

## Serogroup Identification of *Neisseria meningitidis*: Comparison of an Antiserum Agar Method with Bacterial Slide Agglutination

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Received for publication 28 October 1977

A serum agar method for serogrouping *Neisseria meningitidis* is described and compared with conventional bacterial slide agglutination. There was 93% agreement for 300 strains examined individually by each method. Among strains from serogroups A, B, C, Y, and W135, there was 100% correlation, whereas strains from serogroup 29E (Z') had only 67% correlation. The serum agar method was rapid, as well as easy to perform and interpret. The potential benefits of this method for epidemiological studies and reference laboratories processing large numbers of meningococcal isolates are emphasized.

*Neisseria meningitidis* is classified into serogroups based on immunologically and chemically distinct capsular polysaccharides (Table 1). In 1954, the *Neisseriaceae* Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies recommended that *N. meningitidis* be classified into serological groups A, B, C, and D (28) based on the findings of Branham (6). This classification held until 1961, when Slaterus described three new serogroups which he labeled X, Y, and Z. These strains, and a fourth (Z') were not agglutinated by antisera to group A, B, C, or D (26, 27). Evans et al. in 1968 found three additional serogroups provisionally identified as Bo (Boschard), W135, and 29E (10), of which Bo and 29E correspond to Slaterus groups Y and Z', respectively (10, 11). Later, Vedros described an E group (29), but this was later recognized as identical to group Y. Although the nomenclature for classifying meningococci is somewhat confusing, serogroup identification and the more recently described subgrouping or serotyping procedures (13, 14, 16) have provided important epidemiological data on carrier rates and disease causation.

There are no internationally recognized standard procedures or antisera for serogrouping meningococci. Although hemagglutination inhibition (8) and staphylococcus coagglutination (21) have been described, bacterial slide agglutination is most commonly used. A common problem with bacterial agglutination (BA) is nonspecific cross-reactivity and inability to serogroup spontaneously agglutinable strains. Petrie (22) demonstrated in 1932 that meningococci

formed precipitin halos around the colonies when grown on agar containing anti-meningococcal sera. This procedure was employed later to evaluate therapeutic anti-meningococcal sera (15, 23, 24) but was not used for serogroup identification. Recently, Robbins et al. have used serum agar plates to identify other encapsulated bacteria and strains cross-reactive with *N. meningitidis* (5, 15, 25).

This communication compares 300 meningococcal isolates, serogrouped by the antiserum agar (ASA) method at the Bureau of Biologics, with BA performed at the Upstate Medical Center, State University of New York (SUNY), Syracuse. A high degree of correlation was found between the two techniques.

### MATERIALS AND METHODS

**Strains.** Three hundred lyophilized strains were carefully selected by H.A.F. to be a representative cross section of meningococcal strains. Isolates included cultures from the throat, nasopharynx, blood, and cerebrospinal fluid. Another 115 case and carrier isolates were obtained from several university hospitals. These strains had been originally submitted to the Bureau of Biologics for serotyping.

**Cultivation and storage.** Cultures were inoculated onto brain heart infusion (Difco Laboratories, Detroit, Mich.) agar containing 1% normal horse serum and then placed in a 5% CO<sub>2</sub> incubator at 37°C for 5 to 7 h. Bacteria were removed with a cotton-tipped applicator and transferred to vials containing Greaves solution (5% [wt/vol] bovine serum albumin and 5% [wt/vol] monosodium glutamate in 10% [vol/vol] glycerol) and stored at -70°C.

**Preparation and titration of antisera.** The strains used for antiserum preparation are listed in Table 2. To prepare the vaccine, actively growing

meningococci were transferred from a brain heart infusion agar plate to 400 ml of tryptic soy broth (Difco) and incubated for 5 to 6 h on a shaker at 37°C. The cells were recovered by centrifugation at  $10,000 \times g$  for 20 min, suspended in 20 ml of phosphate-buffered saline (pH 7.2) containing 0.5% Formalin, and stored at 4°C. A new batch was prepared for each week of immunization (Table 3).

Five horses and one burro were immunized by a variation of the method of Alexander et al. (1) (Table 2). Animals were bled 4, 6, and 10 days after the last immunization. The sterile sera were pooled, divided, and stored at -20°C. The animals were reimmunized a few months later.

At least two courses of immunization were required to obtain antisera of sufficient titer. For titration of each antiserum, petri dishes containing 1:5 to 1:40 concentrations of specific antiserum were inoculated with strains from six different meningococcal serogroups. The dilution which gave the strongest halo without cross-reactions was selected for use. Final antiserum dilutions for this study varied from 1:10 to 1:40.

**ASA plates.** The ASA plates were prepared by adding tryptic soy broth (30 g/liter; Difco) and Noble agar (14 g/liter; Difco) to a flask of distilled water. The

medium was autoclaved and allowed to cool to 50°C. A specific amount of pretitrated, heat-inactivated horse antiserum was added to each flask and mixed, and 60 ml was poured into each large petri dish (25 by 150 mm) or 15 ml was poured into each small petri dish (15 by 100 mm). The plates were dried for 24 h at room temperature before inoculation. Unused plates were stored at 4°C in sealed plastic bags for up to 4 months.

**BA.** Bacterial slide agglutination was carried out at SUNY as previously described (12). Anti-meningococcal sera were supplied by the Center for Disease Control, Atlanta, Ga., and the Division of Bacterial Diseases, Walter Reed Army Institute of Research (WRAIR), Washington, D.C.

## RESULTS

**Interpretation of ASA plates.** Agar plates prepared with antiserum to meningococcal group A, B, C, Y, W135, or 29E were spot-inoculated with a cotton-tipped applicator which had been rotated three to four times over the surface of the strain frozen in Greaves solution. Up to 32 strains could be inoculated onto the larger (25 by 150 mm) petri dishes, and at least 12 strains could be placed on the smaller (25 by 100 mm) petri dishes. After incubation at 37°C in a 5% CO<sub>2</sub> incubator for 24 h, the ASA plates were removed and examined for halos against a dark background with indirect light (Fig. 1). These halos were intensified by an additional 24 h at 4°C and were similar for all serogroups tested. Optimal results were obtained by using thin ASA plates and properly titrated antiserum as described in Materials and Methods. For this study each ASA plate was read by two people and the results were recorded daily.

**Meningococci serogrouped by BA and ASA performed in experienced laboratories.** To establish ASA as a reliable serogrouping method, 300 strains previously serogrouped at SUNY by conventional BA were sent to the Bureau of Biologics for serogrouping by ASA. At the end of the study, the results of the two methods were compared (Table 4). Overall, there was 93% agreement and, for serogroups A, B, C, Y, and W135, there was 100% agreement. Among the 29E strains identified by BA, only 20 of 30 (67%) were serogrouped as such by ASA.

TABLE 1. Classification of *N. meningitidis* serogroups and their group-specific polysaccharide composition

Serogroup	Chemical composition of capsular polysaccharide	Reference
A	<i>N</i> -acetyl-3- <i>O</i> -acetyl mannose phosphate ( $\alpha$ 1-6)	16
B	<i>N</i> -acetyl neuraminic acid ( $\alpha$ 2-8)	17
C	<i>N</i> -acetyl and <i>O</i> -acetyl neuraminic acid ( $\alpha$ 2-9) or <i>N</i> -acetyl neuraminic acid ( $\alpha$ 2-9)	17
D	Unknown	1
Y	<i>N</i> -acetyl neuraminic acid: glucose	3
W135	<i>N</i> -acetyl neuraminic acid: galactose	3
29E (Z')	3-Deoxy-D-manno-oligosonic acid: <i>N</i> -acetyl galactosamine	2
X	<i>N</i> -acetyl glucosamine phosphate ( $\alpha$ 1-4)	5
Z	Unknown	

TABLE 2. Preparation of group-specific anti-meningococcal serum for the ASA method: immunizing strains

Group	Animal	Strain <sup>a</sup>	Source
A	Horse 49	M1024 (A-1)	Sara Branham
B	Horse 46	99 Misc (B-11)	Walter Reed (WRAIR)
C	Burro 211	60 EUR (C-11)	Walter Reed
Y	Horse 52	6304 (135M)	Walter Reed
W135	Horse 54	S4383	Harry Feldman
29E	Horse 53	6312 (61 Misc)	Walter Reed

<sup>a</sup> The Walter Reed number is given in parentheses.

The remaining 10 strains were nongroupable. In addition, five of seven strains identified by BA as group Z gave a positive reaction with 29E serum used for ASA. Since the only discrepancy between the two methods was with serogroup 29E, both methods were repeated on all strains in question. When a higher-titered 29E antiserum was used in the repeat ASA procedure, cross-reactions with groups D and X were not observed. Otherwise, the initial results (Table 4) were unchanged.

**ASA results of 115 meningococcal isolates previously serogrouped by BA in university hospital laboratories.** Serogrouping meningococci by BA appears simple but requires proper controls and standardized antisera and may be difficult for inexperienced personnel to interpret correctly. Most hospitals in the United States serogroup small numbers of meningococci per year and use a variety of commercial antisera. Having previously established the reliability of the ASA technique (Table 4), we examined 115 meningococcal isolates previously serogrouped in university hospital laboratories (Table 5). Overall, there was 77.4% agreement. There were 26 strains in which ASA did not agree with BA, and 25 of these represented serogroups identified perfectly by ASA in the previous table.

### DISCUSSION

Observation of immunoprecipitates surrounding meningococcal colonies provides a simple, easy to interpret procedure for rapid serogroup identification of *N. meningitidis*. The utility of this technique for identification of groups A, B, and C was demonstrated during the recent meningococcal epidemic in Finland (A. Sivonen, O. Renkonen, and J. B. Robbins, *J. Clin. Pathol.*, in press). Our studies show that the ASA method may be used to serogroup strains of groups Y, 29E, and W135 as well.

Petrie first used halo formation in antiserum agar as a means of differentiating rough and smooth strains of meningococci (22). The halo

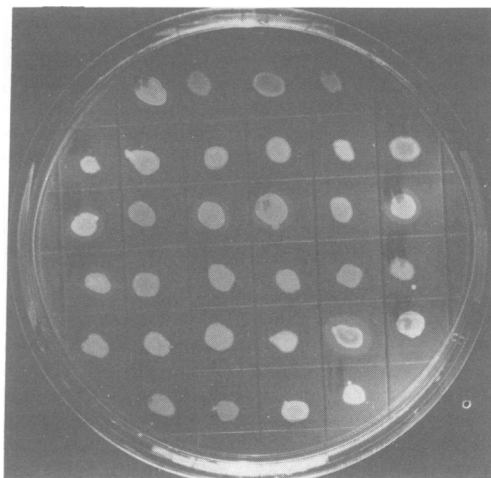


FIG. 1. Examination of 32 meningococcal strains by the ASA method. Plate contains Trypticase soy agar with antiserum to serogroup C polysaccharide (1:40 dilution). The halos around the colonies (after 48 h of incubation) represent seven serogroup C strains.

which he observed was the result of capsular polysaccharide diffusing from the colony into the agar and forming a precipitate with the anticapsular antibody. The addition of group-specific antiserum (Table 1) to the agar is the basis for ASA serogrouping. Halos were frequently present after 24 h of incubation and were intensified by an additional 24 h at 4°C. Plates examined after 48 h may show nonspecific cross-reactions caused by the release of other antigens. Additional difficulties can result from cross-reactions with other encapsulated bacteria (25) or from the use of improperly titrated antimeningococcal sera. There was a consistent cross-reaction between serogroups Y and W135 due to the antigenic similarity of their capsular polysaccharides. Group Y strains produced strong halos on both the group Y and W135 plates, whereas W135 strains produced halos only on the W135 antiserum plate. Thus, even with this cross-reaction, it is easy to distinguish between strains belonging to either the Y or W135 serogroup.

There was complete agreement between ASA and BA methods for serogrouping A, B, C, Y, and W135 strains. However, only 20 of 30 strains identified as 29E by BA were so identified by ASA. These results were confirmed by BA and by ASA with a higher-titered antiserum. The discrepancy between the results from the two methods may be due to differences in the antisera and/or antigenic differences in the 29E capsular polysaccharides. Recent unpublished data

TABLE 3. Preparation of group-specific anti-meningococcal serum for the ASA method: immunization schedule

Week	Vaccine dose <sup>a</sup> (ml) on:		
	Monday	Wednesday	Friday
1	1.0 s.c.	1.5 s.c.	1.0 i.v.
2	1.0 i.v.	2.0 i.v.	3.0 i.v.
3	3.0 i.v.	4.0 i.v.	5.0 i.v.
4	5.0 i.v.	6.0 i.v.	7.0 i.v.

<sup>a</sup> s.c., Subcutaneous injection; i.v., intravenous injection.

TABLE 4. Correlation for 300 strains of *N. meningitidis* serogrouped independently by ASA and BA methods

Serogroup re- sults with ASA <sup>a</sup>	Serogroup results with BA <sup>b</sup>											Total
	A	B	C	Y	W135	29E (Z')	D	X	Z	SA <sup>c</sup>	NG <sup>d</sup>	
A	42	— <sup>e</sup>	—	—	—	—	—	—	—	—	—	42
B	—	50	—	—	—	—	—	—	—	—	—	50
C	—	—	42	—	—	—	—	—	—	—	—	42
Y	—	—	—	35	—	—	—	—	—	—	—	35
W135	—	—	—	—	25	—	—	—	—	—	—	25
29E (Z')	—	—	—	—	—	20	1	2	5	2	—	30
NG <sup>c</sup>	—	—	—	—	—	10	1	23	2	17	23	76
Total	42	50	42	35	25	30	2	25	7	19	23	300

<sup>a</sup> Performed at Bureau of Biologics.<sup>b</sup> Performed at SUNY, Syracuse.<sup>c</sup> SA, Saline agglutinable.<sup>d</sup> NG, Nongroupable.<sup>e</sup> —, None found.

from our laboratory suggests that some strains identified as 29E by BA are nonencapsulated.

Five of seven strains classified by BA as group Z reacted strongly with ASA plates containing 29E antiserum, suggesting the presence of a cross-reactive antigen or chemical similarities between the two capsular polysaccharides. In 1970, Devine and Hagerman (9) found that 29E antiserum agglutinated group Z strains but that Z antisera did not react with 29E strains. A similar relationship was described by Slaterus et al. for Z and Z' strains (27). Recently, Fallon demonstrated that groups Z' and 29E were identical serologically (11). However, further study is needed to clarify the differences within the 29E (Z') serogroup and its relation to group Z.

To obtain an indication of the accuracy of meningococcal serogrouping in the hospital setting, we reexamined 115 meningococcal strains previously typed by BA in university hospital laboratories and found only 77.4% agreement. Since ASA results from the 26 strains in question were reconfirmed by ASA, we concluded that they were incorrectly identified by BA. Because most American hospitals process relatively few meningococcal isolates and depend on a variety of commercial antisera, there is a need for standardized reagents as well as a simple serogrouping method to improve these results. The ASA method is easy to perform and requires little experience for correct interpretation. The ease of interpretation was shown by inviting inexperienced individuals to read the ASA plates. In its present state of development, ASA would be most beneficial to laboratories serogrouping large numbers of meningococcal isolates. In addition, it is a useful tool for epidemiological studies and may be used to determine the incidence of individuals carrying more than one

TABLE 5. Examination of 115 meningococcal strains by ASA method which had been previously serogrouped in university hospitals by BA

Serogroup	ASA re- sult	BA result		Agree- ment (%) <sup>a</sup>
		Positive correlation	Negative correlation	
A	13	12	1	92.3
B	34	28	6	82.3
C	32	25	7	78.1
Y	10	9	1	90
29E (Z')	3	3	0	100
NG <sup>b</sup>	23	12	11 <sup>c</sup>	52.2
		89		77.4
Total	115	89	26	77.4

<sup>a</sup> BA/ASA × 100.<sup>b</sup> NG, Nongroupable.<sup>c</sup> BA results were four serogroup A, two group B, one group C, three group Y, and one group 29E (Z').

meningococcal serogroup. We recently employed the ASA technique for surveillance studies of meningococcal carriers in a military population and were able to obtain group-specific meningococcal carrier rates within 48 h (manuscript in preparation).

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