Journal of Food Protection, Vol. 48, No. 12, Pages 1019-1021 (December 1985)

Medium to Culture and Differentiate Coagulase-Positive and -Negative Staphylococci from Bovine Milk

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(Received for publication April 30, 1985)

ABSTRACT

A medium which incorporates CAMP factor produced by *Streptococcus agalactiae* (group B) into sheep blood agar was used to culture and identify coagulase-positive staphylococci from bovine milk. Of 506 staphylococcal isolates from bovine milk, 92.5% of coagulase-positive organisms produced a wide zone of complete hemolysis, whereas 98.9% of coagulase-negative organisms did not. The agreement of this one-step culture and identification test with the standard tube coagulase test was higher than that of the deoxyribonuclease test medium, Baird-Parker egg yolk medium, tellurite glycine medium and slide coagulase tests.

The practice of identifying types and numbers of various pathogenic bacteria from bulk tank milk samples is being increasingly used as an aid in determining the infection status of a herd and determining the degree of exposure to environmental contaminant organisms. For this information to be of greatest value the number of *Streptococcus agalactiae*, non-agalactiae streptococci, *Staphylococcus aureus*, other staphylococci, and coliforms must be determined. It is especially desirable if the determination can be made accurately from a differential plate without use of biochemical tests. The TKT plate has been used extensively for presumptive identification of *S. agalactiae* and MacConky's plate provides adequate results for enumeration of coliforms. There are media available for differentiation of staphylococci but results of these correlate well with those of the coagulase test, which is usually as the index of pathogenicity in mastitis work.

For this reason an attempt was made to develop a medium which would separate coagulase-positive and -negative staphylococci with reasonable accuracy, and which could be used as a primary counting plate as well as a method of separation for bulk tank cultures. Coagulase-positive staphylococci are among the most common causes of bovine mastitis. These staphylococci are represented predominantly by *S. aureus*, but also by some strains of *Staphylococcus intermedius*, and *Staphylococcus hyicus* subsp. *hyicus (13)*. The coagulase-positive staphylococci isolated from bovine mastitis are generally beta hemolysin producers (*10,16*).

CAMP factor produced by *S. agalactiae* and separated and filtered (group B), when incorporated into sheep blood agar, completes the hemolytic reaction of beta hemolysin making it highly visible (*2,5,12*). Sheep blood agar with CAMP factor (Staphylococcal CAMP medium) was used to culture and identify beta hemolysin-producing staphylococci from bulk and quarter milk samples. Results of the staphylococcal CAMP medium were compared with those of the tube coagulase test, deoxyribonuclease test medium, Baird-Parker egg yolk medium, tellurite glycine medium, and the slide coagulase (clumping factor) test.

MATERIALS AND METHODS

Preparation of crude streptococcal CAMP factor

S. agalactiae (Cornell strain #44) was used because of its reproducible CAMP reaction. Investigators have shown that the CAMP reaction of *S. agalactiae* was enhanced when the organism was grown in the presence of maltose (*8*). Therefore, Brain Heart Infusion (BHI) broth with 10 g of maltose/L was selected for production of CAMP factor (2). The starter culture was initiated by inoculating *S. agalactiae* (Cornell strain #44) into 5 ml of BHI. Following 16 h of incubation at 37°C, 1 ml of the starter culture was inoculated into 1 L of BHI containing 10 g of maltose. This culture was incubated 24 h on a rotary shaker (100 rpm) and divided into four 250-ml portions, then filtered through a 0.22-, μm membrane filter. The filtrate, which contained the CAMP factor, was subsequently referred to as crude CAMP factor. This crude CAMP factor was stored at 4°C, with no significant loss in activity during 1 year.

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Activity assay of crude streptococcal CAMP factor

To Trypticase Soy Agar (TSA) (4 g/100 ml) was added 5-ml of thrice-washed sheep erythrocytes (*13*) to give a 5% concentration. Crude CAMP factor was then added to a final concentration of 1, 5, and 10%. *S. aureus* (T-19) obtained from the Department of Veterinary Pathobiology, College of Veterinary Medicine, St. Paul, Minnesota, was inoculated for isolation onto the various media. Plates were incubated for 24 h at 37°C. The 10% CAMP factor medium produced the best CAMP reaction and was subsequently used as the test medium. Various concentration and purification procedures did not significantly improve these results.

Tube coagulase test

Lyophilized rabbit plasma (Difco) was rehydrated in sterile distilled water. Three tenths ml of a 1:2 dilution of plasma was inoculated with a 24-h-old culture and incubated at 37°C (*4*). Readings were made at 1-h intervals up to 4 h and then at 24 h. Formation of any degree of clot was called positive, a negative was recorded if the plasma remained liquid (*17*).

Deoxyribonuclease (DNAse) agar

Staphylococcal isolates were inoculated onto DNAse test agar as a 2.5-cm streak. Each plate contained eight staphylococcal isolates, a positive and a negative control. Each incubation at 37°C for 24 h, plates were flooded with 1 *N* hydrochloric acid. DNAse-positive isolates showed a distinct clear zone surrounding the culture streak (*15*).

Baird Parker egg yolk (BPEY) medium

Baird-Parker agar with egg-yolk tellurite enrichment was inoculated with staphylococcal isolates as previously described and incubated for 24 h at 37°C. A positive BPEY test was recorded when the culture streak appeared black, shiny, convex, and was surrounded by a clear zone 2-5 mm in diameter. A duller-colored streak with irregular edges, surrounded by a large opaque zone was called a negative test (*1*).

Tellurite Glycine (TG) agar

Tellurite glycine agar was inoculated with staphylococcal isolates as previously described, and incubated for 48 h at 37°C. A black culture streak was called positive, whereas a gray to dull black streak was called negative (*20*).

Staphylococcal CAMP medium

This medium was prepared from TSA with a 10% final concentration of crude CAMP factor. Plates were inoculated with staphylococcal isolates as previously described. A positive CAMP reaction was a zone of complete hemolysis of the erythrocytes, 8-18 mm in diameter, around the colony streak. A very narrow zone, 1-8 mm diameter, was called a negative CAMP reaction (see Fig. 1).

Testing of the staphylococcal CAMP medium

A total of 506 staphylococcal isolates were used in this comparative study. These isolates represented all staphylococci isolated from clinical and sub-clinical mastitis samples submitted to the Mastitis Laboratory, College of Veterinary Medicine, University of Minnesota, during the period of this study, and represented 354 dairy cows in 56 Minnesota herds. The test medium was compared with the tube coagulase tests, slide coagulase test, deoxyribonuclease medium, Baird-Parker Egg Yolk medium, and Tellurite Glycine medium.

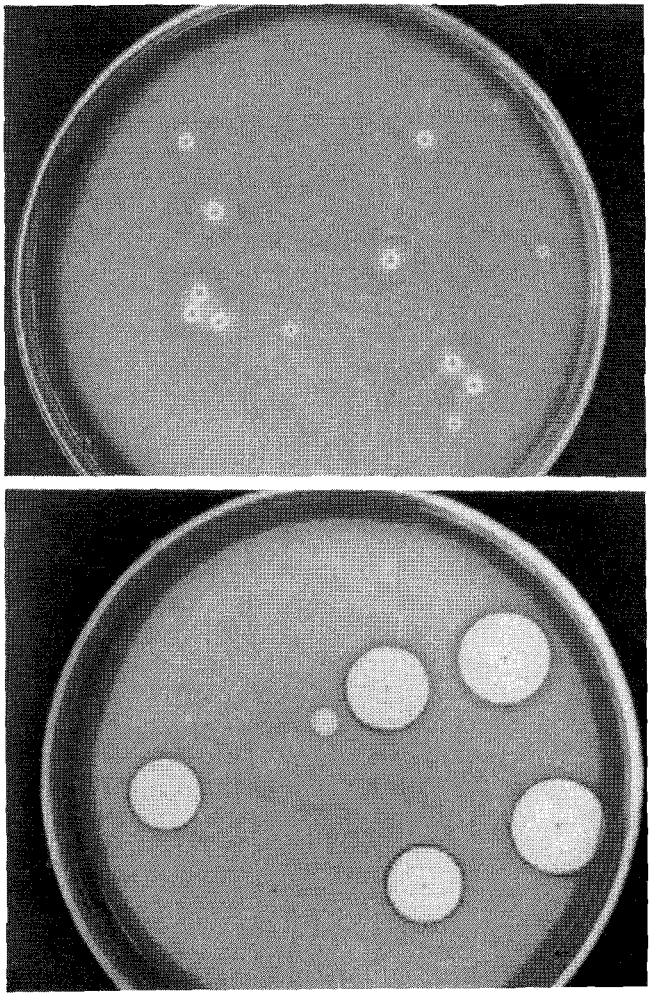

Figure 1. *(Top)* Staphylococcus aureus *(T-19) on sheep blood agar. (Bottom)* Staphylococcus aureus *(T-19) on Staphylococcal CAMP medium.*

RESULTS

Results obtained using the staphylococcal CAMP medium were in overall agreement with those of the tube coagulase test for 96% of the bovine staphylococcal isolates tested. Of the 506 staphylococci isolates, 226 were tube coagulase-positive and 280 were tube coagulase-negative. Table 1 shows the correlation between each of the methods tested and the tube coagulase test. The staphylococcal CAMP medium showed the highest overall agreement at 96%, while the DNAse and slide coagulase tests showed high agreement with the coagulase-positive isolates, 99.1% and 92.9% respectively. Numerous false-positives were noted for the DNAse and slide coagulase tests, while the BPEY and TG media gave numerous false-negatives.

DISCUSSION

The CAMP reaction is a distinct visual reaction occurring to sheep erythrocytes when simultaneously exposed

TABLE 1. *Coagulase-positive vs. coagulase-negative results using CAMP medium, DNAse medium, slide coagulase test, BPEY medium and T6 medium.*

Test	Number of isolates (Percent) Positive reaction	Negative reaction	Overall agreement
CAMP medium	209 (92.5%)	277 (98.9%)	96%
DNAse medium	224 (99.1%)	187 (66.8%)	81%
Slide coagulase test	210 (92.9%)	136 (48.6%)	68%
BPEY medium	53 (23.5%)	219 (79.2%)	54%
TG medium	38 (16.8%)	232 (82.9%)	53%
Total	226 (100%)	280 (100%)	

to staphylococcal beta hemolysin and group B streptococcal CAMP factor (*5,12*). The interaction of staphylococcal beta hemolysin with streptococcal CAMP factor for identification of *S. agalactiae* was first shown in 1947 by Munch-Peterson et al. (*5*). The staphylococcal CAMP medium incorporates *S. agalactiae* CAMP factor into a blood agar medium. When this medium is exposed to staphylococcal beta hemolysin, a CAMP reaction results. Thus the reliability of the medium is solely based on production of beta hemolysin by staphylococci.

Staphylococcal beta hemolysin has been studied quite extensively (S. K. Maheswaren, Ph.D. thesis, University of Minnesota, Saint Paul, 1967) (*19*) and found to be produced either alone, in combination with other hemolysins, or not at al. Loken and Hoyt (*10*) characterized staphylococci isolated from bovine foremilk samples. They found 95% of the coagulase-positive strains isolated produced beta hemolysin either alone or in combination with alpha and/or delta hemolysins. They also suggested that detection of beta hemolysin production would be an adequate cirterion to determine potential pathogenicity in routine mastitis control studies.

Slanetz et al. (*17*) and Smith (*18*) found 97.7% and 92%, respectively, of staphylococcil isolated from cattle that would produce beta hemolysin either alone or in some combination with other hemolysins. Before this, Minnet (*11*), and later Burns and Holtman (*3*), reported production of beta hemolysin by staphylococci to be more characteristic of strains from animals than those isolated from humans. The literature plus the recent work of Jasper et al. (*9*) supports the concept that determining beta hemolysin would be an adequate criterion for identifying coagulase-positive staphylococci. This modified medium should be an accurate means of one-step culture and identificatioin of coagulase-positive staphylococci from bovine foremilk samples.

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