# *Escherichia coli* mastitis in the Dairy Bovine

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Treatment and Diagnosis of *Escherichia coli* Mastitis in the Dairy Bovine

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(ABSTRACT)

Diagnosis techniques and treatments for *Escherichia coli* mastitis in the dairy bovine were evaluated in two experiments. The first experiment evaluated eosin methylene blue agar as a method of distinguishing *E.coli* from other gram-negative mastitis pathogens. *Escherichia coli* will usually produce a green metallic sheen on eosin methylene blue agar. One hundred and twenty-nine milk samples or gram-negative isolates from milk samples were used to compare eosin methylene blue agar to a commercial biochemical test strip (the accepted standard). There was an intermethod agreement of 96.9% and a κ-value of 93.7% indicating excellent agreement beyond chance between test methods. Eosin methylene blue agar is a reliable method for differentiation of *E. coli* from other gram-negative mastitis pathogens. The second experiment evaluated the efficacy of frequent milk-out as a treatment for *E. coli* mastitis. Sixteen Holstein dairy cows were divided into 2 blocks and randomly assigned to 1 of 4 treatment groups: 1) non-infected, not frequently milked-out, i.e. not treated (NI-NT), 2) experimentally infected with *E. coli*, not treated (EC-NT), 3) non-infected, frequently milked-out (NI-FMO), and 4) experimentally infected with *E. coli,* frequently milked-out (EC-FMO). Hours to bacterial, clinical and systemic cure were not different between the EC-NT and EC-FMO treatment groups. Serum α-lactalbumin concentrations were evaluated between treatment groups as a measure of udder health. Serum α-lactalbumin concentrations were higher in cows in the EC-NT treatment group than cows in the NI-

NT, NI-FMO and EC-FMO treatment groups at 12 hours post-experimental challenge. Serum α-lactalbumin concentrations were higher in cows in the NI-FMO treatment group than in cows in the NI-NT, EC-NT and EC-FMO treatment groups at 36 hours post-experimental challenge. Results from this study do not support frequent milk-out as a treatment for *E. coli* mastitis.

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# Chapter 1

## Introduction

Mastitis is an economically important disease on U. S. dairy farms. Mastitis pathogens include both contagious and non-contagious microorganisms. Non-contagious pathogens include environmental *Staphylococcus* spp., environmental *Streptococcus* spp., and coliforms. The coliforms include gram-negative pathogens such as *Escherichia coli*, *Klebsiella* spp., *Serratia* spp., and *Enterobacter* spp. Coliform mastitis often accounts for 20-80% of acute clinical mastitis cases on many dairies.

Clinical cases of coliform mastitis typically occur in the first 70 days of a cow's lactation and usually persist for less than 7 days although chronic cases have been reported. Clinical signs of coliform mastitis range from mild, where the cow is not systemically ill, to death. Most of the clinical signs are produced by bacterial endotoxins. Pyrexia, depression, anorexia, weakness, recumbancy, diarrhea, rumen stasis, watery mammary secretions, tachycardia and tachypenia are some of the signs associated with clinical coliform mastitis. These clinical signs are not exclusive to coliform mastitis, so rapid identification usually through milk culturing is necessary to determine the appropriate treatment protocol.

Treatments generally focus on counteracting the effects of endotoxin release. Supportive therapies such as oral and/or intravenous fluids, anti-inflammatories and systemic antibiotics are standard practice. Intramammary antibiotics are controversial as there is no effective product labeled for intramammary treatment of mastitis caused by coliform bacteria. Frequently milking-out of infected quarters with the aid of oxytocin is

also a commonly recommended practice. The theory behind this practice is that frequent removal of bacteria and their toxins will result in a quicker recovery.

## Chapter 2

## Literature Review

Microbiology

*Escherichia coli*, a lactose fermenting, gram-negative facultative aerobic rod associated with bovine mastitis, is a member of the coliform group of bacteria. The coliform group includes other mastitis pathogens such as *Klebsiella* spp., *Serratia* spp., and *Enterobacter* spp.[1] There are four main serotypes of *E. coli* identified based on antigens O, K, H, and F.[2] Serotypes O, K, and H have been associated with clinical cases of bovine mastitis.[3] Serum resistance is the only apparent virulence characteristic related to pathogenicity of *E. coli* isolated from acute mastitis cases.[4,5] Barrow and Hill experimentally challenged lactating cows intramammarily with serum resistant and serum sensitive *E. coli*. Cows challenged with serum resistant *E. coli* developed clinical mastitis and the organism was recovered from the mammary gland. Serum sensitive *E. coli* did not produce clinical mastitis and was not recovered from the mammary gland.[5]

The cell wall of gram-negative bacteria is composed of a lipopolysaccharide (LPS) layer which consists of three components: a hydrophobic Lipid A layer, a middle oligosaccharide layer and an outer hydrophillic polysaccharide layer, where the O or K antigen is located. In general, the O antigen plays a role in serum resistance of *E. coli* possibly by preventing the membrane attack complex (MAC) from inserting into the cell membrane or by altering it's formation inhibiting lysis of the cell. The K antigen forms a capsule preventing phagocytosis. The LPS, also referred to as endotoxin, is responsible for clinical signs associated with gram-negative sepsis.[5]

Host Defenses

The teat canal and teat sphincter provide physical barriers to mastitis pathogens, damage to either of these barriers can lead to mastitis. Clinical mastitis in recently calved cows can be induced when a few colonies of *E. coli* are injected into the teat canal.[6] Teat canal diameter, depth, composition and distribution of the keratin layer also play a role in protecting cows from mastitis pathogens.[7] Plasma cells in the keratin layer and Furstenbergs rosette may also provide a local defense mechanism during the initial stages of bacterial invasion.[8]

Somatic cells are the next defense system in the mammary gland. Somatic cells in the noninfected mammary gland primarily consist of macrophages followed by neutrophils, lymphocytes and epithelial cells.[9] Neutrophils are the predominant cell type during the early dry period followed by macrophages and lymphocytes.[10] A few days prior to parturition macrophages become the most predominant cell type followed by neutrophils and lymphocytes.[11] The low number of neutrophils[12] and/or poor recruitment of neutrophils into the mammary gland during an infection could account for an increased incidence of *E. coli* mastitis during the periparturient period.[13, 14]

Once bacteria have invaded the mammary gland, humoral substances in the milk become activated. The LPS (endotoxin) in the cell wall of *E. coli* activates the complement system which recruits neutrophils and causes opsonization or lysis of the bacteria.[15] Encapsulated coliform bacteria are resistant to the complement system.[16] Lactoferrin is another anti-bacterial defense mechanism, since it binds iron which is essential for growth of *E. coli*. Concentrations of lactoferrin are higher in dry cow

secretions and during infections than in normal milk.[17] Normal milk also contains higher concentrations of citrate which inhibits lactoferrin activity,[18] perhaps this explains why *E. coli* infections are more common around parturition. Mammary immunoglobulins are of limited value in cases of *E. coli* mastitis although they do assist in the phagocytic process.[19]

The speed and magnitude of the neutrophil response determines the severity of *E. coli* mastitis.[20] A quick response will usually result in mild clinical disease which may go undetected, conversely an inadequate neutrophil response may allow rapid bacterial multiplication, endotoxin release and severe to fatal systemic disease.[21]

Clinical Disease

Mastitis caused by coliform bacteria can result in mild, moderate or severe disease. Most cases of coliform infections result in clinical mastitis (80-90%)[22] but only a small portion (10%)[23] result in severe disease. Approximately 70% of coliform intramammary infections occur in cows less than 30 days in lactation.[22] Infections are typically less than 7 days duration though approximately 13% can become chronic, existing for greater than 100 lactation days. Chronic coliform infections are typically caused by pathogens other than *E. coli*.[22] Most coliform infections are eliminated spontaneously and approved antibiotics are seldom useful.[24] Prevalence of intramammary coliform infections on a dairy farm is typically less than 2%.[22]

Severe disease is usually characterized by an acute onset and associated with cows in early lactation.[22] Clinical signs include pyrexia, depression, anorexia, rumen stasis, tachycardia, tachypenia, diarrhea, weakness, dehydration and in some cases

recumbancy.[25] Affected quarters may not be apparent for a few hours after the onset of clinical signs. Clinical signs associated with the mammary gland may include swelling and firmness with a serum-like or watery secretion with or without clots. Many of the clinical signs are in response to endotoxin release.[25]

Endotoxin is released during bacterial killing or multiplication. Once released it is a neutrophil chemoattractant, recruiting neutrophils from the circulating pool into the mammary gland, initiating a complex cascade of immunological events.[26] Once neutrophils migrate into the mammary gland, they participate in phagocytosis of bacterial cells which is enhanced by opsonization. Opsonization is the immunological recognition of bacteria by attachment of antibodies, particularly IgG and IgM.[27] Neutrophils then engulf and kill the bacteria,[27] later dying and releasing their contents. Locally produced cytokines (interleukin-1, interleukin-6 and tumor necrosis factor-α) are responsible for some of the local (swelling, redness, pain) and systemic (pyrexia, depression, increased production of inflammation-reducing proteins by the liver) reactions.[24] This immune response may not function very effectively during early lactation and may be another reason why severe infections are noted more often during that time period. Cows in early lactation that have reduced liver function or abnormalities, such as ketosis or hepatic lipidosis are more likely, if infected, to exhibit signs of severe coliform mastitis. The liver is a major endotoxin detoxification center in addition to producing inflammation reducing proteins.[20, 24] Additionally cows with low somatic cell counts and impaired neutrophil migration into the mammary gland, such as occurs in early lactation, tend to exhibit more severe disease.[12] Endotoxin also activates the cyclo-oxygenase and

lipoxygenase enzymatic pathways producing arachadonic acid metabolites which initiate additional immunological responses.

Coliform mastitis can also result in mild or moderate disease. Affected cows may not be severely systemically ill, or not apparently even sick. Abnormal secretions, mammary gland swelling, anorexia and pyrexia may occur. Some cows can be chronically infected, usually with no continuous systemic signs of disease, but they often exhibit periodic flare ups.[28,29]

Leukopenia, neutropenia and lymphopenia with a left shift are typical findings in cows with severe coliform mastitis.[30] Neutropenia is due to pooling of neutrophils in the udder and/or due to a reduced number of circulating neutrophils secondary to cortisol release during stress.[31] Some cows in early lactation may experience a neutrophilia. This could be the result of reduced neutrophil recruitment and movement into the mammary gland.[30] The more severe the left shift the more likely the cows are to be bacteremic, although this parameter is not useful
as a prognostic indicator, some biochemical parameters can be used in this manner.[30]

Hemoconcentration, uremia, high aspartate aminotransferase levels, hypokalemia, hyponatremia, hypochloridemia, acid-base derangements[29] and hyocalcemia[31] are common biochemical abnormalities in cows with coliform mastitis. Hemoconcentration is likely due to dehydration or a response to protein sequestration in the mammary gland[32] or gastrointestinal tract.[30] Uremia is usually a consequence of dehydration and/or renal insufficiency due to endotoxic shock.[33] Elevated blood urea nitrogen and creatinine values suggest a poor prognosis.[31] Acid-base abnormalities vary depending on the severity of mastitis. Cows that survive typically have a metabolic alkalosis,

hyponatremia, hypokalemia, and hypochloridemia.[30] Cows that die as a result of coliform mastitis are often acidotic due to reduced cardiac function.[30] Hypocalcemia is due to anorexia and gastrointestinal stasis (impaired absorption).[25] The number of bacteria in the milk can also be a prognostic indicator. Milk with bacterial numbers greater than $10^6$/ml will likely result in a more severe mastitis because neutrophil function is often impaired.[21] Conversely bacterial numbers less than $10^3$/ml suggest adequate neutrophil function and generally result in a mild or moderate case of mastitis.[21]

Health and function of the mammary gland can be measured using a variety of methods. Somatic cell counts are the most common and readily available measurement to evaluate mammary gland health and function. Cows that have or have had coliform mastitis will usually have elevated somatic cell counts for a several weeks post-infection.[34, 35] Alpha-lactalbumin has been used in several studies as a measure of mammary gland integrity.[36, 37] Alpha-lactalbumin is a milk protein that is part of the lactose sythetase pathway which occurs in the alveolar epithelial cells.[36] As somatic cell count increases, so follows α-lactalbumin (r=0.60).[37] Serum α-lactalbumin can also be used to assess mammary gland function. Days of gestation, stage of mammary development, and milking frequency alter serum α-lactalbumin concentrations. Serum α-lactalbumin concentrations were measured in primiparous Holstein cows pre, during and postpartum. Concentrations were low (<300 ng/ml) until the day of parturition when they rapidly rose to over 900 ng/ml. Concentrations rapidly decreased after parturition but did not return to prepartum levels by day 4 postpartum.[38] Cows milked twice daily as compared to cows milked three times daily had higher serum α-lactalbumin concentrations, suggesting intramammary pressure affects α-lactalbumin absorption.[38]

Alpha-lactalbumin has been thought to move into circulation by several methods, leakage through tight junctions, absorption through the basolateral membrane, reabsorption, and leakage through areas devoid of alveolar epithelial cells.[38] The relevance of elevated serum α-lactalbumin concentrations in clinical mastitis remains unclear.

Diagnosis

Diagnosing the etiology (gram-negative versus gram-positive) of an individual case of mastitis on the farm, at the time of treatment, is not accurate (62%).[39] Choosing an appropriate therapy based on an inaccurate diagnosis can result in the choice of an ineffective antibiotic that may result in an unnecessary drug expense and unnecessary milk withhold for the dairy producer.

The accuracy of predicting the etiologic agent (gram-negative or gram-positive) of cows with a clinical cases of mastitis using a diagnostic decision tree was evaluated in a study by White et al.[39, 40] Variables evaluated in the decision tree included: history of previous mastitis in the affected quarter, weakness, clear or white color of the milk, swelling of the mammary gland, watery consistency of the milk, lack of previous mastitis in the other quarters, lack of palpable mammary gland abscesses, and a high body temperature.[39,40] Clinicians evaluated cases of clinical mastitis that had not been treated with antibiotics within the previous 24 hours, recorded information about the variables listed above, their prediction of the etiologic agent (gram-negative or gram-positive) and collected milk samples for culture.[39, 40] In the training set, 78% of the clinical mastitis cases were classified correctly using the decision tree .[40] Applying this system to another population of cows with clinical mastitis resulted in an accuracy of 71% and clinician

prediction accuracy of 62%, both significantly better than chance.[39] Another study compared clinicians prediction of clinical mastitis caused by gram-negative or gram-positive bacteria to culture results. Clinicians correctly predicted the cause of mastitis only 51% of the time.[41] Level of experience, greater or less than 3 years, did not significantly change the prediction accuracy.[41] The best way to determine the cause of clinical mastitis is to perform a bacteriological culture of a milk sample.

There are many methods for identifying mastitis caused by gram-negative pathogens. The Production Management Medicine Department at the Virginia-Maryland Regional College of Veterinary Medicine (Figure 2-1) uses a protocol with which results can be obtained within 24 - 36 hours. The potassium hydroxide (KOH) test can be used to differentiate gram-negative from gram-positive colonies. A few drops of a 3% solution of KOH is placed on a glass slide, then a bacterial colony is transferred into the KOH and mixed. Thickening of the KOH is a positive reaction indicating the bacterial colony is gram-negative.[42] An isolated colony is then plated on to MacConkey and eosin methylene blue (EMB) agars and cultured for further identification. *Escherichia coli* and *Klebsiella* spp. organisms will usually appear pink on MacConkey. *Escherichia coli* colonies will typically produce a green metallic sheen when plated on EMB agar whereas *Klebsiella* spp. will typically lack the green metallic sheen.[43]

There are several other methods, used more often in food safety laboratories, for distinguishing *E. coli* from other gram-negative pathogens. A common biochemical test profile, IMVic, is often used to identify *E. coli*.[44] Tests within this profile includes assays for bacterial utilization of indole, methyl red, Voges-Proskauer and citrate. The typical pattern seen with *E. coli* is (++--) or (-+--), however if the test organism is contaminated

with other coliform organisms the test is unreliable.[44] The beta-glucuronidase test has also been used to identify *E. coli*. Researchers compared the traditional IMVic test to the β-glucuronidase test to identify suspect *E. coli* colonies isolated on EMB agar from raw meat and meat by-products. The β-glucuronidase test was more rapid (3 hours) and more sensitive (97.1%) than the IMVic tests (up to 4 days and 80.9%, respectively).[45] Beta-glucuronidase, in combination with the colony morphology on MacConkey agar, indole, and oxidase tests, is an inexpensive and rapid method of identifying *E. coli* isolates from other gram-negative pathogens in veterinary medicine.[46]

Several different media, EMB, violet red bile agar (VRBA), Petrifilm High Sensitivity Coliform Count plates (PHSCCP), Trypticase soy agar with a violet red bile overlay (TSA/VRBA), were evaluated for use in discriminating *E. coli* from other coliform bacteria in apple cider. *Escherichia coli* was the easiest to identify using the PHSCCP. Eosin methylene blue agar was not very effective in identifying *E. coli* as the acidic pH of apple cider may have altered the reaction of the agar resulting in a large number of false positives.[47] The dyes in EMB agar, eosin and methylene blue, combine and form a precipitant (green metallic sheen) at an acid pH.[43] One study reported atypical reactions of *E. coli* on EMB agar. Atypical reactions included complete lack or inconsistent production of the green metallic sheen. These reactions were likely due to varying pH levels within the EMB agar.[48]

Bacterialogical culturing of the milk sample is an important tool for determining the bacterial etiology of clinical mastitis. Rapid results are possible allowing an appropriate treatment plan to be formulated in a reasonable amount of time.

Treatment

Treatment protocols for clinical coliform mastitis are varied and controversial. Historically systemic and/or intramammary antibiotics, oral and/or intravenous fluids, anti-inflammatory drugs and frequent milk-out of the affected quarter with the aid of oxytocin have been used in various combinations based on the severity of disease. The use of antibiotics is probably one of the most controversial aspects of treatment.

Many coliform infections are self-limiting and will exhibit a spontaneous cure without antibiotics. Moreover, there are cows that may die or become unproductive members of the herd irrespective of antibiotic therapy.[24] Two antibiotics that coliform bacteria are sensitive to in *vitro*, polymixin B and gentamicin are not approved for use in food animals.[49] Ceftiofur is an antibiotic that is effective against coliform bacteria and is approved for use in food animals but is not currently approved for intramammary use.[49] Often by the time the cow exhibits clinical signs of mastitis, the host response has already reduced or eliminated the bacterial load and antibiotic therapy is of little value.[50] Antibacterial therapy should be designed to inhibit the growth of bacteria so as to minimize the cows exposure to more endotoxin release and to prevent the infection from becoming chronic.[49]

The success of an antibiotic depends on it's ability to reach the mammary gland at an effective concentration for an adequate amount of time.[51] The ability of a systemic drug to passively cross the blood milk barrier depends on three factors, lipid solubility, degree of ionization and protein-binding ability. The more lipid soluble, less ionized, and the less protein bound the antibiotic the quicker the antibiotic will enter the mammary gland.[52] The extent to which a drug ionizes is dependant on the dissociation

constant of the drug and the pH of its surroundings. Normal milk is more acidic than blood. Antibiotics that are weak bases tend to accumulate in higher concentrations in milk than in blood, while antibiotics that are weak acids usually reach lower concentrations in milk than in blood. Mastitic milk has a pH similar to that of blood allowing antibiotics to obtain concentrations in the mammary gland similar to those in blood.[53] Additionally inflammation in the mammary gland may prevent the intramammary antibiotic from being distributed effectively into the mammary gland.[52] Concentration gradients also play a role, the larger the concentration gradient, the quicker an antibiotic should diffuse into the mammary gland. Half-life also influences the effectiveness of an antibiotic.[52]

Systemic use of oxytetracycline, ampicillin or ceftiofur as initial therapy in cases of clinical mastitis suspected to be caused by coliform bacteria is common practice. Currently there is only one intramammary antibiotic preparation, Hetacin-K®, labeled for use in mastitis caused by gram-negative organisms.[54]

There have been several studies that have investigated the use of antibiotics as a therapy for coliform mastitis. Ceftiofur has been shown to have excellent activity against gram-negative bacteria *in vitro*, however it does not obtain therapeutic levels in the mammary gland and is not approved for intramammary use.[55] There may be some benefit to using ceftiofur to reduce the incidence of bacteremia.[56] Gentamicin is another antibiotic that has been frequently studied as a treatment for coliform mastitis, although it is not an approved antibiotic for use in food animals. Eight mid-lactation Holstein cows were experimentally challenged with *E. coli* in one quarter post-milking. Half of the cows received an intramammary infusion of 500 mg gentamicin at each milking for 4

treatments beginning14 hours post-experimental challenge. The remaining 8 cows were not treated and served as controls. Gentamicin did not affect the severity or duration of the experimental infection.[57, 58] Jones and Ward (1990) evaluated cows with naturally occurring mastitis caused by gram-negative organisms. The cows were placed into three treatment groups, intramuscular gentamicin, intramuscular erythromycin and no intramuscular antibiotics. All cows were treated with flunixin meglumine and the affected quarters were milked out several times a day and treated with intramammary cephaparin. Cows that were dehydrated received either intravenous or oral fluids and electrolytes. There was no difference in recovery between cows treated with systemic antibiotics and those not treated with systemic antibiotics at either 24 hours or 4 weeks post-experimental challenge.[59] Twelve cows in a 1994 study were experimentally challenged with *E. coli* in two quarters, three to four weeks apart. Cows were randomly assigned to one of two treatment groups, systemic trimethoprim-sulfonamide or non-treated control. This study did not find any benefit to using antibiotic therapy for treatment of experimentally induced *E. coli* mastitis.[60] In 1993 Guterbock et al. randomly assigned mild cases of clinical mastitis on three California dairies to one of three treatment groups; intramammary amoxicillin, intramammary cephapirin and intramuscular oxytocin at milkings. Twenty-six percent of the pre-treatment isolates were coliform bacteria. There were no differences in clinical cure rate (return of quarter and milk to normal) or bacterial cure of coliform mastitis cases between treatment groups.[61]

Frequent milk-out has been recommended as a treatment for mastitis since at least as early as 1869, “The bad milk should be drawn three to four times a day, for by

remaining in the bag, it tends to increase inflammation."[62] There are several early references recommending frequent milk-out as a therapy for mastitis or garget.[63, 64, 65, 66] Today frequent milk-out is still part of the recommended therapy for coliform mastitis though there are very few studies that have evaluated the efficacy of this practice.[21] Intramammary cephapirin and amoxicillin treatments were compared to oxytocin at milking time for cows with naturally occurring mild (no systemic involvement) cases of clinical mastitis. Cows that became worse or did not improve were considered treatment failures and were removed from the study. There were no differences between treatments in clinical cure rates by the ninth milking or bacterial cure rates by day 21 between treatments.[67] In another study, Morin et al evaluated cows with naturally-occurring clinical mastitis. The cows were given a severity score and assigned to a treatment group. Control cows were frequently milked-out on varying schedules based on their severity score and those with severe mastitis also received oral or intravenous fluids and flunixin meglumine. Cows in the antibiotic treatment groups were evaluated, given a severity score and also assigned to a treatment group. The antibiotic treatment groups were treated the same as the control cows with the addition of intramammary cephapirin or intravenous oxytetracycline based on severity score. Cows with coliform mastitis that received antibiotics had higher clinical cure rates by the tenth milking than cows that did not receive antibiotics and were just frequently milked out.[68] In a 1994 study, intramammary antibiotics were compared to frequent milk-out treatments in cows with naturally occurring clinical mastitis. Cows were randomly assigned to one of two treatment groups, intramammary cephapirin or oxytocin and frequent milk-out. There

was no difference in bacterial cure between treatment groups.[69] All of these studies lack a non-treated control group for comparison.

There are only a few reported studies that have compared frequent milk-out to no treatment for cases of coliform mastitis. In one study, cows with abnormal milk were evaluated and placed in one of two treatment groups, frequent milk-out or no frequent milk-out. Cows in the frequent milk-out group were given intramuscular oxytocin and milked-out by hand for a total of 4 times between regular milkings. Cows with coliform mastitis that were frequently milked-out as compared to those cows not frequently milked-out had longer days to clinical and bacteriologic cures suggesting there may be no advantage of frequently milking-out cows with coliform mastitis.[70] A Swedish study evaluated the efficacy of frequent milk-out with the administration of oxytocin in cases of clinical mastitis. According to this study there were no positive effects of frequent milk-out in the treatment of acute clinical mastitis.[71]

Supportive therapy for cases of moderate to severe coliform mastitis is not as controversial as antibiotic use and frequent milk-out therapy. Many cows with moderate to severe coliform mastitis are dehydrated, hypocalcemic and in shock due to the effects of endotoxin release. Intravenous fluids are optimal as gastrointestinal motility in cows with endotoxic shock is reduced. As much as 20-60 liters of a balanced electrolyte solution may be necessary to replace deficits and ongoing losses.[72] It is impractical for field veterinarians to carry this volume of fluids on their trucks and it is just as impractical to administer this volume of fluids on the farm. Hypertonic saline (7.5%) has become a practical alternative.[73] Hypertonic saline is thought to be of clinical benefit because it expands circulating blood volume and tissue perfusion due to redistribution of

body fluids.[74] There are no apparent undesired side-effects of this treatment.[74, 75] Most cows will voluntarily consume oral fluids post hypertonic saline administration. There are many recipes for oral fluids available, most recipes contain a combination of alfalfa meal, calcium, yeast, potassium, sodium, chloride and a glucose precursor.

Anti-inflammatory drugs, flunixin meglumine, dexamethasone, ketoprofen, flurbiprofen, carprophen, ibuprofen, phenylbutazone and aspirin have been used to treat cows with coliform mastitis.[76] Flunixin meglumine is probably the most commonly used anti-inflammatory for cases of moderate to severe coliform mastitis.[77] Phenylbutazone, aspirin and flunixin meglumine are non-steroidal anti-inflammatory drugs that inhibit cyclooxygenase and the formation of arachadonic acid metabolites, reducing inflammation, pyrexia and pain. In one study twelve cows were inoculated intramammarily with endotoxin, half of the cows received flunixin meglumine (1.1 mg/kg every eight hours beginning two hours post-inoculation) while half received saline on the same schedule. Pyrexia was significantly reduced and attitude was significantly improved in cows that were treated with flunixin meglumine. Other parameters that appeared to be improved by the administration of flunixin meglumine were quarter temperature, edema, swelling and pain.[78] A study by Dascanio et al in 1995 compared treatment with flunixin megluime or phenylbutazone to no treatment. Cows with naturally occurring acute clinical mastitis received intramammary gentamicin and were assigned to 1 of 3 treatment groups, intravenous saline, intravenous flunixin meglumine or intravenous phenylbutazone. Cows were evaluated and treated within 6 hours of detection of mastitis. All 3 groups had significantly reduced rectal temperatures in 24 hours. Milk production losses were not different between treatment groups.[79]

Dexamethasone is a steroidal anti-inflammatory that has been studied as a treatment for cases of coliform mastitis. Steroids act to reduce inflammation by interfering with the release of arachadonic acid, reducing clinical signs such as udder edema and swelling.[76] Several studies have evaluated the efficacy of steroids as a treatment for coliform mastitis. Six cows were experimentally challenged with *E. coli* in the rear quarters, three cows were given dexamethasone intramuscularly and the remaining three cows received saline immediately post-challenge. Dexamethasone treated cows milk production reduction, signs of inflammation, reduction in rumen contractions and neutropenia were less severe than cows not treated with dexamethasone. However dexamethasone treated cows had more severe pyrexia than cows not treated with dexamethasone.[80] A study by Anderson and Hunt in 1989 did not obtain similar results. Twenty-one lactating cows were experimentally challenged with endotoxin and assigned to one of three treatment groups; saline, one intravenous dose of dexamethasone (0.44mg/kg), and two intravenous doses of flunixin meglumine (1.1mg/kg) two hours post-challenge. Dexamethasone was responsible for the greatest reduction in rectal temperature, a decrease in milk production and an increased number of circulating leukocytes. Flunixin meglumine did not affect milk production or leukocyte response.[81] Both of these studies administered the anti-inflammatory drugs in close proximity to the experimental challenge rather than waiting until clinical signs were evident which would be a more realistic approach.

Cows with moderate to severe coliform mastitis are often hypocalcemic.[76] Cows with severe coliform mastitis are at a greater risk of calcium induced fatal cardiac arrhythmias than cows with uncomplicated postparturient hypocalcemia or milk fever.

Calcium borogluconate can be administered intravenously or subcutaneously. Oral calcium therapy has also been used.[82]

Treatment of coliform mastitis is varied, however fluid and anti-inflammatory therapy are generally considered beneficial. Antibiotic therapy and the use of frequent milk-out remain controversial. As with many disease conditions prevention is preferred to treatment.

Prevention

Coliform organisms are everywhere in a cow's environment. Control programs should focus on three areas, housing/environment, milking procedures and vaccination. Cows are most likely to obtain a coliform infection near parturition or during the very early dry period.[22] Special attention should be placed on maternity pens and close-up dry cow/heifer facilities. These areas should be well bedded, dry and not over crowded.[83] Sand has become the preferred bedding, as it does not support bacterial growth. Unfortunately the manure management systems on many dairy operations are not designed to handle the abrasive nature of sand. Sawdust and shavings are more commonly used bedding materials on a dairy operation. There are higher numbers of coliform organisms, particularly *Klebsiella* spp., isolated in sawdust and shavings than in straw.[84] Numbers of coliform bacteria from clean sawdust or shavings range from 4.4 x $10^6$ to 6 x $10^6$ cfu/gm of bedding.[85] Daily removal of wet bedding and the addition of clean, dry bedding can reduce the buildup of coliform organisms and provide a cleaner environment for the cows.[85] The addition of lime to the bedding or to the rear of the

stalls has been suggested to help keep stalls dry, however this is not a substitute for good stall management.[85]

Milking time hygiene is another area where control measures can be instituted. Coliform infections typically occur between milkings as a result of an unclean environment contaminating the teats. Vaccination against coliform mastitis is not very common on dairy operations. Approximately 18% and 27% of producers vaccinate heifers and cows, respectively with an *E.coli* mastitis vaccine.[86] Vaccination usually reduces the severity of clinical disease, not the incidence of coliform mastitis.[87] The core antigen (lipopolysaccharide) of gram-negative bacteria is immunogenic and similar between different species of gram-negative bacteria.[21] These characteristics have been used in the development of a vaccination for gram-negative mastitis. A mutant strain of *E. coli* O111:B4 (J5) which has an exposed core antigen, is used in two commercially available vaccines, J-VAC® (Merial) and *Escherichia coli* Bacterin J5 Strain® (Pharmacia and Upjohn). The manufacturer of J-VAC® recommends two vaccinations, one at dry off and another two weeks prior to parturition. The J5 Bacterin® manufacturer recommends an additional vaccination at parturition. A study compared the efficacy of these two vaccines against experimental *E. coli* challenge. Twenty-four Jersey cows were divided into three treatment groups, J-VAC®, J5 Bacterin® and a non-treated control. There were no differences in the severity of clinical signs, severity of mastitis or bacterial clearance between the two vaccination treatment groups, however the bacterial numbers isolated from the vaccinated groups were lower than those isolated from the non-vaccinated group at 144 hours post-challenge.[88] In another study, *Escherichia coli* Bacterin J5® strain was evaluated in primigravid heifers. Ten heifers were vaccinated with J5 Bacterin® at 60

and 48 days prior to parturition and again within 48 hours postpartum. Four heifers were untreated controls. All heifers were experimentally challenged with *E. coli*. Vaccinated heifers had a reduced severity of clinical signs and a reduced duration of intramammary infection as compared to the non-vaccinated heifers.[89] Similar results were obtained in a study using multiparous cows.[90] While vaccination appears to lessen the severity and duration of coliform mastitis, it is not a substitute for good management practices.

Diagnosis and treatment of coliform mastitis are the focus of the following two experiments. Rapid identification of *E. coli* mastitis is important for establishing a prudent treatment plan. Eosin methylene blue agar evaluated as a method of distinguishing *E. coli* from other gram-negative mastitis pathogens. It is hypothesized that bacteria which produce a green metallic sheen when inoculated on to EMB agar is a reliable indicator that the bacteria is *E. coli*. Frequent milk-out as a treatment for *E. coli* mastitis has not been extensively studied yet it has been promoted as a beneficial. One study by Roberson suggests there is no benefit to this practice.[70] The objective of the second study has was to evaluate the efficacy of frequent milk-out as a treatment of *E. coli* mastitis. It is hypothesized that there will be no benefit to using frequent milk-out for cows with experimentally induce *E. coli* mastitis.

Figure

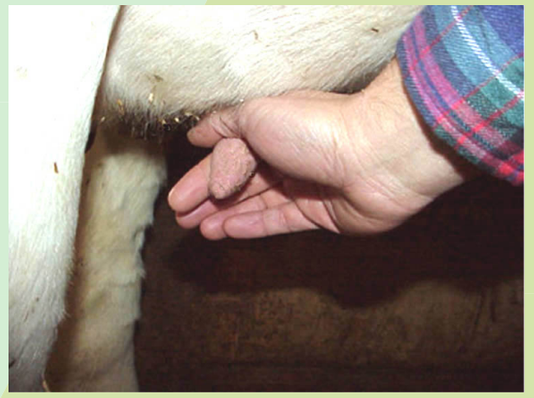
2-1

Milk culturing on the farm.

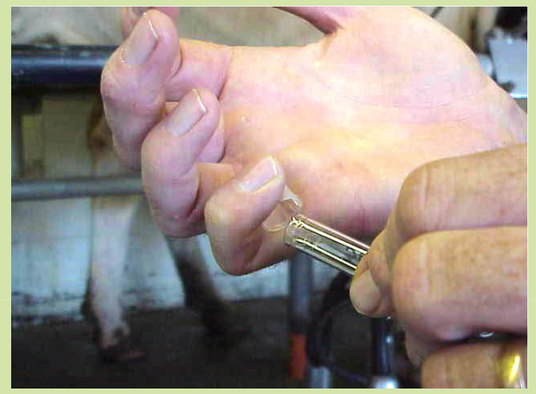
# Milk Culture Flow Chart

## How to Take a Culture

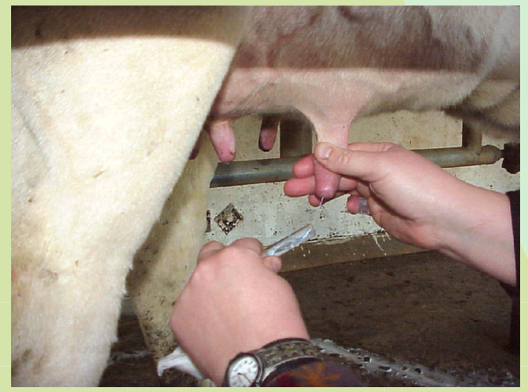
Disinfect the teat, especially the teat end with an alcohol soaked gauze pad.



Pre-strip



Grasp the lid with the pinky of your milking hand

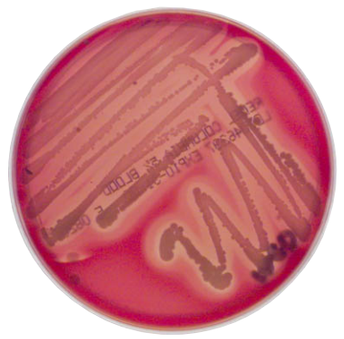


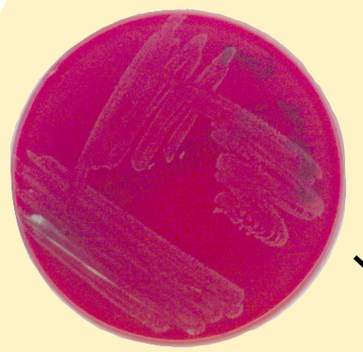
Holding the culture tube parallel to the ground, fill and replace the cap.

Plate on blood agar and incubate overnight.

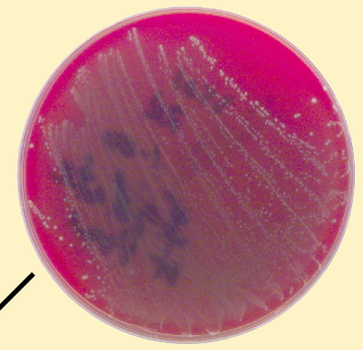
## Colony Size After 18 – 24 Hours of Incubation

**Hemolysis (clear zone)**

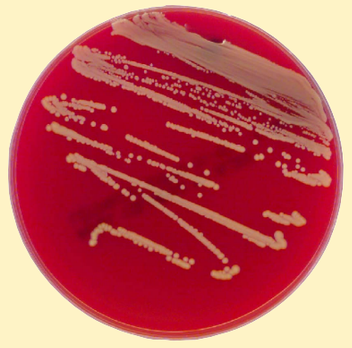




Tiny



Small



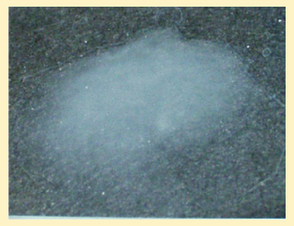
Medium

**KOH Test :** (test for gram-negative bacteria like coliforms) Place a bacterial colony on a slide and add KOH. A positive test will be "stringy".

Large

**Catalase Test :** Place a bacterial colony on a slide and add a drop of hydrogen peroxide.

Negative

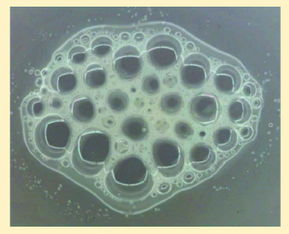


No bubbles

***A. pyogenes***

Slow growing
Smelly milk

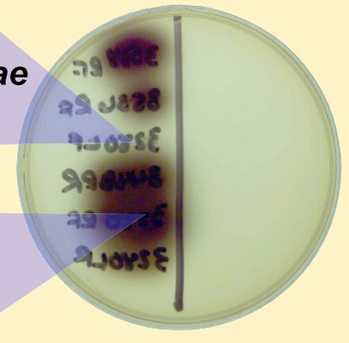
Positive



Bubbles

Yeast
***C. bovis***

**Esculin agar**



Negative - clear
***Strep. dysgalactiae***
***Strep. agalactiae***

Positive - black
***Strep. uberis***

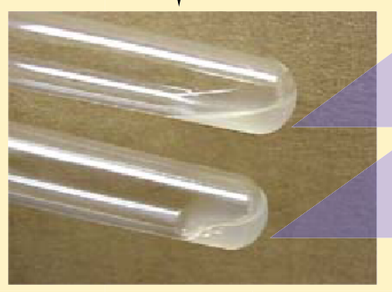
**CAMP Test**

Positive - clear
***Strep. agalactiae***
***Strep. uberis***

Negative - no arrowhead
***Strep. dysgalactiae***
***Strep. uberis***

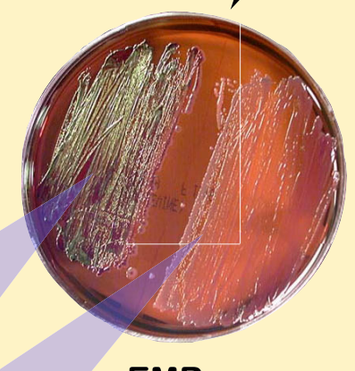
**CAMP Test :** Streak ***Staph. aureus*** down the center of a blood agar plate. Streak a bacterial colony perpendicular to the streak of ***Staph. aureus***.

**Coagulase Test :** Place a bacterial colony in a test tube containing coagulase medium.



Negative - no gel
Coagulase negative ***Staph.***

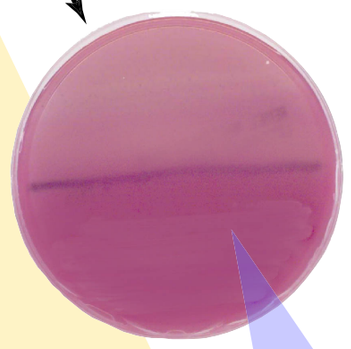
Positive - gel
***Staph. aureus***



Positive - green sheen
***E. coli***

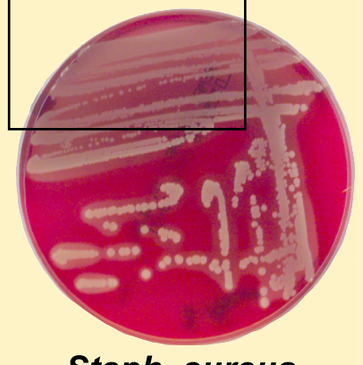
Negative - no green sheen
***Klebsiella***

EMB



MacConkey agar

Positive - pink
***E. coli***
***Klebsiella***



***Staph. aureus***

Coagulase negative ***Staph.***

***Klebsiella***

E. coli


***Dagny Leininger, DVM***
***Jerry Roberson, DVM, PhD***
***VA MD Regional College of Veterinary Medicine***

## Chapter 3

## Using Eosin Methylene Blue Agar to Distinguish Between *Escherichia coli* and Other Gram-Negative Mastitis Pathogens


Abstract

Mastitis is a continuous concern for dairy producers in the US because of its economic consequences. Coliform mastitis accounts for 20-80% of acute clinical mastitis cases and includes gram-negative pathogens *Escherichia coli*, *Klebsiella* and *Enterobacter* species. Rapid identification of the causative organism is essential to implement a prudent treatment plan. *Escherichia coli* can be rapidly identified with Eosin Methylene Blue (EMB) agar based on the occurrence of a green-metallic sheen that appears on the surface of the bacterial colonies. Frozen milk samples from which gram-negative bacteria had been isolated and gram-negative bacterial isolates from milk samples were received from eight states. Samples were grown on 5% sheep's blood agar. Isolated colonies were then plated on EMB agar. Time from inoculation and to first visible green-metallic sheen was recorded. Isolates were identified using (API 20E) biochemical test strips. One hundred and twenty-nine isolates or milk samples were received. Nine species of gram-negative bacteria were identified by the use of biochemical test strips. Of 63 *E. coli* isolates, 61 were EMB positive, and of 66 non-*E. coli* gram-negative isolates, 64 were EMB negative, for an intermethod agreement of 96.9% and a κ-value of 93.7% indicating excellent agreement beyond chance between identification of *E. coli* with biochemical test strips and EMB agar. The minimum and maximum time to first visible sheen were 3.3 hours and 10 hours respectively, for a mean

(standard deviation) of 5.7 (1.5) hours and a median of 5.2 hours. Eosin Methylene Blue agar is a reliable, simple, and rapid method to differentiate *E. coli* from other gram-negative mastitis pathogens.

Introduction

Coliform mastitis accounts for 20-80% of acute clinical mastitis cases[91] and is a continuous concern for U.S. dairy producers because of its economic consequences.[91, 92] Coliform mastitis pathogens are gram-negative usually lactose fermenting bacilli and include *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp.[72] Other gram-negative organisms which can be isolated from the mammary gland include *Serratia* spp., *Pasteurella* spp., *Proteus* spp. and *Pseudomonas* spp.[42] Rapid identification of the causative organism is essential to implement a timely and prudent treatment plan.

Coliform bacteria generally grow rapidly when plated on 5% sheep blood agar and, following overnight incubation, usually provide an adequate amount of bacterial growth for follow-up work.[8] *Escherichia coli* can be identified with eosin methylene blue (EMB) agar based on the occurrence of a green-metallic sheen (Figure 3-1) that appears on the surface of the bacterial colonies.[43] The dyes in EMB agar, eosin Y and methylene blue, are pH indicators, inhibitors of gram-positive bacteria, and at an acid pH, combine to form a green-metallic precipitate (sheen).[43]

The food industry has been using various culturing methods to enumerate the numbers of *E. coli* O157:H7 in meat products and unpasteurized apple cider following several outbreaks of *E. coli* O157:H7 and *Salmonella* spp.[47, 93-95] Media evaluated for culturing heat or cold stressed *E. coli* included EMB agar, violet red bile agar, modified

sorbitol MacConkey agar, sorbitol MacConkey agar supplemented with 4-methylumbelliferyl-β-D-glucuronide, and tryptic soy broth.[47, 94-96] MacConkey and EMB agars are used in some mastitis laboratories to identify and differentiate gram-negative mastitis pathogens.

The primary goal of this study was to evaluate the use of EMB agar as a method for early differentiation of *E. coli* from other gram-negative mastitis pathogens. The secondary goal was to determine the time needed for the first visible sheen to develop.

Materials and Methods

Frozen milk samples from which gram-negative bacteria had been isolated were received from Maryland, New York and North Carolina. Gram-negative bacterial isolates from milk samples were received from Georgia, Illinois, Michigan, and Utah. Milk samples were also obtained from the Virginia Tech Dairy Science Complex.

One hundred and twenty-nine milk samples or isolates from milk samples were received. Fifty microliters of milk were plated on 5% sheep blood agar and incubated aerobically for 18 hours at 37°C. Bacterial cultures were recultured on 5% sheep blood agar and incubated aerobically for 18 hours at 37° C. A single colony from the 5% sheep blood agar was then plated on EMB agar and incubated aerobically at 37° C. The plates were checked every half hour. Time was recorded at inoculation on EMB agar and at first visible sheen. Isolates were identified using biochemical test strips. [a]

Results

Nine species of gram-negative bacteria were identified by the use of biochemical test strips (Table 3-1). Of 63 *E. coli* isolates, 61 were EMB positive, and 64 of 66 non-*E. coli* gram-negative isolates were EMB negative, for an intermethod agreement of 96.9% and a κ –value[96] of 93.7% indicating excellent agreement beyond chance between identification of *E. coli* with biochemical test strips and EMB agar. The minimum and maximum times to first visible sheen were 3.3 hours and 10 hours, respectively, with a mean of 5.7 hours (standard deviation: 1.5 hours) and a median of 5.2 hours.

Discussion

Eosin methylene blue agar provides a rapid and accurate method of distinguishing *E. coli* from other gram-negative mastitis pathogens. Coliform bacteria grow rapidly on blood agar[97] and can be identified within 24 hours of initial plating. Colonies are large enough after 12 hours of incubation on 5% sheep blood agar to streak a colony on EMB agar and obtain results within 18 hours of initial plating, given the observed mean time to first visible sheen of 5.7 hours. Direct inoculation of milk on EMB agar does not allow differentiation of coliform mastitis pathogens. In this situation *E. coli* generally does not produce a green-metallic sheen. Sheen production appears to be sensitive to changes in pH and the lack of sheen production could be due to the alkalinity of mastitic milk[24] interfering with the acidic requirement of EMB agar for production of the green-metallic sheen. Conversely, acidic pH of apple cider enhanced sheen production and has led to false-positive results when unpasteurized apple cider was plated directly on EMB agar.[47]

False-negative reactions have also been related to pH variation within areas of the EMB agar plates,[48] but should be minimal in commercially prepared EMB agar.

MacConkey agar is commonly used to differentiate *E. coli* from other gram-negative mastitis pathogens. MacConkey agar, like EMB agar, inhibits the growth of most gram-positive organisms.[43] Lactose fermenting organisms produce pink colonies[42] and can be differentiated through the level of color change in conjunction with colony morphology. However, additional biochemical tests are needed following isolation for confirmation of *E. coli* and other gram-negative mastitis pathogens. These may include a motility test, an acid production test on triple sugar iron agar, and a citrate utilization test on Simmons citrate agar,[42] which require overnight incubation. More rapid for identification of *E.coli* are positive indole and β-glucuronidase tests,[45, 46, 98] which however require additional equipment.[45, 46] Lactose fermenting organisms appear yellow on Tergitol-7 agar, another differentiating medium used for detection of coliform bacteria, and additional tests have to be performed to differentiate between *Klebsiella* spp. and *E. coli* which both ferment lactose.[8] Several studies comparing modified EMB agar and modified sorbitol MacConkey agar for the recovery of *E. coli* O157:H7 from food products suggested that EMB agar produced significantly higher recovery rates than MacConkey agar.[93-95]

Eosin methylene blue is simple to use and therefore can be applied by veterinarians performing milk cultures in private practice when fast turnaround time is required. It is a very economical method of differentiation, with the materials priced less than one dollar per sample. Two samples can be plated on one blood agar plate ($0.21

per sample) and up to eight isolates can be plated on one EMB agar plate ($0.10 per sample).[b]

Rapid differentiation of *E. coli* from other gram-negative mastitis pathogens is important for the initiation of an appropriate treatment plan. Cows with mild to moderate *E. coli* mastitis will usually self-cure within a few days without intramammary antibiotic therapy, while mild to moderate cases of *Klebsiella* spp. mastitis may evolve into chronic infections and warrant intramammary antibiotic therapy.[97, 99] Intramammary antibiotic therapy in most cases of mild clinical mastitis can be safely delayed until bacterial culture results are obtained. Severe cases can be treated systemically with supportive therapy (fluids, anti-inflammatories, systemic antibiotics, calcium), regardless of the causative agent, until milk culture results are obtained.

In this study, 4 of 129 (3.1%) isolates were misclassified (Table 3-1). Misclassification by EMB agar of *E. coli* cases as non-*E. coli* cases would result in antimicrobial treatment of those cows even though their mastitis may have resolved without the use of intramammary antibiotics. Consequently, the dairy producer would have incurred unnecessary costs for treatment, milk withdrawal, and additional risk of antimicrobial residues in milk. The misclassification of the *Klebsiella* sp. isolate as *E. coli* would have resulted in withholding treatment from that cow and thus put her at an increased risk of developing chronic mastitis.[99] However, the benefit of not using antimicrobial therapy in the other 63 *E. coli* cases may outweigh the possible loss due to withheld therapy in 1 *Klebsiella* spp. case. Mastitis caused by *Serratia* spp. responds poorly to antibiotic therapy[42, 97] and misclassification as *E. coli* may actually be beneficial

in that withholding treatment from *Serratia* spp. allows the option to cull a residue free animal. Although 19 *Serratia* spp. isolates were included in this study, mastitis caused by *Serratia* spp. is rare.[99, 100]

Losses due to mastitis have been estimated at $35 to $295 per cow per year.[101] These estimates include production loss, replacement costs, discarded milk, drugs and veterinary costs.[101] Treatment of clinical mastitis is the most common reason for antibiotic use and residue violations on dairy farms.[91, 92] Use of antibiotics may also favor the appearance of antibiotic resistant organisms. The veterinarian's goal of reduced antibiotic usage may be supported by the use of culture results that allow judicious selection of antimicrobial treatment. In addition, the amounts of discarded milk and the likelihood of residue violations will be reduced.

In conclusion, EMB agar is a reliable, simple, rapid and inexpensive medium for the differentiation of *E. coli* from other gram-negative mastitis pathogens. Given the rapid growth of coliform organisms and a mean time to first visible green-metallic sheen of 5.7 hours, identification of *E. coli* within 24 hours is possible, allowing an appropriate treatment plan to be formulated in a reasonable amount of time.

Data Table

Table 3-1

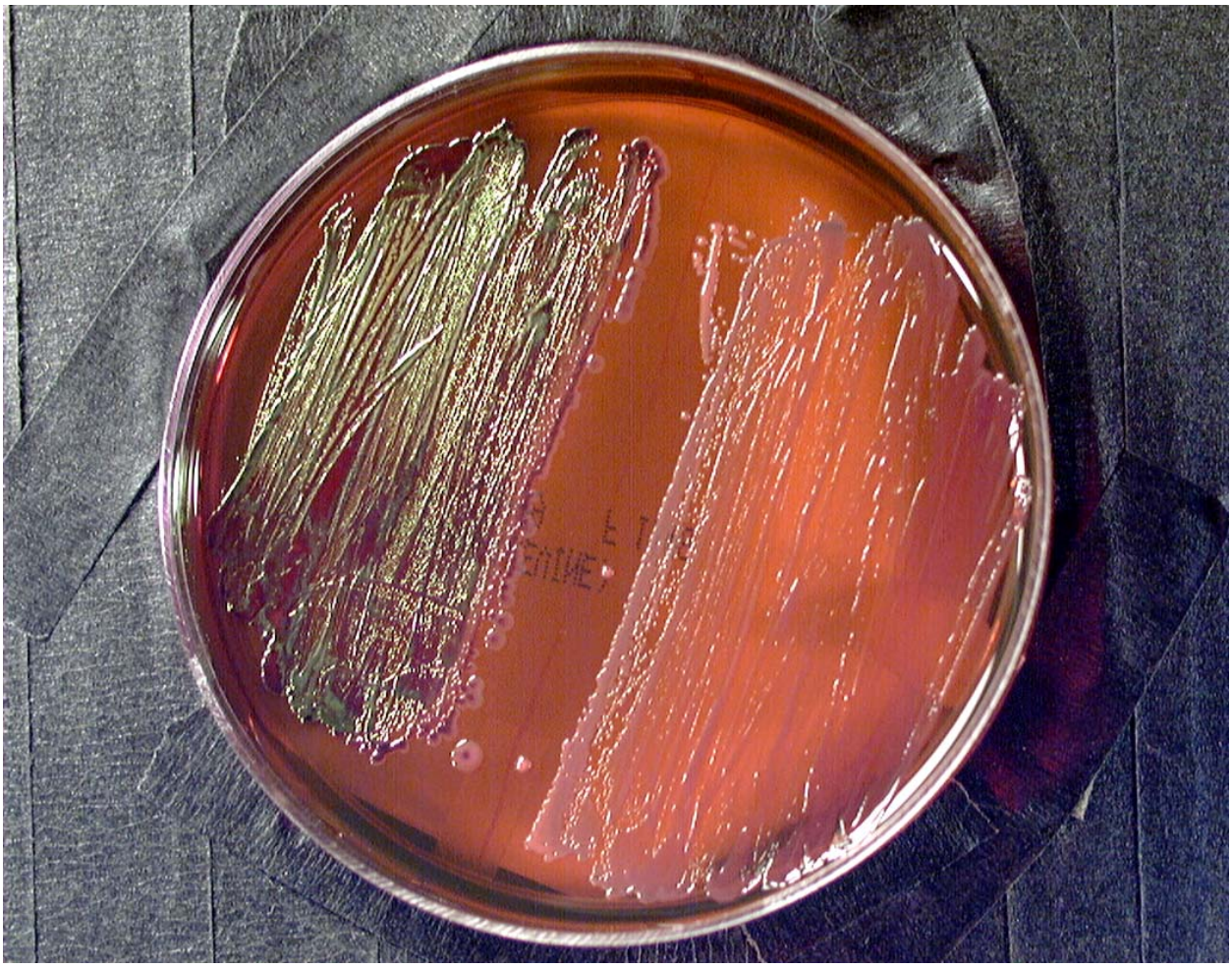
Bacterial identification by biochemical test strip and production of a green-metallic sheen by isolates on eosin methylene blue agar.

| Organism | Number of Isolates | |
|---|---|---|
| | Total | Production of green-metallic sheen |
| *Aeromonas spp.* | 2 | 0 |
| *Burkholderia spp.* | 1 | 0 |
| *Escherichia coli* | 63 | 61 |
| *Enterobacter spp.* | 8 | 0 |
| *Klebsiella spp.* | 25 | 1 |
| *Pantoea spp.* | 4 | 0 |
| *Pasteurella spp.* | 2 | 0 |
| *Pseudomonas spp.* | 5 | 0 |
| *Serratia spp.* | 19 | 1 |

Figure

Figure 3-1

*Escherichia coli* (left) and *Serratia spp.* (right) on Eosin Methylene Blue agar. Notice the green-metallic sheen on the left (*E. coli*).



## Chapter 4

### Efficacy of Frequent Milk-Out as a Treatment for Experimentally Induced *Escherichia coli* Mastitis


Abstract

Frequent milk-out is a commonplace treatment for coliform mastitis. The non-verified rationale for frequent milk-out is that frequent removal of bacteria and/or toxins may improve the clinical outcome. Frequent milk-out was evaluated as a treatment in cases of experimentally induced *Escherichia coli* mastitis. Sixteen Holstein dairy cows were randomly assigned in equal numbers to 1 of 4 treatment groups and were either 1) non-infected, not frequently milked-out, i.e. not treated (NI-NT), 2) experimentally infected with *E. coli*, not treated (EC-NT), 3) non-infected, frequently milked-out (NI-FMO), or 4) experimentally infected with *E. coli*, frequently milked-out (EC-FMO). Frequent milk-out did not affect somatic cell counts or times to bacterial, clinical and systemic cure of experimentally infected cows. Results from this study do not support frequent milk-out as a treatment for *E. coli* mastitis.


Introduction

Mastitis is an economically important disease and costs of clinical mastitis have been estimated at more than $100 per case.[102] On dairy farms with bulk tank milk somatic cell counts of less than 150,000 cells/ml, 35 to 55% of cows experienced at least one episode of clinical mastitis,[103-106] and the greatest proportion of bacteriologically positive milk samples from those cows yielded coliform bacteria.[107, 110, 111]

There is no consensus for the most appropriate treatment of coliform mastitis. Frequent milk-out has been encouraged for treatment of mastitis as early as 1869 in Sloan's Complete Farrier and Cattle Doctor: "The bad milk should be drawn three to four times a day, for by remaining in the bag, it tends to increase the inflammation".[62] Through history this practice, often facilitated through injection of oxytocin, has come in and gone out of favor. Antibiotic use for treatment of coliform mastitis has been discouraged in recent years. Reasons are the lack of proven efficacy, the risk of antibiotic residues in bulk tank milk and resulting penalties, and more recently, the recognition of the development of antimicrobial resistance of bacterial pathogens.

Current teaching encourages supportive therapy (fluids, calcium, anti-inflammatories) and the potential but not proven removal of endotoxins through frequent milk-out.[76] In a recently completed study the outcome of naturally occurring clinical coliform mastitis was not improved in 10 cows that were frequently milked out following administration of oxytocin compared to 9 cows that received no frequent milk-out.[70] In this study we explored the possibility that frequent milking out of quarters experimentally infected with *E.coli* did not enhance the return of affected udders to normal normal health status, and, specifically, did not affect or consistently improve times to either bacteriologic, clinical or systemic cure.

## Materials and Methods

Subjects - Sixteen Holstein cows from the Virginia Tech Dairy Center were equally and randomly assigned to one of two blocks of 8 cows in a randomized complete block experimental design. Cows were selected from 212 cows based on days in milk and

current lactation free of clinical mastitis. Selected cows were free of udder infection (quarter cultures) in 4 samples collected prior to being placed in the study. Average days in milk were 172 days (range: 56-243 days), average lactation number was 2 (range: 1-5), and average daily milk production was 69.5 pounds (range: 46-88 pounds). Cows in each block were randomly assigned, by choosing numbers out of a hat, to one of four treatment groups: 1) cows that were not experimentally infected, not-frequently milked-out (non-infused, not-treated; NI-NT), 2) cows experimentally infected with *E. coli*, but not treated (EC-NT), 3) cows that were not experimentally infected, but were frequently milked-out (NI-FMO) and 4) cows that were experimentally infected with *E. coli* and frequently milked-out (EC-FMO). The experiment was initiated at the beginning of July 1999 for cows in block 1 and the beginning of August 1999 for cows in block 2. Cows were housed separate from the main herd in freestalls bedded with sawdust, milked twice a day at 6 a.m. and 6 p.m. in stanchions with a bucket milker and fed a total mixed ration in a drive through bunk. Cows were given a 3-day acclimation period to the new environment and milking system prior to experimental challenge**.** An *E. coli* vaccination program has not been used in this herd.

Experimental Infection – The *Escherichia coli* 727 suspension for intramammary challenge was prepared using a published procedure, [108] modified as described below: An isolated colony of *E. coli* 727[10] was placed on 5% sheep blood agar and incubated at 37°C for 24 hours. One isolated colony was transferred into brain-heart infusion broth and incubated at 37°C for 12 hours. The broth culture was centrifuged at 5000 rpm for 5 minutes and the pellet resuspended in sterile phosphate buffered saline (PBS). Serial dilutions of the bacterial suspension were made in PBS, and 50 μl of each dilution was

plated on McConkey agar and incubated for 24 hours at 37°C. Bacterial suspensions with colony counts ranging from 50 to 100 cfu/ml were selected. Five aliquots of 1 ml each were prepared, of which 4 aliquots were used for infusion into the right front quarter of challenge cows, and 250 μl of the 5$^{th}$ were immediately plated on MacConkey's agar for determination of bacterial challenge dose. Bacterial count in the 5$^{th}$ aliquot representing the challenge dose of cows in block 1 was 84 cfu/ml, and of cows in block 2 it was 48 cfu/ml.

Frequent Milk - Out Schedule - The right front quarters of the NI-FMO and EC-FMO cows were milked out by hand at 4-hour intervals from 12 to 36 hours post-challenge and at 6-hour intervals from 36 to 84 hours post-challenge. Oxytocin (2 ml, 20 IU/ml) was administered generally intravenously at each milk-out to facilitate milk removal.

Sampling Protocol - Monitoring and sampling schedules are presented in Table 4-1. Physical exams (PE) included measurements of rectal temperature, pulse rate, respiration rate, hydration status, evaluation of rumen motility and strength, and appearance of milk or udder secretion. Cows were considered systemically ill if two or more of the clinical parameters listed above were abnormal.[70] Milk samples for culture and somatic cell counts, prior to experimental challenge, were collected at the morning milking. Production data were recorded on -3, -2, -1 and 0 days prior to challenge and at each treatment and milking post-challenge.

Microbiological Procedures - All milk samples were collected aseptically after wiping the teat ends with a gauze pad soaked in isopropyl alcohol. Samples were collected in sterile milk sample vials. Teats were dipped with a 1% iodine-based barrier

teat dip following sample collection and milking. Milk samples (50 μl) were plated on 5% sheep blood agar and incubated for 24 hours at 37° C. The numbers of colony forming units per ml were determined. Identification of isolates from initial milk cultures were confirmed by biochemical test strips.[a]

Somatic Cell Counts - Somatic cell counts (SCC) were determined by Fossomatic 360[c] at the Dairy Herd Improvement Association laboratory at Virginia Tech. California Mastitis Test (CMT)[109, d] results were used when electronic counts were missing, mostly due to insufficient secretion, and the resulting variable for analysis was designated adjusted SCC (SCCA).

Alpha-lactalbumin Testing - Blood for α-lactalbumin (ALAC) testing was drawn from the coccygeal vein with an 18-ga 1-inch needle into a 10 ml red-topped vacutainer tube. The blood was centrifuged, serum removed and frozen for later analysis. Alpha-lactalbumin concentrations were determined by radio-immunoassay as described elsewhere.[110]

Time to Cure – Time to bacterial cure (BC) was defined as the time interval in hours from experimental challenge to the first milk sample that was culture negative, and from which time on all consecutive milk samples were negative. Time to clinical cure (CC) and time to systemic cure (SC) were defined as the time intervals from experimental challenge to return to consistent clinical normalcy of the challenged quarter, and to return to normal of all PE values, respectively.[70]

Statistical analysis - Effects of *E. coli* infection and frequent milk-out on hours to BC, CC and SC were tested by analysis of variance using the GLM procedure of SAS.[111, e] Log-transformed SCCA and ALAC data were subjected to a repeated measures analysis

of variance using the MIXED procedure of SAS. Only values at times when all treatment groups were represented were used in the analysis. Least squares means are presented throughout the report. Covariation among repeated measurements on the same animal were modeled using a first order autoregressive model. Significant interactions were evaluated using the SLICE option to test for simple main effects with a Bonferroni correction to maintain $\alpha \leq 0.05$. Analysis of study power, i.e. the calculation of the probability of finding a significant difference at a predetermined alphalevel (here $\alpha=0.05$) for BC, CC and SC was done using PASS[112, f] version 6.0.

Results

Cows experimentally challenged with *E. coli* became systemically ill 14.5 hours (range: 12-20 hours) post-experimental challenge. Mean peak temperature was 40.9 (standard deviation: 0.83)°C, mean peak pulse rate was 92 (standard deviation: 27.8) beats/min and mean peak respiration rate was 66.5 (standard deviation = 15.3) breaths/min during the time period when cows were systemically ill. Cows in the NI-NT and NI-FMO groups did not become systemically ill nor did they have abnormal milk, except for one cow in the NI-NT group in block 2 with elevated SCC starting at hour 84, from which no microbial agent was isolated.

Cows experimentally challenged with *E. coli* in block 1 were all severely systemically ill[70] by 12 hours post-experimental challenge with abnormal milk noted between 12 and 20 hours post-experimental challenge. Cows experimentally challenged with *E. coli* in block 2 became moderately to severely systemically[70] ill within 20 hours post-experimental challenge, with abnormal milk noted between 16 and 20 hours post-

challenge. Cows in block 2 had been challenged with a reduced dose (48 cfu/ml) to achieve moderate systemic involvement in order to avoid the severe systemic effects seen in cows infected with 84 cfu/ml in block 1. Neither cows in block 1 nor in block 2 were treated with systemic or intramammary therapy. Milk yields were reduced in cows infected with *E.coli* ($P$=0.004) but were not affected by frequent milk-out. All experimentally challenged cows recovered completely.

Mean times to BC, CC and SC were not different between treatment groups EC-NT and EC-FMO (Figure 4-1). Mean times for EC-NT and EC-FMO respectively for BC were 203 and 159 hours ($P$=0.53), for CC 276 and 360 hours ($P$=0.64) and for SC 144 and 159 hours ($P$=0.95). The power to declare differences of the reported magnitude for BC, CC and SC as significant was 7%, 9.3% and 5.3%.

The adjusted somatic cell counts in cows infected with *E. coli* were higher than in cows not infected with *E. coli* from 24 through 156 hours post-experimental challenge ($2.49 \times 10^6$ vs. $0.096 \times 10^6$ cells/ml; $P < 0.001$; Figure 4-2), irrespective of milk-out treatment, but were not affected by frequent milk-out ($P$=0.34). There were no differences between treatment groups at ≤ 12 hours post-experimental challenge irrespective of frequent milk-out, while SCCA in the later weekly samples behaved differently over the 4 weeks depending on FMO group, irrespective of *E. coli* infection group ($P$=0.02).

Serum α-lactalbumin concentrations were higher in EC-NT cows than in NI-NT, NI-FMO and EC-FMO cows at 12 hours post-experimental challenge ($P < 0.001$; Figure 4-3), and higher in NI-FMO cows compared to cows in the other treatment groups at 36 ($P < 0.001$) and 60 ($P = 0.02$) hours post-experimental challenge.

Discussion

Frequent milk-out is assumed to improve the clinical outcome of mastitis by removing bacteria and/or their toxins. This study does not support this practice. Frequent milk-out with administration of oxytocin did not affect hours to BC, CC or SC in cows with experimentally induced *E. coli* mastitis. Frequent milk-out did not affect somatic cell counts in the treatment groups experimentally challenged with *E. coli*.

No benefits of frequent milk-out following oxytocin administration had been reported when frequent milk-out and no frequent milk-out were compared in 19 cows with naturally occurring *E. coli* mastitis,[70] or when the use of oxytocin and frequent milk-out were applied in cases of acute mastitis.[113] These reports, which appear to be the only studies in which the effect of frequent milk-out was the only factor of interest, support our results.

Frequent milk-out also appeared as good as treatment with intramammary antibiotics.[69] Four of 4 clinical *E. coli* mastitis cases that received frequent milk-out following oxytocin administration and 2 of 3 cases treated with antibiotics were bacterially cured by 3-4 weeks post-infection.[69] Treatment had no significant effect when cows with naturally occurring *E. coli* mastitis received either systemic gentamicin, systemic erythromycin, or no systemic antibiotics, with supportive therapy including frequent milk-out with oxytocin administration several times a day and intramammary antibiotics (cephapirin).[59] Differences in CC or BC were not reported in cows with mild clinical mastitis that were treated with either intramammary antibiotic therapy or oxytocin at milking to improve milk-out at regular milking times.[61]

Cows with naturally occurring *E. coli* mastitis which were treated with varying milk-out frequencies following oxytocin administration, and either intramammary antibiotic therapy (cephapirin) or intramammary and systemic (oxytetracycline) antibiotic therapy, based on mastitis severity showed no differences in BC or CC between antimicrobial treatment groups.[68] There was no significant difference in the number of cows treated with antibiotics that were still clinically infected by the 10$^{th}$ milking after onset of clinical mastitis to those that were not treated with antibiotics. When clinical cure was evaluated in cows from which streptococci or coliform bacteria were isolated, antibiotic treated cows had a higher clinical cure rate by the 10$^{th}$ milking than did non-antibiotic treated cows. Most quarters with coliform bacteria were bacteriologically negative by 14 days, irrespective of treatment group. These studies lend support to our results in that irrespective of treatment, no significant differences in the outcome of cows with experimentally or naturally occurring *E. coli* mastitis were recorded.[68]

Most of the studies listed above, as well as our study reported here, were done on low numbers of cows of varying parities and stages of lactation. The power of this study to detect statistically significant differences in time to cure was very low. However, our goal was to find out if the practice of frequently milking out an *E.coli* infected, clinically mastitic quarter would lead to a clinically relevant and economically feasible improvement of the outcome, such that, based on our findings, we could design and execute a larger study to eventually prove the perceived benefit of frequent milk-out. The obtained results do not encourage us to either carry out or recommend more extensive experiments. Observed differences in median or mean times to cure should have been large and consistent to warrant further studies or recommend the practice to the dairy

producer. Only one of the 3 measured times to cure, the time to bacterial cure, was numerically (non-significantly) shortened by frequent milk-out, while times to clinical and systemic cure were numerically lengthened, leading us to believe that any enhanced study would only confirm the lack of relevant benefit of frequent milk-out to the dairy producer.

As an adjunct to this study we investigated the behavior of α-lactalbumin (ALAC) in serum of experimentally infected cows. Alpha-lactalbumin is a protein found in milk, that can be used as an indicator of udder development and udder health.[37] It enters the bloodstream either through compromised tight junctions, which are located between the mammary epithelial cells that line the alveolar lumen, or through damaged alveolar epithelial cells, or through gaps left by sloughed epithelial cells.[37] Stage of gestation or lactation, frequency of milking and udder health status affect leakage of ALAC into blood.[38] Serum ALAC concentration is positively correlated ($r = 0.60$) with milk somatic cell counts, and cows challenged with *E. coli* endotoxins had increased somatic cell counts as well as increased serum ALAC concentrations.[37] We expected elevated serum ALAC concentrations in cows that were experimentally challenged with *E. coli* and had elevated SCC. However, the highest ALAC levels were measured in the NI-FMO treatment group at times 36 and 60 hours post-experimental challenge (Figure 3, Panel C). Lower ALAC levels were expected in FMO cows, since cows that were milked three times a day had been reported to have lower serum ALAC concentrations than cows milked twice a day.[114] Increased intramammary pressure due to the frequent stimulation,[38] but selective milk-out of one quarter only, may have led to increased serum ALAC concentrations in NI-FMO cows.

Although this study was performed on a small number of cows, we conclude that frequent milk-out as a treatment for *E. coli* mastitis does not improve mastitis outcome. Neither does it appear to have a negative effect, which is consistent with an earlier study of naturally occurring *E. coli* mastitis.[70] Only a large and / or consistent reduction in times to cure would justify the labor intensive practice of frequent milk-out.

Data Table

Table 4-1

Treatment and sampling protocol of 16 cows assigned in equal numbers to either one of 4 treatment groups that were experimentally non-infected (NI) or infected intra-mammarily with *E. coli* (EC), and either not treated (NT) or treated with frequent milk-out (FMO). (a) represents times when udders were tested by California Mastitis Test, and milk samples were collected for bacterial culture and somatic cell counts; for (b) blood samples were collected for serum α-lactalbumin determination in addition to procedures in (a); for (c) a physical exam was performed on cows in addition to samples as in (b), while (d) represents physical exams only.

| Treatment Groups | Time | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pre-challenge | | | | Post-challenge | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | With Administration of Frequent Milk-out | | | | | | | | | | | | | | Follow-up | | |
| | Hours Relative to Experimental Infection | | | | | | | | | | | | | | | | | | | | | | | |
| | -168 | -72 | -48 | -24 | 0 | 4 | 8 | 12 | 16 | 20 | 24 | 28 | 32 | 36 | 42 | 48 | 54 | 60 | 66 | 72 | 78 | 84 | 96 | 108 | 156-828* |
| NI-NT | a | a | a | b | c | d | d | c | | | c | | | c | | c | | c | | c | | c | c | c | a* |
| EC-NT, NI-FMO, EC-FMO | a | a | a | b | c | d | d | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | c | a* |

* Cows sampled every 168 hours (7 days) from 156 through 828 hours

Figures

Figure 4-1

Time to bacterial, systemic and clinical cure of cows experimentally challenged with *E. coli* that were not or were frequently milked out. Open symbols represent cows that were not frequently milked out, closed symbols represent cows that were frequently milked out. Mean times to cure did not differ between cows that were and were not frequently milked out ($P>0.5$).

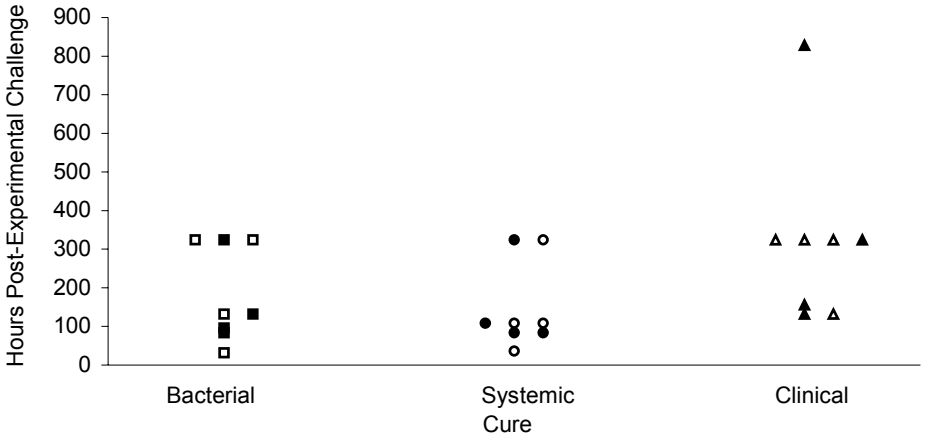


Figure 4-2

Adjusted Somatic Cell Counts (SCCA) by treatment group across time post-experimental infection. Antilogs of least squares means of logarithmically transformed SCCA are presented. The standard error of each presented log mean is 0.2490. Cows were either 1) non-infected, not frequently milked-out, i.e. not treated (NI-NT; Δ), 2) experimentally infected with *E. coli*, not treated (EC-NT; ○), 3) non-infected, frequently milked-out (NI-FMO, ▲), or 4) experimentally infected with *E. coli*, frequently milked-out (EC-FMO; ●). From 24 to 156 hours post-experimental challenge, SCCA of cows infected with *E. coli* were higher than SCCA of cows not infected with *E. coli* ($P < 0.001$).

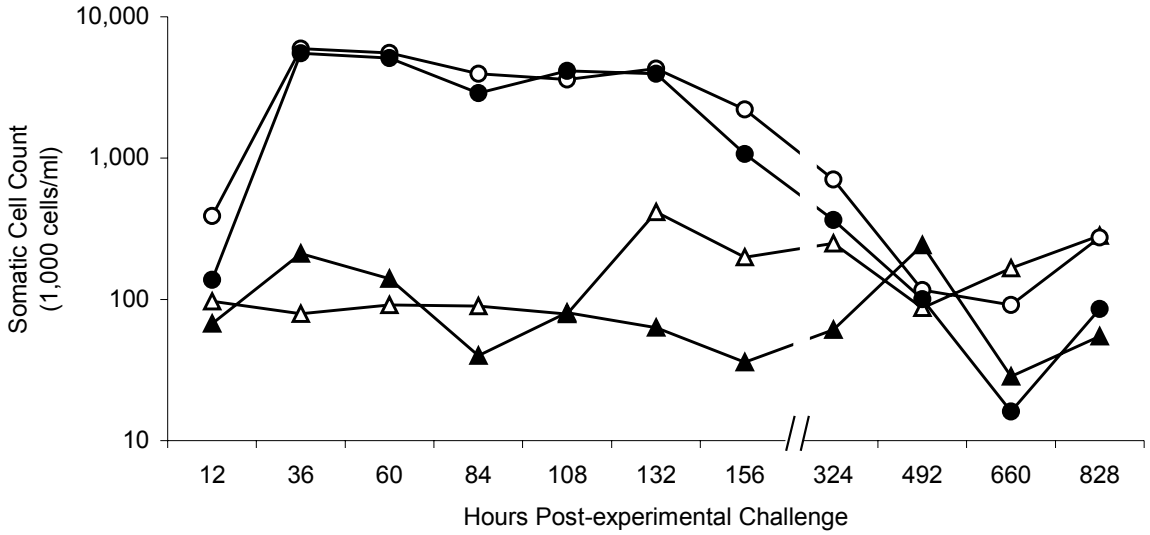
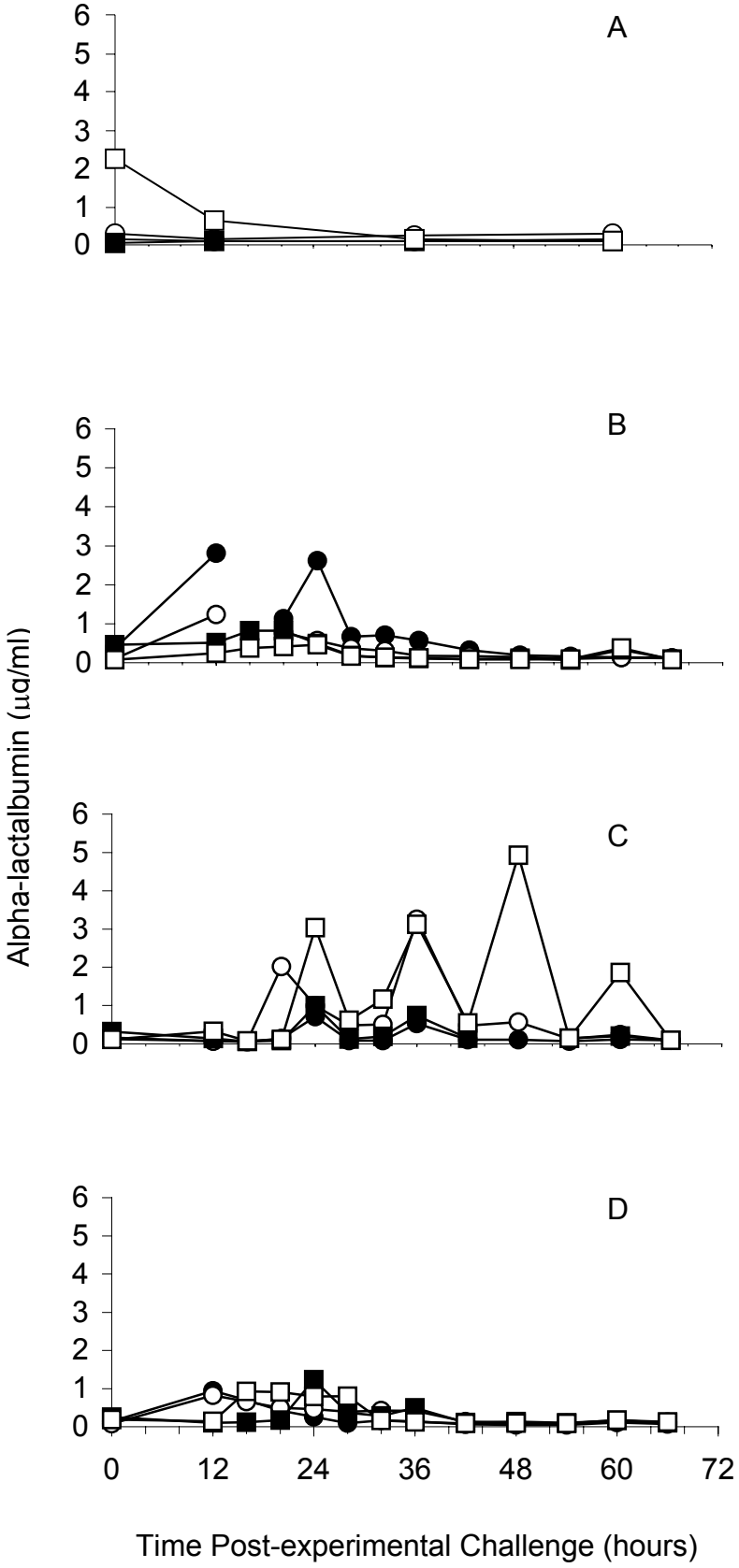


Figure 4-3

Serum α-lactalbumin in blood of individual cows that were either 1) non-infected, not frequently milked-out, i.e. not treated (NI-NT; panel A), 2) experimentally infected with *E. coli*, not treated (EC-NT; panel B), 3) non-infected, frequently milked-out (NI-FMO, panel C), or 4) experimentally infected with *E. coli*, frequently milked-out (EC-FMO; panel D). Circles represent cows in block 1, squares represent cows in block 2.


A
B
C
D
6
5
4
3
2
1
0
0
12
24
36
48
60
72
Alpha-lactalbumin (µg/ml)
Time Post-experimental Challenge (hours)

# Chapter 5

## Summary and Conclusions

Coliform bacteria, *Escherichia coli*, *Klebsiella* spp., *Serratia* spp., and *Enterobacter* spp., are gram-negative mastitis pathogens which account for 20-80% of cases of acute clinical mastitis. Clinical cases of coliform mastitis range from a mild infection to death. Most of the clinical signs associated with coliform mastitis are due to endotoxin release. Etiologic diagnosis of clinical mastitis in the farm is not practical or accurate. Experienced clinicians can predict if the etiologic agent is either gram-negative or gram positive approximately 50% of the time. Inaccurate etiologic diagnosis can result in inappropriate or unnecessary antibiotic therapy.

Rapid identification of *E. coli* from other gram-negative pathogens is more desirable than just identifying the etiologic agent as gram-negative or gram-positive. Mastitis caused by *E. coli* will generally cure with out the aid of intramammary antibiotics and seldom become chronic. In contrast mastitis caused by *Klebsiella* spp. is more likely to become chronic and may benefit from intramammary antibiotic therapy. Identification of *E. coli* from other gram-negative mastitis pathogens within 24-36 hours is possible using eosin methylene blue agar. *Escherichia coli* will usually produce a green metallic sheen on eosin methylene blue agar as compared to other gram-negative mastitis pathogens which produce no green metallic sheen.

Milk samples are initially cultured on 5% sheep blood agar and incubated at 37°C overnight for 18-24 hours. Coliform bacterial typically grow fast enough that colonies

are large enough to work with after 18-24 hours of incubation. An isolated colony is placed on eosin methylene blue agar (EMB) and incubated at 37°C. The average time to first sheen is 5.7 hours.

Eosin methylene blue agar was compared to a biochemical test strip (API E20) using 129 gram-negative milk cultures or gram-negative isolates from milk cultures. There was a high agreement between the results indicating that EMB agar is an accurate method for the identification of *E. coli* from other gram-negative mastitis pathogens. Once an accurate diagnosis is made appropriate therapy can be initiated.

Therapy for coliform mastitis centers on counteracting the effects of endotoxin release. Oral and/or intravenous fluids are standard, non-controversial therapy. Systemic antibiotics though controversial, are commonly administered to prevent septicemia. Anti-inflammatories are also common practice. Flunixin meglumine is the most popular anti-inflammatory used for cases of coliform mastitis. Other anti-inflammatories that have been used include phenylbutazone, aspirin and dexamethasone.

Frequently milking-out infected quarters between regular milking has been recommended in the literature as early as 1869. This practice has gone in and out of favor and is currently a recommended practice. Frequent milk-out was evaluated using 16 Holstein dairy cows. Cows were randomly assigned to 1 of 4 treatment groups, no treatment, *E. coli*, *E. coli* with frequent milk-out, and frequent milk-out only. All *E. coli* cows became moderately to severely systemically ill. These cows were not treated with any supportive therapy other than frequent milk-out or no frequent milk-out. All cows recovered and there was no difference in times to clinical, bacterial and systemic cure between cows infected with *E. coli* that were frequently milked-out to those that were not

frequently-milked-out. While there appears to be no harm in using frequent milk-out as a treatment for cows with *E. coli* mastitis, there does not appear to be any benefit either. A possible sequella to frequent milk-out treatment is the potential for new intramammary infections to establish in non-infected quarters. It is common for non-milked out quarters to leak milk during and post-frequent milk-out treatment, predisposing that quarter to a new intramammary infection.

In summary, eosin methylene blue agar can be used to rapidly differentiate *E. coli* from other gram-negative mastitis pathogens allowing prudent treatment recommendations to be made in a timely fashion. Frequent milk-out as a treatment for cows with *E. coli* mastitis does not appear to be beneficial though it does not appear to be detrimental either.

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## Footnotes

a. Analytical Profile Index, 20E, bioMerieux, France.

b. Remel, Lenexa, KS

c. Foss North America, Eden Prairie, MN

d. California Mastitis Test, Jorgensen Laboratories, Inc., Loveland, CO

e. SAS, ver 7.1, SAS Institute Inc., Cary NC

f. PASS, ver 6.0, Power and Sample Size, NCSS, Kaysville, UT

# Vita

Dagny Jayne Leininger was born on September 2, 1964 in Bellefonte, Pennsylvania. She graduated from Wyomissing Area High School in Wyomissing, Pennsylvania in 1982. Dagny graduated from Harcum Junior College in 1984 with an A. S. in Animal Health Technology and was employed as a Licensed Veterinary Technician at a private practice in Reading, Pennsylvania. Dagny received her B. S. in Animal Bioscience from Pennsylvania State University in 1988 and was employed by North Carolina State University College of Veterinary Medicine as a Licensed Veterinary Technician in the Large Animal Ambulatory Service for 3 years. Dagny received her Doctor of Veterinary Medicine from North Carolina State University College of Veterinary Medicine in 1996.

Dagny was an associate veterinarian at Casselman Veterinary Services in Grantsville, Maryland from 1996 to 1998. Since that time she has been completing a Food Animal Production Medicine Residency at the Virginia-Maryland Regional College of Veterinary Medicine in Blacksburg, Virginia.