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GENETIC STRUCTURE OF *NEISSERIA MENINGITIDIS* SEROGROUP C EPIDEMIC STRAINS IN SOUTH BRAZIL

Claudio Tavares SACCHI (1), Maria Lúcia Cecconi TONDELLA (1), Maria Cecília Outeiro GORLA (1), Ana Paula Silva de LEMOS (1), Carmo Elias A. MELLES (1), Maria Vaneide de PAIVA (1), Dauri Santos RODRIGUES (2), Antonio Joaquim F. ANDRADE (3), Marta Osório RIBEIRO (4) & Alethea SPERB (5).

SUMMARY

In the present study we report the results of an analysis, based on serotyping, multilocus enzyme electrophoresis (MEE), and ribotyping of *N. meningitidis* serogroup C strains isolated from patients with meningococcal disease (MD) in Rio Grande do Sul (RS) and Santa Catarina (SC) States, Brazil, as the Center of Epidemiology Control of Ministry of Health detected an increasing of MD cases due to this serogroup in the last two years (1992-1993). We have demonstrated that the MD due to *N.meningitidis* serogroup C strains in RS and SC States occurring in the last 4 years were caused mainly by one clone of strains (ET 40), with isolates indistinguishable by serogroup, serotype, subtype and even by ribotyping. One small number of cases that were not due to an ET 40 strains, represent closely related clones that probably are new lineages generated from the ET 40 clone referred as ET 11A complex. We have also analyzed *N.meningitidis* serogroup C strains isolated in the greater São Paulo in 1976 as representative of the first post epidemic year in that region. The ribotyping method, as well as MEE, could provide useful information about the clonal characteristics of those isolates and also of strains isolated in south Brazil. The strains from 1976 have more similarity with the actual endemic than epidemic strains, by the ribotyping, sulfonamide sensitivity, and MEE results. In conclusion, serotyping with monoclonal antibodies (C:2b:P1.3), MEE (ET 11 and ET 11A complex), and ribotyping by using *Cla*I restriction enzyme (Rb2), were useful to characterize these epidemic strains of *N.meningitidis* related to the increased incidence of MD in different States of south Brazil. It is mostly probable that these *N.meningitidis* serogroup C strains have poor or no genetic corelation with 1971-1975 epidemic serogroup C strains. The genetic similarity of members of the ET 11 and ET 11A complex were confirmed by the ribotyping method by using three restriction endonucleases.

KEYWORDS: *N. meningitidis* C; Ribotyping; Multilocus Enzyme Electrophoresis.

INTRODUCTION

Meningococcal disease (MD) is a significant cause of mortality and morbidity throughout the world[24,28]. Epidemiological data on MD have primarily been based on the classification of *N.meningitidis* into serogroups that

(1) Bacteriology Division, Adolfo Lutz Institute, São Paulo, Brazil.
(2) Bacteriology Division, Central Laboratory of Santa Catarina State, Brazil.
(3) Center of Epidemiological Surveillance of Parana State, Brazil,
(4) Bacteriology Division, Central Laboratory of Rio Grande do Sul State, Brazil.
(5) Center for Epidemiological Surveillance of Rio Grande do Sul State, Brazil.
Correspondence to: Claudio T. Sacchi: Bacteriology Division, Adolfo Lutz Institute, Av. Dr. Arnaldo 351, 01246-902 São Paulo, Brazil. Phone: 55.11.8510111. Fax:55.11.8513505. Electronic mail address: sacchi@usp.br

depend on the difference in the capsular polysaccharide antigens, with serogroups A, B, and C accounting for about 90% of all cases[24,28]. The serogroups are further assigned to serotypes and subtypes on the basis of antigenic characteristics of the major outer membrane proteins[12,13] and to lipopolysaccharide immunotypes[1,22]. However, the serogroups and serotypes, so far characterized, not ever provide a complete description of the relationships among meningococcal strains.

Studies have indicated that bacteria posses a clonal population structure[1,22] and individual epidemics are usually due to a single clone, although genetic variants of the epidemic clone can occasionally be isolated even during the course of an epidemic[6,23]. Studies describing the genetic relationships among meningococcal strains determined by multilocus enzyme electrophoresis (MEE) have demonstrated that populations of serogroups A, B, and C organisms are clonal[8] and that a group of closely related clones constituent of the *N.meningitidis* serogroup B ET 5 complex strains is associated with several serotypes[6,7] being responsible for at least four known outbreaks occurred in Norway, Chile, Cuba, and Brazil in the last 10 years[7,10,11,25].

In early 1970's two large urban epidemics occurred in the greater São Paulo, São Paulo State, Brazil, one caused by a clone of *N.meningitidis* serogroup C sulfonamide-resistant and the other one by clone III-I of *N.meningitidis* serogroup A strains[19,23]. The peaks of incidence of MD cases were in August and September of 1974 when there was an overlapping of the two epidemic waves caused by serogroups C and serogroup A strains, respectively. In that year, the annual incidence of meningococcal meningitis in the greater São Paulo reached 179 per 100,000 inhabitants (17.873 MD cases)[16,17,19], although only 10% of all cases were caused by serogroup C strains[24]. Since 1976, the first pos-epidemic year, the incidence of MD has been low in the greater São Paulo, however, the prevalence of serogroup C strains increased again in the period from 1989 to 1991 and serotype 2b:P1.3 isolates were responsible for that, representing approximately 18 and 70% of the serogroup C strains isolated in these years respectively[25].

Although immunological methods have revealed extensive variation in *N.meningitidis* with relevance for epidemiology and host immunity, the MEE has been used to study genetic relationships among meningococcal strains, since it yields reliable estimates of the extent of variation in the genome, as a whole, and measures genetic relatedness among strains[6]. Our previous study subtyped the *N.meningitidis* serogroup C strains isolated in the greater São Paulo into 48 distinctive multilocus enzyme genotypes that were divided into 13 different complexes, being the complex 11 strains responsible for the serogroup C epidemic occurred during the period from 1989 to 1990[25]. We have further verified that members of this complex have colonized other parts of Brazil and were responsible for the outbreak in Paraná State with an incidence of 10.45 per 100,000 inhabitants in the same period[27]. The ET 11 complex is primarily represented by strains C:2b:P1.3 (82.6%) thus the predominance of this serotype might provide a simple approach for tracing the appearance of this complex[25], nevertheless, analysis based on surface antigens may give an unrealistic picture of the genetic correlation between meningococcal strains. The typing method based on restriction fragment lenght polymorphism (RFLPs) of rRNA genes (ribotyping) has been successfully applied to study the molecular epidemiology of *N.meningitidis*[15,21,32]. The ribotyping methods could provide, as well as MEE, a useful information about the clonal characteristic of the *N. meningitidis* serogroup B strains isolated during the epidemic occurred in the greater São Paulo, Brazil in 1989-1990[32]. This method also confirmed the genetic similarity of *N.meningitidis* serogroup C ET 11 complex strains by using *Eco*RI and *Cla*I endonuclease restriction enzymes[27].

In the present study we report the results of an analysis, based on serotyping, MEE, and ribotyping of *N.meningitidis* serogroup C strains isolated from patients with MD in Rio Grande do Sul (RS) and Santa Catarina (SC) States as the Center of Epidemiology Control of Ministry of Health detected an increasing of MD cases due this serogroup in the last two years (1992- 1993). The isolates from these two states were analyzed to evaluate the possibility of the high levels of MD cases be caused by C:2b:P1.3 ET 11 complex strains. We have also analyzed *N.meningitidis* serogroup C strains isolated in the greater São Paulo in 1976 as representative of the first pos epidemic year in that region. The goal of this study was to verify the possible genetic linkage between epidemic strains from those epidemics with the actual ones responsible for the continuing increasing of MD cases in different parts of Brazil.

MATERIAL AND METHODS

Meningococcal strains. We analyzed 52 strains from Santa Catarina (SC) and 13 from Rio Grande do Sul (RS) States that represent the total of strains received from both States during the period of 1989 to 1993 (Table 1). These strains were recovered from blood or cerebrospinal fluid of patients with systemic disease and are stored in the Adolfo Lutz Institute collection (National Reference Center for *Neisseria meningitidis*). Data related to the incidence of MD in these two States were obtained from Santa Catarina and Rio Grande do Sul epidemiological surveillance (Table 1).

Thirty one *N.meningitidis* serogroup C strains isolated in the greater São Paulo in 1976, the first post-epidemic year (1971-1975), were used to investigate the possible genetic linkage with the serogroup C strains isolated during 1989-1993. All these strains were analyzed by MEE and ribotyping by using 3 different restriction endonucleases *Cla*I, *Eco*RV, and *Acc*I.

Eleven strains used as control for MEE and ribotyping analysis, representing each ET of ET 8 complex

(endemic strains, n=5) and ET 11 complex (epidemic strains, n=6)[25], are listed in Table 2. They were selected based on the quality of the results obtained by MEE, so that they were considered representative strains of the groups from which they were chosen. The ET 8 complex was chosen because it is represented by C:2a strains recovered during several different years of the endemic period in the greater São Paulo, 1976-1988[25]. These strains were also recovered from blood or cerebrospinal fluid of patients with systemic disease.

TABLE 1

Number and incidence per 1,000,000 inhabitants of meningococcal disease due to *N. meningitidis* serogroup C strains from Santa Catarina and Rio Grande do Sul States in the period from 1989 to 1993.

			Serotyped Strains		% of Serotype[d]		
Year	Cases[a]	CI[b]	No.	(%)[c]	2a	2b	others[e]
Santa Catarina State							
1989	3	0.7	2	66.7	0	50	50
1990	4	0.7	4	100	25	75	0
1991	11	2.3	3	27.3	0	25	75
1992	22	4.7	22	100	0	73	27
1993	30	6.3	21	70	0	100	0
Rio Grande do Sul State							
1989	2	0.2	0	-	-	-	-
1990	2	0.2	0	-	-	-	-
1991	7	0.7	0	-	-	-	-
1992	10	1.0	2	20	0	100	0
1993	36	3.9	11	30.5	0	100	0

a Cases of meningococcal disease (MD) due to *N. meningitidis* serogroup C.

b Annual coefficient of incidence per 1,000,000 inhabitants of MD caused by *N. meningitidis* serogroup C.

c Percentage of strains that were serotyped in relation to the total of MD cases by serogroup C in the same year.

d Percentage of C: 2a, 2b, or other serotypes in relation to the total of typed strains.

e Others serotypes include serotype 4, 8, and nontypable strains.

We also included in this study, as an outgroup of strains, epidemic strains (n=6) of *N.meningitidis* serogroup B ET 5 complex[8,10], isolated during epidemics in Norway, Cuba, Chile, and Brazil (H44/76 and H355/75, CU385/83, BB393, N.44/89 and N.150/88 respectively). In addition, serotyping prototype strains M1080, B16B6, 2996, 2396, 126E, M981, M992, M990, M978, M982, M136, S3032, S3446, 60E, 6557, 190I, 35E, and M1027 were analyzed by MEE and ribotyping.

Serogrouping and serotyping. The 103 selected *N.meningitidis* strains were serotyped and subtyped by using monoclonal antibodies and whole-cell suspensions as described previously[25]. Monoclonal antibodies for types 8, 15, 16 and, subtypes P1.2, P1.3, P1.16 were provided by ZOLLINGER & FRASCH from Walter Reed Army Institute of Research and Food and Drug Administration, USA respectively. Monoclonal antibodies for types 2a, 2b, 4, 17 and subtype P1.7, P1.14, P1.15 were produced at Adolfo Lutz Institute.

Multilocus Enzyme Electrophoresis (MEE). Methods for protein-extract preparation, starch-gel electrophoresis, and enzyme detection have been described previously[29]. Each isolate was characterized by its combination of alleles for 13 enzymes that were assayed: malic enzyme, glucose-6phosphate dehydrogenase, peptidase, isocitrate dehydrogenase, aconitase, NADP-linked glutamate dehydrogenase, NAD-linked glutamate dehydrogenase, alcohol dehydrogenase, fumarase, alkaline phosphatase, indophenol oxidases 1 and 2, and adenylate kinase. Electromorphs (allozymes of each enzyme) were equated with alleles at the corresponding structural gene locus. Distinctive combinations of alleles with the 13 enzyme loci (multilocus genotypes) were designated as ETs. These alleles are correlated with those described previously as well as with standards for ETs[25,27].

Ribotyping. Cells were harvested from two plates of Trypticase Soy Agar (Difco) with 1% of horse serum into 10 ml of a solution containing 0.1M NaCl, 0.05M EDTA, and 0.05M Tris, pH 8.0. Cells were lysed, and high-molecular-weight DNA was extracted and purified as described by BRENNER et al[5]. Preliminary experiments were conducted with 9 enzymes (*Cla*I, EcoRI, *Eco*RV, *Sal*I, *Bgl*II, *Acc*I, *Hind*III, *Kpn*I, and *Xho*I) and a small subset (n=22) of *N. meningitidis* serogroup B and C strains to determine the most appropriate enzyme for restriction digestion (Table 2). An aliquot of each DNA sample was examined spectrophotometrically to determine concentration and purity. Approximately 5µg of DNA was digested with each of the restriction enzymes in a volume of 25µl for a total of 18 h at the ideal temperature in accordance with the protocol of the supplier (New England BioLabs, Beverly, MA). The pKK3535 plasmid DNA was used as a probe[2]. The probe was labeled with digoxigenin-11-dUTP by the random primed method of the Genius 1 DNA labeling and detection kit (catalog no. 1093657; Boehringer GmbH, Mannhein, Federal Republic of Germany). Hybridization of Southern blots to digoxigenin-labeled probe and colorimetric detection were done with the same kit as described by the manufacturers. The lack of hybridization of the pBR322 plasmid vector with *N. meningitidis* serogroup B chromosomal DNA (strain N.44/89) was confirmed previously[32]. No homology between this plasmid and the genome of *N.meningitidis* serogroup B was present. Fragment sizes, based on migration distances of fragments and *Haemophilus aegyptius* 3031 *Eco*RI fragments used as references[14], included in each gel, were calculated by using the DNASTAR program (DNASTAR, Inc., Madison, Wis.). For strains with identical patterns were given the same band-

TABLE 2

Characteristics of the 15 *N. meningitidis* serogroup C strains used as reference of the ET 8, ET 11, and ET 11A complex, and other 7 international strains of *N. meningitidis*.

Strain number	State or Country	Serogroup: Serotype: Subtype	ET[c]	ET Complex	Variable allele designation for[a]													Banding pattern no. obtained with[b]				
					MAE	PG6	PEP	IDH	ACO	GD1	GD2	ADK	ALK	FUM	IP1	IP2	ADH	*Cla*I	*Eco*RV	*Acc*I	Ribotype	SF[e]
N. 1/82	SP	C:2a:P1.2	24	8	4	1	2	2	2	2	2	1	1	1	1	1	1	4	2	1	A	R
N.156/86	SP	C:2a:P1.2	25	8	4	1	2	2	0	2	2	1	1	1	1	1	1	4	1	1	B	S
N.16/76	SP	C:2a:P1.2	26	8	1	1	2	2	0	2	2	1	1	1	1	1	1	4	2	2	C	R
N.76/90	SP	C:2a:P1.2	27	8	1	1	2	2	1	2	2	1	1	1	1	1	1	4	2	3	D	S
N.286/89	SP	C:2a:P1.2	28	8	1	1	2	2	2	2	2	1	1	1	1	1	1	4	2	1	A	S
N.46/91	SP	C:2b:nt	35	11	1	1	2	1	0	2	2	1	1	1	1	1	1	2	1	4	E	S
N.883/90	SP	C:2b:P1.3	36	11	1	1	2	1	1	2	2	1	1	1	1	1	1	2	1	4	E	S
N.91/91	SP	C:2b:P1.3	37	11	1	1	2	1	2	2	2	1	1	1	1	1	1	2	1	4	E	S
N.76/91	SP	C:2b:nt	38	11	1	1	2	1	0	1	2	1	1	1	1	1	1	2	1	4	E	S
N.1337/90	SP	C:2b:nt	39	11	1	1	2	1	1	1	2	1	1	1	1	1	1	3	1	6	F	S
N.1002/90	SP	C:2b:P1.3	40	11	1	1	2	1	2	1	2	1	1	1	1	1	1	2	1	4	E	S
N.151/91	PR	C:2b:nt	49	11A	1	1	2	2	2	1	2	1	1	1	1	1	1	2	2	1	G	S
N.1414/90	SC	C:2b:P1.3	50	11A	1	1	2	3	2	1	2	1	1	1	1	3	1	2	1	4	E	S
N.106/94	SC	C:2b:P1.3	57	11A	1	1	2	3	2	1	2	1	1	1	1	1	1	2	1	4	E	S
N.97/94	SC	C:2b:P1.3	59	11A	1	1	2	3	2	1	2	1	3	1	1	1	1	2	1	5	H	S
2996	Netherlands	B:2b:P1.2,5	40	11	1	1	2	1	2	1	2	1	1	1	1	1	1	2	1	4	E	S
H44/76	Norway	B:15:P1.16	1	1	2	2	1	1	2	1	1	1	2	1	1	1	1	1	3	7	I	R
H355/75	Norway	B:15:P1.15	1	1	2	2	1	1	2	1	1	1	2	1	1	1	1	1	3	7	I	R
BB.393	Chile	B:15:P1.3	1	1	2	2	1	1	2	1	1	1	2	1	1	1	1	1	4	8	J	R
CU385/83	Cuba	B:4:P1.15	2	1	2	2	1	2	1	1	1	1	2	1	1	1	1	1	3	8	K	R
N.44/89	Brazil	B:4:P1.15	2	1	2	2	1	2	1	1	1	1	2	1	1	1	1	1	3	7	I	R
N.150/88	Brazil	B:4:P1.15	1	1	2	2	1	1	2	1	1	1	2	1	1	1	1	1	3	7	I	S

[a] Variable enzymes: MAE, malic enzyme; G6P, glucose-6-phosphate dehydrogenase; PEP, peptidase; IDH, isocitrate dehydrogenase; ACO, aconitase; GD1, glutamate dehydrogenase-1; GD2, glutamate dehydrogenase-2;ADK, adenylate kinase; ALK, alkaline phosphatase; FUM, fumarase; IP1, indophenol oxidase-1; IP2, indophenol-oxidase-2; ADH, alcohol dehydrogenase. These alleles are correlated with those previously described as well as standards for electrophoretic types (Ets)[25,26].

[b] Derived as explained in Material and Methods.

[c] ET-1 complex has been defined as ET-5 complex by D.A. CAUGANT et al.[6,7].

[e] Sulfonamide susceptibility: (R) resistant, (S) sensitive.

ing pattern number referred as ribotype number. When banding pattern numbers for the four restriction endonucleases were combined, each unique group was designated a separate ribotype and was given a letter of the alphabet referred as ribotype letter.

GD. The genetic distance (GD) between pairs of ETs was expressed as the proportion of enzymes loci at which dissimilar alleles occurred among the 13 enzymes tested[29]. For ribotyping, GD was calculated by determining the total number of unique bands produced by each restriction endonuclease and then assembling these as a set of bands of decreasing size. The banding patterns of each strain were compared with this total set of bands (master set), and for each band in a strain profile that matched a band in the master set, the number 1 was placed in that position. When a band was missing, the position was marked with a 0. The patterns of sequences of 1's and 0's for each strain were compared with the patterns for the other strains, and a relatedness index was calculated as the proportion of mismatches of bands [32].

Statistical methods. The genetic diversity (h) at an enzyme locus was calculated as $h=1-\Sigma xi^2[n/(n-1)]$, where xi is the frequency of the i^{th} allele, and *n* is the number of isolates[29].

Sulfonamide susceptibility testing. Disc tests were performed with 300ug sulfonamide discs (CEFAR, Brazil). After overnight growths of test strain on Mueller-Hinton chocolate agar tubes, whole cell suspensions in phosphate-buffered saline were adjusted to a density equal to a 1.0 McFarland standard and were swabbed onto the surface of petri dish (100 mm in diameter) containing 20 ml of Mueller-Hinton agar. After the agar had dried for 3 to 5 min, discs were applied to the plate with a sterile forceps and gently pressed onto the agar surface to ensure good contact. Inverted plates were incubated for 48 h at 37°C in a candle jar, and inhibition zones were then read against a dark background. All measurements were made to the nearest millimeter and included the disc.

RESULTS

Overall genetic diversity by MEE. In the collection of 65 isolates examined by MEE (RS, *n*=13, and SC *n*=52), 10 enzyme loci were polymorphic for 2-4 alleles. The average number of alleles per locus was 2.1. The highest frequency of null alleles occurred at the glutamate dehydrogenase-1 locus, for which 6.1% of the isolates lacked activity. The electrophoretic enzyme profiles of isolates were compared with the 49 ETs previously described by SACCHI et al[25] and 11 new ETs were found, ET 50 to ET 60. ETs were numbered consecutively according to their positions in the dendrogram (not shown). The ETs were segregated into three subgroups, labeled initially as I, II, and III. The closely related ETs in subgroup I, ET 38 (*n*=2) and ET40 (*n*=48) represented genotypes previously designated as belonging to the ET 11 complex[25]. Subgroup II were represented by ET 49 (*n*=1) and 3 other closely related new ETs, ET 50 (*n*=1), ET 57 (*n*=2), and ET 59 (*n*=1). These 4 ETs have a GD of 0.08 to 0.15 among them and components of this group have a GD of 0.15 to 0.31 with members of the ET 11 complex. However, strains of subgroup II had a GD that varied from 0.08 to 0.15 when it was compared only with the ET 40. Since the highest GD between ET 40 and subgroup II was 0.15 and ET 40 is the representative ET of the greater São Paulo epidemic (67.5% of the MD cases), and Curitiba city epidemic (71.2% of the MD cases)[25,27], this closely related but distinctive group of clones were considered as a lineage generated from ET 40 clone being referred from now as ET 11A complex, Table 3 hachured column.

TABLE 3
Genetic Distance diagram obtained by MEE of representative ETs of ET 11 and ET 11A complexes[a].

	GD[b]									
	ET 11 complex						ET 11A complex			
ETs	35	36	37	38	39	40	49	50	57	59
35	0									
36	0.08	0								
37	0.08	0.08	0							
38	0.08	0.15	0.15	0						
39	0.15	0.08	0.15	0.08	0					
40	0.15	0.15	0.08	0.08	0.08	0				
49	**0.23**	**0.23**	**0.15**	**0.15**	**0.15**	**0.08**	0			
50	**0.31**	**0.31**	**0.23**	**0.23**	**0.23**	**0.15**	0.15	0		
57	**0.23**	**0.23**	**0.15**	**0.15**	**0.15**	**0.08**	0.08	0.08	0	
59	**0.31**	**0.31**	**0.23**	**0.23**	**0.23**	**0.15**	0.15	0.15	0.08	0

[a] Numbers in boldface type represent the GD with members of the ET 11 complex. The hachured numbers represent the GD in relation to the ET 40.

[b] GDs were estimated as explained in Material and Methods.

Subgroup III were represented by 10 isolates distributed into 9 different ETs with GD of 0.23 to 0.54 among them. Strains of subgroup III had low genetic relationships with ETs of ET 11 or ET 11A complexes (mean GD = 0.38). Among the 9 ETs subgroup III, 8 were represented by single isolates (ETs 31, 52, 53, 54, 55, 56, 58, and 60), while 1 (ET 51) was recovered from two patients. The mean GD among ETs of subgroup III was 0.30 that reflects the occurrence of dissimilar alleles at an average of 4 loci.

Restriction endonuclease digestion of DNA. The *Cla*I, *Eco*RI, and *Acc*I were the only enzymes tested that were capable of restricting DNA extracted from all strains tested in Table 2. Digestion did not occur when *Kpn*I, *Bgl*II, and *Sal*I were used; few bands were demonstrated following restriction with *Hind*III and *Xho*I. Good digestion was generally seen when *Eco*RI was

used; however, the resulting patterns following hybridization with the rRNA probe did not always produced good distribution of fragments. Suitable digestion and discrimination were seen when *Cla*I, *Acc*I, and *Eco*RV were used. However, an ideal group discrimination was always seen when *Cla*I was used. Thus, it appears that *Cla*I is the most appropriate enzyme for ribotyping of *N.meningitidis* serogroup C clones (Fig.1); strains that demonstrate identical *Cla*I ribotype patterns may be further subdivided by using a second enzyme, such as *Acc*I, or *Eco*RV. The discriminatory ability of these last two endonucleases was not the same with the ribotype patterns obtained with *Eco*RV being less informative than those generated by *Acc*I.

Ribotyping and Serotyping. The 65 *N. meningitidis* serogroup C strains from Santa Catarina and Rio Grande do Sul States were analyzed by serotyping and ribotyping. These results are presented in Table 4. Almost all analyzed strains from these two States were serotype 2b (84.6%) and 48 of them were C:2b:P1.3 (87.3%). The ribotyping method using *Cla*I endonuclease could divide these 65 strains into 4 different ribotypes (Rb), (Rb2, Rb4, Rb5, and Rb6). The most prevalent ribotype was Rb2, present in 84.6% of all cases. The serotype 2b and the Rb2 were very closely related since all C:2b strains were also Rb2 and vice versa. The Rbs 4, 5 and 6 are represented by 9 *N.meningitidis* C:NT:nt and 1 C:2a:nt strains isolated on SC. The designation Rb1 is reserved to describe the ET 5 complex strains defined by CAUGANT et al.[6,7], and proposed by TONDELLA et al. [31].

The 31 serogroup strains from 1976 could also be divided into different serosubtypes and ribotypes, Table 5. By using 3 restriction endonuclease, *Cla*I, *Eco*RV, and *Acc*I, these strains were subtyped into 8 different ribotype letters. When 15 selected strains representing each ET of the ET 8 (n=5), ET 11 (n=6), and ET 11A (n=4) complex, the prototype strain for serotype 2b, and the 6 *N.meningitidis* B epidemic strains were analyzed by using 3 different restriction enzymes, four different ribosomal DNA restriction profiles were obtained with *Cla*I and *Eco*RV, and 8 were obtained with *Acc*I, Table 2. When the ribosomal DNA restriction profiles for all four restriction endonucleases were considered together, 11 different ribotypes were obtained, Table 2. By using four restriction endonucleases, different GDs were obtained allowing further discrimination of the ET 11 and ET 11A complex.

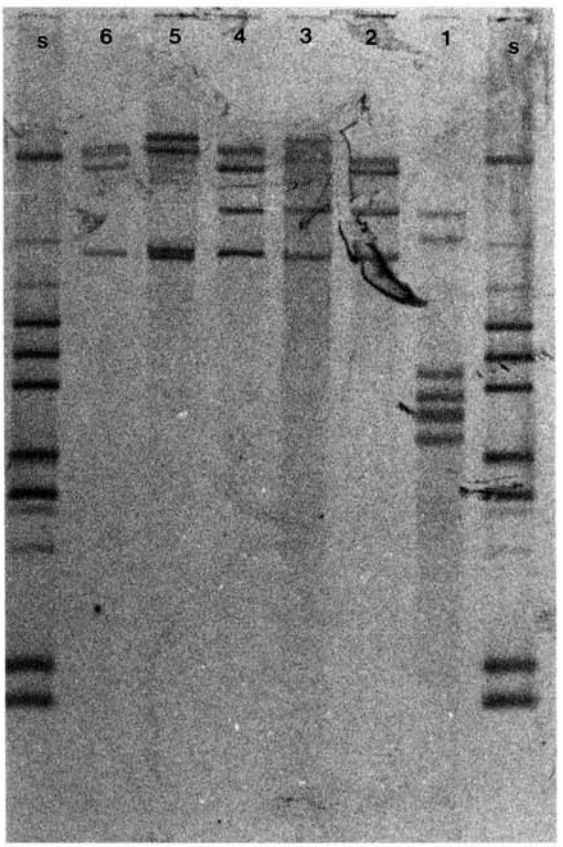

Fig. 1 - *Cla*I restriction fragments of 6 representative *N. meningitidis* strains hybridized with pKK3535 plasmid DNA labeled with digoxigenin-11-dUTP. Lane 1, N.44/89 serogroup B (Rb1); lane 2, N. 1002/90 serogroup C (Rb2); lane 3, N.1337/90 serogroup C(Rb3); lane 4, N.16/76 serogroup C (Rb4); lane 5, N.691/93 serogroup C (Rb5); lane 6, N.97/92 serogroup C (Rb6); lane *S. H. aegyptius* 3031.

TABLE 4

Serotype: subtype distribution of *N. meningitidis* serogroup C strains isolated in Santa Catarina and Rio Grande do Sul in relation to Ribotype, MEE, and Sulfonamide susceptibility.

Number of Isolates	Serotype: Subtype	Ribotype[a]	ET complex 10	11	11A	Others	Sulfadiazine susceptibility[b]
48	2b:P1.3	2	0	43	5	0	S
3	2b:P1.15	2	0	3	0	0	S
4	2b:nt	2	0	4	0	0	S
1	2a:nt	4	1	0	0	0	S
8	NT:nt	5	0	0	0	8	R
1	NT:nt	6	0	0	0	1	R

[a] Ribotyping numbers were defined by using only *Cla*I restriction enzyme.

[b] Sulfonamide susceptibility: (R) resistant, (S) sensitive.

Relationships among serotypes, ET complex, ribotypes, and sulfonamide susceptibility. The 17 prototype strains used in the serotyping scheme were included in this study. Each of these strains had a distinctive genotype by MEE and Rb by using *Cla*I restriction enzyme (data not shown). Only the prototype strain for serotype 2b (2996 strain), belonged to the ET 40 and ribotype 2, Table 2. Thus, no attempt was giv-

en to the others prototype strains that did not match with those patterns obtained for ET 11 or ET 11A complex by MEE, or for Rb 2 by ribotyping. Both Norwegian strains H355/75 and H44/76, Chilean BB.393, and Brazilian N.150/88 were classified as ET 1 and Rb 1 as previously described by TONDELLA et al.[32]. The Cuban CU385/83 and Brazilian N.44/89 strains were classified as ET 2 and Rb1. There were dissimilar alleles only at isocitrate dehydrogenase and aconitase enzymes loci being all grouped at the same ET 1 complex. However, those same strains were additionally subdivided by using 4 restriction enzymes into 3 different ribotypes I, J, and K by the presence of two new bands, one obtained with *Eco*RI and other with *Acc*I restriction enzyme. There were good correlation between sulfonamide sensitivity and ET 11 and ET 11A complex strains, Tables 2, 4 and 5 while all meningococal serogroup B ET 1 complex except one (N.150/88), were sulfonamide resistant, Table 2.

TABLE 5

Characteristics of 31 strains of *N. meningitidis* serogroup C isolated in São Paulo - SP in 1976 and analyzed by ribotyping using 3 different restriction enzymes.

Strain	Serotype: Subtype	Banding pattern no. obtained with			Ribotypes
		*Cla*I	*Eco*RV	*Acc*I	
N.1	NT: P1.2	4	2	2	C
N.2	2a: nt	7	2	2	L
N.3	2a: P1.2	4	2	2	C
N.5	2a: P1.9	4	2	2	C
N.6	2a: P1.2	4	2	2	C
N.8	NT: P1.2	4	2	2	C
N.9	2a: P1.2	4	2	2	C
N.10	NT: P1.7	10	5	10	M
N.12	NT: P1.2	4	2	2	C
N.13	2a: nt	4	2	2	C
N.14	2a: P1.2	4	2	2	C
N.15	2a: P1.2	4	2	2	C
N.19	2a: P1.2	4	2	2	C
N.20	2a: P1.2	4	2	2	C
N.21	2a: P1.2	4	2	2	C
N.24	2a: nt	4	2	2	C
N.25	2a: nt	11	2	2	N
N.39	2a: nt	4	2	2	C
N.42	NT: P1.7	10	5	11	O
N.45	2a: P1.2	4	2	9	P
N.47	2a: P1.2	4	2	9	P
N.49	NT: P1.7	8	5	10	Q
N.52	2a: P1.2	9	2	2	R
N.62	2a: P1.2	4	2	2	C
N.64	2a: nt	4	2	2	C
N.70	2a: P1.2	4	2	2	C
N.87	2a:nt	9	2	2	R
N.98	2a: P1.2	11	2	2	N
N.95	2a: P1.2	11	2	2	N
N.108	2a: nt	4	2	2	C
N.113	NT: P1.2	4	2	2	C

DISCUSSION

The analysis of multilocus genotypes of the isolates did not demonstrate extensive diversity of clones causing meningococcal disease in south Brazil during the period 1989-1993, with only 3 (21%) of the 14 clones identified more than once in the sample.

Our strain collection currently comprises ≈ 700 *N.meningitidis* serogroup C isolates in the south region of Brazil in the same period. This extensive collection enabled us to follow clonal replacement and evolution occurring in meningococcal serogroup C population causing disease in this area during the last 5 years. Clonal replacements associated with increases in incidence of disease and occurrence of epidemics have been demonstrated in several parts of the world, including Norway[15,16], and Brazil[25,26]. However, these changes in clonal composition have been associated with a change in one or more phenotypic properties of the strain, such as serogroup, serotype, or susceptibility to sulfonamide[16,29,30]. During the last 3 years the annual coefficient of incidence per 1,000,000 inhabitants of MD in SC State shows an increasing that was followed by high percentages of C:2b:P1.3 strains, and in RS State 100% of the analyzed strains belong to this serotype: subtype.

Here we have demonstrated that the MD due *N.meningitidis* serogroup C strains in RS and SC States, Brazil occurring in the last 4 years were caused mainly by one clone of strains (ET 40), with isolates indistinguishable in serogroup, serotype, subtype and even by ribotyping. One small number of cases that were not due to ET 40 strains, represent closely related clones that probably are new lineages generated from ET 40 clone referred as ET 11A complex.

The meningococcal serogroup C responsible for the current increases in incidence of systemic disease in the south of Brazil are shown to be members of a genetically distinctive complex of clones having no close relationships to other epidemic serogroup B or C endemic organisms. We have further discovered that strains belonging to these 2 closely related but distinct group, ET 11 and ET 11A complex had poor genetic correlation with ET 15 clone of strains that have been responsible for outbreaks in Canada. This clone was defined by ASHTON, et al.[4,30] and belongs to the ET 37 complex defined by CAUGANT et al.[6,7] (data not shown).

WOODS et al.[32] in their study showed that ribotyping gave a greater discriminating capacity than MEE for subtyping *N.meningitidis* serogroup C using three endonuclease restriction enzymes, however they did not use epidemiological well-characterized isolates. Here the ribotyping method was successfully applied and we could conclude that ribotyping as well as MEE can be used to search for epidemic *N.meningitidis* serogroup C strains.

Most isolates of the ET 11 complex from Brazil (1989-1993), are characterized by an association of several phenotypic properties: they are sulfonamide-sensitive, serogroup C, and serotype 2b:P1.3[10,25,26,27]. The first indication of reduced sensitivity of *N. meningitidis* to sulfonamides were seen soon after the discovery of this class of drugs in 1932[24]. The meningococcal resistant strains belong to serogroup B or C and in the group C epidemic in São Paulo, Brazil in 1971-1973, 95% of group C and 33% of sporadic group B strains were sulfonamide resistant[24]. In 1975 MUNFORD et al.[20] studying meningococcal strains by bactericidal test and agar gel double diffusion using rabbit antisera found that 73% of *N. meningitidis* serogroup C strains isolated in São Paulo in 1972 were serotype 2 and this serotype was associated with sulfonamide resistence. Unfortunately, we do not have any meningococcal serogroup C strains from the epidemic period (1971-1975) to make it possible to correlate them with the ET 11 or ET 11A complex strains. However, we were able to analyze 31 meningococcal C strains from the first pos-epidemic year, 1976. Seventh-seven of those are serotype 2a and 79% of them are sulfonamide resistant being different from ET 11 and ET 11A complex strains. Sixteen of them have been previously characterized by MEE as ETs that do not belong to the ET 11 complex[25].

The ribotyping method, as well as MEE, could provide useful information about the clonal characteristics of *N.meningitidis* serogroup C strains isolated in greater São Paulo in 1976 and also strains isolated in south Brazil. The strains from 1976 have more similarity with the actual endemic strains by the ribotyping, sulfonamide sensitivity, and MEE results. Although it was not possible to establish genetic linkage between the epidemic ET 11 complex strains with meningococcal C strains from the 1970's epidemics occurred in greater São Paulo, it is probable that they are very different taking into account the analyzed strains from 1976 that can represent at least in part clones present during those epidemic years.

In conclusion, serotyping with monoclonal antibodies (C:2b:P1.3), MEE (ET11 and ET 11A complex), and ribotyping by using *Cla*I restriction enzyme (Rb2), were useful to characterize these epidemic strains of *N. meningitidis* related to the increased incidence of MD in different States of south Brazil. It is mostly probable that these *N.meningitidis* serogroup C strains have poor or no genetic correlation with 1971-1975 epidemic serogroup C strains. The genetic similarity of members of the ET 11 and ET 11A complex were confirmed by the ribotyping method by using three restriction endonucleases. The number of ribotypes may change, depending on the number of restriction endonucleases used; however, the epidemiological significance of this additional degree of discrimination may not be necessary for epidemiological purposes.

RESUMO

Estrutura genética de cepas epidêmicas de *Neisseria meningitidis* Sorogrupo C do Sul do Brasil

No presente estudo, nós reportamos os resultados de uma análise, baseada na sorotipagem, multilocus enzimático (MEE) e ribotipagem de *N. meningitidis* sorogrupo C isoladas de paciente com doença meningocócica no Rio Grande do Sul (RS) e Santa Catarina (SC), onde o Centro de Controle Epidemiológico do Ministério da Saúde detectou um aumento do número de casos de doença meningocócica (DM) devido a este sorogrupo nos últimos 2 anos (1992-1993). Nós demonstramos que a DM devido a cepas de *N. meningitidis* sorogrupo C no RS e SC que ocorreram nos últimos 4 anos foi devido principalmente por um clone (ET 40), com isolados indistinguíveis por sorogrupo, sorotipo, subtipo e até por ribotipagem. Um pequeno número de casos que não foram devidos a cepas do ET 40 representaram um grupo geneticamente relacionado, que provavelmente é uma nova linhagem gerada do clone ET 40, referido como complexo ET 11A. Nós também analisamos cepas de *N. meningitidis* sorogrupo C isoladas na grande São Paulo em 1976 como um grupo representativo do primeiro ano pós-epidêmico na região. A ribotipagem, bem como MEE, puderam fornecer informações sobre as características clonais das cepas isoladas no período pós-epidêmico e também no Sul do Brasil. As cepas de 1976 possuem mais similaridades com as cepas endêmicas atuais do que com as cepas epidêmicas (1992-1993) por ribotipagem, sensibilidade a sulfonamida e MEE. Em conclusão, sorotipagem com anticorpos monoclonais (C:2b:P1.3), MEE (complexo ET11 e ET11A) e ribotipagem usando a enzima de restrição *Cla*I, foram úteis em caracterizar estas cepas epidêmicas de *N. meningitidis* relacionadas com o aumento da incidência da DM em diferentes estados do sul do Brasil. É muito provável que estas cepas de *N. meningitidis* sorogrupo C possuam pouca ou nenhuma correlação genética com as cepas epidêmicas sorogrupo C de 1971-1975. A similaridade genética dos membros do complexo ET 11 e ET 11A foram confirmadas por ribotipagem usando-se 3 enzimas de restrição.

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