G	G+ cocci	+	-	N/A	N/A	N/A
855 LF	CNS	528	gold	N/G	G+ cocci	+	-	N/A	N/A	N/A
913 RR	Yeast	746	small white	N/G	G+ budding cells	N/A	N/A	N/A	N/A	N/A
743 RR	K. pneumoniae	857	large, wet cream	large, wet, light pink	G- rods	N/A	N/A	N/A	N/A	N/A

Table 39: Reactions of *Streptococcus* and Gram-negative rod species present in milk samples from Farm 10 (Oldham County, KY).

Sample Name	Species ID	Esculin	Inulin	Lactose	Raffinose	Salicin	Sorbitol	Sucrose	Glycogen	Hippurate	Litmus milk	Latex Agg
953 LF	S. uberis	+	+	+	-	+	+	+	-	+	A/C	No group
956 LR	S. dysgalactiae	-	-	+	-	-	+	+	+	-	A	Group C
871 RR frozen	S. dysgalactiae	-	-	+	-	-	+	+	+	-	A	Group C
		Sulfur	Indole	Motility	Urea	Bile	LIA	Cit	TSI	H_2S		
743 RR	K. pneumoniae	-	-	-	+	+	K/K	+	A/Ag	-		

Table 40: Kirby-Bauer test results for species identified in milk samples from Farm 10 (Oldham County, KY).

Sample Name	Species	AM	CZ	XNL	CF	E	K	OX	P	P10/NB	PRL	TE
953 LF	S. uberis	S	S	S	S	S	R	R	S	S	S	R
956 LR	S. dysgalactiae	S	S	S	S	S	R	S	S	S	S	R
871 RR frozen	S. dysgalactiae	S	S	S	S	S	I	S	S	S	S	R
914 RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
912 RF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
903 RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
958 LR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
874 RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
958 RR	S. aureus	S	S	S	S	S	S	S	S	S	S	S
914 RF	S. aureus	S	S	S	S	S	S	S	S	S	S	S
855 RF	CNS	S	S	S	S	S	S	S	S	S	S	S
956 LR	CNS	S	S	S	S	S	S	S	S	S	S	R
958 LR	CNS	S	S	S	S	S	S	S	S	S	S	S
958 RR	CNS	S	S	S	S	S	S	S	S	S	S	S
855 LF	CNS	S	S	S	S	S	S	S	S	S	S	S
743 RR	K. pneumoniae	R	S	S	S	R	S	R	R	R	R	R

Note: Antimicrobial susceptibilities reported were based on the Clinical and Laboratory Standards Institute guidelines.
S = Susceptible; I = Intermediate; R = Resistant