Identification of Bacterial Pathogens of Bovine Mastitis by the Practicing Veterinarian

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CULTURING TECHNIQUES ARE CONSIDERED complicated and time consuming by many veterinarians. Control of mastitis is often attempted with out detection of subclinical cases which serve as reservoirs of infection for the entire herd. Effective control of bovine mastitis must necessarily be based upon the detection of every infected quarter in a herd. Identification of the etiologic agents avoids hit or miss methods, gives indications for specific therapy, aids in prognosis and furnishes information for a definite plan of control.

The purpose of this paper is to present a procedure whereby the practitioner may determine the etiology of mastitis cases in his own laboratory. The laboratory equipment need not be elaborate or expensive. A compound microscope with an oil immersion lens is necessary. The other essential equipment can be obtained at a cost of less than $100.00[2]

GENERAL PROCEDURE

First 24 Hours

(1) Collect sample. (2) Make direct smear (Gram's stain) in acute cases.* (3) Incubate milk samples. (4) Streak blood agar plates. (5) Incubate inoculated plates.

*Sufficient evidence may be obtained at these points to warrant a tentative diagnosis.

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Second 24 Hours

(6) Examine bacterial growth, noting colony characteristics and hemolysis.* (7) Transfer selected colonies to appropriate media. (8) Incubate all new cultures.

Third 24 Hours

(9) Examine cultures, note reactions and, when indicated, inoculate carbohydrate media. (10) Incubate newly inoculated cultures. (11) Make final observations of media.

DETAILS OF PROCEDURE

1. COLLECT SAMPLE

Proper collection of specimens is important to avoid contamination which may prevent the diagnosis or increase the time necessary to isolate the pathogens. All tubes must be sterile and may be rubber or cork stoppered, or screw-capped. Sterilization by autoclaving (pressure cooker) at 15 pounds pressure for 15 minutes is effective.

Fore milk samples are the best for culturing. After properly cleansing the test and orifice (1), use the strip cup before collecting the milk specimen. Avoid contamination of the tube by holding it in a near-horizontal position during filling and by protecting the exposed lower end of the stopper. An indicator such as bromcresol purple may be added to the tubes before sterilization so that Hotis test results and specimen for culturing can be obtained from a single sample.[1]

2. DIRECT SMEAR

A clean glass slide is essential. Prepare a thin smear of milk or udder exudate on the slide. Allow the smear to air dry and

fix lightly with heat. Flood the slide with xylol (xylene) for 30 seconds, then pour off the excess and allow the slide to air dry. Proceed with the Gram stain as follows:

a. Hucker's Gentian (crystal) violet, one minute.
b. Wash with distilled water.
c. Gram's iodine, one minute.
d. Pour off excess fluid.
e. Acetone alcohol (30-70 mixture), six to ten seconds.*
f. Wash with distilled water.
g. Safranin, one minute.
h. Wash with distilled water.

* This time must be adjusted according to the thickness of the smear.

Blot the slide dry and observe under oil immersion. Examination of the smear may reveal the morphology and Gram reaction of the pathogen.

3. INCUBATE MILK SAMPLES

The milk samples are incubated at 37° C for 12 to 16 hours.

4. STREAK BLOOD AGAR PLATES

A loopful of incubated milk is streaked onto the surface of one quarter of a blood agar plate. Five percent bovine blood agar is suggested for this purpose, but whole blood of the horse, sheep or rabbit may also be used. The same kind of blood should always be used as there is variation in the hemolytic patterns produced by pathogens on agar prepared from the blood of different species.

5. INCUBATE INOCULATED PLATES

The plates are incubated in an inverted position at 37° C for 20 to 24 hours. Most pathogenic bacteria will form visible colonies in this time. *Corynebacterium pyogenes* is one possible exception to this rule and plates should be incubated for a minimum of 48 hours when this organism is suspected.

6. EXAMINE BACTERIAL GROWTH

(See colony characteristics in Table I)

TABLE 1: COLONY CHARACTERISTICS

ORGANISM	HEMOLYSIS	SIZE	SHAPE	SURFACE	COLOR
Streptococcus agalactiae	alpha or beta	small	round, entire	smooth	none (translucent)
Streptococcus dysgalactiae	alpha or beta	small	round, entire	smooth	none (translucent)
Streptococcus uberis	alpha	small	round, entire	smooth	none (translucent)
Streptococcus zooepidemicus	beta (wide)	small	round, entire	smooth	none (translucent)
Staphylococcus aureus	usually double zone	med. to large	round, entire	smooth	white or yellow
Pseudomonas aeruginosa	beta	large	irreg.	smooth to rough	green pigment
Escherichia coli	none	med. to large	round, entire	smooth	white to gray
Paracolobactrum sp.	none	med. to large	round, entire	smooth	white to gray
Klebsiella pneumoniae	none	med. to large	round, entire	smooth	usually gray
Aerobacter aerogenes	none	med. to large	round, entire	smooth	usually gray
Corynebacterium pyogenes	narrow beta	small	round, entire	smooth	white

TABLE 2: BIOCHEMICAL REACTIONS OF MASTITIS STREPTOCOCCI

	LITMUS MILK	SUCROSE	SALICIN	RAFFINOSE	INULIN	TREHALOSE
Streptococcus agalactiae	AC	+	+*	−	−	+
Streptococcus dysgalactiae	A	+	−	−	−	+
Streptococcus uberis	ACR	+	+	−	+	+

A-acid, C-coagulation, R-reduction
* occasional strains negative

TABLE 3: BIOCHEMICAL REACTIONS OF COLIFORM ORGANISMS

	DEXTROSE	LACTOSE	SUCROSE	LITMUS MILK	INDOL	MOTILITY
Escherichia coli	+*	+	−x	ACR	+	+xx
Aerobacter aerogenes	+*	+	+	AC	−	+
Klebsiella pneumoniae	+*	+	+	AC	−	−
Paracolobactrum sp.	+*	+**	+	AC	−	+

*All fermentations accompanied by gas formation
**Slow reactions, 2-14 days
xOccasional strains positive
xxOccasional strains negative

Staphylococcus aureus may be identified on the blood agar plate by the characteristic double-zone hemolysis. Likewise, beta hemolysis, green pigment formation and the odor of trimethylamine are sufficient to identify *Pseudomonas aeruginosa*.

7. TRANSFER COLONIES

a. A suspected streptococcus colony is carefully selected and transferred to sterile brain-heart infusion broth. b. If there is doubt of the identification of *Staph. aureus* on blood agar, a suspected colony is transferred to a mannite-salt agar slant. c. A colony suspected of being *Pseudomonas aeruginosa* is transferred to a tube of litmus milk. d. A suspected colony of the coliform group (i.e., *Escherichia, Aerobacter, Klebsiella* or *Paracolobactrum*) is transferred to brain-heart infusion broth. e. When *C. pyogenes* is suspected, several colonies are transferred to a tube of litmus milk.

8. INCUBATE CULTURES

All cultures are incubated at 37° C for 20 to 24 hours.

9. EXAMINE CULTURES

a. Suspected streptococci; smear and stain to determine morphology and Gram reaction and to establish the purity of the culture. Then transfer broth inoculum to a series of caryohydrate media as indicated in Table 2. b. Suspected staphylococci; abundant growth on the salt agar slant with fermentation of the mannite (color change to yellow) is indicative of *Staph. aureus*. A loopful of colony growth is transferred to coagulase test medium. c. *Pseudomonas aeruginosa* in litmus milk wil first acidify, then (by 20 hours) will usually decolorize the litmus, partially digest the casein and show an alkaline reaction due to the protein breakdown. Sometimes green pigment will be seen. The milk is usually not coagulated. d. Suspected coliform cultures are smeared and

stained as described above and then, if pure, transferred to a battery of carbohydrate media as indicated in Table 3. e. Litmus milk cultures of *C. pyogenes* will usually not show any change in 24 hours, so incubation should be continued.

10. INCUBATE NEW CULTURES

Incubate all newly inoculated cultures at 37° C for 20 to 24 hours.

11. MAKE FINAL OBSERVATIONS

a. Streptococci; all fermentations are observed and interpreted according to Table 2. As a rule of thumb, all raffinose positive cultures should be discarded as non-pathogens. b. Staphylococci; the coagulase test is read. A positive test is partial or complete coagulation of the plasma while a negative test is the absence of any coagulation. Many strains of *Staph. aureus* will coagulate rabbit plasma within six hours. c. Suspected coliform cultures; complete the test for indol by addition of a few drops of chloroform followed by a few drops of Kovac's reagent to the SIM medium (Difco). A positive test is indicated by the appearance of a pink to red color. Observe the fermentations and compare with Table 3 for identification of the organism. d. Suspected *C pyogenes;* if characteristic changes are not present in the litmus milk at this time, incubation and observation must be continued for an additional 24 to 48 hours. *Corynebacterium pyogenes* typically attacks litmus milk in the following sequence; acidification, coagulation and finally digestion.

CULTURE MEDIA USED IN IDENTIFYING MASTITIS PATHOGENS

Commercially prepared dehydrated media will be convenient as well as economical for use in the practitioner's laboratory. Such media are prepared for use by adding distilled water, dispensing and sterilizing.

The following is a suggested list of media for use in mastitis diagnosis: (1) Blood agar base (2) Brain-heart infusion broth (3) Mannite-salt agar (4) Litmus milk (5) SIM (Difco) (6) Phenol Red broth base (carbohydrate-free) (7) Selected purified carbohydrates.

Blood agar is preferred to other solid media because the hemolytic patterns are of much value in identifying pathogens. Sterile citrated whole blood is added to previously autoclaved and cooled blood agar base to give a final concentration of 5 per cent.

Sterile blood may be collected by jugular venipuncture of the sheep, cow and horse or cardiac puncture of an anesthetized rabbit. The area should be prepared by shaving and disinfecting with iodine. The collection equipment and citrate solution must be sterile (autoclave). The amount of sodium citrate used should give a final concentration of one percent in the collected blood. The blood may be dispensed into sterile tubes after collection so that the contents of one tube is the proper amount for mixing with one flask of blood agar base. Sterile whole blood may be refrigerated for two or three months before hemolysis becomes objectionable.

Material for the coagulase test may be collected and the medium prepared in the following manner. Using a sterile pipette, draw the plasma from a tube of sterile citrated rabbit blood (1-2% sodium citrate) Dilute the plasma with sterile physiologic saline solution (0.85% NaCl) in the proportion of one part plasma to four parts saline. Dispense into sterile tubes in 0.5 ml. amounts and refrigerate until used.

The article by Peterson, *et al,* gives details of the procedure for pouring plates of blood agar or other solid media. Slants may be made by dispensing two or three ml. amounts of the melted medium into sterile glass tubes and allowing the agar to solidify with the tubes in a slanted position. All broth media should be dispensed in three ml. amounts in standard test tubes, stoppered with non-absorbent cotton or solid plugs and sterilized. All media should be refrigerated until used in order to minimize dehydration.

At times the use of a selective medium may be indicated to isolate a certain bacterial pathogen from a contaminated specimen, however such media have limited application because of their selectivity.

Formulae for two of these media follow.

Edward's Medium

Meat extract agar (pH 7.4)	1000 ml.
Crystal violet (0.1% aqueous solution)	2 ml.
Esculin*	1 gm.
Citrated or defibrinated ox blood	50 ml.

*The esculin is put into solution by boiling it in a small quantity of water and adding it to the melted agar at the same time as blood.

This medium inhibits the growth of the staphylococci but allows the growth of streptococci and coliform organisms. *Streptococcus agalactiae* colonies are purple, colonies of *Strep. dysgalactiae* are blue-gray and those of *Strep. uberis* are brown. Coliform colonies are black.

Sodium azide-crystal violet blood agar

Tryptose agar (pH 6.8)	1000 ml.
Crystal violet (0.1% aqueous solution)	2 ml.
Sodium azide*	0.5 gm.
Citrated or defibrinated ox blood	50 ml.

*Add the sodium azide and crystal violet at the same time as the blood.

This medium permits the growth of streptococci and *E. rhusiopathiae* but inhibits the growth of most other micro-organisms.

SUMMARY

A minimal procedure is outlined for identification of common mastitis pathogens in the office laboratory of the practicing veterinarian. Materials and methods utilized are discussed and/or listed.

Literature Cited

1. Merchant, I. A. and Packer, R. A., Handbook of the etiology, diagnosis and control of infectious bovine mastitis. Burgess Publishing Company, Minneapolis, 1952.
2. Peterson, H. J., Ramsey, F. K. and Collier, J. R., Identification of bacterial pathogens of poultry by practicing veterinarians. Iowa State College Vet. 15:73-77. 1953.

The sheep industry of the West involves roughly 25 million sheep, which annually produce about two-thirds of the nation's lamb tonnage and about three-quarters of its total wool production—40,000 or more carloads of market sheep and about 5,000 carloads of wool.