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Use of eosin methylene blue agar to differentiate *Escherichia coli* from other gram-negative mastitis pathogens

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Coliform mastitis accounts for 20–80% of acute clinical mastitis cases[2] and is a continuous concern for US dairy producers because of its economic consequences.[2,3] Coliform mastitis pathogens are gram-negative, usually lactose-fermenting bacilli and include *Escherichia coli, Klebsiella* spp., and *Enterobacter* spp.[1] Other gram-negative organisms that can be isolated from the mammary gland include species of *Serratia, Pasteurella, Proteus,* and *Pseudomonas.*[9] Rapid identification of the causative organism is essential for implementing 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.[7] *Escherichia coli* can be identified with eosin methylene blue (EMB) agar based on the occurrence of a green-metallic sheen (Fig. 1) that appears on the surface of the bacterial colonies.[12] The dyes in EMB agar, eosin Y and methylene blue, are pH indicators and inhibitors of gram-positive bacteria and at an acid pH combine to form a green-metallic precipitate (sheen).[12]

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* infection.[4,5,8,18] 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.[4,5,8,18] 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 culture time needed for the first visible sheen to develop.

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 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 0.5 hour. Time was recorded at inoculation on EMB agar and at first visible sheen. Isolates were identified using biochemical test strips.[a]

Nine species of gram-negative bacteria were identified by the use of biochemical test strips (Table 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[6] of 93.7%, indicating excellent agreement beyond chance between biochemical test strips and EMB agar for identification of *E. coli.* The minimum and maximum times to first visible sheen were 3.3 hours and 10 hours, respectively, with a mean of 5.7 hours (SD = 1.5 hours) and a median of 5.2 hours.

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[16] 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[17] interfering with the acidic requirement of EMB agar for production of the green metallic sheen. Conversely, the 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.[18] False-negative reactions have also been related to pH variation within areas of the EMB agar plates[14] 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.[12] Lactose-fermenting organisms produce pink colonies[9] 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 tests may include a motility test, an acid production test on triple sugar–iron agar, and a citrate utilization test on Simmons citrate agar,[9] all of which require overnight incubation. More rapid for identification of *E. coli* are positive indole and β-glucuronidase tests,[10,11,13] which re-

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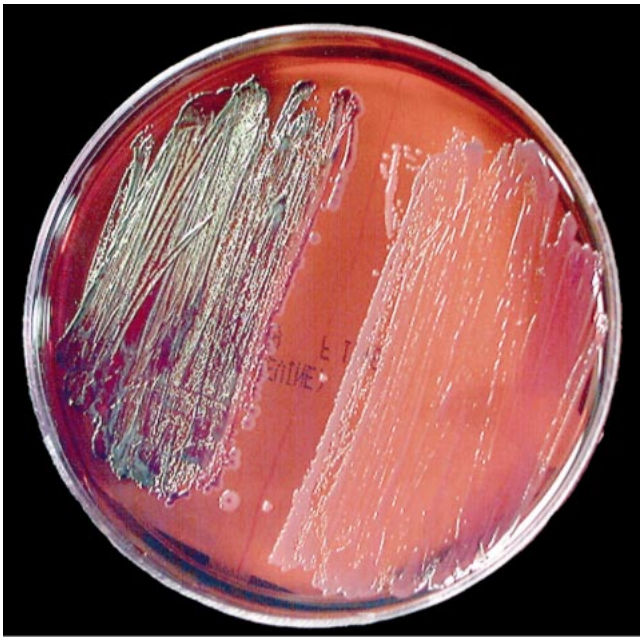

Figure 1. *Escherichia coli* (left) and *Serratia* spp. (right) on eosin methylene blue agar. Notice the green metallic sheen on the left (*E. coli*).

quire additional reagents.[10,11] 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.[7] 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 did MacConkey agar.[4,5,8]

Eosin methylene blue agar is simple to use, and therefore veterinarians can perform milk cultures in private practice when fast turnaround time is required. It is a very economical method of differentiation, with the materials priced at less than $1 per sample. Two samples can be plated on 1 blood agar plate ($0.21 per sample) and up to 8 isolates can be plated on 1 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, whereas mild to moderate cases of *Klebsiella* mastitis may evolve into chronic infections and warrant intramammary antibiotic therapy.[16,19] 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 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* isolate as *E. coli* would have resulted in withholding treatment from that cow and thus would have put her at an increased risk of developing chronic mastitis.[19] 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* case. Mastitis caused by *Serratia* responds poorly to antibiotic therapy,[9,16] and misclassification as *E. coli* may actually be beneficial in that withholding treatment from a cow infected with *Serratia* allows the option to cull a residue-free animal. Although 19 *Serratia* isolates were included in this study, mastitis caused by *Serratia* species is rare.[19,20]

Table 1. Identification of bacterial isolates by biochemical test strip and by production of a green metallic sheen on eosin methylene blue agar.

Organism	Total no. isolates	No. isolates with 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

Losses due to mastitis have been estimated at $35 to $295 per cow per year.[15] These estimates include production loss, replacement costs, discarded milk, drugs costs, and veterinary costs.[15] Treatment of clinical mastitis is the most common reason for antibiotic use and residue violations on dairy farms.[2,3] 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.

Eosin methylene blue 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.

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