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Superoxol and Amylase Inhibition Tests for Distinguishing Gonococcal and Nongonococcal Cultures Growing on Selective Media

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Two inexpensive screening tests were evaluated singly and in tandem for distinguishing *Neisseria gonorrhoeae* from other oxidase-positive microorganisms growing on selective gonococcal media. In tests of 728 cultures, including 460 *N. gonorrhoeae*, 4 *Neisseria lactamica*, 257 *Neisseria meningitidis*, and 7 *Branhamella catarrhalis*, both Superoxol (30% H_2O_2; J. T. Baker Chemical Co., Phillipsburg, N.J.) and amylase inhibition tests were 100% sensitive (positive) for 20-h cultures of *N. gonorrhoeae*. Singly, the Superoxol test was 92.7% specific for *N. gonorrhoeae*, compared with a specificity of 82.3% for the amylase inhibition test. By using tandem screening tests to distinguish gonococci, we achieved an overall specificity of 98.6%. Group A meningococci were the primary source of error in the Superoxol test, with 97% (37 of 38) strains producing gonococcal like reactions for catalase. From 5 to 20% of *N. meningitidis* serogroups X, Y, Z, and Z' and nontypable strains, as well as about 50% of *B. catarrhalis* and *N. lactamica* strains, were also strong catalase producers.

The development of selective gonococcal (GC) media has significantly improved laboratory techniques for isolating *Neisseria gonorrhoeae*, even though other microorganisms, including *Neisseria meningitidis*, *Kingella denitrificans*, some *Branhamella catarrhalis* strains, and some other *Neisseria* species, can grow on selective media (1, 3, 13). Although confirmatory biochemical and immunological tests are available for identifying *N. gonorrhoeae*, no single test has consistently proven 100% sensitive and specific. In addition, the cost and time required for some confirmatory tests may limit their routine use. Inexpensive screening tests may provide an alternative by helping laboratory workers distinguish GC and non-GC cultures early in the identification process.

The Superoxol (30% H_2O_2; J. T. Baker Chemical Co., Phillipsburg, N.J.) test, a rapid screening method for identifying gonococci growing on selective media, was reportedly 100% sensitive and 99% specific in tests with over 200 GC and meningococcal cultures (11). However, about 50% of *B. catarrhalis* and *Neisseria lactamica* strains gave GC-like reactions for catalase. Because these microorganisms are often confused with *N. gonorrhoeae* (1, 7), we proposed to further evaluate the Superoxol test as well as a recently described amylase inhibition test (8) for screening gram-negative diplococci capable of growing on selective media. Results for using each test singly and in tandem to distinguish GC and non-GC cultures are presented.

MATERIALS AND METHODS

Bacteria. Microorganisms of known identity were obtained from the Sexually Transmitted Diseases and Special Bacteriology Laboratories at the Centers for Disease Control, Atlanta, Ga., and consisted of 460 *N. gonorrhoeae* strains (including 60 beta-lactamase-producing strains, as determined by the starch paper technique [9]), 257 serogrouped *N. meningitidis* strains, and 11 *N. lactamica* and *B. catarrhalis* strains. Four meningococcal strains were maltose negative by carbohydrate utilization tests (7) and were part of a group of *N. gonorrhoeae* cultures provided to the Centers for Disease Control by state health laboratories as "problem" cultures (1). Ten *N. gonorrhoeae* strains were vancomycin sensitive and grew poorly on selective modified Thayer-Martin medium (MTM) (13).

Media. All cultures were initially grown on chocolate GC agar (BBL Microbiology Systems, Cockeysville, Md.) and then transferred to plates (100 by 15 mm) of GC selective screening medium (GC-SSM), which consisted of 20.0 ml of hemoglobin-free MTM base containing 1.0% IsoVitaleX as well as 3 μg of vancomycin, 7.5 μg of colistin, and 12.5 μg of nystatin per ml (13). Each GC-SSM plate was treated with two concentrations of alpha-amylase type VI A (Sigma Chemical Co., St. Louis, Mo.). For preparation of the amylase, a stock solution of 10 mg/ml was made in 1.0 mM calcium chloride. The stock solution was stored at −20°C until being used for making further dilutions in sterile distilled water. Amylase concentrations of 0.1 and 0.25 mg/ml were found optimal for treating plates in this study. One drop (0.05 ml) of each concentration was placed in two different areas on the agar plates and allowed to dry for about 6 h at 21°C. The amylase-treated plates were then stored at 4°C until about 24 h before being used. Holding the medium at this temperature prevented the area of amylase-mediated starch hydrolysis from enlarging beyond the usual 20-mm diameter. For testing vancomycin-sensitive organisms, the selective inhibitors vancomycin, colistin, and nystatin, along with hemoglobin, were deleted from the MTM.

Amylase inhibition test. Test organisms were picked from chocolate agar by using a sterile cotton swab or wire loop and streaked over the surface of the GC-SSM plates. The size of the test inoculum used was not critical, although results were more easily interpreted when about 10^5 CFU was used. After inoculation, the plates were incubated at 36°C for 20 h or longer in an atmosphere of 5% CO_2. The amylase-treated areas were observed, and the degree of growth inhibition was recorded as complete (Fig. 1), incom-

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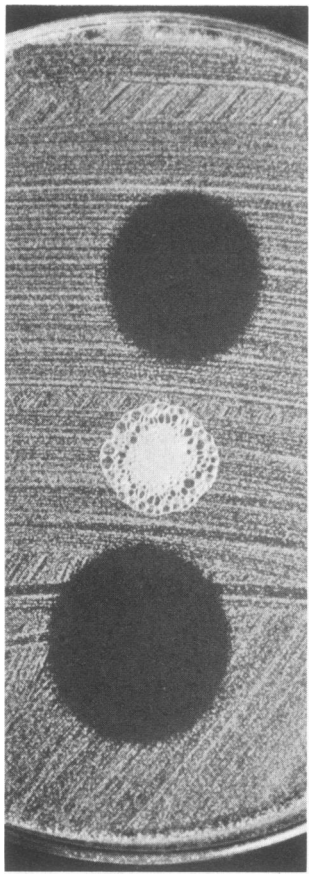

FIG. 1. *N. gonorrhoeae* showing strong Superoxol reaction (center) and two zones of complete inhibition in areas of medium exposed to alpha-amylase.

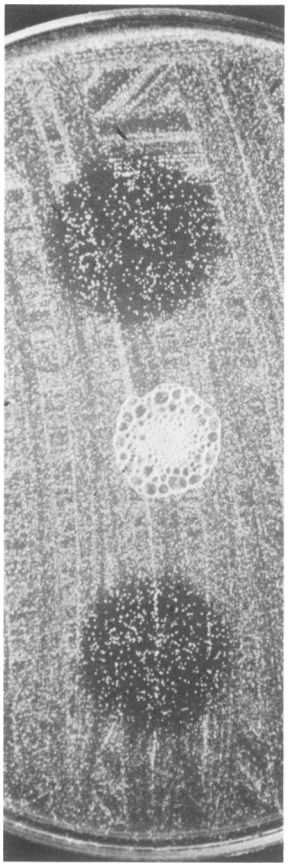

FIG. 2. Group A *N. meningitidis* showing strong Superoxol reaction (center) and two areas of incomplete amylase inhibition.

plete (Fig. 2), or unremarkable (Fig. 3). *N. gonorrhoeae* cultures were distinguished by two large (>20-mm) zones of complete growth inhibition in the amylase-treated areas (Fig. 1 and 4). Because zone size is influenced by the concentration of amylase used and the incubation period, the primary factor used for differentiating cultures in this test was the presence or absence of growth in the amylase-treated area.

Superoxol test. Hydrogen peroxide (30%) was used as described by Saginur et al. (11) to detect catalase activity in cultures growing for 24, 48, 72, and 96 h on chocolate medium and GC-SSM. About 0.05 ml was dropped on colonies of test organisms and observed for the immediate, brisk bubbling which characterized *N. gonorrhoeae* and other strong catalase-producing microorganisms. Reactions delayed for 3 s or longer or weak bubbling was considered negative for *N. gonorrhoeae*, provided that the culture had not been incubated for longer than 24 h.

Confirmatory tests. Microorganisms producing conflicting results in the two screening tests were reidentified by using the micro-carbohydrate utilization (14) and Phadebact GC-coagglutination (Pharmacia Fine Chemicals, Piscataway, N.J.) tests.

Statistical tests. Differences in laboratory findings were tested for significance by the chi-square test (12).

RESULTS

All 728 test organisms except 10 vancomycin-sensitive GC strains grew on both chocolate GC agar and hemoglobin-free GC-SSM plates. Identical results were obtained for Superoxol tests performed on both media. All 460 *N. gonorrhoeae* strains that were less than 24 h old showed strong catalase activity. However, in tests performed with *N. gonorrhoeae* cultures that had been incubated for longer than 48 h, we observed a substantial decline in catalase activity in areas of confluent colony growth (Table 1). When 30% hydrogen peroxide was applied near the edge of growth surrounding the amylase-treated area (Fig. 4), however, strong catalase activity was detected for up to 96 h. Suboptimal growth conditions in this area of the medium may have resulted in slower degradation or increased catalase activity, or both. The overall specificity of the Superoxol test for *N. gonorrhoeae* in this study was 92.7%. The major source of error in

TABLE 1. Effects of culture age on Superoxol tests performed on areas of confluent growth and at the periphery of amylase inhibition

Test area	% of *N. gonorrhoeae* strains positive[a] after:			
	24 h (n = 460)	48 h (n = 47)	72 h (n = 47)	96 h (n = 35)
Confluent growth	100	78.7	21.3	8.6
Periphery of amylase zone	100	95.7	93.6	91.4
P	NS[b]	<0.02	<0.001	<0.001

[a] A positive test result consisted of immediate strong bubbling upon exposure of colonies to 30% hydrogen peroxide.
[b] NS, Not significant.

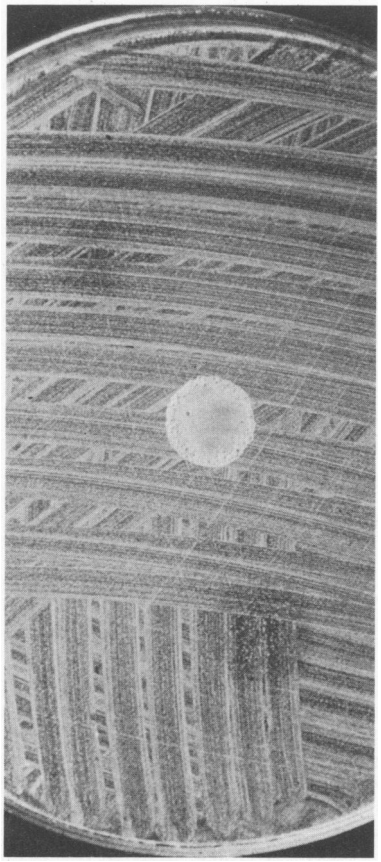

FIG. 3. *N. lactamica* showing positive Superoxol reaction and unremarkable inhibition in amylase-treated areas of medium.

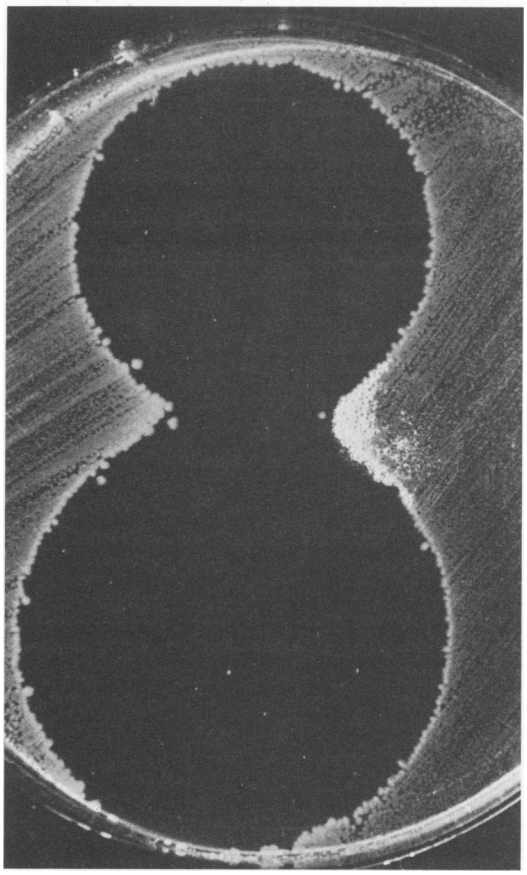

FIG. 4. *N. gonorrhoeae* incubated for 72 h and showing positive Superoxol reaction only around the periphery of the amylase-inhibited area.

the test was group A meningococci that gave GC-like reactions with 97% (37 of 38) of the strains tested. *N. meningitidis* serogroups X, Y, Z, and Z' and nontypable strains gave GC-like reactions with 5 to 20% of the strains. Over 50% (6 of 11) of the *B. catarrhalis* and *N. lactamica* strains were also strong catalase producers (Table 2).

In the amylase inhibition test, all 460 *N. gonorrhoeae* strains showed large (>20-mm) zones of complete growth inhibition. From 8 to 80% of different meningococcal serogroups showed varying degrees of growth inhibition in the amylase test (Table 2 and Fig. 2 and 3). As a group, only 47% of meningococci could be distinguished from *N. gonorrhoeae* by growth of colonies in the amylase-treated area of the medium (Fig. 2). The amylase test was, however, significantly ($P < 0.001$) better than the Superoxol test for distinguishing group A meningococci, *B. catarrhalis*, and *N. lactamica* strains, which helped in achieving an overall specificity for *N. gonorrhoeae* of 98.6% for the tandem of tests. The specificity of the amylase test alone for *N. gonorrhoeae* was 82.3%.

DISCUSSION

Both the Superoxol and amylase inhibition tests gave positive results with all *N. gonorrhoeae* strains tested (100% sensitivity). However, their individual specificities were 92.7 and 82.3%, respectively, compared with 98.6% for the tandem of tests. Although the two criteria, sensitivity and specificity, are important measures of reliability, these values are greatly influenced by the type and combination of cultures being tested. In tests where the predominant cultures were *N. gonorrhoeae* and group B and C meningococci, the Superoxol test achieved a high degree of specificity (about 99%), as previously reported (11). As shown in this study, however, the presence of large numbers of group A meningococci or *B. catarrhalis* and *N. lactamica* strains in the test population substantially reduced the specificity of the Superoxol test, and tandem use with the amylase inhibition test provided significant ($P < 0.01$) improvement.

We previously showed the effects of amylase to be directed at altering the growth medium and not at attacking GC cells per se. It appears that the inhibitory effects produced by exposing media to amylase result from the degradation of starch, which otherwise provides a detoxifying effect by neutralizing fatty acids and possibly other inhibitors in the medium. Serum and other blood products (hemoglobin) may have similar detoxifying effects and can interfere with the effects produced by amylase (8). In this study, translucent, hemoglobin-free medium was used exclusively for performing the amylase test. Commercial development of a similar translucent medium could greatly expedite use of the amylase procedure and could provide added benefits by allowing better visualization of colony characteristics which help to distinguish GC and non-GC isolates (6). As shown by other investigators, hemoglobin-free MTM as used in this study appears to be significantly ($P < 0.05$) more sensitive than conventional MTM for detecting *N. gonorrhoeae* in primary culture specimens (5, 10).

The Superoxol and amylase inhibition tests were shown in

TABLE 2. Percentage of non-GC strains distinguished from *N. gonorrhoeae* by using amylase inhibition or Superoxol screening test, or both[a]

Species and serogroup	No. of strains tested	% Distinguished by:		
		Amylase test alone[b]	Superoxol test alone[c]	Tandem of tests
N. meningitidis				
A	38	92.1	2.6	92.1
B	92	53.8	97.8	97.8
C	40	30.0	100.0	100.0
W135	28	42.9	96.5	100.0
X	5	20.0	80.0	100.0
Y	34	14.7	91.2	94.2
Z	5	40.0	80.0	100.0
Z'	10	40.0	90.0	90.0
Nontypable	5	20.0	80.0	80.0
B. catarrhalis	7	57.1	28.6	85.7
N. lactamica	4	100.0	75.0	100.0

[a] Tests were performed with 18- to 24-h growth of cells on a hemoglobin-free selective GC medium.

[b] Non-GC cultures were distinguished by uninhibited or incomplete growth in amylase-treated areas of the medium as compared with complete inhibition of *N. gonorrhoeae*.

[c] Non-GC cultures were distinguished by delayed or weak bubbling after exposure to 30% hydrogen peroxide as compared with immediate, brisk bubbling by *N. gonorrhoeae*.

this study to be highly specific (98.6%) when used in tandem for identifying *N. gonorrhoeae*. However, because other organisms capable of growing on selective media can occasionally give GC-like reactions in both tests, cultures appearing to be *N. gonorrhoeae* should be confirmed by conventional tests (1). The greatest value, at present, for using the combined screening tests appears to be for early presumptive recognition of non-GC microorganisms growing on selective media and for detection of *N. gonorrhoeae* and *N. meningitidis* strains having unusual biochemical or immunological characteristics that would cause them to be misidentified by conventional tests (2, 4, 7). Although the Superoxol test can be performed on primary culture plates, the amylase inhibition test has been evaluated only for screening of subcultured microorganisms. Its value for screening of primary culture plates has yet to be determined.

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