JOURNAL OF BACTERIOLOGY, Jan. 1968, p. 1-4 Vol. 95, No. 1
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Serological Studies of Ungroupable *Neisseria meningitidis*

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Received for publication 7 October 1967

Verification that Slaterus' *Neisseria meningitidis* serotypes X, Y, and Z are groups distinct from each other and from groups A, B, C, and D was made by use of the tube agglutination test on absorbed and unabsorbed antisera. A significant number of meningococcal strains in this country, which could not be classified serologically with standard antisera prepared to Branham's neotype A, B, C, and D strains, were grouped specifically with antisera prepared to the Slaterus types. The strains grouped as X, Y, and Z were from various geographical areas of the United States and were isolated from both carriers and cases. Over a 2-year period, the cultures tested ranged in predominance in descending order as follows: group B, C, Y, X, Z, A, and D. It is recommended that Slaterus' types should be considered as standard groups and follow in alphabetical order with the standard A, B, C, and D groups; i.e., X would be designated as group E, Y as group F, and Z as group G. It was observed that false-grouping cross-reactions could be greatly reduced by reconstituting the lyophilized grouping antisera in 50% glycerol-water. Of 99 cultures which could not be specifically grouped with antisera reconstituted in distilled water, 19 were specifically grouped with antisera reconstituted in 50% glycerol-water.

Since 1954, when the *Neisseriaceae* subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies (8) recommended the classification of *Neisseria meningitidis* into serological groups, the designations group A, B, C, and D have been used in this country. Branham (2) reviewed the serology of the meningococci and described the four groups as broad serological groups, each having its own epidemiological significance. In 1956, Jyssum (5) studied 13 meningococcal strains and found 5 strains which failed to react with antisera prepared to group A, B, C, and D strains. He called this type N. However, when Branham studied the N strains, she found that they reacted with her group B antisera. In 1957, after studying approximately 2,200 strains of *N. meningitidis*, Branham (3) selected neotype strains to represent the major serological groups. She recommended that antisera for grouping be made from strains which were antigenically "broad" and specific to their serogroup.

Slaterus (6), using the micro-precipitin gel method, tested meningococcal strains in Holland. He found three strains which failed to react with antisera prepared against Branham's A, B, C, and D strains. Absorption of antisera prepared to the new types which Slaterus had provisionally labeled as X, Y, and Z indicated that the specific precipitation lines remained after absorption with strains not belonging to these types.

In view of a significant number of strains which could not be classified serologically in our laboratory with standard antisera prepared to Branham's neotype A, B, C, and D strains, a study was undertaken to determine whether some of these strains might be identified serologically with sera prepared to the Slaterus X, Y, and Z strains. Results of these tests and some observations of the effect of the fluid used for reconstituting the lyophilized *N. meningitidis* sera are given in this report.

MATERIALS AND METHODS

Strains. Strains were obtained from 35 states, Israel, and the Philippines.

Antisera. All strains were tested with group A, B, C, and D antisera prepared by the Biological Reagents Section, National Communicable Disease Center (NCDC), and a serum prepared from a strain which reacted with Slaterus' X serum when tested by him (6).

Antisera were prepared to the Slaterus X, Y, and Z strains (kindly sent to us by G. W. Slaterus). These

antisera were used to test all the smooth ungroupable strains.

Antisera to groups A, B, C, and D were purchased from two commercial sources and were used to confirm the negative reactions of the Slaterus strains and the ungroupable strains which failed to react with any other antisera.

Rabbit antisera preparation. The strains which were preserved in sterile defibrinated rabbit blood were removed from deep freeze storage and re-examined morphologically, biochemically, and serologically. Transfers were made from Heart Infusion Agar (Difco) slants to which a drop of rabbit blood had been added to slants of Heart Infusion Agar containing 5% rabbit blood and 1% glucose. The slants were incubated under CO_2 at 37 C. Growth from approximately 15 hr slants was harvested with 0.5% Formalin-treated physiological saline and allowed to stand at room temperature for at least 1 hr. The harvested cell suspension was then adjusted with 0.5% Formalin-treated saline to a density equivalent to a McFarland no. 3 standard as determined with a spectrophotometer. The inoculum was made each week on the day of the first injecton and used for the injections of that week only. Sera from the preimmunized rabbits were nonreactive. An immunization schedule used by the Diagnostic Reagents Section, NCDC, was followed (Harrell et al., NCDC Procedure Manual for Bacteria and Fungal Diagnostic Reagents, PHS 32–34). This schedule consisted of three successive intravenous injections per week for 3 weeks: 1st week, 0.25, 0.25, 0.50 ml; 2nd week, 0.50, 0.50, 1.0 ml; 3rd week, 1.0, 1.0, 2.0 ml. Five days after the last injection, animals were bled by cardiac puncture. The titers of the final immune sera when checked by the tube agglutination test were as follows: X, 1:160; Y, 1:180; and Z, 1:160. Because a second series of immunizations gave lower titers and some cross-reactions, only sera from the first series were used.

Serological procedures. Branham's tube agglutination test (3) and the slide agglutination described by the American Public Health Association Subcommittee on Diagnostic Procedures and Reagents (1) were used. The micro-precipitin agar-gel method of Crowle (4) was employed. Antigens used for all tests were Formalin-treated 18- to 24-hr cultures. These preparations usually did not remain stable after 24 hr.

Absorption. One loopful (2 mm) of viable 18-hr culture from a 5% rabbit blood-Heart Infusion Agar plate was emulsified in 0.5 ml of the undiluted antiserum, incubated at 37 C for 1 hr, and centrifuged at 600 × *g* for 10 min. The serum was then removed and tested by the slide agglutination method. Sometimes the absorptions were repeated several times to assure complete absorption.

When it was necessary to repeat absorption with the homologous strain, comparative absorptions were also made with the heterologous strains. After the last absorption, all sera were tested by the tube agglutination method. The neotype strains of Branham and Slaterus were used for absorption, agar-gel, and tube agglutination studies.

RESULTS

Titrations of antisera of groups A, B, C, D, X, Y, and Z with the homologous and heterologous antigens were performed by the tube agglutination procedure. All antisera reacted only with their homologous antigens except for antisera to group B (prepared at NCDC), which also reacted with the Z antigen. However, tube agglutination tests with group B antisera from two commercial sources were both negative with the Z antigen. All three of the group B antisera failed to react with the Z strain in the slide agglutination test.

Agar-gel precipitin reactions indicated that antisera to Slaterus' X, Y, and Z strains formed specific bands with their homologous antigens which were not seen with heterologous antigens.

The Slaterus types were proved to be distinct by absorption tests of X, Y, and Z antisera with the homologous and heterologous strains of *N. meningitidis* (A, B, C, D, X, Y, and Z). These results are shown in Table 1. Absorption of X, Y, and Z antisera by heterologous strains did not lower the titers of these sera when tested with their homologous antigens; however, absorption with the homologous strains (X, Y, and Z) removed the specific agglutinins in their respective antisera.

Slaterus X, Y, and Z antigens were also used to absorb group A, B, C, and D antisera (prepared at NCDC). The agglutination titers of these sera with their homologous antigens were not reduced by the Slaterus X, Y, and Z antigens.

From June 1965 to June 1966, 299 cultures of *N. meningitidis* were tested serologically by slide agglutination (Table 2). When originally tested, 54 of these strains were ungroupable: 4 were too rough for testing, 21 gave cross-reactions with two or more antisera, and 28 failed to

TABLE 1. *Titers of Slaterus X, Y, Z homologous antisera after absorption with homologous and heterologous strains*

Serogroup of strain used for absorption	X serum	Y serum	Z serum
A[a]	160[b]	80	160
B	320	80	160
C	320	80	160
D	320	80	80
X	—	80	160
Y	320	—	160
Z	160	80	—
Unabsorbed homologous titer	160	80	160

[a] Branham and Slaterus neotype strain used for absorption.
[b] The reciprocal of the dilution.

agglutinate. Twenty-eight ungroupable smooth strains, which had been stored in a deep freeze, were retested with A, B, C, D, X, Y, and Z antisera. Of the 28 strains, 13 reacted with the Y antiserum, 6 did not react, and 9 were too rough for testing.

Some strains of *N. meningitidis* tended to show cross-reactions in one or more of the A, B, C, and D grouping sera which had been lyophilized and then reconstituted in distilled water; however, these same strains were usually smooth or negative in two antisera which had been preserved by mixing equal parts of antiserum and glycerol. This observation prompted an evaluation of 50% glycerol as a reconstituting fluid for the standard grouping sera.

TABLE 2. *Neisseria meningitidis serological results by slide agglutination (June 1965–June 1966)*

Serogroup	No.	Per cent
A	0	—
B	207	69.2
C	32	10.7
D	1	0.3
X	6	2.0
Y	13	4.3
Z	0	—
Ungrouped		
Smooth (negative)	15[a]	5.0
Cross-reactions	21[b]	7.0
Rough	4	1.3
Total	299	

[a] Not typable with X, Y, and Z sera after storage in a deep freeze. Nine were too rough and six were unreactive.

[b] Tested prior to the introduction of serum in 50% glycerol-water.

A study was made comparing antisera reconstituted in distilled water with antisera reconstituted in 50% glycerol. Of 99 cultures, 19 could not be grouped with antisera reconstituted in distilled water because of nonspecific agglutinations. These ungroupable cultures could be specifically grouped when tested with antisera reconstituted in 50% glycerol. Since this comparative study was completed, the 50% glycerol solution has been used routinely in our laboratory. Of 250 strains tested, only 2 were ungroupable due to cross-agglutination.

Twenty-five cultures were negative by the slide agglutination test when group A, B, C, and D antisera were used. Twenty-two of these cultures reacted with group B or C conjugates in the fluorescent-antibody (FA) procedure (performed by the Special Projects Unit, Bacteriology Section, NCDC), and, when these 25 cultures were later tested by the slide agglutination test with X, Y, and Z antisera, they were grouped as Y. When FA testing was repeated with a conjugate prepared from Y antiserum, the group Y cultures stained more deeply than previously seen with the group B and C conjugates.

Table 3 illustrates that, during a 2-year period, 9 strains were serogrouped as Slaterus X, 46 as Y, and 5 as Z. These strains were received from 17 different states, including Hawaii, and from one foreign country.

TABLE 3. *Number of strains serogrouped as Slaterus X, Y, and Z (June 1965–June 1967) by geographical origin and source*

Geographical origin	Clinical source										Total
	Spinal fluid or blood			Nasopharynx			Sputum		Other		
	X	Y	Z	X	Y	Z	X	Y	X	Y	
Areas of U.S.											
North East		1			1		3	1			6
Middle Atlantic		1	1			1		2			5
E. North Central		3		1		1				2	7
W. North Central									1		1
South Atlantic	1	2		1	5	1					10
E. South Central		2		1							3
W. South Central		1									1
Mountain					2						2
Pacific		10			2		1	1		4	18
Israel					5	1				1	7
Total	1	20	1	3	15	4	4	4	1	7	60

DISCUSSION

Tube agglutination tests on absorbed and unabsorbed antisera indicate that Slaterus' types X, Y, and Z are distinct from one another and from Branham's groups A, B, C, and D.

The majority of previously serologically unclassified strains can be grouped by use of antisera to the Slaterus strains. In agreement with Slaterus' findings on cultures isolated in the Netherlands, we found group X and Y meningococcal isolates from meningococcal infections and carriers in the United States. In 1963, Slaterus reported (7) that he had isolated group Z strains only from carriers, and recently he confirmed that this is still the case (*personal communication*). We have, however, received one group Z culture from spinal fluid in addition to cultures from nasopharyngeal sources.

We observed that false-grouping cross-reactions were greatly reduced by reconstituting the grouping antisera in 50% glycerol-water. It is therefore recommended that this procedure be used to reconstitute lyophilized meningococcal grouping sera.

In the cultures tested in this laboratory from June 1965 to June 1967, the strains found were, in descending order of frequency, groups B, C, Y, X, Z, A, and D. (Of approximately 1,000 cultures tested since 1950, only one group D strain has been received.)

As yet, no epidemic has been reported where the meningococcal strains were found to be Slaterus X, Y, or Z serogroups. The cultures received in this laboratory have been from sporadic cases or carriers. Even though the epidemiological significance of the Slaterus serogroups is not fully understood at this time, attempts to categorize strains of *N. meningitidis* cultures should include antisera to X, Y, and Z.

Slaterus labeled the X, Y, and Z strains as "types"; however, our results suggest that the term "group" should be used. We recommend that Slaterus' types X, Y, and Z should be considered as standard groups and follow the present groups A, B, C, and D in alphabetical order; i.e., X would be designated as group E, Y as group F, and Z as group G.

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