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Comparison of Methods for Identifying *Staphylococcus* and *Micrococcus* spp.

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Three methods employed to distinguish staphylococci from micrococci were compared, using clinical and environmental strains. When these methods are used, misinterpretation of results, as well as erratic results, may occur, and suggestions for eliminating these problems are provided. The most sensitive test that combines ease of use and speed in obtaining results for distinguishing the two genera is the lysostaphin susceptibility test. Two other tests, facultatively anaerobic growth in semisolid thioglycolate agar and fermentation of dextrose, may also be used to distinguish these two genera, but results are often slow in developing, are subject to technical difficulties, and may lead to incorrect assignment of certain species of staphylococci and micrococci to their proper genera.

Over the past several years, increased emphasis has been placed upon identification of potentially pathogenic coagulase-negative staphylococci. Renewed interest of clinical laboratory investigators was prompted by reports concerning the description and distribution of new species (12, 13, 14, 21) and the need for determining their significance in human infections. The role of *Staphylococcus epidermidis* in infectious processes is established (4, 7, 18, 19, 23, 25). However, other species have been implicated as potential pathogens as well. One such species, *Staphylococcus saprophyticus*, has been found to be associated with urinary tract infections in humans, and the need for its identification was partially responsible for the renewed interest in coagulase-negative staphylococci (4, 7, 9, 18, 19, 25). It was originally assigned to *Micrococcus* subgroups I through IV of Baird-Parker on the basis of a weakly fermentative reaction in the oxidative and fermentative (OF) medium of Hugh and Leifson (1, 4, 8, 17). More recently, overall deoxyribonucleic acid base composition analyses have demonstrated that *S. saprophyticus* more closely resembles staphylococci than micrococci. *S. saprophyticus* is now considered to be a member of the genus *Staphylococcus*, as evidenced by the classification of this organism provided in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (2).

Many methods have been suggested for differentiating micrococci from staphylococci. The most traditional of these tests is determination of the ability of an isolate to produce acidic by-products when grown anaerobically in the presence of dextrose, i.e., fermentation of dextrose. In this test, most staphylococci are fermentative and most micrococci are either oxidative or asaccharolytic. Difficulties in detection of acid production from glucose by the traditional Hugh and Leifson OF test led to the development of a more sensitive "staphylococcus OF medium." The medium was recommended by the Subcommittee on Taxonomy of Staphylococci and Micrococci (STSM) in 1965 (24) and was modified by Davis and Hoyling (3) by raising the agar content from 0.2 to 0.35%. The medium differed from the Hugh and Leifson OF medium in meeting the nutritional requirements of staphylococci and micrococci for growth and by substitution of the indicator, bromcresol purple, which is yellow at pH 5.2, for bromthymol blue, the indicator in the Hugh and Leifson OF medium, which is yellow at pH 6.0. Even with these refinements, certain micrococci and staphylococci are misidentified with the STSM standard OF test. Some strains of *Micrococcus kristinae* may weakly ferment dextrose and a number of species of staphylococci, such as *S. saprophyticus*, *S. cohnii*, *S. xylosus*, *S. haemolyticus*, and *S. sciuri*, either may fail to grow in the anaerobic portion of the OF medium or may not produce sufficient quantities of acid to be detectable with bromcresol purple. In such cases, *M. kristinae* would be misidentified as *Staphylococcus* sp., and the staphylococci would be misidentified as *Micrococcus* spp. (14-16, 21, 22).

An alternative differential test medium was

proposed by Evans and Kloos (5) and was based on the ability of an isolate to grow anaerobically in thioglycolate medium containing 0.35% agar. Staphylococci were reported to grow in both aerobic and anaerobic portions of the medium, but micrococci were reported to grow only in the aerobic portion. As with the OF test, certain staphylococci and micrococci may be misidentified with this test. Some strains of *M. kristinae* may grow as individual tiny colonies in the anaerobic portion of the medium, giving the appearance of *Staphylococcus* spp., and *S. hominis*, *S. haemolyticus*, *S. xylosus*, *S. warneri*, and *S. sciuri* may, on occasion, produce such an insignificant amount of growth in the anaerobic portion of the medium that test results could be misinterpreted as being representative of *Micrococcus* spp. (14-16, 21).

In 1968, Klesius and Schuhardt (10) introduced the use of the enzyme lysostaphin for differentiation of staphylococci from micrococci. It has been reported that results of the lysostaphin susceptibility test provide groupings of strains which very closely approximate those based on deoxyribonucleic acid base composition analyses for the family *Micrococcaceae* (13, 14, 16, 21). Most *Staphylococcus* spp. were reported to be susceptible to lysostaphin, and most *Micrococcus* spp. were reported to be resistant to lysis by the enzyme. As with the other two tests, exceptions are known to occur. Approximately 10% of *Micrococcus* spp. have been reported to be either susceptible or weakly susceptible to lysostaphin, and occasional strains of *S. epidermidis*, *S. haemolyticus*, *S. warneri*, *S. capitis* and *S. hominis* have been found to be slightly resistant (20, 21).

The purpose of the present study was to determine the accuracy and reliability of the tests for dextrose fermentation, facultative growth in semisolid thioglycolate agar, and lysostaphin susceptibility in providing generic differentiation of the family *Micrococcaceae* isolated from both clinical and environmental samples.

MATERIALS AND METHODS

The gram-positive cocci used in this study were isolated from clinical specimens collected at Walter Reed Army Medical Center (WRAMC), Washington, D.C., and from several marine sampling sites in the Chesapeake Bay and the Atlantic Ocean. The reference strains of staphylococci were obtained from D. W. Kloos, North Carolina State University, Raleigh, N.C., and included the following: *S. capitis* ATCC 27840, *S. epidermidis* ATCC 14990, *S. hominis* ATCC 27844, *S. warneri* ATCC 27836, *S. haemolyticus* DSM 20263, *S. saprophyticus* CCM 883, *S. xylosus* DSM 20266, *S. simulans* ATCC 27848, *S. cohnii* DSM 20260, *S. aureus ATCC 12600*, *S. sciuri* subsp. *lentus* ATCC 29070, and, *S. sciuri* subsp. *sciuri* ATCC 29062. The reference strains of micrococci were obtained from J. K. Keiser, WRAMC, and included the following: *M. roseus* WRAMC 16 and *M. luteus* WRAMC 252. Identification of strains for the purposes of this study was accomplished by using the simplified scheme and methods of Kloos and Schleifer (13) for *Staphylococcus* spp. Strains identified as members of the genus *Micrococcus* were not identified to species level in this study. Results of a more thorough taxonomic study of these strains, employing numerical taxonomy, will be reported elsewhere (Gunn and Colwell, manuscript in preparation).

The OF medium employed was a modification of the Hugh and Leifson OF medium (3, 24). The composition of the STSM OF medium was as follows: tryptone, 15 g; yeast extract, 1.5 g; dextrose, 15 g; agar, 5.25 g; 1.6% bromcresol purple in 95% alcohol (wt/vol), 3.8 ml; and 1.5 liter of distilled water. The medium was adjusted to pH 7.0, heated to boiling, and dispensed into tubes, with each tube containing media to at least two-thirds total volume. The tubes of media were autoclaved for 15 min at 115°C. Before use, the tubes of media were steamed to remove dissolved oxygen and allowed to resolidify in an upright position. After solidification, the tubes were stab inoculated to the bottom of the medium. Each tube was covered with ca. 15 to 20 mm of sterile paraffin oil. A tube similarly inoculated, but not covered with oil, served as the aerobic control. The inoculated tubes of media were incubated for five days at 35°C. Yellow color formation in the medium was considered positive for dextrose utilization.

The thioglycolate medium was a modification (3) of that used by Evans and Kloos (5). The composition of the semisolid medium was as follows: thioglycolate broth base (BBL Microbiology Systems, thioglycolate medium without resazurin or dextrose, no. 11727), 36 g; 0.1% resazurin in distilled water, 1.5 ml; dextrose, 7.5 g; agar, 4.1 g; yeast extract, 7.5 g; and distilled water, 1.5 liter. The medium was adjusted to pH 7.0, boiled, and dispensed into tubes, with each tube filled to two-thirds total volume. The tubed media were autoclaved for 15 min at 115°C. Before use, the tubes of media were steamed, cooled, and inoculated, as described above. The tubes were not covered with paraffin oil after inoculation but were incubated for 5 days at 35°C. Growth to the bottom of the tubes was recorded as positive for facultatively anaerobic growth. Care was taken during inoculation to ensure that the inoculum was light, i.e., not visible upon withdrawal of the inoculating needle from the medium. The OF medium, conversely, was heavily inoculated.

Determination of lysostaphin susceptibility was performed by the method of Hajek (6). A 10× stock solution of lysostaphin was prepared by dissolving 10 mg of lysostaphin (Sigma Chemical Co., L-8255, lot 18C-0426) in 40 ml of phosphate buffer (0.02 M) adjusted to pH 7.4. To this mixture was added NaCl, to a final concentration of 1% (wt/vol). The final concentration of lysostaphin in the mixture was 250 μg/ml. The stock solution was dispensed in 1-ml volumes, frozen, and stored at −60°C. A working dilution was prepared by diluting 1 ml of the lysostaphin stock

solution with 9 ml of the 0.02 M phosphate buffer (pH 7.4). The lysostaphin susceptibility test was performed by adding 5 drops of an actively growing heart infusion broth (BBL) culture of the test organism to a small tube containing 5 drops of lysostaphin solution. A control tube was prepared by adding 5 drops of phosphate buffer to the cell suspension in lieu of the enzyme solution. The tubes were placed in a 35°C dry block and observed at 30, 60, and 120 min for lysis or clearing of the mixture containing enzyme and cells. Extent of lysis was recorded as 0, 1+, 2+, 3+, 4+, with 0 representing no lysis or clearing and 4+ representing complete lysis of the cells in suspension. Two laboratory strains, a lysostaphin-resistant *Micrococcus* sp. and a lysostaphin-susceptible *Staphylococcus* sp., served as controls.

RESULTS

Four hundred and ninety-one strains representing the family *Micrococcaceae* were tested. Forty-seven were *Micrococcus* spp., and the remainder were *Staphylococcus* spp. The small number of micrococci in the sample set is a direct reflection of their frequency of occurrence in clinical and environmental specimens, relative to numbers of staphylococci. Criteria used to distinguish the two genera have been reported elsewhere (3, 5, 6, 12, 24). For the most part, *Staphylococcus* spp. produced acid from glycerol, grew at 45°C, were oxidase negative, grew relatively rapidly, forming raised to slightly convex colonies, were susceptible to lysostaphin, grew anaerobically in semisolid thioglycolate medium, and fermented dextrose. Certain species of staphylococci are known to possess characteristics that are exceptions to the above, e.g., the *S. sciuri* strains examined in the present study were oxidase positive. Most *Micrococcus* spp. did not produce acid from glycerol or grow at 45°C, were resistant to lysostaphin, did not grow in semisolid thioglycolate medium, grew slowly as moderate to highly convex colonies, and did not ferment dextrose. Approximately one-half of the micrococci were oxidase positive. Strains were further characterized by 62 additional tests, and the results were analyzed in a numerical taxonomy study (Gunn and Colwell, manuscript in preparation).

The most prevalent *Staphylococcus* sp. was *S. epidermidis* (227 strains), followed by *S. hominis* (99), *S. warneri* (26), *S. haemolyticus* (15), *S. saprophyticus* (56) and other species. Most of the *S. hominis* strains and approximately one-fourth of the *S. epidermidis* strains were derived from samples of seawater. The scheme of Kloos and Schleifer (13) used for identification of the staphylococci was originally developed for staphylococci isolated from humans and was not intended for use with environmentally derived strains. For the purposes of this study, however, the environmental strains were biotyped by the Kloos and Schleifer simplified scheme, and they did fit the descriptions of the species given by other investigators (14, 15, 21) (Table 1). As previously mentioned, a more definitive description of the environmental strains will be reported. *S. saprophyticus* strains were isolated only from samples of human urine. Nine isolates could not be identified by using the simplified scheme of Kloos and Schleifer (13).

Seventy-two percent of the 444 *Staphylococcus* spp. were lysed by lysostaphin, fermented dextrose, and grew anaerobically in semisolid thioglycolate agar. An additional 11% differed in

TABLE 1. *Reactions of selected strains of Staphylococcus and Micrococcus spp. to three presumptive tests*

Species	No. of stains tested	Lysostaphin susceptibility[a]	OF dextrose, fermentation	Facultative growth
Staphylococcus spp.[b]	320	+	+	+
S. epidermidis	20	+	+	–
S. saprophyticus	8			
S. hominis	7			
S. warneri	4			
S. cohnii	1			
S. haemolyticus	1			
Staphylococcus spp.	6			
S. hominis	1	+	–	+
S. simulans	1			
S. epidermidis	34	–	+	+
S. hominis	11			
S. saprophyticus	5			
S. warneri	5			
S. haemolyticus	1			
S. simulans	1			
Staphylococcus spp.	3			
S. epidermidis	1	–	–	+
Micrococcus sp.	1			
S. epidermidis	9	–	+	–
S. saprophyticus	4			
S. hominis	1			
Micrococcus spp.	2	+[w]	–	–
Micrococcus spp.	44	–	–	–

[a] +, Positive reaction; +[w], weakly positive reaction.
[b] Includes *S. epidermidis, S. haemolyticus, S. simulans, S. saprophyticus, S. capitis, S. cohnii, S. hominis, S. warneri, S. xylosus,* and *S. aureus.*

the three tests by showing no growth in the anaerobic portion of the thioglycolate agar. In contrast, 14% differed in being resistant to lysostaphin, and 17 strains of staphylococci yielded different patterns (Table 1).

All but three of the *Micrococcus* spp. tested in this study demonstrated the following reactions: lysostaphin resistant, oxidative or asaccharolytic with dextrose, and aerobic growth in semisolid thioglycolate agar. Two of the aberrant *Micrococcus* isolates differed from other micrococci in being weakly lysed by lysostaphin. The third differed in being able to grow in the anaerobic portion of the thioglycolate agar tube.

Of the 444 staphylococci tested, 9% required incubation for more than 2 days before a visible change in pH of the OF medium occurred. Fourteen of the strains were clinical isolates. Four were identified as *S. saprophyticus*, and the remainder were identified as *S. epidermidis*. The other 25 strains examined were isolated from seawater or samples collected from the marine environment and comprised the species *S. epidermidis* (15 strains), *S. hominis* (5), *S. cohnii* (1), and *S. warneri* (4). All but 3 of the 39 strains which required longer than 2 days to effect a visible pH change gave a strongly positive reaction within 5 days. These three strains included two environmental strains and one clinical strain of *S. epidermidis*.

Fifteen strains were judged to be resistant to lysostaphin, but were observed to be very weakly susceptible when compared with the enzyme-free control tube. Five were *S. saprophyticus*, five were *S. epidermidis*, and the remainder represented other *Staphylococcus* spp. and two strains of *Micrococcus* spp. Thirty strains were recorded as being either 1+ or 2+ in susceptibility to lysostaphin.

Fifteen strains were concluded to be strict aerobes, but were recorded as yielding very weak growth in the anaerobic region of thioglycolate agar. Five were *S. saprophyticus*, six were *S. epidermidis*, two were *S. hominis*, one was *S. cohnii*, and one was a *Micrococcus* sp. Another 31 strains produced either 1+ or 2+ growth in the anaerobic portion of the agar. The proportion of species included in this group was similar to the mixture described above for the weakly lysostaphin-susceptible strains.

DISCUSSION

Sources of variability affecting intra- and interlaboratory reproducibility of the tests compared in this study were sought. Approximately one-eighth of the isolates were inoculated simultaneously into a test medium or enzyme solution, and the reactions were read as a group to reduce subjectivity in the reading of test results.

It has been suggested that ≥50% reduction in turbidity after incubation for 2 h in the presence of lysostaphin comprises a positive test for susceptibility (6), representing in this study a reading of ≥2+. Three percent of the staphylococci tested were recorded as 1+, thereby being misidentified, if the above criterion had been used in this study and if lysostaphin susceptibility was the only test used to distinguish staphylococci from micrococci. Overall, 83% of the staphylococci and 4% of the micrococci examined were found to be susceptible to lysostaphin. Results obtained in this study for *Micrococcus* spp. were similar to those reported in the literature (20). But, the proportion of susceptible strains of *Staphylococcus* spp. was lower than that reported by other investigators (20), due, in part, to the large numbers of environmental isolates tested in this study. Of the 61 lysostaphin-resistant staphylococci, 40 were isolated from seawater, and, if they were excluded from the study, 90% of the staphylococci would have been lysostaphin susceptible. In addition, the method of testing for susceptibility used in this study (6) was different from the spot test employed by Kloos et al. (16). Differences between their results and those presented here could be accounted for by the methods used. Thus, the accuracy of the lysostaphin susceptibility test, as a single distinguishing test for staphylococci and micrococci, may vary according to source of the isolate, the method used for testing, and the criteria used to assess susceptibility. It should be stressed that staphylococci grown in serine-rich media possess cell walls that are more resistant to lysostaphin than staphylococci grown in glycine-rich media (20). Media containing peptones prepared from meat are high in glycine, whereas, peptones from casein are high in serine (20). Thus, the medium-dependent action of lysostaphin should be taken into consideration when the lysostaphin susceptibility test is used to distinguish *Staphylococcus* spp. from *Micrococcus* spp.

Only 3 of the 444 strains of staphylococci were found to be negative for fermentation of dextrose. None of the 47 micrococci were fermentative, although many were oxidative. How rapidly the test can be read in the laboratory is a direct function of the size of the inoculum used, the growth rate of the isolate, and the optimum temperature for growth. Environmental isolates, in contrast to clinical isolates, were, when first isolated, observed to grow as smaller colonies on agar media at 35°C and to show enhanced growth at 25°C. In general, an agreement with

extent of acid formation in the OF test and amount of growth in the anaerobic portion of thioglycolate agar was observed. Ninety-two percent of the *Staphylococcus* spp. examined in this study were fermentative within 2 days, and most of these strains were positive within 18 to 24 h.

For best results, the OF test medium should always be heated to drive off dissolved oxygen, and the tubes should be allowed to cool in an upright position before they are inoculated heavily. In our experience, failure to follow this procedure is the most important cause of inconsistent and equivocal results. If dissolved oxygen is not removed, oxidative micrococci may be able to acidify dextrose under oil and yield a "fermentative" reaction. Oxygen can diffuse into the top centimeter of the tubed medium within 18 to 24 h, whether the medium is stored under refrigeration or at ambient temperature.

Eighty-six percent of the 444 *Staphylococcus* spp. and 2% of the *Micrococcus* spp. grew in the anaerobic portion of thioglycolate agar. Only one-fourth of the aerobically growing staphylococci were environmentally derived. Other studies have reported similar results and have identified those species in which misidentifications occur when this medium is used to differentiate the two genera (14-16, 21). As in the case of the OF test, the ability of staphylococci to grow anaerobically in thioglycolate agar is a function of source of the isolate, species variation, incubation temperature, and growth rate. The test medium used in the present study is different from that reported by Evans and Kloos (5) and, again, as in the case of the lysostaphin susceptibility test, differences may also be attributed to the use of different test methods. Unlike the OF test medium, this medium is optimally employed if inoculated lightly. Otherwise, it is very difficult to distinguish a weakly positive reaction from an inoculation residue line after incubation for 5 days. As in the case of the OF medium, it is absolutely necessary to use either freshly prepared medium or freshly boiled medium to test for anaerobic growth in semisolid thioglycolate agar. A suggestion has been made (5) that, after the temperature of freshly boiled medium is lowered to ca. 50°C, a loopful of broth culture can be inoculated into the medium and thoroughly mixed, yielding better results than with an inoculating needle. This procedure was successful in our hands; however, if large numbers of cultures are to be inoculated at one time, and into different types of media simultaneously, needle inoculation is preferable.

In conclusion, three methods can be successfully employed in the diagnostic laboratory to distinguish between *Staphylococcus* spp. and *Micrococcus* spp. The single most accurate test, which combines ease of use and speed in obtaining results for distinguishing between the two genera, is the lysostaphin susceptibility test. The test for fermentation of dextrose, in this study, yielded results more closely corresponding to genus identification, but it is a test that is known to be frequently slow in yielding results. Furthermore, several species can produce only enough acid from dextrose to affect marginally the pH indicator present in the medium. Since the number of strains of *Micrococcus* spp. isolated from both clinical and marine environments is low, in comparison to *Staphylococcus* spp., positive lysostaphin susceptibility tests will be common, and, consequently, false-positive reactions associated with micrococci will be low in expected overall frequency of occurrence. Regardless of the test used to distinguish between *Staphylococcus* spp. and *Micrococcus* spp., when problems arise, all three tests and an array of other tests will be necessary to distinguish between the two genera.

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