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Comparison of Various Methods for Differentiation of Staphylococci and Micrococci

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A modified oxidase test (Remel, Lenexa, Kans.) and susceptibility to furazolidone and lysostaphin (Remel) were evaluated in conjunction with the Staph-Ident strip (Analytab Products, Plainview, N.Y.) to accurately differentiate between staphylococci and micrococci. A total of 414 clinical isolates of catalase-positive, gram-positive cocci were each tested with the Staph-Ident strip and by glucose fermentation, acid production from glycerol, susceptibility to furazolidone and lysostaphin, and the oxidase test. Based on the reference methods of glucose fermentation and acid production from glycerol, 396 (95.6%) of the organisms were classified as *Staphylococcus* species and 18 (4.4%) were classified as *Micrococcus* species. Of the staphylococci, 99% were oxidase negative and susceptible to furazolidone; 82% were susceptible to lysostaphin. All of the micrococci were oxidase-positive and resistant to furazolidone and lysostaphin. Of the staphylococci, 99% were identified to species by the Staph-Ident strip. However, six (33%) of the micrococci were incorrectly identified as *Staphylococcus* species (three each of *Staphylococcus hominis* and *Staphylococcus saprophyticus*). Because of the demonstrated sensitivity and specificity of the oxidase and furazolidone susceptibility tests, it is suggested that either of these methods be used in the clinical laboratory to accurately differentiate between staphylococci and micrococci. It is also suggested that when working with the Staph-Ident strip, additional testing such as furazolidone susceptibility or oxidase activity should be performed to provide increased accuracy in the differentiation and characterization of members of the family *Micrococcaceae*.

Demonstrating the ability of the staphylococci to ferment glucose has served as the basis for differentiating these organisms from the micrococci, a closely related genus of catalase-positive, gram-positive cocci. However, the limitations of this method have been emphasized in various reports (5, 10, 11). The need for a reliable and practical laboratory method to differentiate between *Staphylococcus* and *Micrococcus* species is based on their morphological similarities and the differences in their pathogenic potential. The micrococci are not considered significant human pathogens. The clinical significance of the staphylococci has been reviewed extensively (1, 12–14, 17).

Alternate laboratory methods for the purpose of differentiating the staphylococci from the micrococci have been addressed by several groups of investigators. Schleifer and Kloos (18) recommend that the differentiation be based on demonstrating the ability of the staphylococci to produce acid from glycerol (1%) in the presence of 0.4 μg of erythromycin per ml and the susceptibility of the staphylococci to lysostaphin (200 μg/ml) and resistance to lysozyme (25 μg/ml). Although the micrococci vary in their response to lysozyme, *Micrococcus luteus*, the most commonly encountered species associated with humans, is generally very susceptible.

Other methods that have been developed and evaluated for this purpose include furazolidone susceptibility (4, 20, 21; M. Ducate and D. Florek-Ebeling, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C134, p. 293), modifications of the oxidase and benzidine tests (8), susceptibility to bacitracin (7), and a serological method based on antibodies specific for staphylococcal and micrococcal cell wall peptidoglycans (19). All of these methods have been reported to be accurate for distinguishing between these two genera.

Because of the recent introduction of alternate methods for the purpose of differentiating between staphylococci and micrococci and the documented limitations of the classical methods used for this purpose, I decided to assess several of these methods to suggest a scheme to clinical laboratories that would reliably and rapidly differentiate between the two genera. Thus, I compared glucose fermentation (GF), acid production from glycerol, oxidase, response to furazolidone, and response to lysostaphin. I also assessed the use of the Staph-Ident strip (Analytab Products, Plainview, N.Y.) when working with catalase-positive, gram-positive cocci.

MATERIALS AND METHODS

Organisms. A total of 414 isolates of catalase-positive, gram-positive cocci were randomly selected from clinical specimens obtained from the Clinical Microbiology Laboratory of the Medical Center Hospital of Vermont. Each bacterium was maintained on a nutrient agar slant (BBL Microbiology Systems, Cockeysville, Md.) at 4°C and subcultured to a Trypticase soy agar plate (BBL Microbiology Systems) supplemented with 5% sheep blood (Krutulis Laboratories, Bridgeport, N.Y.) before being tested. Cultures were incubated at 35 to 37°C for 18 to 24 h. Each organism was tested for GF, acid production from glycerol, oxidase, growth characteristics on furazolidone-peptone (FP) agar media, growth inhibition by a furazolidone disk diffusion method, and lysostaphin susceptibility. Additionally, each organism was biochemically characterized by the Staph-Ident strip. Reference strains used in this investigation included *Micrococcus luteus* ATCC 318, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus sciuri* ATCC 29060, *Staphylococcus simulans* ATCC 27851, and *Staphylococcus saprophyticus* ATCC 15305.

GF. GF was determined by the method described by Facklam and Smith (6). The medium was prepared with the following ingredients per liter of distilled water: 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 1 g of yeast extract (Difco), 0.04 g of bromcresol purple (Sigma Chemical

Co., St. Louis, Mo.), 10 g of glucose (Difco), and 2.2 g of agar (BBL). The pH was adjusted to 7.0, and the medium was sterilized and dispensed into screw-top glass tubes (16 by 120 mm; Fisher Scientific Co., Pittsburgh, Pa.) to a depth of 2 in. (ca. 50.8 mm). A heavy inoculum of each organism was tested, and fermentation was determined by the appearance of a yellow butt in the tube which was overlaid with mineral oil and incubated at 35 to 37°C for 24 h. Organisms that did not produce acid were incubated for a total of 5 days before they were recorded as negative for GF.

Acid production from glycerol. Plates used to determine acid production from glycerol were prepared as previously described by Schleifer and Kloos (18) and contained 1 g of $NH_4H_2PO_4$, 0.2 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 g of yeast extract (Difco), 10 ml of glycerol (Sigma), 0.04 g of bromcresol purple (Sigma), 9 g of agar (BBL), and 1 liter of distilled water. After the medium was autoclaved and allowed to cool, 0.4 ml of a 1,000-μg/ml solution of erythromycin (Sigma) was added and allowed to mix. The medium was dispensed into petri dishes (100 by 15 mm; Fisher). Three to four colonies were used to make a single streak several centimeters long on the surface of the agar medium. A positive result was indicated by the appearance of a yellow halo surrounding the streak after 18 to 24 h of incubation. Organisms that did not produce acid were incubated for a total of 72 h before being recorded as negative for acid production from glycerol.

Oxidase. Oxidase activity was determined by using commercially available Microdase disks (Remel, Lenexa, Kans.). The disks are specifically prepared for the differentiation between micrococci and staphylococci and are impregnated with tetramethyl-*p*-phenylenediamine in dimethyl sulfoxide. To test for the presence of the oxidase enzyme, one large colony or several small colonies were picked with a sterile applicator stick, rubbed across the disk, and examined for no more than 30 s. Positive results were indicated by the development of a blue to purple-blue color, whereas no color development was recorded as a negative oxidase result.

FP agar media streak plate method. FP agar medium was prepared as previously described by von Rheinbaben and Hadlok (21). Plates were prepared with furazolidone (Sigma) concentrations equal to 0.02 and 0.03%. Additionally, a growth control plate was prepared using the same protocol without adding furazolidone. These media were prepared with 10 g of peptone (Difco), 5 g of yeast extract (Difco), 5 g of NaCl, 1 g of glucose (Difco), 12 g of agar (BBL), and 1 liter of distilled water. The pH was adjusted to 7.0, and the medium was autoclaved. A 0.2% furazolidone solution was prepared in acetone (Fisher). After the peptone medium was cooled, an appropriate amount of the furazolidone solution was added to achieve the desired concentration in the medium. After a few minutes of mixing, the media were dispensed into petri dishes (100 by 15 mm; Fisher). To test for growth characteristics on these media, each isolate was initially inoculated to Trypticase soy broth (BBL Microbiology Systems) and incubated at 35 to 37°C for several hours, after which turbidity was adjusted to match a no. 0.5 McFarland optical density standard (10^8 CFU/ml). By using a 0.001-ml calibrated inoculating loop, an inoculum was obtained from the Trypticase soy broth and a streak, several centimeters long, was made on the surface of the growth control, 0.02% FP agar, and 0.03% FP agar. At least eight different organisms could be tested on one plate. All plates were incubated at 35 to 37°C for 18 to 24 h and examined for growth. Results were interpreted as either growth or no growth. All cultures that failed to grow on the FP media after 24 h were incubated for an additional 24 h. Organisms that failed to grow after 48 h were recorded as susceptible to furazolidone.

Furazolidone disk diffusion. Susceptibility to a 100-μg furazolidone disk (BBL) was determined by inoculating three to five well-isolated colonies into Trypticase soy broth and incubating them for 3 to 4 h as previously described by Bauer et al. (2). The standardized Bauer-Kirby disk-diffusion method was performed with Mueller-Hinton (Scott Laboratories, Inc., Richmond, Calif.) agar plates (150 by 15 mm; Fisher). Zone sizes of growth inhibition were measured in millimeters after 18 to 24 h of incubation at 35 to 37°C. Each organism was inoculated onto 1/4 of a Mueller-Hinton plate so that a total of four different organisms could be tested on one plate.

Susceptibility to lysostaphin. Susceptibility to lysostaphin was determined by using the commercially available lysostaphin reagent set (Remel) and following the instructions of the manufacturer. To perform the test, 0.2 ml of sterile saline (0.85%) was added to a test tube (13 by 75 mm). A heavy suspension of each organism was made in the saline, after which 0.2 ml of lysostaphin solution was added. All tubes were incubated for 2 h, and results were interpreted as follows: a clearing of the solution indicated susceptibility to the lysostaphin, and a turbid solution indicated resistance to the lysostaphin.

Staph-Ident strip. The Staph-Ident system, previously described by Kloos and Wolfshohl (9), was used to obtain an identity for each isolate studied. Several colonies were transferred to 3 ml of 0.85% saline, and a suspension equivalent to a no. 3 McFarland optical density standard was prepared. By using a sterile Pasteur pipette, 3 drops of the suspension were added to each of the 10 microcupules on the strip (phosphatase, urea, beta-glucosidase, mannose, mannitol, trehalose, salicin, beta-glucuronidase, arginine, and beta-galactosidase). After 5 h of incubation at 35 to 37°C, the first nine tests were recorded according to the instructions of the manufacturer. To interpret the last test (beta-galactosidase), 2 drops of Staph-Ident reagent (0.35% fast blue BB salt [diazotized 4'-amino-2',5'-diethoxybenzanilide, zinc chloride salt] in 2-methoxyethanol) was added to microcupule 10. The development of a purple color after 30 s was interpreted as a positive result. Depending on the positive or negative reactions, a four-digit profile number was derived for each organism, and a species identification was obtained in the Staph-Ident profile list provided by the manufacturer. Identifications for organisms not listed were obtained by a telephone inquiry system provided by Analytab Products.

RESULTS

The results of GF, acid production from glycerol, the oxidase test, and susceptibility to lysostaphin and furazolidone for 414 clinical isolates of catalase-positive, gram-positive cocci are represented in Table 1. For data analysis, an organism was classified as a *Staphylococcus* species if it fermented glucose or produced acid from glycerol. Otherwise, an organism was classified as a *Micrococcus* species. As determined by the Staph-Ident strip, the following distribution of organisms within the genus *Staphylococcus* was encountered: *S. aureus* ($n = 244$), *S. epidermidis* ($n = 87$), *S. haemolyticus* ($n = 22$), *S. warneri* ($n = 13$), *S. capitis* ($n = 3$), *S. sciuri* ($n = 2$), *S. cohnii* ($n = 1$), and *S. simulans* ($n = 1$). Also, according to the Staph-Ident results (both correct and incorrect), 19 isolates were identified as *S. hominis*, and 8

TABLE 1. Comparison of Staph-Ident strip identification, GF, acid production from 1% glycerol in the presence of 0.4 μg of erythromycin per ml, oxidase test, growth characteristics on FP agar media (0.02 and 0.03%), response to a 100-μg furazolidone disk, and response to lysostaphin for 414 clinical isolates of catalase-positive, gram-positive cocci[a]

Staphylococcus sp. as identified by SIS	Total tested	GF		GE		OXD		FP at 0.02 and 0.03%[b]		FD		LYSO	
		Positive	Negative	Positive	Negative	Positive	Negative	Susceptible	Resistant	Susceptible	Resistant	Susceptible	Resistant
S. aureus	244	244	0	244	0	0	244	243[c]	0	244	0	237	7
S. epidermidis	87	87	0	87	0	0	87	86[c]	0	87	0	62	25
S. haemolyticus	22	22	0	22	0	0	22	22	0	22	0	0	22
S. hominis	16	16	0	16	0	0	16	16	0	16	0	13	3
	3	0	3	0	3	3	0	0	3	0	3	0	3
S. warneri	13	13	0	13	0	0	13	13	0	13	0	4	9
S. saprophyticus	5	5	0	5	0	0	5	5	0	5	0	5	0
	3	0	3	0	3	3	0	0	3	0	3	0	3
S. capitis	3	3	0	3	0	0	3	3	0	3	0	2	1
S. sciuri	2	2	0	0	2	0	2	0	2	0	2	2	0
S. cohnii	1	1	0	1	0	0	1	1	0	1	0	1	0
S. simulans	1	1	0	1	0	0	1	1	0	1	0	1	0
Other	12	0	12	0	12	12	0	0	12	0	12	0	12
	1	1	0	1	0	1	0	0	1	0	1	1	0
	1	1	0	0	1	1	0	0	1	0	1	0	1
Total	414	396	18	393	21	20	394	390	22	392	22	328	86

[a] SIS, Staph-Ident strip; GE, acid production from 1% glycerol in the presence of 0.4 μg of erythromycin per ml; OXD, oxidase test; FD, furazolidone disk; LYSO, lysostaphin.

[b] Results for 0.02 and 0.03% FP agar media combined.

[c] One isolate each of *S. aureus* and *S. epidermidis* were unable to grow on the FP control medium after 48 h of incubation.

isolates were identified as *S. saprophyticus*. However, six of these organisms (three each of *S. hominis* and *S. saprophyticus*) failed to ferment glucose and did not produce acid from glycerol. These six organisms were classified as *Micrococcus* species. I was unable to identify the remaining 14 organisms, and they were classified as "other." Of these 14 isolates, 12 gave an identical test reaction pattern: glucose was not fermented nor was acid produced from glycerol. All were oxidase positive and resistant to both furazolidone and lysostaphin. The remaining two organisms both fermented glucose but differed in their ability to produce acid from glycerol and in their susceptibility to lysostaphin. However, both organisms were oxidase positive and resistant to furazolidone.

Based on the reference methods of GF and acid production from glycerol, 396 organisms were designated as *Staphylococcus* species. Of these, 396 (100%) fermented glucose, 394 (99%) produced acid from glycerol, 394 (99%) were oxidase negative, 2 (0.5%) were unable to grow on the FP control medium, and 392 (99%) were susceptible to furazolidone as determined by both the streak plate method and the disk diffusion method. Of the *Staphylococcus* species, 325 (92%) were susceptible to lysostaphin, and 18 (100% of the *Micrococcus* isolates were resistant to lysostaphin.

DISCUSSION

To determine the relative sensitivity and specificity of the oxidase test and susceptibility to furazolidone and lysostaphin as methods to differentiate between *Staphylococcus* species and *Micrococcus* species, the results of these methods were compared to the reference methods of GF and acid production from glycerol as described by Schleifer and Kloos (18). If an organism produced a positive test result with either of these methods, it was classified as a *Staphylococcus* species, whereas a negative result for both methods indicated that it was a *Micrococcus* species. By using these criteria, 396 (95.6%) of the organisms were classified as *Staphylococcus* species, and 18 (4.4%) were classified as *Micrococcus* species. This low percentage of *Micrococcus* isolates corresponds to the low recovery rate of these organisms from clinical specimens.

All of the organisms designated as *Micrococcus* species were oxidase positive and were resistant to furazolidone by both the disk diffusion and streak plate methods, representing 100% sensitivity. There were only two (0.5%) organisms designated as *Staphylococcus* species that were oxidase positive; neither of these organisms could be identified by the Staph-Ident strip, and they are classified as "other." Four (1%) of the organisms classified as *Staphylococcus* species were resistant to furazolidone by both methods tested; each of these organisms fermented glucose, and two were identified as *S. sciuri*. I was unable to identify the remaining two. Although all of the organisms classified as *Micrococcus* species were resistant to lysostaphin, 73% of the lysostaphin-resistant organisms were *Staphylococcus* species, making this particular method less specific.

The oxidase test was observed to be very rapid and accurate for differentiating between staphylococci and micrococci. Color development by the micrococci was rapid (30 s) and easy to interpret. Although *S. sciuri* is reported to

be the only oxidase-positive species of *Staphylococcus* (8), it is interesting to note that the *S. sciuri* isolates ($n = 2$) encountered in this investigation were oxidase negative. In performing the oxidase test for this purpose, it is important to use the tetramethyl-*p*-phenylenediamine reagent dissolved in dimethyl sulfoxide. The dimethyl sulfoxide is necessary as a solvent because it enhances the permeation of the tetramethyl-*p*-phenylendiamine into the bacterial cell. The Microdase disks (Remel) used in this investigation have this modification and are very effective. To maximize the use of the disks, up to five organisms were tested on one disk instead of testing one organism per disk as recommended by the manufacturer.

The furazolidone susceptibility testing provided accurate results but required overnight incubation. A preliminary report (P. A. Ezekiel and J. S. Baker, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C367, p. 372) emphasized the need to standardize the inoculum when performing the streak plate method. By standardizing the inoculum to 10^8 CFU/ml and inoculation 0.001 ml to the surface of the medium, ambiguous results were eliminated. The organisms tested in this investigation either grew or did not grow. However, seven (39%) of the *Micrococcus* species required 48 h of incubation before growth was noted, and two (0.5%) of the *Staphylococcus* species did not grow on the control medium. Both concentrations of furazolidone (0.02 and 0.03%) tested were equally effective. The furazolidone disk diffusion method was also easy to perform and interpret, and all results were available after 24 h of incubation, making it more desirable than the streak plate method.

All of the *Micrococcus* isolates were determined to be resistant to the furazolidone disk (zone sizes of growth inhibition ranged between 6 and 9 mm), and 394 (99.5%) of the *Staphylococcus* isolates were susceptible (zone sizes of growth inhibition were 15 mm or more). The furazolidone disk diffusion method may easily be implemented in the protocol of a clinical microbiology laboratory that is already performing Bauer-Kirby susceptibility testing. No additional media are required, and the furazolidone disks are readily available. For those laboratories performing broth dilution MICs, a single concentration of furazolidone may be used to determine susceptibility or resistance. This would provide a reliable screening result to differentiate between the staphylococci and micrococci.

The lysostaphin susceptibility test (Remel) was not as reliable a method as the others for differentiating between these organisms in this investigation. This is in direct contrast to the spot lysostaphin susceptibility test described by Schleifer and Kloos (18), which has been demonstrated to be quite reliable. Although all of the micrococci were resistant to lysostaphin, the staphylococci varied in their susceptibility to this agent. Of the *S. aureus* isolates, 237 (97%) were susceptible, but a significant number of staphylococci, other than *S. aureus*, were resistant; 29% of *S. epidermidis*, 100% of *S. haemolyticus*, 18% of *S. hominis*, 69% of *S. warneri*, and 33% of *S. capitis* were lysostaphin resistant. On the other hand, *S. saprophyticus* ($n = 5$) and *S. sciuri* ($n = 2$) were 100% susceptible. The number of *Staphylococcus* species other than *S. aureus* encountered in this investigation represents 38% of all the *Staphylococcus* isolates. Since a significant percentage of these isolates may be resistant to lysostaphin, although this method is rapid, it is not adequate for differentiating between staphylococci and micrococci. Although significant differences exist in the peptidoglycan structure between the staphylococci and micrococci, there are also significant differences that exist among the various *Staphylococcus* species, making certain species more susceptible to lysostaphin than others (3, 16).

The micrococci in this investigation demonstrated interesting results when tested with the Staph-Ident strip. Of these organisms, 12 (67%) resulted in the profile number 0000. The information provided by the Analytab Products telephone inquiry system associated this number with "low selectivity" for *S. capitis*, *S. cohnii*, and *S. saprophyticus*. Although this does not lead to a specific identification, information should be made available to the investigator that would indicate such a profile number may be associated with a *Micrococcus* species. This information would enhance the accuracy of the strip when working with catalase-positive, gram-positive cocci. It is also interesting to note that six (33%) of the micrococci were identified as *Staphylococcus* species (three each of *S. hominis* and *S. saprophyticus*). These six organisms clearly fit into the pattern of test results which are characteristic for *Micrococcus* species: glucose was not fermented nor was acid produced from glycerol. All of these organisms were oxidase positive and resistant to furazolidone and lysostaphin. When working with the Staph-Ident strip alone, a misidentification would have resulted in all of these cases.

The oxidase test (Microdase disk; Remel) proved to be the most sensitive (100%) and was sufficiently specific (99%) for providing a rapid means of accurately differentiating between staphylococci and micrococci. By picking a colony from an 18- to 24-h sheep blood culture, the test is easily performed, and results are recorded within 30 s. This is much more desirable than either the GF or acid production from glycerol method. GF requires 5 days before negative results are confirmed, and glycerol requires up to 3 days of incubation before confirming negative results for *Micrococcus* species. Furthermore, in this investigation, eight (2%) of the *Staphylococcus* species required 48 h before acid was detected from GF, and one additional isolate required 72 h.

In summary, by performing a rapid oxidase test or determining susceptibility to a 100-μg furazolidone disk, workers in clinical microbiology laboratories may more easily make the distinction between *Staphylococcus* and *Micrococcus* species. Also, when either of these methods is used in conjunction with the Staph-Ident strip, the accuracy of the strip is improved, and a better identification test system is the result.

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