Nuclease Enhancement of Specific Cell Agglutination in a Serodiagnostic Test for *Neisseria gonorrhoeae*

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Antiserum to a purified type R lipopolysaccharide antigen isolated from *Neisseria gonorrhoeae* was used in a slide agglutination test and compared with conventional carbohydrate utilization and fluorescent antibody tests to confirm the identity of laboratory cultures classified as typical or "atypical" *N. gonorrhoeae*. Cultures of *Corynebacterium vaginalis*, *N. meningitidis*, *N. catarrhalis*, *N. sicca*, and *N. lactamicus* were also tested in the slide agglutination procedure. The addition of both deoxyribonuclease and ribonuclease (1 mg/ml) to the cell suspension medium of phosphate-buffered saline improved the sensitivity and specificity of the agglutination reaction for *N. gonorrhoeae*. Problems relating to the agglutination test as an aid in identification of *N. gonorrhoeae* are discussed.

In studying the antigenic structure of *Neisseria gonorrhoeae*, investigators in Canada isolated a rough type of lipopolysaccharide (R-LPS), common to all strains of *N. gonorrhoeae* (3, 4; M. B. Perry, V. Daoust, K. G. Johnson, B. B. Diena, and F. E. Ashton, submitted for publication). Antibody to the R-LPS has been used in an experimental slide agglutination test (SAT) for rapid identification of *N. gonorrhoeae* (6). Antibodies to the R-LPS antigen raised in leghorn hens specifically agglutinated both piliated and nonpiliated cells of more than 1,000 gonococcal strains (3, 6). We have tested the R-LPS antiserum by the recommended SAT procedure and by experimental modifications of SAT as an aid in the identification of typical and "atypical" gonococcal cultures. Our atypical cultures included those that gave poor or equivocal carbohydrate utilization or fluorescent antibody test results.

MATERIALS AND METHODS

Bacterial culture. Routine Gram stain, oxidase reaction, and morphological characteristics (colony types) were determined for all cultures. When these preliminary test results were compatible with those of *N. gonorrhoeae*, more specific identification procedures were applied. Typical gonococcal strains, including N7, N9, and others from the Center for Disease Control-Venereal Disease Research Branch (CDC-VDRB) culture collection were used as reference strains yielding expected fluorescent antibody (FA) and sugar utilization reactions. Presumptive gonococcal strains giving atypical FA or sugar reactions were submitted by state health laboratories to CDC for confirmation as *N. gonorrhoeae*. Nongonococcal microorganisms including *N. meningitidis*, serogroups B and C, *N. catarrhalis*, *N. sicca*, *N. lactamicus*, and

Corynebacterium vaginalis (*Haemophilus vaginalis*) strains were from the CDC-VDRB culture collection. All strains were grown on plates containing GC base medium (Difco) supplemented with 1% fetal calf serum and IsoVitaleX (Baltimore Biological Laboratories, Baltimore, Md.). Incubation was in a candle jar for 20 h at 36°C.

Hen serum anti-R-LPS. One-milliliter vials of lyophilized anti-R-LPS hen serum were generously provided by B. B. Diena of the Laboratory Centre for Disease Control, Ottawa, Canada and M. Perry of the National Research Council, Ottawa, Canada.

Hen serum agglutination test. The SAT was performed as previously described (6) and with the following modifications. (i) Bacterial cells were removed from the agar surface and suspended in the appropriate buffer with a calcium alginate swab (Calgiswab, Inolex Co., Glenwood, Ill.) in place of a wire bacteriological loop. (ii) A cell suspension medium (phosphate-buffered saline [PBS], pH 7.2), consisting of 6.14 g of Na₂HPO₄, 8.5 g of NaCl, and 5.0 ml of 37% formaldehyde dissolved in 995 ml of distilled water, was prepared as recommended (6). We modified the PBS suspension medium by adding one or both deoxyribonuclease (DNase) and ribonuclease (RNase) (Sigma Chemical Co., St. Louis, Mo.) at 1 mg each per ml of the PBS buffer. For use in routine tests, the PBS buffer containing both DNase and RNase was stored in glass vials at -70°C and thawed immediately before being used in the SAT.

FA test. A rabbit antigenococcal conjugate that had been absorbed with cells of *N. meningitidis* group B and *N. sicca* to improve specificity was obtained from the Biological Products Division at CDC and used in a previously described direct FA procedure with formaldehyde-fixed cells (2).

Carbohydrate utilization tests. Utilization of sugars was tested in cystine Trypticase agar as previously described (5). All lots for media were pretested, and positive and negative controls were included in every run.

RESULTS

Technical as well as biological factors were found to influence the sensitivity and specificity of the SAT. Freshly rehydrated anti-R-LPS serum imparted a milky appearance to the test portion of the slide, making difficult a critical comparison of agglutination with the antigen control portion when agglutinating activities were slight or slow reacting. When rehydrated hen serum, preserved by freezing at -70°C , was thawed precipitates often formed, thus rendering the serum unsuitable for use in the SAT. We had difficulty with the recommended wire bacteriological loop in obtaining uniform cell suspensions with certain test strains, especially when the cultures were older than 24 h. This problem was overcome by our using a calcium alginate swab to remove cells from the culture plate and to make suspensions in the test buffer.

Certain strains of *N. gonorrhoeae* especially from older cultures formed stringy clumps in the PBS buffer, and although adding glycerol 1:1 to the buffer, as recommended (6), reduced the nonspecific clumping of cells, it also slightly decreased the agglutination titer of the hen serum with gonococcal cells (Table 1). As an alternative to glycerol, we found that adding nucleases (DNase and RNase, 1.0 mg/ml) reduced stringy clumping and substantially increased the specific agglutination titer of the hen serum with *N. gonorrhoeae* isolates (Table 1). Adding DNase alone accounted for most of the improvement. The combination of both DNase and RNase appeared, however, to provide the best overall results in the agglutination reaction with gonococcal cells (Table 2).

TABLE 1. Comparison of anti-R-LPS agglutination titers of *Neisseria* species suspended in PBS and in PBS with and without nuclease or glycerol

Test organism ^a	Serum agglutination titer ^b		
	PBS	PBS + glycerol (1:1)	PBS + nuclease ^c
<i>N. gonorrhoeae</i> N9	10	10	82
<i>N. gonorrhoeae</i> 113	4	± 4	>82
<i>N. gonorrhoeae</i> 092	4	± 4	>82
<i>N. gonorrhoeae</i> N7	10	<4	28
<i>N. meningitidis</i>	<4	<4	<4
<i>N. lactamicus</i>	<4	<4	<4

^a Approximately 10^8 colony-forming units of cells suspended in test buffers for 15 min before being mixed (1:2) with dilutions of anti-R-LPS hen serum in disposable serological trays.

^b Data are expressed as reciprocals of the serum titer after incubation for 30 min at 20°C .

^c Both DNase and RNase were added at 1.0 mg/ml of PBS (pH 7.2).

Since the degree of cell agglutination with the anti-R-LPS serum varied with different microorganisms and different gonococcal strains, we found that we needed a graded system of scoring reactions and for recording results. Agglutinations were scored as negative ($-$ or $\pm$), positive ($+$), or strongly positive ($++$) on the basis of the degree of clumping present at 5 min after the test reagents were mixed on the slide. By using the PBS buffer with and without nucleases to test 44 randomly coded bacterial cultures, we found that 3 of 11 strains classified previously by cystine Trypticase agar and FA tests as typical *N. gonorrhoeae* gave $-$ or $\pm$ reactions in the PBS buffer without nucleases, whereas all 11 strains gave $+$ or $++$ reactions in the buffer containing nucleases (Table 3). In addition, the nuclease buffer gave a more uniform cell suspension, which helped eliminate false-positive reactions when testing *N. meningitidis*, *N. sicca*, *N. catarrhalis*, and *C. vaginalis* cultures (Table 3). In testing atypical or presumptive *N. gonorrhoeae* cultures, we scored 19 out of 22 strains $+$ or $++$ in the nuclease buffer and 16 out of 22 $+$ or $++$ in the plain PBS buffer. In tests of nongonococcal microorganisms, only the *C. vaginalis* isolates gave significant cross-agglutination with the R-LPS antiserum. However, only a limited number of nongonococcal microorganisms were tested (Table 3).

Although nuclease treatment improved the agglutination of gonococci in the SAT, we did not find in the FA test that treating cells with nuclease improved the fluorescent staining of gonococci. In the FA test, the brilliance of fluorescent staining appeared to be more affected by the age and subpassage of the gonococcal culture. Younger gonococcal cells, incubated for 14 to 18 h, generally stained more brilliantly

TABLE 2. Effects of nuclease on the agglutination of strain N9 gonococcal cells at increasing dilutions of anti-R-LPS hen serum

Dilution of hen serum	Treatment of gonococcal cells and agglutination results ^a			
	Buffer only	Buffer + RNase	Buffer + DNase	Buffer + RNase and DNase
1:4	$+$ ^b	$+$	$+$	$+$
1:10	$+$	$+$	$++$	$++$
1:28	$-$	$\pm$	$++$	$++$
1:82	$-$	$-$	$\pm$	$+$

^a Cells were suspended at 10^8 colony-forming units in PBS (pH 7.2) with and without 1.0 mg of RNase, DNase, or both enzymes per ml.

^b Agglutination results were read at 5 min and graded as negative ($-$ or $\pm$), positive ($+$), and strongly positive ($++$).

TABLE 3. *Specificity and sensitivity of the serum agglutination test for identification of Neisseria gonorrhoeae*

Category tested	Anti-R-LPS hen serum agglutination with cells suspended in PBS ^a					
	PBS alone			PBS with 1 mg of nuclease/ml		
	(-±)	(+)	(++)	(-±)	(+)	(++)
Typical <i>N. gonorrhoeae</i> ^b	3/11	8/11	0/11	0/11	4/11	7/11
Presumptive <i>N. gonorrhoeae</i> ^c	6/22	10/22	6/22	3/22	11/22	8/22
Nongonococcal microorganisms ^d	4/11	7/11	0/11	1/11	0/11	0/11

^a Numbers are results expected for *N. gonorrhoeae* per number tested.

^b Strains producing expected sugar utilization and fluorescent antibody results.

^c Strains producing sugar utilization or fluorescent antibody results not typical for *N. gonorrhoeae*.

^d Nongonococcal microorganisms included *N. sicca*, *N. catarrhalis*, *N. meningitidis* serogroup B and C, and 7 different isolates of *C. vaginalis*.

than cells from older, more lysed cultures. Subpassage on laboratory medium also improved the staining of certain gonococci which had given equivocal fluorescence in previous tests.

DISCUSSION

The confirmation of *N. gonorrhoeae* cultures becomes increasingly complex as additional tests are applied to the identification process. When multiple tests are performed, it is not unusual for a culture to give the expected reaction in one test but not in others, resulting in a classification of presumptive or atypical *N. gonorrhoeae*. Test results are influenced by a number of factors, including the quality of test reagents, the conditions of the test cultures, and the standards by which comparisons are made. Therefore, new tests must be evaluated under various conditions.

We found that with reagents now available and recommended procedures, the difficulties of the SAT are primarily in specificity and sensitivity. Modifications in the test procedure and adding nuclease to the suspension buffer appeared to improve the specific agglutination of

gonococcal cells. The leakage and polymerization of nucleic acids from old or injured bacterial cells as demonstrated by Catlin with *N. meningitidis* (1) may possibly contribute to the increased viscosity and nonspecific clumping of cells used in the test and therefore may, with certain cultures, interfere with the detection of specific agglutination. Surface nucleic acids did not appear to present a problem in the FA test, since treating cells with nuclease did not improve the fluorescent staining of gonococcal cells. The ineffectiveness of nuclease in improving the FA test may be due to the relatively small size of the fluorescein-conjugated antibody used to detect gonococcal surface antigens and to the fact that, in fluorescent staining, close cell-to-cell contact is not required. Such contact is, of course, required in agglutination reactions.

The identification and isolation of R-LPS as a common antigen of *N. gonorrhoeae* may make possible the development of a new generation of simple serodiagnostic tests for the identification of this organism. However, the specificity and sensitivity of the present SAT procedure and the methods for preserving test reagents need to be improved.

LITERATURE CITED

1. Catlin, B. W. 1960. Transformation of *Neisseria meningitidis* by deoxyribonucleates from cells and from culture slime. *J. Bacteriol.* **79**:579-590.
2. Deacon, W. E., W. L. Peacock, E. M. Freeman, and A. Harris. 1959. Identification of *Neisseria gonorrhoeae* by means of fluorescent antibodies. *Proc. Soc. Exp. Biol. Med.* **101**:322-325.
3. Diena, B. B., F. E. Ashton, A. Ryan, R. Wallace, and M. B. Perry. 1978. The lipopolysaccharide (R-type) as a common antigen of *Neisseria gonorrhoeae*. I. Immunizing properties. *Can. J. Microbiol.* **24**:117-123.
4. Perry, M. B., V. Daoust, B. B. Diena, F. E. Ashton, and R. Wallace. 1975. The lipopolysaccharides of *Neisseria gonorrhoeae*. Colony types 1 and 4. *Can. J. Biochem.* **53**:623-629.
5. Vera, H. D. 1948. A simple medium for identification and maintenance of the gonococcus and other bacteria. *J. Bacteriol.* **55**:531-536.
6. Wallace, R., F. E. Ashton, A. Ryan, B. B. Diena, C. Malysheff, and M. B. Perry. 1978. Use of hen antiserum to gonococcal lipopolysaccharide in a rapid slide test for the identification of *N. gonorrhoeae* from primary isolates and secondary cultures. *Can. J. Microbiol.* **24**:124-128.