Rapid Serogroup Identification of *Neisseria meningitidis* by Using Antiserum Agar: Prevalence of Serotypes in a Disease-Free Military Population

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Nasopharyngeal cultures from 414 Marines were plated directly onto antiserum agar containing the antibiotics vancomycin, colistin, and nystatin for meningococcal isolation and serogroup identification. Meningococci were isolated from 267 Marines, giving a carrier prevalence of 64.5%. A total of 58% of the isolates could be placed into serogroups; of these 22.3% were group B, 4.7% were group C, 25.7% were group Y, 24.3% were group W135, and 23.0% were group 29E. No serogroup A organisms were recovered. Serotyping by agar gel double diffusion was performed on 148 strains. More than 70% of these strains were nontypable, and the disease-associated serotype 2 was present only in two group Y isolates. The same 148 isolates were also classified by major outer membrane protein patterns after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis pattern IV was most common among all serogroups. The data demonstrate the effective use of antiserum agar for meningococcal surveillance and document the frequency of specific serotypes and polyacrylamide gel electrophoresis types among carrier isolates obtained from a nonrecruit military population.

In the United States the attack rate of meningococcal disease has been about 1 case per 100,000 (5), whereas asymptomatic carriage ranges from 5 to 10% in a normal civilian population (20) to more than 20% in military personnel (2, 29). Many carrier isolates and nearly all case isolates of meningococci have chemically and immunologically distinct capsules composed of linear polymers of mono- and disaccharides (18). These capsular polysaccharides provide a scheme for classifying strains into serogroups denoted A, B, C, D, X, Y, Z, W135, and 29E (Z'). Of these, serogroups B, C, and Y account for most meningococcal disease in the United States (27).

Meningococci may also be characterized by serotypes based on immunologically specific outer membrane proteins and lipopolysaccharides (11, 21, 25, 38). In the Frasch-Chapman system there are 15 recognized protein serotypes represented by Arabic numbers (11). Although this system has been used primarily to classify group B and C isolates, these serotypes are also present in other serogroups (14). Certain serotypes are associated with asymptomatic carriage, whereas others, such as serotype 2, are isolated primarily from patients (10, 13, 21, 22-24).

There have been numerous studies of meningococcal carriage in military personnel (2, 3, 7, 8, 19, 21, 26, 29, 34). However, in the past 5 years, there have been few studies concerning the incidence of group-specific carriage or prevalence of specific serotypes within military personnel (21, 34). The use of agar plates containing group-specific antisera (antiserum agar [ASA]) and the antibiotics vancomycin, colistin, and nystatin (VCN) to inhibit competitive throat flora (35) provides a rapid and accurate method for the isolation and serogrouping of pharyngeal meningococci (6, 32).

This communication describes (i) the use of ASA to isolate and determine serogroups of meningococci from carriers in a single operation, (ii) the distribution of serotypes in a disease-free nonrecruit military population, and (iii) the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) patterns for strain classification.

MATERIALS AND METHODS

Study population. In 1977, 414 Marines, living in barracks at Camp Lejeune, N.C., were surveyed for meningococcal carriage. Their time in service ranged from 4 months to 11 years, with an average of 2.7 years and a median of 2.0 years. Approximately 67% of the

study participants were white, 28% were black, and 5% were other races.

ASA plates. ASA plates, using antisera produced in horses and burros against whole meningococci (6), were modified by the addition of VCN inhibitor (BBL Microbiology Systems, Cockeysville, Md.). A 6-ml amount of ASA was dispensed with a Manostat variable pump (Manostat Corp., New York, N.Y.) onto each third of tri-partitioned petri dishes (Y-plate; Fisher Scientific, Silver Spring, Md.) to provide uniform levels of ASA. Two triplates containing ASA for groups A, B, C, Y, W135, and 29E were streaked for each volunteer.

Specimens. Specimens were taken orally from the nasopharynx behind the uvula by one of the authors using flexible cotton-tipped applicators, swabbed directly onto ASA plates, and incubated for 18 h at 37°C in the presence of 5% CO₂. Plates were examined for precipitin halos by three observers. All meningococcal strains (groupable and nongroupable) were checked with 15-cm diameter ASA plates which accommodated 32 strains per plate (6). ASA nongroupable strains were examined for serogroups D, X, and Z by slide agglutination at the Bureau of Biologics and by Harry Feldman, Upstate Medical Center, Syracuse, N.Y. (9).

Identification of meningococci. Meningococcal strains were identified by colonial morphology, positive oxidase reaction, Gram stain, and sugar fermentation. Sugar fermentations were performed by a modification of the method of Sanders et al. (31), using petri dishes containing, in grams per liter: tryptone (Difco Laboratories, Detroit, Mich.), 17; NaCl, 5; yeast extract (Difco), 10; phenol red indicator, 0.025; and Noble agar (Difco), 14. The pH was 7.3. A lawn of organisms from the original culture plate was spread liberally over the agar, and disks containing glucose, maltose, sucrose, and lactose (Difco) were placed on the agar surface. A positive fermentation was identified by a red to yellow color change at 6 to 8 h and by enhanced growth around the disks at 24 h. Cystine tryptophan agar (36) with the same four sugars was used to check the results of the agar-disk technique.

Serotyping. Strains were serotyped as previously described (12). Briefly, 148 randomly selected strains were grown overnight in tryptic soy broth (Difco) and centrifuged; the cells were extracted with 0.2 M lithium chloride in 0.1 M sodium acetate, and cell-free extracts were ultracentrifuged to obtain serotype antigens. The serotype antigen from each strain was tested by agar gel double diffusion with specific antisera produced in rabbits.

Sodium dodecyl sulfate-PAGE types. Serotype antigen preparations used for serotyping were examined by sodium dodecyl sulfate-PAGE on slab gels containing 10% acrylamide and 0.3% bisacrylamide in neutral phosphate buffer; they were electrophoresed as previously described (15).

RESULTS

Two meningococcal carrier surveys were performed at Camp Lejeune in April and December of 1977 to detect the prevalence of meningococcal serogroups and serotypes in a nonrecruit military population. Results of the two surveys

of over 200 volunteers each were similar despite differences in season and section of the base sampled. The prevalence of meningococcal carriage was 64.5% (267 of the 414 Marines cultured). A total of 275 meningococcal strains were isolated from these Marines; 259 (94.2%) were single isolates, and 16 (5.8%) were multiple isolates (including two volunteers with mixed serogroups [B and 29E; Y and 29E] and six groupable and nongroupable pairs of strains). In addition to the 275 meningococci isolated, three strains of *N. lactamica* and three strains of *N. gonorrhoeae* were identified. *N. lactamica* in areas of heavy growth produced a halo on the group 29E ASA, and one isolate produced a light halo on group B ASA but could be easily differentiated from meningococci by colonial morphology and sugar fermentations. In our experience, the sugar agar-disk method was reproducible and more rapid than cystine-tryptophan agar slants. Increased growth around the disk provided a means of checking the indicator results and was helpful for confirming 10 strains that were slow maltose fermenters, a phenomenon also observed by others (P. Olcen, D. Danielsson, and J. Kellander, Acta Pathol. Microbiol. Scand. Sect. B., in press).

Groupable strains accounted for 57.8% of the meningococci isolated (159 of 275 strains); 116 of the 275 isolates (42.2%) were nongroupable. Of the 267 culture-positive Marines, 8 carried both groupable and nongroupable strains or strains from multiple serogroups. Nearly confluent growth on ASA plates was present in over 90% of the cultures, which may have prevented identification of other multiple isolates. Group-specific precipitin halos were often present at 24 h but were more easily visualized at 48 h after inoculation (Fig. 1). A correlation of 96.4% was found between serogroups identified by the direct ASA method and the final ASA serogroup results. Of the groupable strains isolated, 22.3% were group B, 4.7% were group C, 25.7% were group Y, 24.3% were group W135, and 23.0% were group 29E (Table 1). No group A strains were isolated. Eleven group X strains were identified by bacterial slide agglutination screening of the ASA nongroupable strains. All but four of the nongroupable isolates examined by bacterial slide agglutination were either saline agglutinable or autoagglutinable in normal rabbit serum.

Since certain serotypes are associated with carriage and others are associated with disease, we screened 148 Camp Lejeune isolates to determine the prevalence of different serotypes in a nonrecruit population free of meningococcal disease (Table 2). We were specifically interested in the prevalence of disease-associated serotype 2 in this population. Having previously

FIG. 1. Throat culture from a Marine volunteer, swabbed on ASA triplate containing meningococcal antisera to groups A, B, and C in the sections labeled A, B, and C, respectively. Halo formation at 48 h (section C) identifies the strain as a serogroup C organism.

TABLE 1. Results of direct serogroup identification of nasopharyngeal meningococci by ASA

Group	No. isolated	% carriage	% of total meningococcal strains	% of groupable meningococci
A	0	0	0	0
B	33	8.0	12.0	22.3
C	7	1.7	2.5	4.7
Y	38	9.2	13.8	25.7
W135	36	8.7	13.1	24.3
29E	34	8.2	12.4	23.0
X ^a	11	4.1	4.0	7.4
NG ^b	116	28.0	42.2	0

^a Identified by bacterial slide agglutination among strains found nongroupable by ASA.

^b NG, Nongroupable by ASA and bacterial slide agglutination.

serotyped over 2,000 meningococcal strains, we have found serotype 2 only in serogroups B, C, Y, and W135 (14). Of the 90 Camp Lejeune strains examined from these serogroups, only 2 were identified as serotype 2, and both of these were from serogroup Y. In contrast to our experience with case strains, nearly 90% of group B and group C carrier isolates and 82% of all strains tested were non-serotypable.

In addition to serotyping, PAGE typing has been used in our laboratory for several years. Recently, a PAGE typing scheme for classifying

strains based on major outer membrane protein patterns was developed by Frasch and Mocca (C. E. Frasch and L. F. Mocca, manuscript in preparation). Ten different PAGE patterns based upon the two or three major outer membrane proteins and designated by Roman numerals I through X have been described. Five of these patterns (I, II, IV, V, VII) were prevalent among Camp Lejeune isolates (Fig. 2). PAGE pattern IV was most common among all serogroups (Table 3) except group Y, which contained a larger number of PAGE type V strains. The PAGE type I pattern is typical of nearly 85% of the serotype 2 strains, but can also be found in other serotypes. Both group Y serotype 2 strains were PAGE type I. Two strains with PAGE type I patterns were present in groups B and C, compared with four each in groups Y and W135.

DISCUSSION

This study was designed to evaluate meningococcal carriage in a relatively stable military base population free of disease. Most of the volunteers studied lived in barracks on the base and had been in military service for at least 6 months. Such a population would be expected to have the high numbers of carriers necessary to demonstrate the efficacy of ASA for direct serogroup identification. In addition, the incidence of different serotypes and PAGE types in this population could be used for comparison with high-risk recruit populations.

Meningococcal disease has been a serious medical problem for military recruits. Since the introduction of meningococcal group C polysaccharide vaccine in 1970, there has been a significant drop in meningococcal disease in the Armed Forces (1). This vaccine was replaced by a bivalent group A and group C vaccine in 1978. With the exception of a minor outbreak of group Y disease at Fort Dix (33), there has been no

TABLE 2. Prevalence of serotypes among different meningococcal serogroups

Serotype(s)	No. in the following serogroups:					
	B	C	Y	W135	29E	NG ^a
1, 8	5 ^b	0	0	0	1	2
2	0	0	2	0	0	0
15	1	0	1	0	5	14
Other ^c	1	0	1	0	2	4
NT ^d	23	5	27	24	18	12

^a NG, Nongroupable by ASA.

^b Contained three strains with multiple serotypes (1, 8, and 15).

^c Other serotypes.

^d NT, Nontypable.

FIG. 2. Five most common PAGE patterns found at Camp Lejeune (I, II, IV, V, and VII). Molecular weight standards were: a, bovine serum albumin (68,000); b, ovalbumin (45,000); c, chymotrypsinogen A (25,000); and d, ribonuclease A (13,700).

TABLE 3. PAGE type results of meningococcal isolates according to serogroup

PAGE type	No. in the following serogroups:					
	B	C	Y	W135	29E	NG ^a
I	1	1	4	4	1	2
II	1	0	3	2	0	1
IV	28	3	11	11	16	26
V	0	0	12	4	1	0
VII	0	1	1	0	6	1
Other ^b	0	0	0	3	2	2

^a NG, Nongroupable by ASA.

^b Other PAGE types.

increase in disease caused by other serogroups. Protection against meningococcal disease is correlated with serum bactericidal antibody directed against meningococcal group- and type-specific antigens (16, 39). This antibody may be induced by carriage of encapsulated or non-encapsulated meningococci or by other bacteria containing cross-reactive antigens (30). Many

questions related to disease pathogenesis and the epidemiology of carriage must still be answered. ASA with VCN is a useful epidemiological tool to study meningococcal carriage and to maintain periodic surveillance of susceptible populations. During the recent meningococcal epidemic in Finland, ASA was invaluable for serogrouping A, B, and C strains (32). Our study demonstrates that ASA with VCN is a convenient method for directly serogrouping meningococcal isolates of the major (A, B, and C) and minor (Y, W135, and 29E) serogroups. Final results were available within 48 h of nasopharyngeal culture. One of the limitations of ASA is the large amount of antiserum required. The use of tripartitioned plates reduces the amount of antiserum and incubator space needed for large epidemiological studies.

The cross-reaction between serogroups Y and W135 (6) is easy to distinguish and posed no difficulty in this study. Possible cross-reactions of *Escherichia coli* K1 with group B meningococcal antiserum and of *E. coli* K92 with group C antiserum were eliminated by VCN. However, in four cases severe contamination with *Proteus* (resistant to colistin) prevented direct serogrouping.

There were no group A carriers and a low prevalence of group C carriage. Group A carriage and disease in the United States is presently confined to the Pacific Northwest (4), and group C carriage may have been reduced by the routine administration of group C monovalent vaccine to military personnel (17). The incidences of group B and the minor serogroups Y, W135, and 29E were similar. There is little predictive value between group-specific carrier rates and the incidence of meningococcal disease (2). Some observers have suggested that changes in the rate of group-specific carriage or acquisition rates are most important for predicting outbreaks (37), but this hypothesis fails to consider the importance of individual strain virulence within serogroups. Serotyping has provided a means of characterizing strains within serogroups, and serotype 2 has been a marker for virulence (10, 24). This serotype is frequently associated with disease in groups B and C (10, 22, 24, 28). A recent study of a small meningococcal outbreak among Royal Air Force recruits (34) exemplifies the usefulness of both serogrouping and serotyping strains. Despite low carriage (2%) and acquisition rates of group B serotype 2, this serotype was still responsible for the four cases of meningitis. By comparison, other group B serotypes and high carrier and acquisition rates of group W135 serotype 2 strains during this period were not associated with disease, suggesting that both

capsular and noncapsular antigens are important in the pathogenesis of meningococcal disease.

Two serotype 2 isolates were identified among the Camp Lejeune population sampled, and both of these were serogroup Y strains. Low carriage of serotype 2, particularly among serogroups B and C, would be expected in a disease-free population. All isolates examined from the December Camp Lejeune survey were non-serotypable. The rate of nontypable group B and C strains (80%) and the overall nontypable rate (74%) are in sharp contrast to our previous nontypable rate of 30% (13). This difference can be accounted for in part by the fact that most of the strains routinely examined in our laboratory are case isolates. This high rate of nontypable strains among both case and carrier isolates suggests the need to expand our present serotyping scheme.

For several years, this laboratory has been examining PAGE patterns of strains for comparison with the serotype results. Most of the 15 serotypes have a distinct PAGE type, and nearly all PAGE types represent distinct serotypes. On the basis of this work, Frascch and Mocca (manuscript in preparation) have proposed a PAGE typing scheme based on major outer membrane proteins as an additional method of classifying strains. This method circumvents variations in antisera and was a particularly useful addition to serotyping the groupable and nongroupable carrier isolates from Camp Lejeune. There is good correlation between serotype and PAGE type results; therefore, PAGE typing provides an important procedure complementary to the agar gel double-diffusion method used for serotyping. For example, about 85% of serotype 2 strains have a PAGE I pattern, and the PAGE I pattern is found among many disease isolates of groups B and C. In the disease-free population sampled at Camp Lejeune, this pattern was found in 2 of 35 group B and group C isolates. Further examination of more carrier isolates from all serogroups will be necessary to further evaluate the efficacy of the PAGE typing method for other epidemiological studies.

Our results demonstrate that ASA with VCN is a rapid and accurate method for directly serogrouping large numbers of meningococcal isolates. This method would be useful for meningococcal surveillance of outbreaks or for determining carriage before and after meningococcal immunoprophylaxis. Serotyping and PAGE typing demand more materials and effort but provide important additional markers for characterizing disease isolates within specific populations. Further understanding of the dynamics of me-

ningococcal carriage and disease spread within populations will result from the use of such epidemiological tools.

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