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Characterization of Epidemic *Neisseria meningitidis* Serogroup C Strains in Several Brazilian States

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Epidemic strains of the *Neisseria meningitidis* C:2b:P1.3 electrophoretic type 11 complex were responsible for an outbreak in Curitiba, Parana State, Brazil, from 1990 to 1991. Strains of this complex were also isolated in other Brazilian states and were responsible for a meningococcal disease epidemic in São Paulo State in 1990. Serotyping both with monoclonal antibodies and by multilocus enzyme electrophoresis was useful for typing these epidemic strains related to the increased incidence of meningococcal disease. The genetic similarity of members of the electrophoretic type 11 complex was confirmed by the ribotyping method by using *Eco*RI or *Cla*I endonuclease restriction enzymes.

Meningococcal disease (MD) is a significant cause of mortality and morbidity throughout the world (8, 13). The incidence of MD in Brazil has been monitored since serogroup A and C epidemics occurred between 1971 and 1974. In 1974, the incidence was greater than 179 cases per 100,000 inhabitants (6, 7). From 1980 to 1992, the annual incidences of MD ranged from 1.0 to 1.4 per 100,000 inhabitants in different states of Brazil. During the period between 1981 and 1987, the mean proportion of serogroup B isolates identified was about 83%, while serogroup C strains represented only 6% of isolates. In 1988, the incidence of MD in the greater São Paulo area exceeded 4.06 per 100,000 inhabitants, suggesting a new epidemic in that region. This epidemic was different from previous ones because it was caused by serogroup B strains in 1988 and 1989 and serogroup B and C strains in 1990 (4, 9, 10).

The incidence of MD caused by *Neisseria meningitidis* serogroup C in greater São Paulo, São Paulo State, Brazil, has been low since the end of the epidemic situation in 1971 and 1972. In that region, the prevalence of serogroup C strains increased from 4 to 14% and 8 to 32% during the years 1989 and 1990, respectively. Serotype 2b isolates were responsible for most of this increase, representing approximately 22 and 74% of the serogroup C strains isolated in 1989 and 1990, respectively (10).

For epidemiological purposes, *N. meningitidis* serogroup C strains isolated in greater São Paulo from 1976 to 1990 were analyzed by different methods to determine whether the observed increase in the number of cases of MD caused by *N. meningitidis* serogroup C was correlated to a new emerging clone. Our previous study divided these strains into 48 distinctive multilocus enzyme genotypes that were grouped into 13 different complexes (10). Strains of the C:2b electrophoretic type (ET) 11 complex were responsible for much of the increased occurrence of MD (10).

Curitiba, the capital of Parana State, Brazil, also had an outbreak of MD caused by *N. meningitidis* serogroup C during the years 1989 and 1990. The incidence of MD in that city, with a population about 1.6 million, reached 10.45 per 100,000 inhabitants, with 168 cases reported in 1990. Of 67 strains that were serogrouped, 17.9 and 82.1% of them belonged to serogroups B and C, respectively.

We report here the analysis of 151 *N. meningitidis* serogroup C strains isolated between 1989 and 1991 in five Brazilian states. The strains were serotyped, subtyped, and typed by multilocus enzyme electrophoresis (MEE), and some strains were ribotyped. The goals of the study were to determine (i) whether the observed increase in the number of cases of MD caused by serogroup C in the city of Curitiba was caused by C:2b:P1.3 ET 11 complex strains, as was found in greater São Paulo during the same period, and (ii) how widely strains belonging to the ET 11 complex are distributed in representative Brazilian states. We also report here the genetic relatedness of the ET 11 complex strains by using the ribosomal DNA restriction profiles (ribotyping) of a set of isolates representing each ET included in this complex.

MATERIALS AND METHODS

Bacterial strains. We analyzed 73 strains of *N. meningitidis* serogroup C isolated in Curitiba, Parana, Brazil, from 1989 to 1991 (Table 1). These strains were recovered from the blood or cerebrospinal fluid of patients with systemic disease and are stored in the Adolfo Lutz Institute collection (National Reference Center for *N. meningitidis*). Data related to the incidence of MD in this city were obtained from Parana State epidemiological surveillance.

We also analyzed all strains ($n = 78$) of *N. meningitidis* serogroup C isolated in 1990 and 1991 received from Espirito Santo State (21 strains), Goiás State (12 strains), Minas Gerais State (21 strains), and Brasilia, District Federal (24 strains). We chose these states because they represented those in which laboratory resources were best positioned to isolate *N. meningitidis*. Therefore, laboratories in those states sent the greatest number of *N. meningitidis* serogroup C strains to the Adolfo Lutz Institute during the period 1989 to 1991. These strains were recovered from the blood or cerebrospinal fluid of patients with systemic disease.

The strains used in the ribotyping analysis representing each ET of the ET 11 complex are listed in Table 2. They were

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TABLE 1. MD caused by *N. meningitidis* serogroup C and B strains in Curitiba, Parana, Brazil, from 1989 to 1991

Year	No. of MD cases	CI[a]	No. (%) of strains serogrouped	No. (%) of strains of serogroup:			No. (%) of serogroup C strains analyzed
				B	C	Others	
1989	33	2.14	17 (51.5)	12 (70.6)	4 (23.5)	1 (5.9)	2 (50.0)
1990	168	10.45	67 (39.9)	12 (17.9)	55 (82.1)	0 (0)	39 (70.9)
1991	104	6.50	58 (55.8)	17 (29.3)	41 (70.7)	0 (0.0)	32 (78.0)
Total	305		142 (46.6)	41 (28.9)	100 (70.4)	1 (0.7)	73 (73.0)

[a] CI, coefficient of incidence per 100,000 inhabitants.

selected on the basis of the quality of the results obtained by an MEE reaction, so that they were considered representative strains of the groups from which they were chosen.

Serogrouping and serotyping. Serogrouping and serotyping of the 151 *N. meningitidis* strains were performed as described previously (10).

MEE. Methods for the preparation of protein extracts, starch gel electrophoresis, and enzyme detection have been described previously (11). Each isolate was characterized by its combination of alleles for 13 enzymes that were assayed: malic enzyme, glucose 6-phosphate 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 (10). These alleles are correlated with those described previously as well as with standards for ETs (10).

Statistical methods. The genetic diversity (h) at an enzyme locus was calculated as $h = 1 - \Sigma x_i^2[n/(n - 1)]$, where x_i is the frequency of the ith allele and n is the number of isolates (11). Cluster analysis was done by the average linkage method.

Ribotyping. The strains used in the analysis are listed in Table 2. Cells were harvested from a plate of Trypticase soy agar (Difco) with 1% horse serum into 10 ml of a solution containing 0.1 M NaCl, 0.05 M EDTA, and 0.05 M Tris (pH 8.0). Cells were lysed, and high-molecular-weight DNA was extracted and purified as described by Brenner et al. (2). *N. meningitidis* DNA (5 μg) was digested with *Eco*RI or *Cla*I (1 U/μg of DNA), in accordance with the protocol of the supplier (New England BioLabs, Beverly, Mass.). Restricted DNA was electrophoresed in Tris-acetate buffer overnight on a 0.8% agarose gel at 1 V/cm and was transferred by capillary action by Southern blotting onto nylon filters (Nytran; Schleicher & Schuell, Keene, N.H.). pKK3535 plasmid DNA was used as a probe (1). It 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 Genius 1 kit as described by the manufacturers. Fragment sizes were estimated by measuring the migration distances, with *Haemophilus aegyptius* 3031 *Eco*RI fragments used as references for *Eco*RI and *Cla*I digests (5). Ribosomal banding patterns were confirmed by repeated runs, and strains with identical patterns for each restriction endonuclease were given the same banding pattern number. When banding pattern numbers for the two restriction endonucleases were combined, each unique group was designated a separate ribotype and was given a letter of the alphabet.

TABLE 2. Representative *N. meningitidis* strains of each ET analyzed by ribotyping

Strain	Type	ET[a]
H44/76	B:15:P1.16	5
N.44/89	B:4:P1.15	5 complex
N.76/90	C:2a:P1.2	27
N.46/91	C:2b:P1.3	35
N.883/90	C:2b:P1.3	36
N.91/91	C:2b:P1.3	37
N.76/91	C:2b:P1.3	38
N.1337/90	C:2b:nt[b]	39
N.1002/90	C:2b:P1.3	40
N.151/91	C:2b:nt	49

[a] The ET 5 and the ET 5 complex were described by Caugant et al. (3, 4). ETs 27 to 40 were described by Sacchi et al. (10).

GD. The genetic distance (GD) between pairs of ETs was expressed as the proportion of enzyme loci at which dissimilar alleles occurred among the 13 enzymes tested (11). 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 of sequences for the other strains, and a relatedness index was calculated as the proportion of mismatches of bands (12).

RESULTS

Serotyping. The serotyping analysis of *N. meningitidis* serogroup C strains showed that all cases of MD were caused by serotypes 2a and 2b (Table 3). The greatest percentages of serotype 2b strains were found in Curitiba (100%) and in the state of Goiás (58.3%), while serotype 2a strains were the most prevalent in the other three states. In Curitiba, 81.0% of *N. meningitidis* serogroup C strains were C:2b:P1.3 (data not shown).

ETs. The electrophoretic enzyme profiles of 151 *N. meningitidis* serogroup C isolates were compared with those of 48 distinctive multilocus enzyme genotypes described previously (10) to identify whether any strain belonged to the ET 11 complex. Seventy-two strains from Curitiba could be placed in the ET 11 complex (Table 3). However, strain N.151/91 (C:2b:nt, where nt is not typeable) did not match any of the 48 ETs that are already known. The ET 11 complex is represented by six different ETs (ET 35 to ET 40), with a GD of from 0.07 to 0.15 among them. Strain N.151/91 had a GD that varied

TABLE 3. ET (ET 11 complex) distribution of *N. meningitidis* serogroup C strains isolated in five Brazilian states from 1989 to 1991

State[a]	No. (%) of strains of:								Total no. (%) of strains analyzed[b]
	ET 36	ET 37	ET 39	ET 40	Total ET 11 complex	Other ETs	Serotype		
							2a	2b	
ES	0	0	0	3 (14.3)	3 (14.3)	18 (85.7)	19 (90.5)	2 (9.5)	21 (13.9)
MG	0	0	0	1 (4.8)	1 (4.8)	20 (95.2)	17 (81.0)	4 (19.0)	21 (13.9)
DF	0	0	4 (16.7)	4 (16.7)	8 (33.3)	16 (66.7)	13 (54.2)	11 (45.8)	24 (15.9)
GO	0	0	0	5 (41.7)	5 (41.7)	7 (58.3)	5 (41.7)	7 (58.3)	12 (8.0)
PR	1 (1.4)	12 (16.4)	7 (9.6)	52 (71.2)	72 (98.6)	1 (1.4)	0	73 (100.0)	73 (48.3)
Total	1 (0.7)	12 (7.9)	11 (7.3)	65 (43.0)	89 (58.9)	62 (41.1)	54 (35.8)	97 (64.2)	151 (100)

[a] ES, Espirito Santo; MG, Minas Gerais; DF, Brasilia, District Federal; GO, Goiás; PR, Parana.
[b] The percentage is related to the total percentage of strains analyzed.

from 0.07 to 0.23 when it was compared with the six distinct ETs of the ET 11 complex, therefore being very close to the ET 11 complex. Since the genetic distance between ET 40 and strain N.151/91 was 0.07 and ET 40 is the representative ET of the São Paulo epidemic (10), this new ET is classified as ET 49. The presence of ET 11 complex strains in other states was variable, but the ET 11 complex was present in all states analyzed (Table 3).

Ribotyping. When the 10 strains selected for ribotyping were analyzed, five different ribosomal DNA restriction profiles were obtained with *Cla*I (Fig. 1) and six different profiles were obtained with *Eco*RI. However, when ribosomal DNA restriction profiles for the two restriction endonucleases were considered together, seven different ribotypes were obtained (Table 4). The six strains representative of each ET of the ET 11 complex were divided into three ribotypes, ribotypes D, E, and F. Strain N.76/90, of the ET 8 complex, and strain N.151/91 fell into distinct ribotypes, ribotypes C and G, respectively (Table 4). ET 5 and ET 5 complex strains of serogroup B (9) were classified as ribotypes A and B, respectively.

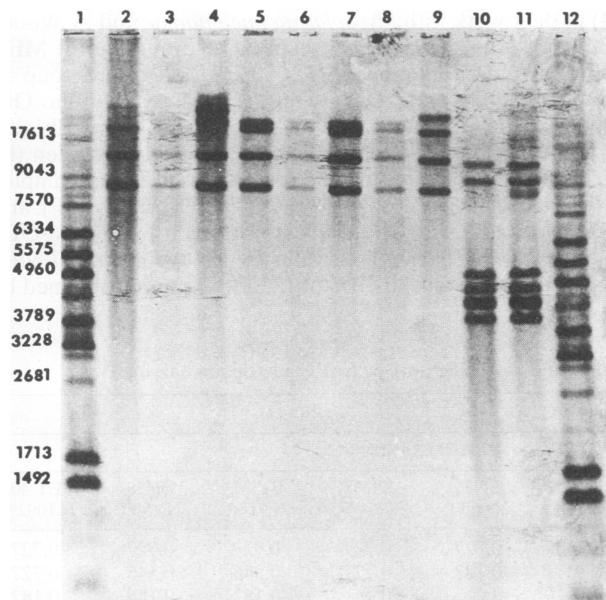

FIG. 1. *Cla*I restriction fragments of 10 *N. meningitidis* strains hybridized with pKK3535 plasmid DNA labeled with digoxigenin-11-dUTP. Lane 1, *H. aegyptius* 3031; lane 2, N.151/91 (*Cla*I banding pattern 5, ET 49); lane 3, N.1002/90 (*Cla*I banding pattern 3, ET 40); lane 4, N.1337/90 (*Cla*I banding pattern 4, ET 39); lane 5, N.76/91 (*Cla*I banding pattern 3, ET 38); lane 6, N.91/91 (*Cla*I banding pattern 3, ET 37); lane 7, N.883/90 (*Cla*I banding pattern 3, ET 36); lane 8, N.46/91 (*Cla*I banding pattern 3, ET 35); lane 9, N.76/90 (*Cla*I banding pattern 2, ET 27); lane 10, N.44/89 (*Cla*I banding pattern 1, ET 2); lane 11, H.44/76 (*Cla*I banding pattern 1, ET 1); lane 12, *H. aegyptius* 3031. Numbers on the left are molecular sizes (in base pairs).

By using two endonuclease enzymes, different GDs were obtained (Table 5). ET 5 and ET 5 complex *N. meningitidis* serogroup B strains were very similar, but they had long GDs from the other ETs analyzed. On the other hand, among the *N. meningitidis* C strains representing the ET 11 complex studied, the differences in GDs were not significant when the *Cla*I endonuclease was used, demonstrating a very high degree of relatedness among them. However, the GD obtained with *Eco*RI showed some degree of heterogeneity among strains in the ET 11 complex, especially for strains N.1337/90 and N.151/91.

DISCUSSION

We analyzed about 75% of all *N. meningitidis* serogroup C strains isolated in Curitiba, Parana, Brazil, during the epidemic period of 1990 to 1991. By analyzing the rate of incidence and the percentage of meningococci that have been serogrouped during this period (Table 1), we can conclude that meningococcal serogroup C strains were responsible for the outbreak in that city. We also found that members of the *N. meningitidis* C:2b:P1.3 ET 11 complex, which were responsible for an MD epidemic in São Paulo State in 1990 (10), were also responsible for the epidemic in Curitiba during the same period, although we do not have enough information to conclude if the strains spread to Curitiba from São Paulo or from other areas. We found that all isolates of serogroup C meningococci from Curitiba analyzed belonged to the ET 11 complex and that 71.2% of them were ET 40.

Members of the ET 11 complex were also present in the other states from which strains were analyzed, but their prevalences were different, because only 14.3 and 4.8% of the serogroup C meningococcal strains from Espirito Santo State and Minas Gerais State, respectively, were of the ET 11 complex. In these two states, the proportions of serotype 2a strains were very high (90.5 and 81.0%, respectively). On the other hand, Brasilia, District Federal, and Goiás State had similar percentages of serotypes C:2a:P1.2 and C:2b:P1.3, with 33.3 and 41.7% of the strains belonging to the ET 11 complex, respectively. It is clear that ET 11 complex is primarily represented by serotype C:2b:P1.3, so because of the predominance of this serotype, a simple approach for tracing the appearance of this complex may be to search for this serotype.

TABLE 4. Patterns obtained by MEE and ribotyping of representative *N. meningitidis* strains of each ET[a]

ET[b]	ET complex	Variable allele designation for[c]:													GD[d]	Banding pattern no. obtained with[e]:		Ribotype[e]
		MAE	G6P	PEP	IDH	ACO	GD1	GD2	ADK	ALK	FUM	IP1	IP2	ADH		*Cla*I	*Eco*RI	
1	5[f]	2	2	1	1	2	1	1	1	2	1	1	1	1	0.385	1	1	A
2	5[f]	2	2	1	2	1	1	1	1	2	1	1	1	1	0.538	1	2	B
27	8	1	1	2	2	1	2	2	1	1	1	1	1	1	0.231	2	3	C
35	11	1	1	2	1	0	2	2	1	1	1	1	1	1	0.154	3	3	D
36	11	1	1	2	1	1	2	2	1	1	1	1	1	1	0.154	3	3	D
37	11	1	1	2	1	2	2	2	1	1	1	1	1	1	0.077	3	4	E
38	11	1	1	2	1	0	1	2	1	1	1	1	1	1	0.077	3	4	E
39	11	1	1	2	1	2	1	2	1	1	1	1	1	1	0.077	4	5	F
40	11	1	1	2	1	2	1	2	1	1	1	1	1	1	0.0	3	3	D
49	11	1	1	2	2	2	1	2	1	1	1	1	1	1	0.077	5	6	G

[a] One representative strain of each ET was ribotyped.
[b] The strain in each ET was as follows: ET 1, H.44/76 B:15:P1.16; ET 2, N.44/89 B:4:P1.15; ET 27, N.76/90 C:2a:P1.2; ET 35, N.46/91 C:2b:P1.3; ET 36, N.883/90 C:2b:P1.3; ET 37, N.91/91 C:2b:P1.3; ET 38, N.76/91 C:2b:P1.3; ET 39, N.1337/90 C:2b:nt; ET-40, N.1002/90 C:2b:P1.3; and ET 49, N.151/91 C:2b:nt.
[c] 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.
[d] The GD was derived as explained in the text. The GD was calculated in relation to the ET 40 electromorph profile.
[e] Derived as explained in the text.
[f] The ET 5 and the ET 5 complex were described by Caugant et al. (3, 4).

However, even with the significant rate of occurrence of C:2b:P1.3 ET 11 complex strains, there were no MD outbreaks in Brasilia or Goiás State during the analysis period, which suggests that some other factors besides the presence of the epidemic strains may be responsible for the increase in the incidence of MD. Nevertheless, we must consider that all strains from Parana State came from a single city. However, because we do not have enough epidemiological information concerning the locations or sources of the isolates from other states, no such inference can be made.

Strains belonging to the ET 11 complex were isolated for the first time in 1989 in the city of São Paulo, and members of this complex have been defined as a group of related *N. meningitidis* strains with a GD of less than 0.15 that is mainly represented by C:2b:P1.3 strains (10). Initially, the ET 11 complex was composed of six distinctive ETs separated by a GD of from 0.07 to 0.15. We report here the inclusion of a new ET in this complex, ET 49, represented by a C:2b:P1.3 strain varying from other ET 11 complex strains by GDs of 0.07 to 0.15.

The results of ribotyping analysis of representative strains of each ET belonging to the ET 11 complex were dependent on the number of restriction endonucleases used. The importance of using more than one restriction enzyme to establish genetic homogeneity or heterogeneity was emphasized by Yogev et al. (14) in their work with *Mycoplasma pneumoniae* and by Woods et al. (12), who described a comparative evaluation of MEE and ribotyping for subtyping *N. meningitidis* serogroup C isolates obtained from Los Angeles County, California. Our analysis of the GDs generated by MEE (Table 4) and ribotyping data (Table 5) revealed a general agreement between the two methods with respect to the relatedness of ET 11 complex strains, although strains N.1337/90 (ET 39, ribotype F) and N.151/91 (ET 49, ribotype G) demonstrated the greatest GDs among the ET 11 complex strains by ribotyping with *Eco*RI (Table 5). However, the GDs of these two strains obtained by

TABLE 5. GD diagram obtained by ribotyping 10 *N. meningitidis* strains by using *Cla*I or *Eco*RI restriction enzyme[a]

Strain	GD[b]									
	ET 5 complex[c]		ET 8 complex, ET 27, N.76/90[d]	ET 11 complex[d]						
	ET 5, H.44/76	ET 5 C, N.44/89[c]		ET 49, N.151/91	ET 39, N.1337/90	ET 35, N.46/91	ET 36, N.883/90	ET 37, N.91/91	ET 38, N.76/91	ET 40, N.1002/90
H.44/76		0.0	0.727	0.636	0.545	0.727	0.727	0.727	0.727	0.727
N.44/89	**0.0**		0.727	0.636	0.364	0.727	0.727	0.545	0.545	0.727
N.76/90	**0.727**	**0.727**		0.273	0.364	0.0	0.0	0.182	0.182	0.182
N.151/91	**0.727**	**0.727**	**0.182**		0.455	0.273	0.273	0.273	0.273	0.273
N.1337/90	**0.727**	**0.727**	**0.364**	**0.182**		0.364	0.364	0.182	0.182	0.364
N.46/91	**0.727**	**0.727**	**0.182**	**0.182**	**0.182**		0.0	0.182	0.182	0.0
N.883/90	**0.727**	**0.727**	**0.182**	**0.182**	**0.182**	**0.0**		0.182	0.182	0.0
N.91/91	**0.727**	**0.727**	**0.182**	**0.182**	**0.182**	**0.0**	**0.0**		0.0	0.182
N.76/91	**0.727**	**0.727**	**0.182**	**0.182**	**0.182**	**0.0**	**0.0**	**0.0**		0.182
N.1002/90	**0.727**	**0.727**	**0.182**	**0.182**	**0.182**	**0.0**	**0.0**	**0.0**	**0.0**	

[a] Numbers in boldface type represent the GDs obtained with *Cla*I, and numbers in non-boldface type represent the GDs obtained with *Eco*RI.
[b] GDs were estimated as explained in the text.
[c] The ET 5 and the ET 5 complex classifications are those of Caugant et al. (3, 4).
[d] The ET 8 complex and the ET 11 complex classifications are those of Sacchi et al. (10).

MEE and ribotyping (*Cla*I) were low enough to justify their inclusion in the ET 11 complex. It is probable that better discrimination of ET 11 complex strains could be obtained by using more restriction enzymes. However, the additional degree of discrimination of ET 11 complex strains may not have epidemiological relevance.

In conclusion, epidemic strains of *N. meningitidis* C:2b:P1.3 of the ET 11 complex were responsible for the epidemic in Curitiba, Parana, Brazil, from 1990 to 1991. Strains of this complex were also isolated in different Brazilian states. Both serotyping with monoclonal antibodies and MEE were useful for typing these epidemic strains related to the increased incidence of MD. The genetic similarity of members of the ET 11 complex was confirmed by the ribotyping method by using two 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 needs further assessment.

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