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Use of Trehalose-Mannitol-Phosphatase Agar to Differentiate *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* from Other Coagulase-Negative Staphylococci

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Using a plate medium containing trehalose, mannitol, and phenolphthalein diphosphate (TMPA), we differentiated significant clinical isolates of *Staphylococcus epidermidis* by their lack of acid production in 18 h from other coagulase-negative staphylococci, with our results having a sensitivity (R. S. Galen and S. R. Gambino, *Beyond Normality: The Predictive Value and Efficiency of Medical Diagnoses*) of 100%, a specificity of 89.9%, and a positive predictive value of 94.8%. With a Taxo A bacitracin disk, which differentiates *Staphylococcus* species from *Micrococcus* species, no zone of inhibition was seen for 96% of all staphylococcal strains, with 5 of 26 strains of *Staphylococcus saprophyticus* exhibiting zone diameters up to 10 mm. By using resistance to a 5-μg novobiocin disk, we differentiated *S. saprophyticus*, with our results having a sensitivity of 100%, a specificity of 97.1%, and a positive predictive value of 83.9% on TMPA. These two species represented 77.8% of coagulase-negative staphylococci isolated. Reference strains of *Staphylococcus* and *Micrococcus* species were differentiated by TMPA. The cost of TMPA was compared with that of another method. TMPA was found to offer an inexpensive, sensitive method for rapidly differentiating coagulase-negative *Staphylococcus* isolates.

Interest in the identification to species level of coagulase-negative staphylococci (C-NS) has recently increased. *Staphylococcus epidermidis* is the predominant C-NS species isolated in human clinical studies, representing over 72% of significant C-NS isolates (6, 14, 17). From urine specimens, *Staphylococcus saprophyticus* is a common C-NS isolate (1, 2, 13), causing urinary tract infections in young females. Together, these species represent a large proportion of the C-NS isolated in clinical specimens. It would be extremely useful for routine clinical laboratories to have a simple, inexpensive protocol to quickly differentiate these two species from other C-NS.

The genus *Staphylococcus* is differentiated from the genus *Micrococcus* by anaerobic acid production from glucose (19) and aerobic acid production from 1% glycerol in the presence of 0.4 μg of erythromycin per ml (16) for which the staphylococci are positive and the micrococci are negative. Resistance to a Taxo A bacitracin disk (4) separates staphylococci from micrococci, which are susceptible. The staphylococci can be identified according to the simplied scheme of Kloos et. al. (10, 11) or by a rapid micromethod such as the API Staph-Ident system. *S. saprophyticus* has been differentiated from the other species by its resistance to novobiocin (NV), as indicated by growth on P agar (9–11) containing 1.6 μg of NV or by having an inhibition zone diameter of less than 16 mm when tested with a 5-μg NV disk (7, 10).

S. epidermidis fails to utilize the carbohydrates trehalose and mannitol (98 and 100%, respectively [9, 15]) and lacks phosphatase activity in only 5% (9) of strains. These features were used in the formulation of a novel differential medium, trehalose-mannitol-phosphate agar (TMPA). For the other C-NS species, the maximum utilization rates of either trehalose or mannitol are (9): *S. capitis*, 94%; *S. cohnii*, 100%; *S. haemolyticus*, 95%; *S. hominis*, 90%; *S. saprophyticus*, 96%; *S. simulans*, 92%; *S. warneri*, 100%; and *S. xylosus*, 96%. These species lack phosphatase activity (9) in 72 to 84% of strains, except for *S. xylosus* and *S. simulans* which lack it in only 20 to 23%. This study investigated the capability of the lack of acid production on TMPA to separate strains of *S. epidermidis* from other C-NS. Further differentiation of other C-NS species was done with 5-μg NV disks. Taxo A bacitracin disks were also applied.

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MATERIALS AND METHODS

Bacterial strains. The 198 strains of C-NS studied were significant isolates from clinical specimens submitted to the Microbiology Laboratory, Department of Laboratory Medicine, St. Joseph's Hospital, Hamilton, Ontario, Canada, and included 65 from peritoneal fluid, 30 from blood, and 103 from urine. They were differentiated from *Micrococcus* species by their ability to produce acid anaerobically from glucose (19) and aerobically from 1% glycerol plus 0.4 μg of erythromycin per ml on purple agar (16). The strains were identified by the simplified scheme of Kloos and Schleifer (10) as being 128 *S. epidermidis*, 26 *S. saprophyticus*, 13 *S. hominis*, 10 *S. warneri*, 7 *S. haemolyticus*, 4 *S. simulans*, 3 *S. cohnii*, and 2 *S. capitis*; 5 were not identified to species level. Also tested were 13 reference C-NS strains (12 species) and 7 *Micrococcus* strains (5 species), which originated from W. E. Kloos of North Carolina State University, Raleigh. Isolates were stored at −50°C in Trypticase soy broth (BBL Microbiology Systems) plus 15% glycerol before testing.

TMPA. TMPA contained (final concentrations) 1% filter-sterilized trehalose, 1% mannitol, and 0.01% phenolphthalein diphosphate in sterilized and cooled purple agar base (Difco Laboratories). Isolates were unfrozen, plated on 5% sheep blood agar (Columbia agar base; Oxoid Ltd.), and incubated at 35°C for 18 h. Four to five well-isolated colonies were touched with a sterile wire loop, transferred to 3 ml of Trypticase soy broth, and incubated at 35°C for 2 to 4 h.

Then, they were diluted by using sterile 0.85% saline to a 0.5 McFarland barium sulfate turbidity standard. Each diluted suspension was swabbed in three directions to one-half of the dried surface of a TMPA plate (15 by 100 mm). To this a 5-μg NV (Becton Dickinson and Co.) disk and a Taxo A bacitracin (ca. 0.04 U; BBL) disk were applied. The inoculum was streaked for isolated colonies on the remainder of the plate. All cultures were incubated at 35°C for 18 h.

Carbohydrate acid production was evaluated after 18 h by the following criteria of Kloos and Schleifer (10): +, moderate to strong acid, yellow color into the medium; ±, weak acid, yellow color under the culture streak but not extending into the medium; −, no acid, very faint to no yellow color under the culture streak. NV susceptibility was determined after 18 h, with resistant strains having inhibition zone diameters of 16 mm or less (10). NV susceptibility was confirmed on P agar plates (11) containing 1.6 μg of NV per ml (10). Resistant strains grew, whereas susceptible ones did not. After noting the acid production on TMPA, phosphatase activity was determined by adding several drops of ammonium hydroxide to the lid of a petri plate and inverting the TMPA over it. Phosphatase-positive colonies became a bright pink. Phosphatase activity was confirmed by the 4-h tube method of Kloos and Schleifer (10) by using 0.005 M phenolphthalein monophosphate (sodium salt) in 0.01 M citric acid-sodium citrate buffer.

Data analysis. For the analysis (5) of the capability of TMPA to differentiate *S. epidermidis* from other C-NS, a true-positive (TP) was classified as a strain of *S. epidermidis* that lacked acid production on TMPA, and a true-negative (TN) was classified as a strain of C-NS, non-*S. epidermidis*, that produced acid. In the analysis (5) of NV resistance on TMPA as a means to differentiate *S. saprophyticus* from other C-NS, a TP was classified as a strain of *S. saprophyticus* that produced an inhibition zone diameter of ≤16 mm (7, 10), and a TN was a non-*S. saprophyticus* strain of C-NS that had zones >16 mm. Sensitivity (5) represents the percentage of TP compared with TP plus false-negatives. Specificity (5) is TN as a percentage of TN plus false-positives. The predictive value (5) of a positive result is TP as a percentage of TP plus false-positives, and the predictive value of a negative result is TN as a percentage of TN plus false-negatives. Efficiency (5) is TP plus TN as a percentage of the total. Prevalence is TP plus false-negatives as a percentage of the total.

RESULTS

Using the criterion of a positive result being the lack of acid production on TMPA after 18 h, we differentiated *S. epidermidis* among the 198 strains of C-NS, with a predictive value (5) of 94.8%. The predictive value for differentiating non-*S. epidermidis* strains was 100%, and the overall efficiency of TMPA was 96.5%. The sensitivity of *S. epidermidis* differentiation was 100%, and the specificity was 89.9%. The prevalence rate of *S. epidermidis* was 64.7%. One strain of *S. capitis* failed to grow well on TMPA in 18 h. The seven non-*S. epidermidis* strains that failed to produce acid on TMPA within 18 h were two *S. warneri*, one *S. hominis*, one *S. haemolyticus*, and one *S. capitis*, with two not identified to species level. Upon reincubation, acid was produced within 24 h by one *S. warneri* strain and one *S. haemolyticus* strain and within 48 h by the other *S. warneri* strain.

On TMPA, 30.5% of the 128 strains of *S. epidermidis* were phosphatase negative. Of these, 2 were phosphatase positive by the tube method and 37 were phosphatase negative by both methods. Six of the seven TMPA acid-negative strains that were non-*S. epidermidis* were phosphatase negative.

The results of using 5-μg NV disks on TMPA to differentiate *S. saprophyticus* from other C-NS are shown in Table 1. All 26 isolates of *S. saprophyticus* were NV resistant, as indicated by inhibition zone diameters of less than 16 mm (10), with a mean zone diameter of 11.0 mm. Five other strains were NV resistant: one *S. epidermidis* (14 mm), one *S. hominis* (11 mm), one *S. warneri* (12 mm), and two *S. cohnii* (9 and 10 mm). All strains that indicated resistance to NV by the disk method were resistant to 1.6 μg of NV per ml in P agar. The remainder of the strains were susceptible to NV by both methods, with a mean inhibition zone diameter of 23.9 mm. Using resistance to an NV disk as a criterion, we differentiated *S. saprophyticus* from other C-NS, with a sensitivity (5) of 100% and a specificity of 97.1%. The predictive value for a positive test was 83.9% and for a negative test was 100%. The prevalence of *S. saprophyticus* was 13.2%. All 26 strains of *S. saprophyticus* were isolated from females, 23 from outpatient midstream urines, 2 from catheter urines, and 1 from blood. For all urines, *S. saprophyticus* represented 24.3% of the C-NS and had a positive predictive value of 92.6%. For midstream urines of outpatient females, 71% of the 31 C-NS isolates were *S. saprophyticus*, and the positive predictive value was 95.7%, with a specificity of 88.9%.

All strains studied were identified as *Staphylococcus* species. They had been differentiated from *Micrococcus* species by their resistance to Taxo A bacitracin (4), anaerobic acid production from glucose, and aerobic acid production on glycerol-erythromycin. There was no zone of inhibition with a Taxo A bacitracin disk on TMPA for 190 (96.5%) of the clinical *Staphylococcus* strains. Five strains of *S. saprophyticus* had zones of 10 mm or less. One strain of *S. warneri* had a zone of 13 mm, and one strain not identified to species level had a zone of 16 mm. One strain, *S. capitis*, failed to grow on TMPA in 18 h. Acid was produced from glucose anaerobically by 88.9% of all strains. No acid was produced by 1 *S. warneri* strain, 2 *S. cohnii* strains, and 19 of 26 *S. saprophyticus* strains. The other seven strains of *S. saprophyticus* produced weak acid reactions within 3 days. For aerobic acid production on glycerol-erythromycin, 92.4% of the strains produced acid, including all *S. saprophyticus* strains. No growth occurred in 7.6% of the strains.

Table 2 shows the results of the reference staphylococcal and micrococcal strains. On TMPA, with a Taxo A bacitracin disk, it was possible to separate most *Micrococcus*

TABLE 1. Inhibition zone diameters of C-NS to 5-μg NV disks on TMPA

Species[a] (no.)	Inhibition zone diam (mm)[b]	
	Mean ± SD	Range
S. epidermidis (127)	24.4 ± 2.0	20.0–31.0
S. hominis (12)	22.2 ± 2.3	19.0–27.0
S. warneri (9)	21.6 ± 1.9	19.0–25.0
Others[c] (18)	22.2 ± 1.3	20.0–25.0
S. saprophyticus (26)	11.0 ± 0.9	9.0–13.0
Others[d] (5)	11.2 ± 1.7	9.0–14.0

[a] One strain of *S. capitis* failed to grow well on TMPA.
[b] Diameters of >16 mm were considered to be susceptible, and diameters of ≤16 mm were considered to be resistant.
[c] Includes the following strains: seven *S. haemolyticus*, four *S. simulans*, one *S. cohnii*, and one *S. capitis*; five were not identified to species level.
[d] Includes the following strains: one *S. epidermidis*, one *S. hominis*, one *S. warneri*, and two *S. cohnii*.

TABLE 2. Differentiation of reference strains of *Staphylococcus* and *Micrococcus* on TMPA

Species and strain[a]	TMPA acid production[a]	Inhibition zone diam (mm)	
		NV (5 μg)	Taxo A bacitracin
S. capitis ATCC 27840	+	23.0	0
S. cohnii ATCC 29974	+	10.0	0
S. epidermidis ATCC 14990	−	24.0	0
S. haemolyticus ATCC 29970	+	22.0	0
S. hominis ATCC 27844	+	23.0	0
S. hyicus ATCC 11249	+	26.0	0
S. lentis ATCC 29070	+	13.0	0
S. saprophyticus ATCC 15305	+	8.0	0
S. saprophyticus KH 231	+	9.0	0
S. sciuri ATCC 29062	+	12.0	0
S. simulans ATCC 27848	+	21.0	0
S. warneri ATCC 27836	+	21.0	0
S. xylosus ATCC 29971	+	10.0	8.0
M. kristinae ATCC 27572	−	25.0	16.0
M. luteus ATCC 27141	−	26.0	20.0
M. lylae ATCC 27567	−	30.0	22.0
M. sedentarius ATCC 27573	−	28.0	19.0
M. sedentarius ATCC 27574	±	24.0	0

[a] *M. roseus* ATCC 186 and ATCC 397 did not grow on TMPA.

species from *Staphylococcus* species. Lack of acid production or resistance to NV differentiated *S. epidermidis* and *S. saprophyticus* from the other C-NS species.

The material cost, in Canadian dollars, to differentiate each strain of C-NS was $0.43 on TMPA (including 5-μg NV and Taxo A bacitracin disks) and $0.80 by the method of Kloos et al. (10, 11), based on four strains tested per carbohydrate utilization plate. For each strain, the time required to dilute, inoculate, read, and record, including media preparation, was 2 min for TMPA (18) and 14.5 min (18) for the Kloos et al. method. The incubation time was 18 h for TMPA and up to 3 days for Kloos et al.

DISCUSSION

In this study the majority of isolates were represented by *S. epidermidis* (64.7%) and *S. saprophyticus* (13.1%). Both species could be differentiated on TMPA, the latter with a 5-μg NV disk, with a sensitivity of 100% and a specificity of ca. 90% or greater. For non-*S. epidermidis* C-NS species, 28.8% of the total strains were expected to utilize trehalose or mannitol at rates of 90 to 96% (9). Thus, only a small proportion of non-*S. epidermidis* isolates would be expected to be TMPA acid negative. Seven (3.5%) such strains were found. In a population with a higher prevalence rate of these strains, the positive predictive value of TMPA would decrease. In human clinical studies, *S. epidermidis* represents over 72% of significant C-NS isolates (6, 14, 17), and TMPA offers a quick, simple means of differentiating these.

Only 5 to 12% of *S. epidermidis* strains are reported to be phosphatase negative (1, 3, 9, 12), whereas non-*S. epidermidis* C-NS species lack phosphatase activity in up to 72 to 84% of strains (9). Phosphatase production was not a key characteristic in the simplified scheme of Kloos and Schleifer (10) for C-NS identification. In this study, 39 of 128 strains of *S. epidermidis* were phosphatase negative on TMPA at 18 h. This high proportion of phosphatase-negative *S. epidermidis* strains limits the usefulness of the phosphatase test in further differentiating strains of *S. epidermidis* from other C-NS species that are TMPA acid negative.

In the current study, no zone of inhibition to the Taxo A bacitracin disk for 190 of 198 strains was seen. With a criterion for resistance set as an inhibition zone diameter equal to or less than 10 mm on TMPA, five strains of *S. saprophyticus* could be classified as resistant. Falk and Guering (4), using the Taxo A bacitracin disk, found no zone of inhibition for 15 *Staphylococcus* species (n = 156). They found a mean zone diameter of 17 mm, with a range of from 10.5 to 25.0 mm, for the eight *Micrococcus* species (n = 70).

Weak or no acid production from glucose under anaerobic conditions for all the strains of *S. saprophyticus* tested indicated a limitation when using this test to separate *Staphylococcus* species from *Micrococcus* species. Schleifer and Kloos (15) found that only 24.3% of their 83 strains of *S. saprophyticus*, when grown under anaerobic conditions in yeast extract-glucose broth, could lower the pH from 6.8 to between 5.0 and 5.4, a weak acid reaction. Bromcresol purple, the pH indicator (16), is yellow at pH 5.2 and purple at pH 6.8. Kloos (8) stated that classic glucose oxidation-fermentation reactions cannot adequately resolve *Staphylococcus* strains that produce low amounts of acid from glucose anaerobically from *Micrococcus kristinae*. For acid production on glycerol-erythromycin, 183 of the *Staphylococcus* strains were positive, with the remaining 15 failing to grow. Schleifer and Kloos (16) found that 98.1% of 319 *Staphylococcus* strains produced acid on glycerol-erythromycin, as did 4.7% of 106 *Micrococcus* strains.

The *S. saprophyticus* strains tested were differentiated from the other C-NS with a high degree of sensitivity and specificity by the 5-μg NV disk on TMPA. Similar results were found by Kloos and Schleifer (10) on P agar and by Goldstein et al. (7) on P agar, 5% sheep blood agar, or Mueller-Hinton agar. In this study, five non-*S. saprophyticus* strains were NV resistant, two of which were *S. cohnii* (10, 11), a known NV-resistant species (8, 9).

Thus the use of TMPA offers a quick, inexpensive method of differentiating *S. epidermidis* from other C-NS. The incorporation of the phosphatase reaction was not useful in the differentiation of TMPA acid-negative strains. When used in conjunction with a 5-μg NV disk, *S. saprophyticus* can be differentiated. By using a Toxo A bacitracin disk to separate *Staphylococcus* species from *Micrococcus* species, 99% of the staphylococcal strains have an inhibition zone diameter equal to or less than 10 mm, with 96.5% exhibiting no zones. If further identification to species level is required, more extensive identification systems (10) can be used.

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