Use of Shake Cultures in a Semisolid Thioglycolate Medium for Differentiating Staphylococci from Micrococci

JAMES B. EVANS AND W. E. KLOOS

Department of Microbiology and Department of Genetics, North Carolina State University, Raleigh, North Carolina 27607

Received for publication 1 November 1971

The standard diagnostic test for differentiating staphylococci from micrococci is based on the ability of the former to produce acid anaerobically in a glucose-containing growth medium. This test has been modified to provide greater convenience, easier interpretation of results, and better correlation with deoxyribonucleic acid (DNA) base composition. In the modified test, shake cultures in Brewer's fluid thioglycolate medium with 0.3% agar added are observed for growth in the anaerobic zone of the tubes. This test was applied to 125 strains of staphylococci and micrococci, and all except two strains gave results that were consistent with other criteria. Of particular interest were eight strains of Micrococcus saprophyticus and three strains of M. lactis that have a DNA composition of 30 to 37% guanine plus cytosine (GC). All 11 of these cultures produced anaerobic growth and thus would be classified as staphylococci. Strains of M. lactis that have a high GC content in their DNA grew only aerobically. Some cultures of staphylococci produced characteristic band patterns of anaerobic growth and other cultures produced only a few anaerobic colonies from an inoculum of $10^8$ to $10^9$ cells. These observations suggest some interesting genetic and metabolic capabilities in such cultures.

At present, the most widely accepted single diagnostic test for separating staphylococci from micrococci is based on the ability of staphylococci to ferment glucose anaerobically and the lack of this ability among micrococci (2, 8, 9). The International Association of Microbiological Societies Subcommittee on Taxonomy of Staphylococci and Micrococci proposed a standard method for this test (8). This method involves stab inoculation of tubes of a semisolid medium with bromocresol purple as the pH indicator, covering with a layer of sterile mineral oil, and observing for a color change throughout the tube. This test is somewhat inconvenient for laboratories using it on a routine basis, and some difficulties are encountered in interpreting the results, particularly when the indicator in only part of the tube turns yellow (3, 5, 6). As with any single test, there is less than a perfect correlation with the results of detailed taxonomic studies (4, 7). However, only when the taxonomic unit is arbitrarily defined on the basis of the test being used should one anticipate a perfect correlation. The standard method is presently considered the most reliable and reproducible method of distinguishing these two genera (9).

Whittenbury (10) proposed the use of tubes of a soft-agar medium to study the aerobic and anaerobic utilization of fermentable substrates by lactic acid bacteria. Staphylococci under anaerobic conditions have the same requirement for a fermentable energy source as do lactic acid bacteria. Thus, Whittenbury's method is applicable to staphylococci. We have modified the method by using a medium containing thioglycolate to improve the anaerobic zone of the deeper portion of the tube, an oxidation-reduction indicator to delineate the anaerobic zone, and an increased agar concentration to prevent the gravitational migration of the inoculum and colonies.

MATERIALS AND METHODS

Cultures. The cultures used in this study included an extensive stock culture collection having

1 Paper no. 3600 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh.
representatives of each of Baird-Parker's (1) sub-
groups of staphylococci and micrococci, as well as a
number of cultures freshly isolated in our laboratory
from skin surfaces of healthy adults and children.
Also included were the host strains for the interna-
tional phage-typing series of Staphylococcus aureus.
The majority of the stock cultures were received
from M. Kocur (Czecholovak Collection of Microor-
organisms), A. C. Baird-Parker (Unilever Research
Laboratory), or the American Type Culture Collec-
tion. The deoxyribonucleic acid (DNA) base com-
position of many of these strains has been listed
in the Czecholovak Collection of Microorganisms
Catalogue of Cultures. We have determined this
composition in other strains by use of the thermal
denaturation method.

Medium. The medium selected for routine use
was Brewer's fluid thioglycolate medium (Difco)
with the addition of 0.3% agar (total of 0.35% agar
in final medium). The medium contains glucose as
an energy source, sodium thioglycolate to help main-
tain a low redox potential, and methylene blue as a redox
indicator. Preliminary trials with other basal media
and growth indicators such as triphenyltetrazolium
chloride or pH indicators did not disclose any ad-
vantage over the thioglycolate medium. The agar
concentration was selected to provide sufficient gel
strength to minimize gravitational settling of the
inoculum without too much reduction in transpar-
ency of the medium.

Inoculation. For routine screening of cultures,
one loopful of a 24-hr culture in Trypticase soy broth
(BBL) was transferred into a tube of the sterile test
medium that had been steamed and cooled to 50 C.
(Approximately 8 ml of medium was used per 16-mm tube.)
After the inoculum had been gently mixed with the loop, the
medium was allowed to solidify at room temperature. For preliminary
studies, inocula with known cell concentrations were
used, but this was considered impractical for a rou-
tine diagnostic test. For screening of fresh isolates, a
 colony may be picked from the isolation medium
with a sterile needle and emulsified in 1 ml of sterile
broth or saline for use as an inoculum.

Incubation. An incubation temperature of 35 C
was selected to provide a rapid response from the
staphylococci without being too high for most of the
micrococci. Overnight incubation was sufficient for
the staphylococci and most of the micrococci, but up
to 72 hr was required for some of the slow-growing
cultures. For better contrast for photographic pur-
pouses, cultures were incubated for as long as 1 week.
This could have resulted from either the downward diffusion of toxic metabolic products or the depletion of essential nutrients. Continued incubation for 1 week did not alter the appearance of such cultures.

Five strains of *S. epidermidis*, one from each of Baird-Parker's (1) subgroups II, III, IV, V, and VI, were tested. All had heavy aerobic growth and moderate to heavy anaerobic growth (Fig. 3 and 4) except one strain that produced only about 100 scattered colonies in the anaerobic zone.

Nine strains recently isolated from human skin and five strains recently isolated from tobacco leaves and identified only as "coagulase-negative staphylococci" were tested. All 14 strains produced heavy aerobic growth; half of them produced heavy anaerobic growth, and the other half produced only scattered colonies anaerobically.

Eight strains of *Micrococcus saprophyticus*, including strains from Baird-Parker's (1) sub-

**Fig. 3. Staphylococcus epidermidis 1512, 24-hr culture.**

**Observations.** The tubes were examined by looking through them toward, but not directly at, a light while slowly rotating the tube. The location and intensity of the zones of growth were noted. Although cultures were classified as either aerobic or facultatively anaerobic on the basis of this test, a variety of patterns of bands of growth were observed that may provide clues to subtle differences in the respiratory systems of the organisms and may also have some taxonomic value.

**RESULTS**

Twenty-three strains of *S. aureus* were included in the test series. These included 16 host strains from the international phage-typing series, 1 of Baird-Parker's (1) subgroup I strains, and 6 fresh isolates from the skin of healthy individuals. All cultures produced moderate to heavy growth throughout the tube within the first 24 hr, as demonstrated in Fig. 2 (compare with the uninoculated control shown in Fig. 1). Some strains had a band of little or no growth just beneath the zone of heavy aerobic growth.
Differdiation of Staphylococci and Micrococci

One produced heavy anaerobic growth and the other two produced only scattered colonies anaerobically. These three cultures apparently differ from *M. saprophyticus* only in their failure to produce acetoin, and they also appear to belong to the genus *Staphylococcus*.

Fourteen strains of *M. lactis*, mostly from the Czechoslovak Collection of Microorganisms, having a DNA composition of 68 to 72% GC were tested. All produced aerobic growth within 24 hr and no anaerobic growth even after 7 days of incubation. Figure 7 depicts one of the more slowly growing strains after 7 days.

Forty-four cultures of *M. luteus* all produced moderate to slight growth aerobically and no growth anaerobically. The middle of this range of results is illustrated in Fig. 8.

Seven cultures of *M. roseus*, all ATCC strains that were originally isolated several decades ago, produced slow and rather weak aerobic growth and no anaerobic growth. They

**Fig. 5.** *Micrococcus saprophyticus* BP-4, 7-day culture.

**Fig. 6.** *Micrococcus saprophyticus* BP-19, 7-day culture.
offers greater simplicity, easier evaluation of results, and a better correlation with the DNA base composition. Omission of the sterile mineral oil seal may make the test more widely accepted and used. Since glucose utilization (fermentation) is detected by direct observation of a growth response, one avoids the problems associated with the diffusion of acid that may cause color changes in a pH indicator at some distance from the site of growth. Media without glucose (or some other fermentable substrate) fail to support anaerobic growth of staphylococci, verifying the validity of using growth response as a measure of their ability to ferment glucose. The agar shake technique enables one to observe aerobic and anaerobic growth in the same tube. This technique also has demonstrated that some cultures of staphylococci contain only a small population of cells, presumably mutants, that are capable of anaerobic glucose utilization. This rather frequent observation suggests that further genetic and metabolic studies of such strains might be highly desirable.

**DISCUSSION**

The test proposed here is not a new criterion for separating staphylococci from micrococci. Rather, it is an improved method for detecting the ability of staphylococci to ferment glucose anaerobically. In comparison with the presently recommended standard method (8), it

Fig. 7. *Micrococcus lactis* 2127, 7-day culture.

gave the same appearance as the more weakly growing strains of *M. luteus*.

Five unclassified micrococci with a DNA composition of 66 to 70% GC (ATCC 533; CCM 314, 547, 810, and 2140) grew aerobically but not anaerobically. These strains are quite similar to either *M. lactis* or *M. luteus*.

In the collection of cultures included in this study, two strains produced anomalous results. An orange-pigmented micrococcus having a DNA composition of 68 to 71% GC (CCM 2142) slowly produced moderately heavy growth both aerobically and anaerobically (Fig. 9). One culture with 37% GC in its DNA (ATCC 8456) grew poorly aerobically and not at all anaerobically.

Fig. 8. *Micrococcus luteus* ISU, 7-day culture.
These other genera may produce a positive test for catalase, it will be much different than the strong positive reaction produced by staphylococci. As with any diagnostic test, there will be some anomalous results. A particular problem may occur with those staphylococci that produce only a few scattered anaerobic colonies. Whether or not one will detect this situation will depend in some instances on the level of inoculum used. True standardization of the inoculum in terms of cells per milliliter seems somewhat impractical for routine use. Use of one loopful of broth from a 24-hr culture in BBL Trypticase soy broth offers some degree of reproducibility.

Many of the obligately aerobic micrococci, particularly some strains of *M. luteus* and *M. roseus*, grow poorly in Trypticase soy broth and very poorly in the thioglycolate test medium. This does not affect the validity of the test, however, and these cultures would be correctly classified as micrococci.

**ACKNOWLEDGMENTS**

This investigation was supported by Public Health Service grants AI-07693 and AI-08255 from the National Institute of Allergy and Infectious Diseases.

We also wish to acknowledge the excellent technical assistance of Sandra Cott and Margaret Musselwhite.

**LITERATURE CITED**

1. Baird-Parker, A. C. 1965. The classification of staphylococci and micrococci from world-wide sources. J. Gen. Microbiol. 38:363–387.

2. Cowan, S. T., and K. J. Steel. 1964. Comparison of differentiating criteria for staphylococci and micrococci. J. Bacteriol. 88:804–805.

3. Gibson, T. 1967. The status of the genus Micrococcus. Int. J. Syst. Bacteriol. 17:231–233.

4. Kiesius, P. H., and V. T. Schuhardt. 1968. Use of lyso- staphin in the isolation of highly polymerized deoxyri- bonucleic acid and in the taxonomy of aerobic Micro- cococcaceae. J. Bacteriol. 96:739–743.

5. Kocur, M., and N. Mortensen. 1967. Comparison of methods for estimation of anaerobic production of acid from glucose and mannitol in staphylococci and micrococci. Acta Pathol. Microbiol. Scand. 71:141–146.

6. Mortensen, N., and M. Kocur. 1967. Correlation of DNA base composition and acid formation from glucose of staphylococci and micrococci. Acta Pathol. Microbiol. Scand. 69:445–457.

7. Rosypal, S., A. Rosypalova, and J. Horejs. 1966. The classification of micrococci and staphylococci based on their DNA base composition and Adansonian analysis. J. Gen. Microbiol. 44:281–292.

8. Subcommittee on Taxonomy of Staphylococci and Micro- cocci. 1965. Recommendations. Int. J. Syst. Bacteriol. 15:109–110.

9. Subcommittee on Taxonomy of Staphylococci and Micro- coccice. 1971. Minutes of meeting. Int. J. Syst. Bacteriol. 21:161–163.

10. Whittenbury, R. 1965. The use of soft agar in the study of conditions affecting the utilization of fermentable substrates by lactic acid bacteria. J. Gen. Microbiol. 32:575–584.

---

**FIG. 9. Orange-pigmented micrococcus CCM2142, 7-day culture.**

This test is particularly useful with cultures that appear to be misclassified by the standard test, i.e., cultures with a low GC content in their DNA and that produce partial acidification of the medium in the standard test. These include cultures that currently would be classified as *M. saprophyticus*, or as *M. lactic* if they fail to produce acetoin. It also includes some cultures of staphylococci that have been maintained in the laboratory for many years, particularly strains of *S. epidermidis*. The positive test for anaerobic glucose utilization by cultures of *M. saprophyticus* and some of *M. lactis* is less surprising if one examines Baird-Parker’s results (1), which indicate that these “glucose oxidizing” micrococci produce a final pH in glucose broth of 4.4 to 5.8.

There are, of course, several limitations to the test. Other genera of gram-positive cocci, such as streptococci and pediococci, are not readily distinguished from staphylococci, so a test for catalase production should be part of any screening program. Even though some of