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Variability in Growth of *Neisseria polysaccharea* on Colistin-Containing Selective Media for *Neisseria* spp.

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In a prospective survey of 773 healthy schoolchildren in southern Alberta, Canada, *Neisseria polysaccharea* was isolated from the pharynxes of only 4 (0.5%) subjects, whereas *Neisseria lactamica* and *Neisseria meningitidis* were isolated from 110 (14%) and 15 (2%) children, respectively. These strains of *N. polysaccharea*, together with three other sporadic isolates from Alberta, Canada, were compared with the type strain from France and strains from Spain and Germany. All strains were phenotypically identical, except that the Canadian and German strains, for which the colistin MICs were 1 mg/liter, failed to grow on Thayer-Martin medium (TMM), whereas the type strain and the Spanish strains, for which the colistin MICs were >7.5 mg/liter, were not inhibited. Multilocus enzyme electrophoresis indicated that six distinct electrophoretic types were present among the seven Canadian strains. Our results show that growth on gonococcal selective media which contain colistin is a variable feature of this taxon.

In 1983, Riou et al. (15) described a new species of *Neisseria*, which they provisionally named *Neisseria polysacchareae* and subsequently renamed *N. polysaccharea*, in accordance with the International Code of Nomenclature of Bacteria (14).

Strains belonging to the new taxon grow on selective media, such as gono-meningo agar (15) and Thayer-Martin medium (TMM) (21), which are used for the isolation of pathogenic *Neisseria* spp. Biochemically, *N. polysaccharea* produces acid from glucose and maltose but not from sucrose, fructose, lactose, or mannitol. Therefore, it could be confused with noncapsulated *N. meningitidis*. However, *N. polysaccharea* differs from *N. meningitidis* in its ability to synthesize a polysaccharide on 5% sucrose agar, lack of γ-glutamyltransferase, cysteine requirement for growth on Catlin's medium, and pigment production (1–3, 15).

The strains of *N. polysaccharea* on which Riou et al. (15) based their description were isolated from throats of healthy children during a study of case contacts of meningococcal disease in which gono-menigo agar alone was used as the primary culture medium. Similarly, Boquete et al. (2) conducted a retrospective examination of strains of putative *N. meningitidis* isolated from previous surveys of *N. meningitidis* carriage in schoolchildren for which only TMM was used as the primary culture medium (2, 17). Of the 216 strains which they examined, 50 conformed to the description of *N. polysaccharea* by Riou and Guilbourdench (14), including growth on selective media. Cann and Rogers (3) reexamined 22 isolates thought to be nongroupable *N. meningitidis* from a previous survey (4) and identified 8 as *N. polysaccharea*. All of these grew on TMM. Berger (1), however, isolated two strains which failed to grow on TMM, in contrast to the findings of earlier studies.

Previous reports of the prevalence *N. polysaccharea* have been based upon retrospective analysis of preserved strains of *N. meningitidis* that were isolated in surveys which used selective media. A systematic study to establish the prevalence of *N. polysaccharea* by using a nonselective medium has not been hitherto undertaken. We undertook a survey of schoolchildren to determine the prevalence of *N. polysaccharea* in relation to the prevalence of *N. lactamica* and *N. meningitidis*.

We then examined the characteristics of the strains of *N. polysaccharea* isolated in this survey and other strains isolated sporadically in Alberta and compared them with the type strain first described in France and strains later isolated in Germany and Spain.

MATERIALS AND METHODS

Isolates. Sixteen strains of *Neisseria* spp. were examined. Three strains (86376, 87042, and 87043), which were isolated from children aged 2 to 6 years, were referred to this laboratory for confirmation as *N. meningitidis*. Four strains (89353, 89354, 89356, and 89357) were isolated in the survey referred to below. Two strains of *N. polysaccharea* (85321 and 85322), which were isolated in Germany and described previously (1), were kindly provided by U. Berger, Department of Bacteriology, University of Heidelberg, Heidelberg, Germany, who also provided strain CIP 100113 (our strain 85323), the type strain described by Riou et al. (14). Three strains (87188, 87189, and 87190), which were isolated in Spain as described previously (2), were kindly provided by J. A. Saez-Nieto, Laboratorio de Referencia de Meningococos, Centro Nacional de Microbiologia, Virologia e Immunologia Sanitarias, Madrid, Spain. One strain each of *N. meningitidis* (ATCC 13077), *N. lactamica* (ATCC 22970), and *N. subflava* (ATCC 14221) were also included.

Survey. A survey to determine the prevalence of *N. polysaccharea* was performed in conjunction with another survey to determine the carriage rate of an outbreak strain of *N. meningitidis* group C in rural and urban communities of southern Alberta, Canada. These communities were selected to serve as a control population for a population in an area of Ontario, Canada, that was affected by an outbreak of *N. meningitidis* group C. Seven hundred seventy-three randomly selected schoolchildren between the ages of 6 months

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and 18 years were included in the survey, after the appropriate consent was obtained from their parents. Details of this survey are published elsewhere (11). The posterior pharynxes of the subjects were swabbed by using cotton-tipped swabs. These were plated directly onto chocolate agar (Difco Laboratories, Detroit, Mich.) and TMM (Difco) containing vancomycin (3 mg/liter), colistin (7.5 mg/liter), trimethoprim (5 mg/liter), and amphotericin B (7.5 mg/liter). Each plate was placed individually in a separate Bio-Bag (Marion Scientific, Kansas City, Mo.) with a CO_2-generating capsule and incubated at 35°C within 2 h of swabbing. After 48 h of incubation, the plates were transferred to the Provincial Laboratory of Public Health, Calgary, where the isolation and identification of *Neisseria* spp. were performed.

Determination of phenotypic characteristics. Production of acid from glucose, maltose, sucrose, fructose, lactose, and mannitol was examined by using cysteine tryptic agar (20); modified oxidation-fermentation medium (10); the medium of Flynn and Waitkins (6), which is routinely used at the Public Health Laboratory; QuadFERM (API Laboratory Products Ltd., St. Laurent, Quebec, Canada); Starch Gel Sugars (Institute Armand-Frapier, Montreal, Quebec, Canada); and RIM (PML Microbiological, Missisauga, Ontario). Nitrate reductase, nitrite reductase, hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside, gelatinase, and production of polysaccharide from 5% sucrose were tested by previously described techniques (12). Detection of prolylaminopeptidase and γ-glutamyltransferase was carried out by the use of Gonocheck II (EY Laboratories, San Mateo, Calif.). A reaction in Gonocheck II that was similar to that for *N. gonorrhoeae* was considered positive for prolylaminopeptidase, whereas the reaction for *N. meningitidis* was considered positive for γ-glutamylaminopeptidase. DNase activity was determined by the plate method (12) and by the use of QuadFERM. Other characteristics examined were hemolysis on Columbia blood agar by using 5% sheep blood agar and growth on chocolate agar at 22°C in CO_2. Growth in the presence or absence of cysteine-cystine in defined medium was determined by the technique of Catlin (5).

Electrophoretic analysis of enzymes. Strains of *N. polysaccharea* were grown on G.C. medium (9) for 24 h at 37°C in the presence of 5% CO_2. Organisms were scraped from the surface of agar plates (150 by 15 mm, two per strain) in 3.0 ml of cold 10 mm Tris–1 mM EDTA–0.5 mM NADP. To each suspension was added 1 ml of glass beads (diameter, 75 to 150 μm; G-3753; Sigma Chemical Co., St. Louis, Mo.), and the suspensions were vortexed at high speed for 3 min. Cell debris and glass beads were removed by centrifugation at 25,000 × *g* for 20 min. Supernatants were sterilized by passage through 0.22-μm-pore-size Millex-GV filters (Millipore Corp., Bedford, Mass.) and stored in aliquots at −70°C until use. Electrophoretic analysis of enzymes was carried out as described by Selander et al. (19).

MICs. The MIC of colistin was determined according to the standard of the National Committee for Clinical Laboratory Standards (13) for the determination of *N. gonorrhoeae* susceptibility by the plate dilution method. Antibiotic concentrations ranging from 0 to 128 mg/liter were incorporated in GC base (Difco) with defined supplement (Dalynn Laboratory Products, Calgary, Alberta, Canada), and the plates were examined after 24 h of incubation at 35°C in CO_2 for growth inhibition according to the standard. Strains in *N. gonorrhoeae* (WHO V) and *Pseudomonas aeruginosa* (ATCC 27853) were included as controls.

RESULTS

Survey. The culture examination of 773 throat specimens yielded a total of 132 strains of *Neisseria* spp. These included 15 *N. meningitidis*, 110 *N. lactamica*, and 4 strains identified as *N. polysaccharea*, according to the reactions listed in Table 1. These four strains, hereafter designated by the numbers 89353, 89354, 89356, and 89357, were isolated on chocolate agar but not TMM. The *N. polysaccharea* strains were isolated only from children under 6 years of age. In no instance was *N. meningitidis* isolated concurrently with *N. lactamica* or *N. polysaccharea*. Both *N. lactamica* and *N. polysaccharea* were simultaneously isolated from one individual.

Phenotypic characteristics. All of the phenotypic characteristics of the seven Canadian isolates of *N. polysaccharea* were identical to those of the type strain CIP 100113 and are listed in Table 1. They were similar to each other and to the Spanish strains (87188, 87189, and 87190) and the German strains (85321 and 85322), except for growth on TMM. None of the seven Canadian strains or the two German strains grew on TMM at 35°C in 5% CO_2, whereas all of the Spanish strains and the French type strain did.

All strains of *N. polysaccharea* required cysteine-cystine for growth and failed to grow on chocolate agar at 22°C in 48 h in 5% CO_2.

The reactions for glucose, maltose, fructose, lactose, and mannitol utilization were appropriate for *N. polysaccharea* by all six methods used. The reaction that was indicative of sucrose utilization varied with different strains in different media. None of the strains demonstrated acid production from sucrose in the QuadFERM test, and one strain (87190) gave a change in pH indicator in modified oxidation-fermentation medium. Various combinations of reactions, ranging from a slight change in the indicator in one reaction to a strong change in up to three of the remaining sucrose reactions, were observed. Precipitation of polysaccharide around colonial growth on sucrose-containing media was detected. With strain 85322, acid production from fructose was noticed only in the medium of Flynn and Waitkins (6) after 48 h of incubation but not in any of the other fructose-containing media. Reactions for *N. meningitidis*, *N. lactamica*, and *N. subflava* were appropriate, as described previously (12).

We could not detect DNase activity by either of the two methods used in this study in any strain of *N. polysaccharea* examined, including the two Germans strains (85321 and 85322), which previously have been shown to possess DNase activity (1).

Susceptibility to colistin. The MIC of colistin for the French isolate of *N. polysaccharea*, one of the Spanish isolates (87188) of *N. polysaccharea*, *N. meningitidis*, *N. gonorrhoeae*, and *N. lactamica* was >128 mg/liter; for two Spanish strains (87189 and 87190) it was 64 mg/liter; and for the seven Canadian and the two German strains of *N. polysaccharea* it was 1 mg/liter, as it was for *N. subflava*. The MIC of colistin for *P. aeruginosa* was 4 mg/liter. The expected colistin MIC range for *P. aeruginosa* ATCC 27853 was 2 to 4 mg/liter (7). The MIC of trimethoprim for all these strains was greater than 5 mg/liter, and the MIC of vancomycin for all strains was greater than 3 mg/liter.

Electrophoretic analysis of enzymes. Of the 12 enzymes examined, phosphoglucomutase produced two bands in the gels with all strains. Each band was considered to be a separate enzyme locus, for a total of 13 enzyme loci. All of the enzymes were polymorphic for from two to seven alleles,

TABLE 1. Characteristics of type strain and Alberta isolates of *N. polysaccharea* and their comparison with *N. meningitidis*

	N. polysaccharea		*N. meningitidis*[c]
	CIP 100113[a]	Alberta isolates[b]	
Acid produced from:			
Glucose	+	+	+
Maltose	+	+	+
Sucrose	−	−	−
Fructose	−	−	−
Lactose	−	−	−
Mannitol	−	−	−
Enzyme activity			
Oxidase	+	+	+
Catalase	+	+	+
β-Galactosidase	−	−	−
Prolyliminopeptidase	+	+	Variable
γ-Glutamyltransferase	−	−	+
DNase	−	−	−
Tributyrinase	−	−	−
Reduction of:			
Nitrate	−	−	−
Nitrite	+	+	Variable
Polysaccharide production in the presence of 5% sucrose	+	+	−
Growth in Catlin's medium without cysteine-cystine	−	−	+
Growth on chocolate agar at 22°C	−	−	−
Growth on TMM	+	−	+

[a] Reference strain described by Riou et al. (14).
[b] Strains 86376, 87042, 87043, 89353, 89354, 89356, and 89357.
[c] From references 3 and 12.

with a mean number of 4.0 alleles per locus. Genetic diversity among the enzyme loci varied from 0.073 to 0.788 (Table 2), with a mean of 0.516 per locus. Eleven electrophoretic types were found among the 13 strains. Two of the Canadian strains, 86376 and 87042, had identical electrophoretic patterns and two of the Spanish strains, 87189 and 87190, had identical electrophoretic patterns which were distinct from those of all other strains. The seven Canadian strains exhibited a common allele for the enzyme fumurase. Strains 87043 and 89353 differed at one enzyme locus (glutamic-oxaloacetic transaminase) only. Otherwise, all strains differed by at least two enzyme loci.

DISCUSSION

A key feature of *N. polysaccharea*, as described by Riou et al. (14), is its ability to grow on media selective for pathogenic *Neisseria* spp. Results of our studies indicate that there are two subsets of *N. polysaccharea* which are characterized by their ability or failure to grow on TMM.

The reason for the failure of Canadian strains to grow on TMM was determined to be their susceptibility to the concentration of colistin used in both gono-meningo agar and TMM, i.e., 7.5 mg/liter. Our strains and the German strains for which the MICs were 1 mg/liter, failed to grow on TMM, in contrast to the French and German strains, for which the MICs were ≥7.5 mg/liter, which did grow. It is noteworthy that for these strains the MIC was either low (1 mg/liter) or high (64 or ≥128 mg/liter), with no tailing in between. All strains of *N. polysaccharea* were resistant to the concentrations of vancomycin and trimethoprim that are used in TMM. Hence, deletion of colistin from TMM offers a selective medium that is suitable for the isolation of both colistin-susceptible and -resistant strains of *N. polysaccharea*.

To eliminate the possibility that the seven strains isolated in Alberta may have been one clone, 12 enzyme loci were analyzed by multilocus enzyme electrophoresis. By this procedure we could demonstrate that only two strains had identical electrophoretic type patterns. Both of these strains were isolated in Calgary during the same month, although a definite link between the two carriers could not be established. Two Spanish strains (87189 and 87190) had identical electrophoretic types but were different from the third Spanish strain (87188) and the type strain. All other strains examined had different patterns. It is noteworthy that for strains 87189 and 87190 the colistin MICs were identical (64 mg/liter), in contrast to the third Spanish strain (87188), for which the colistin MIC was >128 mg/liter.

Antibodies to *N. polysaccharea* and *N. lactamica* cross-react with various serogroups of *N. meningitidis*, and their

TABLE 2. Genetic diversity of 13 enzyme loci of *N. polysaccharea*

EC no.	Symbol	Enzyme	No. of alleles	Genetic diversity
1.1.1.40	ME	"Malic" enzyme	4	0.675
1.1.1.42	IDH	Isocitrate dehydrogenase	7	0.788
1.1.1.49	G6P	Glucose-6-phosphate dehydrogenase	5	0.073
1.4.1.2	GD1	Glutamate dehydrogenase (NAD)	3	0.584
1.4.1.4	GD2	Glutamate dehydrogenase (NADP)	5	0.674
2.6.1.1	GOT	Glutamic-oxaloacetic transaminase	5	0.750
2.7.4.3	ADK	Adenylate kinase	2	0.073
2.7.5.1	PGM1	Phosphoglucomutase 1	2	0.073
2.7.5.1	PGM2	Phosphoglucomutase 2	3	0.584
3.1.3.1	ALP	Alkaline phosphatase	4	0.712
3.4.X.X	PEP	Peptidases	6	0.687
4.2.1.2	FUM	Fumarase	3	0.520
4.2.1.3	ACO	Aconitase	3	0.520

natural colonization of the pharynx has been postulated as a mechanism for natural immunity against *N. meningitidis* (2, 3, 8). In our survey we found that only 0.5% of the subjects carried *N. polysaccharea*, whereas 14% carried *N. lactamica*. We calculate from the results of the two studies of Cann and associates (3, 4) that the carrier rates of *N. polysaccharea* and *N. lactamica* in England are 0.5 and 9%, respectively. The role of cross-immunization against *N. meningitidis* by natural colonization by *N. polysaccharea* therefore is unlikely to be significant when compared with that by *N. lactamica*, in view of their relative prevalences.

The biochemical and enzymatic reactions for all strains examined, with the exception of sucrose utilization, were similar to those described for *N. polysaccharea*. We used six different types of media or kits to test for biochemical reactions. Reactions to acid production from sucrose were variable in different media and with different strains. Riou et al. (14) have also described rare acid production from sucrose. We believe that these inconsistencies are due to a change in the indicator by the various amounts of polysaccharide, which is acidic in nature (16), produced from sucrose by different strains, rather than acid formation resulting from the oxidation of sucrose. Therefore, caution is recommended in the interpretation of the reactions of *N. polysaccharea* for acid production in sucrose-containing media.

N. polysaccharea strains have been isolated only from healthy children and are not known to be implicated in any disease process. However, their phenotypic similarity to *N. meningitidis* strains can interfere with the accuracy of meningococcal prevalence studies in areas where strains of *N. polysaccharea* resistant to colistin are present. Furthermore, genetic transformation studies have shown the possible potential of this microorganism to be a source of the meningococcal resistance gene (18).

Our finding of two distinct subsets of strains suggests that the documentation of this species is incomplete.

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