Mastitis Pathogen Identification, Plates, Media, and Costs

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There are available to the veterinarian a variety of microbiological media for the cultivation of bacteria associated with infectious processes. Even within the more restricted area of bovine mastitis there are a number of selective and differential microbiological media, some of which are included in commercially available kits. The practitioner wishing to do his own microbiology must carefully evaluate each of the media so that meaningful and accurate results are obtained.

Many bacteria have at one time or other been implicated as causative agents of infectious mastitis. From a bacteriological standpoint, the veterinarian doing his own culturing should attempt to recognize only the more common of these. The organisms in this category would include *Streptococcus agalactiae* (beta hemolytic streptococcus), *Staphylococcus aureus, Escherichia coli, Klebsiella* and perhaps *Corynebacterium pyogenes* and *Streptococcus uberis.* For organisms which are sporadically involved or require specialized media, e.g. *Mycoplasma,* assistance of other laboratories should be sought.

It is commonly accepted that the gram-positive cocci, including *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis* and *Streptococcus dysgalactiae,* comprise the most important group of mastitis pathogens. As previously mentioned *S. aureus and Str. agalactiae* are of primary importance.

Coliform mastitis characterized by acute inflammation of the mammary gland, with high fever, diarrhea, shock, and frequently death is commonly associated with an environmental source of the organisms. *Enterobacter* and *E. coli* are frequently associated with unsanitary conditions where fecal material is thought to be the major source of the organisms. Recently, *Klebsiella* mastitis has been associated with the use of sawdust for bedding.

Corynebacterium pyogenes mastitis is characteristically a chronic sporadic disease in which the milk frequently has a foul odor, which results from the presence of an anaerobic gram-positive coccus along with *C. pyogenes.*

Media Useful for Cultivation of Mastitis Pathogens

Blood agar is the most important and useful bacteriological media. All of the bacteria commonly associated with mastitis grow readily on this medium. Importantly, those bacteria which have characteristic hemolytic patterns are more readily recognized. *S. aureus* will be 2-4 mm in diameter and typically be surrounded by a zone of complete hemolysis near the colony and an incomplete zone to the outside of the clear, complete zone. This type of hemolysis is commonly referred to as double zone hemolysis. Less frequently *S. aureus* may not have a zone of hemolysis or it may be surrounded only by a zone of complete hemolysis or a zone of incomplete hemolysis.

Streptococcus agalactiae, which is a beta-hemolytic streptococcus, is 1-2 mm in diameter in 24-48 hours. Some isolates of *Str. agalactiae* are non-hemolytic when cultivated on blood agar. These isolates can be presumptively identified by performing a CAMP test on sheep blood agar plates.

Growth of *Corynebacterium pyogenes* may not be observed until 48 hours of incubation. At this time isolated colonies will be pinpoint and some hemolysis may be noted where the growth is heavier. Typical colonies will not be seen until 72-96 hours of incubation. One millimeter dome shaped colonies surrounded by a zone of clear hemolysis will then be observed.

With the exception of swarming *Proteus*, hemolytic *E. coli*, and sometimes *Pseudomonas,* the gram-negative rods are difficult to distinguish on blood agar. MacConkey agar is, however, useful for the differentiation of some of these organisms.

The coliforms are lactose fermenters and typically form red colonies. *E. coli* form flat, non-mucoid, red colonies which are surrounded by a zone of red, precipitated bile. *Klebsiella* are more mucoid and characteristically the colonies have colorless rings surrounding a red center. This is particularly observable when the colonies are viewed through the bottom of the petri dish. The non-lactose fermenting bacteria such as *Proteus* and *Pseudomonas* will form colorless colonies.

It may be presumed that bacteria which grow on MacConkey are gram-negative because with few exceptions gram-positive organisms do not grow on MacConkey medium. Conversely, if a bacterium grows on blood agar but not MacConkey, it may be

predicted that the organisms are probably gram-positive.

A number of media may be used for the selective isolation and presumptive identification of streptococci (Fig. 1), particularly *Streptococcus agalactiae.*

The most recent modification of the streptococcal selective media is TKT/FC[1]. TKT/FC is an acronym for basic ingredients toxin, crystal violet, thallous acetate and ferric citrate. Thallium and crystal violet are inhibitors which allow streptococci to grow but inhibit the growth of most other bacteria. The toxin utilized in this medium is the staphylococcal beta (incomplete hemolysis) hemolysin which is involved in the CAMP reaction. The incorporation of the toxin into the medium along with sheep red cells at the time the medium is prepared, results in sensitization of the red cells, and obviates the need for streaking a beta-hemolytic staphylococcus on the medium as is done in the conventional CAMP test. Iron salts such as ferric citrate or ferric ammonium citrate serve as indicators of esculin utilization. Organisms which utilize esculin produce black colonies, which are frequently surrounded by a brownish zone. Streptococci in this category include *Str. uberis, Str. lactis,* and the enterocci or fecal streptococci. Esculin negative organisms, such as, *Str. agalactiae* and *Str. dysgalactiae* appear as bluish-grey colonies. *Str. agalactiae* will, however, be surrounded by a clear zone of hemolysis because it is CAMP positive. *Str. uberis* will sometimes give a positive CAMP type reaction, but unlike *Str. agalactiae,* their colonies will be black.

While TKT/FC is a good medium it has one serious drawback. The staphylococcal toxin which is added to sensitize the sheep red blood cells is quite labile. If the medium is improperly made or stored for too long before use, the red cells will not be sensitized and therefore not give the CAMP reaction.

Occasionally isolates of *Str. agalactiae* do not produce a zone of hemolysis when cultivated on blood agar without sensitized red blood cells. These strains can readily be detected on TKT/FC or by the conventional CAMP test. Because of the difficulty in making TKT/FC it is best purchased in the form of prepared plates or kits from commercial sources.

Edward's medium (modified)[2] is another selective and differential medium for the cultivation of streptococci. It is similar to TKT/FC, but there are some important differences. Staphylococcal toxin is not added to the medium so there is no CAMP reaction. Except for the non-hemolytic strains, *Str. agalactiae* will appear as a beta-hemolytic organism. Esculin is found in the medium, but there is no iron salt indicator. In our laboratory we added ferric ammonium citrate (0.5 gm/liter) prior to autoclaving the medium so that the esculin reaction may be observed directly.

Streptosel[3] is a commonly used selective medium for streptococci. It is not as highly inhibitory as TKT/FC or Edward's medium in that organisms other than streptococci will grow on the medium. *Pseudomonas* and *Proteus* are two examples of such organisms. A gram-stain would be very useful in differentiating these organisms, which are gram-negative rods.

Just as there are selective media for streptococci, a number of selective and differential media are available for the identification staphylococci (Fig. 2). Two of the more commonly used media are Mannitol-Salt[4] and Vogel-Johnson[5].

In mannitol-salt, a concentration of 6.5% sodium chloride acts to inhibit many bacteria. Bacteria which will grow on the medium in addition to staphylococci are yeasts, enterococci and some members of the genus *Bacillus,* making interpretation of results difficult at times. *Bacillus* organisms may be recognized because they usually form mucoid, watery type colonies. In addition, *Bacillus* and yeasts may be differentiated from staphylococci in a gram-stain, *Bacillus* being a large gram-positive rod; the yeasts are also gram-positive and show budding. Enterococcal colonies are smaller and less opaque than those of the staphylococci. The pathogenic staphylococci form colonies which are 2-4 mm in diameter and are commonly associated with a change in the color of the medium from pink to yellow indicating mannitol is being utilized. *Staphylococcus epidermidis* does not utilize mannitol and will change the color of the medium to a more red color. Unfortunately not all *S. aureus* utilize mannitol, therefore, some of them will be misidentified as *S. epidermidis* if the mannitol reaction is used as the sole basis for differentiation.

Vogel-Johnson is a mannitol containing medium that is more inhibitory than mannitol-salt for organisms other than *S. aureus.* Because of the incorporation of potassium tellurite, *S. aureus* colonies will be black. These colonies may be surrounded by yellow zones indicating the utilization of mannitol. Thus, black colonies surrounded by a yellow zone may be identified as *S. aureus. S. epidermidis* will grow, but the colonies are smaller; as with mannitol-salt the medium becomes more red. Non-staphylococci do not grow in the 24-48 hour period after inoculation of the medium. All of the previously described media are available from a variety of commercial sources as individual plates. In Fig. 3 are examples of the appearance of mastitis pathogens on some commonly used bacteriological media.

Recently Pitman-Moore has released a number of kits incorporating some of the previosly described media. Bactassay[6] is a five chambered culture system employing brain heart infusion (BHI) agar with

[1]*Gibco Diagnostics, P.O. Box 4385, Madison, Wisconsin 53713.*

[2]*Flow Laboratories, P.O. Box 226, Rockville, Maryland 20852.*

[3]*BioQuest, P.O. Box 243, Cockeysville, Maryland 21030.*

[4]*Difco Laboratories, Detroit, Michigan 48232 or BioQuest.*

[5]*Difco Laboratories or BioQuest.*

[6]*Pitman-Moore, Inc., Washington Crossing, New Jersey 08560.*

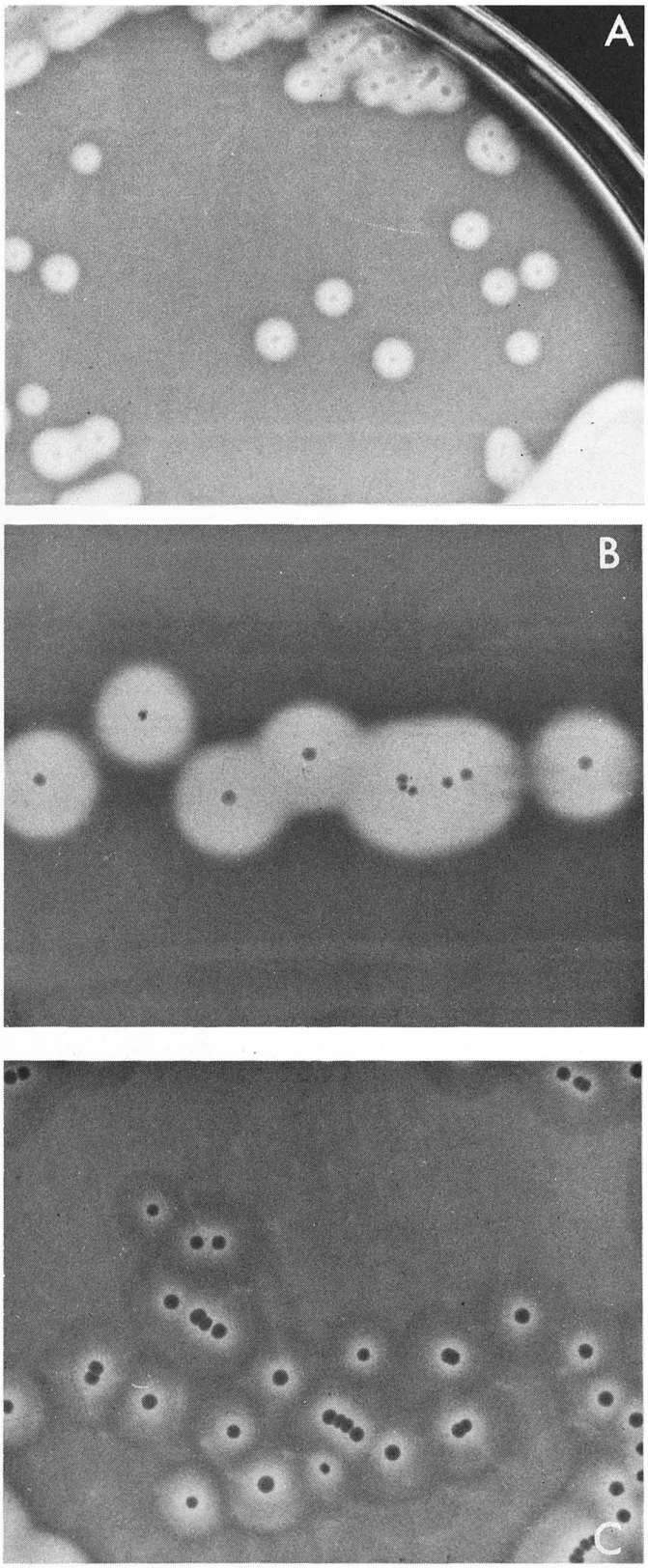

Figure 1. Media for Streptococci. A. TKT/FC. Toxin - Beta (incomplete) toxin of Staphyloccuc aureus (Modified Camp Test). Crystal violet - Inhibits gram-positive bacteria except streptococci. Thallous acetate - Inhibits gram negative organisms. Esculin and Ferric Citrate - Esculin positive organisms have black colonies surrounded by a dark zone. Esculin negative organisms have blue-gray colonies. Esculin positive organisms - Streptococcus uberis, Streptococcus lactis, and Enterococcus. B. Streptosel. C. Edward's Medium.

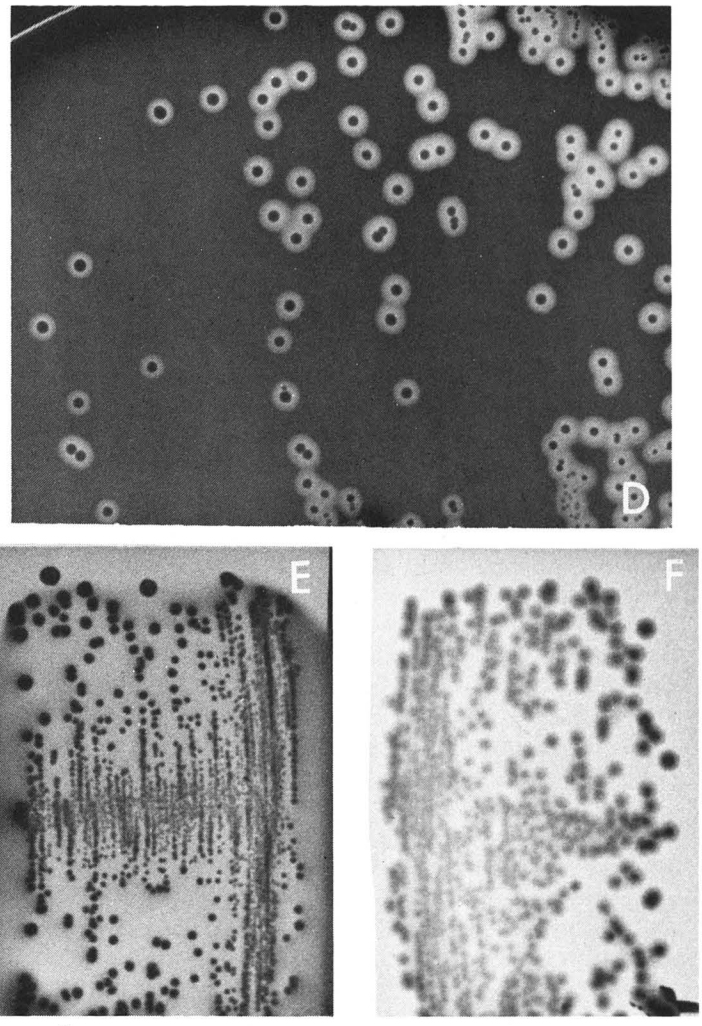

Figure 2. Media for Staphylococcus Aureus. Vogel-Johnson. Tellurite Inhibitory. Staphylococcus aureus - Black colony, yellow zone. Most other organisms inhibited. Mannitol Salt. 6.5% salt inhibitory for many bacteria. Staphylococcus aureus - Yellow zone. Staphylococcus epidermidis - Red zone. Yeast and Bacillus will grow.

Figure 3. Colonial Characteristics of Common Mastitis Pathogens. A. Streptococcus agalactiae on blood agar at 84 hours. A zone of beta (clear) hemolysis surrounds the colony. B. Streptococcus agalactiae on TKT/FC at 24 hours. C. Staphylococcus aureus on blood agar at 24 hours. Zones of complete hemolysis are in the area immediately around the colony. A zone of beta or incomplete hemolysis occurs outside the zone of complete hemolysis. D. Corynebacterium pyogenes on blood agar at 96 hours. E. Escherichia coli on MacConkey agar at 24 hours. A precipitate which is red in color may be seen around some of the colonies. F. Klebsiella on MacConkey at 24 hours. Colonies are mucoic, having a red center and clear outer ring.

serum, MacConkey agar, mannitol-salt and streptosel in the four smaller chambers for the isolation of bacteria from clinical specimens. The fifth larger chamber contains Muller-Hinton with serum to be used for the determination of susceptibility of organisms to chemotherapeutic agents. Brain heart infusion agar with serum is a general purpose, non-selective culture medium which permits the growth of

most of the common clinically important bacteria. This medium is included in the kit as a replacement for blood agar. By making this substitution the shelf life of the medium is extended, however, this creates an important negative condition, i.e. the inability to determine hemolytic patterns of bacterial isolates. A comparative pictorial chart of important veterinary pathogens is supplied by the manufacturer as an aid in the identification of isolates. Care must be taken that proper interpretation of results is made, particularly when more than one organism is observed in the BHI chamber. Gram-staining of individual isolates is essential. Each of the smaller chambers should be streaked with a loop, not a swab, for best isolation of bacterial colonies.

The chamber used for susceptibility testing should not be inoculated at the same time that the smaller chambers are inoculated with the clinical specimen. Only after individual colonies are obtained should appropriate isolates be selected for antibiotic susceptibility testing. Not only is direct susceptibility testing an example of poor technique, but it may also be very expensive if negative results are obtained. Bactassay costs about $3.00 per kit and is best suited for evaluation of the mastitis status of the individual cow[7].

A new three chambered kit, Mastassay-D[8], containing blood agar, MacConkey agar and TKT/FC was recently marketed. The common mastitis pathogens grow on one or more of the media. As with Bactassay a pictorial chart is provided. The kit may be used for culturing milk from an individual cow, or if care is exercised three, or possibly four, composite samples may be streaked on each section. By evaluating the results on the three chambers the presumptive identification of the important pathogens may be made.

A second three chambered kit in which each of the chambers contains TKT/FC (Mastassay-S[9]) is also available from Pitman-Moore. The kit is of value only in those herds where *Streptococcus agalactiae* is a problem. Each of the chambers may be used for cultivation of milk from individual quarters or by dividing each chamber into three or four additional sections composite samples from 9-12 animals may be cultured on each plate. The cost per plate for Mastassay-S and Mastassay-D is approximately three dollars. As with Bactassay instructions for use of the Mastassay kits are provided and should be consulted by the practitioner for more details.

In our laboratory the individual cases of mastitis are processed as routine cases through the diagnostic laboratory by cultivation on blood agar and MacConkey. Broth cultures are also inoculated to be used as necessaryfor subculturing.

When milk from a large number of animals is being cultured we have used TKT/FC, or modified Edward's medium with added ferric ammonium citrate for selective isolation of streptococci, and Vogel-Johnson for the isolation of staphylococci. These media are used where there is a herd problem because we are mainly interested in the isolation of *Streptococcus agalactiae* and *Staphylococcus aureus.* The media are poured in large petri dishes (150mm x 15mm) which are divided into sections, usually 12 in number. Composite quarter samples from individual cows are spread over each section. Results are recorded after 24 and 48 hours of incubation at 37°C. Because these media are highly selective, the presumptive identification of *Str. agalactiae* and *S. aureus* may be made with minimal effort and reasonable reliability. Our cost for materials to prepare TKT/FC in the large plates is approximately one dollar, while Vogel-Johnson costs approximately thirty cents.

The proper use and interpretation of the results obtained from the use of bacteriological media can provide valuable information as to the specific causes of bovine mastitis. If the veterinarian learns to recognize the appearance of the major pathogens more precise control and therapeutic regimens may be initiated. Implicit in the obtaining of good bacteriological results in the use of good technique in the collection of samples and streaking of media.

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[7]*A kit similar to Bactassay, except that BHI and Streptosel come with added sheep blood, and media come as conventional plates or biplates, is available from Pharmacologic Supply Co., 175 Union St., Centerburg, Ohio 43011.*

[8]*Pitman-Moore, Inc.*

[9]*Pitman-Moore, Inc.*