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Prevalence of Maltose-Negative *Neisseria meningitidis* Variants During an Epidemic Period in Spain

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We studied the prevalence of maltose-negative variants of *Neisseria meningitidis* in Spain from 1978 to 1980. Sugar utilization studies were performed with both CTA medium and Mueller-Hinton medium; bromothymol blue was used as the indicator in Mueller-Hinton medium. Of 1,714 isolates of *N. meningitidis* recovered from the cerebrospinal fluid or blood of patients with meningococcal infections, 64 (3.7%) were maltose-negative variants; 13 (3.3%) of the 363 isolates found in carriers had the same characteristic. All maltose-negative cultures isolated from both patients and carriers belonged to serogroup B and were resistant to sulfadiazine at a minimal inhibitory concentration, 10 μg/ml or more. Serotype 2 isolates were the most prevalent isolates in patients (68.8%), followed by nontypable isolates (20.3%). Only serotype 2 isolates (66%) and nontypable isolates (33%) were found in carriers.

Most clinical microbiology laboratories use sugar utilization tests to differentiate among the species of the genus *Neisseria*, particularly those recognized as being pathogenic for humans (*Neisseria gonorrhoeae* and *Neisseria meningitidis*). The two pathogenic species differ in the production of acid from maltose. This reaction is usually positive in *N. meningitidis* and negative in *N. gonorrhoeae* (3, 15).

The existence of maltose-negative variants of *N. meningitidis* has been described by Kingsbury (13), who found that 93% of the strains that were resistant to more than 50 μg of sulfadiazine per ml were maltose negative because they lacked the enzymes necessary to ferment this sugar.

The isolation of a strain of *N. meningitidis* with this characteristic from a patient with meningitis has been described recently. Granato et al. (11) have suggested that studies were needed to determine the prevalence of these maltose-negative variants in order to know the epidemiological scope of these strains and to ascertain the extent of the problem of differentiating between *N. meningitidis* and *N. gonorrhoeae*, particularly in the many laboratories that base this differentiation on sugar utilization.

In this paper we describe the prevalence of maltose-negative variants during a 3-year period that coincided with the period of the greatest incidence of cases of meningococcal infection in Spain (J. A. Sáez-Nieto et al., submitted for publication).

MATERIALS AND METHODS

Isolates. We studied 1,714 *N. meningitidis* isolates recovered from the cerebrospinal fluid or blood of patients with meningococcal infections. In addition, we studied 201 isolates from carriers who were in contact with patients and 162 isolates from the general population.

The identities of the isolates were confirmed in our laboratory by Gram stains, oxidase tests, and sugar utilization tests. The serogroup and serotype of each isolate were also determinated.

Sugar utilization tests. Two media were used to study sugar utilization. The first was the medium normally used in our Meningococci Reference Laboratory and consisted of Mueller-Hinton medium (Difco Laboratories) containing bromothymol blue as the indicator (MHBT medium) (19). Lactose, glucose, maltose, and sucrose were added to the basal medium at 1% concentrations of 1%.

Dense suspensions in phosphate-buffered saline (pH 6.9; 66.6 ml of 0.15 M Na_2HPO_4 per liter, 33.3 ml of 0.5 M KH_2PO_4 per liter, 8.5 g of NaCl per liter) were made from 18-h cultures that were grown on blood agar plates and were incubated at 37°C in a 5% CO_2 atmosphere. Each suspension was inoculated onto plates containing MHBT medium over an area 0.5 cm in diameter. As many as 20 isolates could be placed on each petri dish. Then these plates were incubated for 72 h in a humid atmosphere, and they were read every 24 h.

The second medium used was CTA medium (Difco); the above-mentioned sugars were added to this medium at concentrations of 1 and 10%. CTA medium was the only medium used for those isolates that were maltose negative on MHBT medium. A sugar concentration of 10% was used for the isolates which gave

TABLE 1. Maltose-negative variants of *N. meningitidis* isolated from patients during 1978, 1979, and 1980[a]

Year	No. of isolates	No. of maltose-negative isolates
1978	369	12 (3.3)[b]
1979	861	38 (4.4)
1980	484	14 (2.9)

[a] January to September.
[b] The numbers in parentheses are percentages.

TABLE 2. Maltose-negative variants of *N. meningitidis* isolated from carriers during 1978, 1979, and 1980[a]

Year	No. of isolates		No. of maltose-negative isolates[b]
	Total	From contacts	
1978	121	71	6 (5.0)[c]
1979	198	102	6 (3.0)
1980	44	28	

[a] January to September.
[b] All of the maltose-negative isolates were from contacts.
[c] The numbers in parentheses are percentages.

negative reactions with a concentration of 1%. The media containing the sugars were placed into screw-capped tubes, and these tubes were not opened during incubation. Tubes were inoculated with dense suspensions and then incubated for 5 days at 37°C in a humid 5% CO_2 atmosphere. Readings were made every 24 h with this medium.

Serogrouping. Serogrouping was performed by slide agglutination. The sera used were produced in our laboratory in rabbits by using the inoculation protocol of Vedros (18). The strains used to produce antisera to serogroups A, B, C, X, Y, Z, and W135 were obtained from N. E. Vedros *Neisseria* Repository, Berkeley, Calif., and the strains used to produce antisera to serogroups D and 29E (Z') were obtained from the International Meningococci Reference Center, Marseille, France.

Serotyping. The specific antigens of the serotypes were extracted from cultures of meningococci in 250 ml of tryptic soy broth (Difco) which were incubated overnight with agitation at 37°C.

Cells were recovered by centrifugation at 10,000 × *g* for 20 min. The packed cells were suspended in 5 ml of 0.2 M LiCl–0.1 M sodium acetate (pH 5.8) and heated at 50°C for 2 h in a shaking water bath.

The specific antigens of the serotypes were obtained by ultracentrifugation at 100,000 × *g* for 2 h (10).

The serotyping method used was the Ouchterlony technique under the conditions described by Frasch and Chapman (9).

Type-specific antisera were produced in rabbits as previously described (8).

Sulfadiazine sensitivity test. Sulfadiazine sensitivity was tested on plates containing Mueller-Hinton agar (Difco) by adding 1, 5, 10, 25, 50, and 100 μg of sulfadiazine per ml to the medium. Meningococcal suspensions were prepared in phosphate-buffered saline (pH 6.9) from overnight cultures on blood agar. Each of these suspensions was adjusted to a concentration of 10^5 cells per ml.

Sulfonamide-containing agar plates were inoculated with this suspension by using a 2-mm loop to make a streak 2 cm long. After incubation for 18 h at 37°C in a humid atmosphere, the plates were read, and the lowest concentration that produced at least 80 to 90% inhibition of growth was considered the minimal inhibitory concentration (MIC). All tests included one plate of Mueller-Hinton agar without sulfadiazine as a control, in which confluent growth of the strain was required.

The isolates were placed into the following three groups according to their MICs: sensitive strains (MIC, ≤1 μg/ml), moderately resistant strains (MIC, 5 to 10 μg/ml), and resistant strains (MIC, 25 μg/ml or more).

RESULTS

Of 1,714 isolates recovered from patients, 64 (3.7%) were maltose negative. Of 363 isolates recovered from carriers, 12 (3.3%) were maltose negative (Tables 1 and 2).

The results obtained with the two culture media used (MHBT medium and CTA medium) were generally consistent; we found only six isolates that were maltose negative on MHBT medium but positive on CTA medium. The results with these isolates were not included in the tables. We found no significant differences in the proportion of maltose-negative isolates in these two media over the 3 years of the study.

Table 3 shows the sulfadiazine sensitivities of these isolates. No isolate from any patient or carrier had an MIC of less than 10 μg/ml. Table 3 also shows the distribution of sulfonamide MICs in the maltose-positive population. The proportion of strains with an MIC of 10 μg/ml or more was 95%.

The distribution of serotypes is summarized in Table 4. Maltose-negative strains were found only in group B; however, this group was the most prevalent group among isolates from patients and constituted 85% of all such isolates. Maltose-negative strains constituted 4.4% of all serogroup B isolates recovered from patients.

Type 2 isolates were the most prevalent isolates (69%), followed by nontypable isolates (20%). We also found two isolates of serotype 1, three of serotype 8, one of serotype 15, and one that reacted with types 1, 8, and 15. Only serotype 2 and nontypable isolates were found in carriers.

DISCUSSION

Normally, *N. meningitidis* and *N. gonorrhoeae* colonize different anatomical sites and cause clearly different infectious processes. However, isolations of both of these microorganisms from unusual sites are being described increasingly.

TABLE 3. Resistance to sulfadiazine in maltose-negative and maltose-positive isolates from patients and carriers

Source	Strains	Total no. tested	No. with the following MICs of sulfadiazine:					
			1 μg/ml	5 μg/ml	10 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml
Patients	Maltose negative	64			7 (10.9)[a]	34 (53.1)	9 (14.1)	14 (21.9)
	Maltose positive	1,650	28 (1.7)	55 (3.3)	172 (10.4)	743 (45.0)	476 (28.9)	176 (10.7)
Carriers	Maltose negative	12			2 (16.7)	5 (41.6)	2 (16.7)	3 (25.0)
	Maltose positive	351	73 (20.8)	66 (18.8)	40 (11.4)	86 (24.5)	53 (15.1)	33 (9.4)

[a] The numbers in parentheses are percentages.

Meningococci are found mainly in cerebrospinal fluid, blood, and the nasopharynx, although they are also occasionally found in the urogenital tract, either as a cause of infection (1, 5) or as a saprophyte (6, 14).

On the other hand, apart from their typical locations in association with gonorrhea and prostatitis (15), gonococci have been found in numerous other infectious processes, including pharyngitis, meningitis, arthritis, and others (4, 7, 12, 16), and in sites without any clear involvement in infection (2, 17).

It is clear that since meningococci and gonococci are being isolated from unusual sites, correct differentiations between these species must be made, particularly in those laboratories in which differentiation is based solely on sugar utilization.

The appearance of maltose-negative strains of *N. meningitidis* detected by Kingsbury (13) and recently by Granato et al. (11) increases the importance of a study of the occurrence of these strains in the natural environment. In this work we determined the proportion of maltose-negative variants during the period from 1978 to 1980 (January to September), which coincided with a period of peak incidence of meningococcal infections in Spain (6,618 cases in 1979, constituting a rate of 17.6 cases per 100,000 inhabitants) (Sáez-Nieto et al., submitted for publication).

The proportion of maltose-negative variants among the meningococci isolated from patients was 3.73% (Table 1). The isolates from the carriers studied revealed a frequency of 3.3% maltose-negative variants (Table 2). We confirmed the observation of Kingsbury (13) that all isolates from patients and carriers were resistant to sulfadiazine; 89% of the isolates from patients and more than 80% of the isolates from carriers had MICs of at least 25 μg/ml, and the rest of the isolates from both groups had MICs of at least 10 μg/ml (Table 3).

All maltose-negative isolates belonged to serogroup B, which may be explained by the fact that this serogroup is clearly predominant in both patients and carriers in Spain. On the other hand, as isolations of maltose-negative variants belonging to serogroups Y and C have been described (11), this characteristic is not unique to a single serogroup.

Serotype 2 was the most prevalent type among isolates from patients and carriers, followed by the group of nontypable isolates. This distribution may have been due to the fact that these are the two most prevalent serotypes in Spain (Sáez-Nieto et al., manuscript in preparation).

We think that the prevalence of maltose-negative variants that we found probably reflects the general situation since the isolates which we analyzed represented a large proportion of the isolates found in patients in Spain.

Maltose negativity could be useful as an epidemiological tool since it could serve as one more strain marker in addition to the more important markers, such as serogroup and serotype, particularly in investigations of outbreaks, secondary cases, and family contacts.

It is necessary to have alternative means of differentiation between *N. gonorrhoeae* and *N. meningitidis* in addition to sugar utilization. Although the difference between these two species is clear from the growth and appearance of the colonies, the location of these species in unusual sites may lead to mistaken identifications arising from the possible appearance in clinical samples of maltose-negative variants of *N. meningitidis*.

TABLE 4. Serotypes of maltose-negative *N. meningitidis* group B isolates from patients and carriers

Serotype	No. of patients	No. of carriers
1	2 (3.1)[a]	
2	44 (68.8)	8 (66.6)
8	3 (4.7)	
15[b]	2 (3.1)	
Others[c]	0	0
Nontypable	13 (20.3)	4 (33.3)

[a] The numbers in parentheses are percentages.

[b] Including one type 15 strain and one type 1, 8, and 15 strain.

[c] Including types 4 through 6, 9, and 11 through 14.

In conclusion, we believe that other studies should be performed in different countries to determine the real prevalence of these maltose-negative strains.

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