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Evaluation of Methods for Differentiation of Coagulase-Positive Staphylococci

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The purpose of this study was to determine the minimum number of tests that could be used to differentiate between the coagulase-positive strains of staphylococcus: *Staphylococcus aureus*, *Staphylococcus hyicus*, and *Staphylococcus intermedius*. Eighty coagulase-positive strains of each of the three species were examined. The five tests conducted were growth on modified Baird-Parker agar, growth on P agar supplemented with acriflavin, production of acetoin, anaerobic fermentation of mannitol, and presence of β-galactosidase. Positive test percentages for *S. aureus* were 100% for growth on modified Baird-Parker agar, 100% for growth on P agar supplemented with acriflavin, 94% for production of acetoin, 99% for anaerobic fermentation of mannitol, and 0% for presence of β-galactosidase. Positive test percentages for *S. intermedius* were 0% for growth on modified Baird-Parker agar, 0% for growth on P agar supplemented with acriflavin, 1% for production of acetoin, 0% for anaerobic fermentation of mannitol, and 100% for presence of β-galactosidase. *S. hyicus* isolates were negative in all five tests. Results from the 240 coagulase-positive staphylococcus strains tested would suggest correct identification of coagulase-positive staphylococci with P agar supplemented with acriflavin and the β-galactosidase test. These two tests are simple to conduct and result in quick and easy differentiation of the three coagulase-positive staphylococcal species.

The latest edition of *Bergey's Manual of Determinative Bacteriology* (14) lists three coagulase-positive staphylococcus (CPS) species: *Staphylococcus aureus*, *Staphylococcus hyicus*, and *Staphylococcus intermedius*. *S. aureus* is a major agent of bovine mastitis (12). The prevalence and pathogenicity of *S. hyicus* and *S. intermedius* as agents of bovine mastitis remain uncertain. Although it has been suggested that *S. intermedius* is an agent of bovine mastitis, this issue has not been fully resolved (16). Some of the strains in the aforementioned study (16) were later determined to be atypical strains of *S. aureus* (17). Other studies found no *S. intermedius* among isolates from the milk of dairy cows (10, 12). The development of a simple procedure differentiating between species of CPS would be a valuable aid in determining the prevalence of *S. intermedius* as an agent of bovine mastitis.

Presumptive differentiation of CPS species by visual appraisal of growth on blood agar cannot be reliably made. While the majority of *S. aureus* strains are pigmented and produce hemolysins, *S. aureus* strains without one or both of these properties could be confused with *S. intermedius* and coagulase-positive strains of *S. hyicus*. *S. intermedius* is nonpigmented and may produce hemolysins, and *S. hyicus* is negative for both pigment and hemolysins (14). Currently the easiest method of identifying CPS to the species level is through commercial miniaturized biochemical test systems. However, none have shown 100% accuracy in identifying the bovine strains of CPS. Some laboratories may not be readily able to differentiate the relatively rare CPS species that cause bovine mastitis. A system which could make such a differentiation at minimal cost of time and money would be desirable. There are a few simple tests that could be incorporated into a CPS differentiation system. Two agars, modified Baird-Parker agar (MBP) and P agar supplemented with acriflavin, support growth of *S. aureus* but inhibit the growth of the other CPS (4, 8). The ability of *S. aureus* to aggressively ferment mannitol anaerobically is in sharp contrast to the other CPS, which normally do not. Likewise, the production of acetoin from glucose metabolism is characteristic of *S. aureus* but not characteristic of *S. hyicus* or *S. intermedius* (14). Finally, *S. intermedius* expresses the enzyme β-galactosidase, whereas the other CPS strains typically do not. The present study was conducted to determine the minimum number of tests that could be used to differentiate between the CPS species: *S. aureus*, *S. hyicus*, and *S. intermedius*.

MATERIALS AND METHODS

Bacterial isolates. *S. aureus* and coagulase-positive *S. hyicus* strains were isolated from samples collected from university and commercial dairy herds in the northwest. *S. intermedius* strains were obtained from various culture collections throughout the United States. The origins for the samples are summarized in Table 1. Eighty strains per species were tested. *S. aureus* Neave (ATCC 27543) and the *S. intermedius* type strain (ATCC 29663) were used as controls for the five tests, as these strains are known to have typical reactions for their respective species. Additionally, 20 strains of coagulase-negative *S. hyicus* were tested.

Experimental procedure. By the tube coagulase test (Difco Laboratories, Detroit, Mich.), all strains studied except for the 20 coagulase-negative *S. hyicus* strains were positive for the enzyme coagulase by 24 h. Strains were isolated on Columbia agar with 5% sheep blood (BBL, Becton Dickinson, Cockeysville, Md.) prior to testing. Confirmation of

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TABLE 1. Sources of *Staphylococcus* species test strains

Origin	No. of *Staphylococcus* isolates		
	S. aureus[a]	*S. hyicus*[a]	*S. intermedius*
Bovine			
Milk	64	66	1
Skin	6	14	0
Human	5	0	5
Other	5[b]	0	4[c]
Other animal	0	0	59[d]
Unknown	0	0	11
Total	80	80	80

[a] All isolates were isolated from dairies.
[b] Two air isolates, one grain isolate, one bedding isolate, and one fly isolate.
[c] Four isolates from human food.
[d] Fifty-three canine isolates, four feline isolates, one squirrel monkey isolate, and one raccoon isolate.

species identification for *S. aureus* and both coagulase-positive and coagulase-negative *S. hyicus* was by the API STAPH Trac system (Analytab Products, Plainview, N.Y.). The API STAPH-IDENT system (Analytab Products) was used to confirm the identification of *S. intermedius* strains.

A pure culture of each strain was inoculated into 2 ml of tryptone soy broth. After the broth was vortexed, an aliquot was removed and streaked on MBP (4). Plates were incubated at 37°C for 48 h. Growth of glistening jet-black colonies indicated a positive test of growth on MBP.

P agar was prepared as described previously (13). Before plates were poured, P-agar medium was supplemented with 7 μg of acriflavin per ml (8). The procedure was identical to that for MBP except that a 24-h incubation time was used. Growth on P agar supplemented with acriflavin was indicative of a positive test.

Anaerobic fermentation of mannitol was tested as previously described (15). Briefly, the medium was steamed for 15 min and then solidified by placing the medium in iced water. A tube was then immediately inoculated with the isolate by using a wire loop and making sure that the inoculum reached the bottom of the tube. The surface of the medium was covered with 2 cm of sterile paraffin oil and incubated for 5 days at 37°C. A positive test was indicated by a color change from purple to yellow.

The acetoin test was performed as previously described (6) but with slight modifications. The medium was prepared with methyl red Voges-Proskauer medium (Difco Laboratories). Briefly, a few well-isolated colonies were inoculated into the methyl red Voges-Proskauer medium. After a 24-h incubation at 37°C, 0.6 ml of α-naphthol and 0.2 ml of 40% potassium hydroxide were added. The lids were left off, and the tubes were gently shaken to expose the medium to air. A pink to red color appearing in the upper layer of the medium indicated a positive test.

The medium for the β-galactosidase test was prepared as previously described (9) except that the substrate 2-naphthyl-β-D-galactopyranoside was used in place of *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma Chemical Co., St. Louis, Mo.). After inoculation, the tubes were incubated for 1 h at 37°C. After incubation, 1 drop of fast blue 4-benzoylamino-2,5-diethoxybenzenediazonium chloride (Sigma Chemical Co.) was added. A mauve color appearing after a few minutes indicated a positive test.

TABLE 2. Differentiation of coagulase-positive *Staphylococcus* species by five tests

Staphylococcus species[a]	% Positive by:				
	P agar	MBP	β-Galactosidase	Acetoin	Anaerobic fermentation of mannitol
S. aureus	100	100	0	94	99
S. hyicus	0	0	0	0	0
S. intermedius	0	0	100	1	0

[a] Eighty isolates of each species were tested.

RESULTS

The results for the 80 strains of each of the three CPS species are presented in Table 2. Every *S. aureus* strain grew on the acriflavin-supplemented agars, whereas no strains of coagulase-positive *S. hyicus* or *S. intermedius* grew on these agars. Coagulase-positive *S. hyicus* and *S. intermedius* strains were consistently unable to ferment mannitol anaerobically, whereas 99% of the *S. aureus* strains were positive for anaerobic mannitol fermentation. One *S. aureus* isolate from bovine milk did not ferment mannitol anaerobically. The percents positive for the acetoin test were 1, 94, and 0% of *S. intermedius* (one canine strain), *S. aureus* strains (four bovine isolates and one human isolate were test negative), and coagulase-positive *S. hyicus*, respectively. All of the *S. intermedius* strains were positive for β-galactosidase, while all other CPS species were negative. All 20 coagulase-negative *S. hyicus* strains were negative in the five tests.

DISCUSSION

In determining the minimum number of tests that could be used to differentiate between CPS, two tests, growth on P agar supplemented with acriflavin and the β-galactosidase test, should allow quick and easy differentiation of these organisms. Results from the two tests of the 240 CPS tested in this study demonstrated 100% accuracy.

Growth on P agar supplemented with 7 μg of acriflavin per ml combined with the tube coagulase test and presence of hemolysis has been suggested to be helpful in confirming the identity of *S. aureus* (8). Harmon and coworkers (8) found that 155 of 156 *S. aureus* isolates from bovine milk grew on P agar, 1 of 10 *S. intermedius* (canine) isolates gave slight growth, and no coagulase-positive *S. hyicus* isolates grew. Findings from the present study demonstrated similar results, as all 80 strains of *S. aureus* grew on P agar. In the present study, no *S. hyicus* or *S. intermedius* strains showed any growth on P agar when inoculated as described. The method of agar inoculation by Harmon et al. (8) is not known. However, in developing our technique, it was noted that when the agar was inoculated from colonies rather than broth, slight growth was present. The higher concentration of organisms in one location may have diminished the concentration of acriflavin that the upper organisms were exposed to, which could have allowed for slight growth. For the purpose of differentiation among CPS, P agar is preferred to MBP because it is equally effective and less expensive and requires a shorter incubation time.

One of the key tests used by the STAPH-IDENT system to differentiate *S. intermedius* from *S. aureus* is the β-galactosidase test. Studies have demonstrated that 100% of *S. intermedius* strains are positive for β-galactosidase while the other CPS species do not show β-galactosidase activity (1,

11). This test is also known in most microbiology texts as the ONPG test because it is based on the substrate ONPG (9). A few *S. aureus* strains were tested by the methods and the substrate ONPG as described elsewhere (9). These strains appeared to be positive for β-galactosidase. However, a yellow color of the test is interpreted as positive, possibly explaining why this test cannot be performed on yellow-pigmented organisms, such as some *S. aureus* strains. The API STAPH-IDENT literature (1) suggested that the substrate 2-naphthyl-β-D-galactopyranoside is an acceptable substitute for ONPG. There was a need to develop the β-galactosidase test to a standard tube test level as described in Materials and Methods. This test proved quite successful in differentiating *S. intermedius* strains, which are positive, from the other CPS species, which were all negative for the enzyme, consistent with the API STAPH-IDENT literature (1). The β-galactosidase test not only was the easiest test to perform but also has the shortest incubation (1 h) of all five tests.

The National Mastitis Council (2) recommends that the acetoin test (Voges-Proskauer test) be used as an additional means to differentiate *S. aureus* from coagulase-positive *S. hyicus* and *S. intermedius*. *S. intermedius* and *S. hyicus* do not produce acetoin (14), while the majority of *S. aureus* strains do (8). The test of acetoin production as a tool to differentiate CPS species may be substrate dependent. When pyruvate is used as the substrate, 99% of *S. intermedius* strains and 86% of *S. aureus* strains were positive (3). The results presented here are in close agreement with results of other studies that demonstrated that greater than 90% of *S. aureus* strains produce acetoin from glucose (8, 14). Although 94% of *S. aureus* strains were positive, the acetoin test may be the least desirable method to differentiate between *S. aureus* and *S. intermedius* (1% test positive). The test is subject to weak positives and is more time-consuming than the agar and the β-galactosidase tests.

The use of anaerobic utilization of mannitol to distinguish *S. aureus* from other staphylococci has been recommended (15). Strains of *S. intermedius* do not utilize mannitol anaerobically (7). A report states that *S. aureus* from dogs and pigeons did not ferment or only weakly fermented mannitol anaerobically (5). However, most of these strains were *S. aureus* biotypes E and F, which are now known as the species *S. intermedius* (7). In the current study, 99% of *S. aureus* strains were positive for anaerobic fermentation of mannitol whereas no *S. intermedius* or *S. hyicus* strains were positive, which is in close agreement with results of other reports (5, 7, 14). While the sensitivity and specificity of anaerobic fermentation of mannitol are relatively high for identifying *S. aureus*, some *S. aureus* strains could be misidentified. Additionally, anaerobic fermentation of mannitol requires more labor and time than the other tests, and weak positive reactions do occur. Therefore, the agars supplemented with acriflavin were superior to anaerobic fermentation of mannitol in differentiating *S. aureus* from the other CPS species.

Results from the 240 CPS strains tested would suggest that use of P agar supplemented with acriflavin and the β-galactosidase test accurately differentiate the three coagulase-positive species. These tests are meant to supplement rather than replace the coagulase test. Both tests are easily performed, easily interpreted, and relatively inexpensive and require little time. *S. aureus* strains are positive on P agar and negative for β-galactosidase. *S. intermedius* strains are negative on P agar and positive for β-galactosidase. Both coagulase-positive and coagulase-negative *S. hyicus* strains are negative on both tests. Atypical strains may necessitate additional tests, but of the CPS isolates we tested, all were identified to the species level by these two tests alone. Coagulase-negative and coagulase-positive *S. hyicus* strains react identically in the five tests conducted, which is consistent with results in *Bergey's Manual of Determinative Bacteriology* (14). However, it remains unknown whether these tests will differentiate coagulase-negative *S. hyicus* from other coagulase-negative staphylococcal species.

While the motivation behind this research was to obtain an accurate, simple system to differentiate CPS strains of bovine origin, a simple means to differentiate CPS species would be beneficial to any researcher or microbiologist who deals with the staphylococcal species.

REFERENCES

1. **Anonymous.** 1985. STAPH-IDENT staphylococcal system. API Analytab Products, Division of Sherwood Medical, Plainview, N.Y.
2. **Anonymous.** 1990. Microbiological procedures for the diagnosis of bovine udder infections. National Mastitis Council, Inc., Arlington, Va.
3. **Anonymous.** 1986. STAPH Trac. API Analytab Products, Division of Sherwood Medical, Plainview, N.Y.
4. **Devriese, L. A.** 1981. Baird-Parker medium supplemented with acriflavin, polymyxins, and sulphonamide for the selective isolation of *Staphylococcus aureus* from heavily contaminated materials. J. Appl. Bacteriol. **50**:351–357.
5. **Devriese, L. A., and P. Oeding.** 1976. Characteristics of *Staphylococcus aureus* strains isolated from different animal species. Res. Vet. Sci. **21**:284–291.
6. **Finegold, S. M., and E. J. Baron.** 1986. *Enterobacteriaceae*, p. 413. *In* S. M. Finegold and E. J. Baron (ed.), Bailey and Scott's diagnostic microbiology, 7th ed. The C. V. Mosby Co., St. Louis.
7. **Hajek, V.** 1976. *Staphylococcus intermedius*, a new species isolated from animals. Int. J. Syst. Bacteriol. **26**:401–408.
8. **Harmon, R. J., B. E. Langlois, and K. Akers.** 1991. A simple medium for the verification of identity of *Staphylococcus aureus* of bovine origin. J. Dairy Sci. **74**(Suppl. 1):202.
9. **Hendrickson, D. A.** 1985. Reagents and strains, p. 1093–1107. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
10. **Jasper, D. E., F. Infante, and J. D. Dellinger.** 1985. Relationships among the results of coagulase, staphylococcal toxin, and thermonuclease tests on staphylococci from cow milk. J. Clin. Microbiol. **21**:582–584.
11. **Kloos, E. W., and J. F. Wolfshohl.** 1982. Identification of *Staphylococcus* species with the API Staph-Ident System. J. Clin. Microbiol. **16**:509–516.
12. **Langlois, B. E., R. J. Harmon, and K. Akers.** 1983. Identification of *Staphylococcus* species of bovine origin with the API Staph-Ident System. J. Clin. Microbiol. **18**:1212–1219.
13. **Phillips, E., and P. Nash.** 1985. Culture media, p. 1051–1092. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and H. J. Shadomy (ed.), Manual of clinical microbiology, 4th ed. American Society for Microbiology, Washington, D.C.
14. **Schleifer, K. H.** 1986. Gram-positive cocci. Family I. *Micrococcaceae*, p. 999–1032. *In* P. H. A. Sneath, N. S. Mair, and M. E. Sharpe (ed.), Bergey's manual of determinative bacteriology, 9th ed. The Williams & Wilkins Co., Baltimore.
15. **Subcommittee on Taxonomy of Staphylococci and Micrococci.** 1965. Recommendations. Int. Bull. Bacteriol. Nomencl. Taxon. **15**:109–110.
16. **Watts, J. L., S. C. Nickerson, and J. W. Pankey.** 1983. Preliminary report on mastitis in beef cows. Dairy research report, p. 253–282. Hill Farm Research Station, Homer, La.
17. **Watts, J. L., J. W. Pankey, and S. C. Nickerson.** 1984. Evaluation of the Staph-Ident and STAPHase systems for identification of staphylococci from bovine intramammary infections. J. Clin. Microbiol. **20**:448–452.