Epidemiol. Infect. (1991), **106**, 143–150
Printed in Great Britain

Genetic relationships among strains of *Neisseria meningitidis* causing disease in Italy, 1984–7

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(*Accepted 18 September 1990*)

SUMMARY

Since 1984 a change in the epidemiological pattern of meningococcal disease in Italy has occurred with a predominance of *Neisseria meningitidis* of serogroup C (76%), serotype 2a and a high proportion of strains resistant to sulphonamides (71%). In order to understand better the epidemiology of the group C *N. meningitidis* strains responsible for the disease over the last years in Italy, we studied the genetic features of phenotypically closely related strains, by enzyme electrophoresis. The results showed that the genetic and the phenotypic characteristics of the 57 strains studied were similar, suggesting the spread of a single clone during recent years in our country.

This result is in agreement with the circulation of strains typical of epidemic situations, despite the decreasing incidence of meningoccal disease in Italy.

INTRODUCTION

Beginning in 1984 a change in the epidemiological pattern of meningococcal disease in Italy has occurred [1, 2]. Whereas in previous years, as in most other western European countries [3–5], *Neisseria meningitidis* of serogroup B was the leading cause of disease [6], a shift to a predominance of serogroup C, serotype 2a, sulphonamide-resistant strains have then been observed [1, 2]. By distinguishing between phenotypically similar bacteria, analysis of the chromosomal genotype of *N. meningitidis* isolates by multilocus enzyme electrophoresis has demonstrated the high resolving power of that method for epidemiological purposes [7, 8].

To identify the genetic features of the phenotypically closely related strains recently causing meningococcal disease in Italy, we studied the multilocus enzyme genotypes, as well as the serogroups, serotypes, and sulphonamide susceptibility of 57 strains recovered from patients in the years 1984–7.

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MATERIALS AND METHODS

Bacterial strains

The 57 *N. meningitidis* strains studied were isolated from the cerebro-spinal fluids of patients in various parts of Italy in the years 1984–7. These strains represented a random sample of all isolates sent to the National Institute of Health, Rome, during that period.

Serogrouping

Meningococci were serogrouped by slide agglutination with antisera against capsular polysaccharides of serogroups A, B, C, Y, Z, W135, 29E (Wellcome Diagnostics).

Serotyping

Strains were serotyped for protein antigens by the agar gel double diffusion method using LiCl EDTA extracts [9]. Antisera were prepared as described for serogroup B prototype strains by immunizing rabbits with formalin killed meningococci [10], as monoclonal antibodies are not available in Italy. The antisera against serotype 2a, 2b and 2c strains M1011, 2996 and 2396 respectively were adsorbed with the heterologous type 2a, 2b and 2c strains as described [11].

Sulphonamide susceptibility testing

Minimal inhibitory concentrations (MICs) of sulphadiazine were determined in duplicate for each strain by the agar dilution method as previously described [12]. Isolates with MIC $\geqslant$ 100 mg/l were considered resistant.

Electrophoresis of enzymes

Methods of starch-gel electrophoresis and selective enzyme staining were similar to those described by Selander and co-workers [13]. The 14 enzymes assayed were malic enzyme (ME), glucose 6-phosphate dehydrogenase (G6P), peptidase (PEP), isocitrate dehydrogenase (IDH), acotinase (ACO), NADP-linked glutamate dehydrogenase (GD1), NAD-linked glutamate dehydrogenase (GD2), alcohol dehydrogenase (ADH), fumarase (FUM), alkaline phosphatase (ALK), two indophenol oxidases (IP1 and IP2), adenylate kinase (ADK), and an unknown dehydrogenase (UDH).

Electromorphs of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding structural gene locus. Numerical allele designations were cognate with those previously recorded in *N. meningitidis* [8]. Each isolate was characterized by its combination of alleles at the 14 enzyme loci and distinctive multilocus genotypes were designated as electrophoretic types, ETs.

ETs were numbered sequentially according to the position in the dendrogram (Fig. 1). Except for ET-5, ET numbers were not cognate with those previously assigned [8].

Statistical analyses

Genetic diversity at an enzyme locus among ETs or isolates was calculated as $h = (1-\Sigma x_i^2)\ (n/n-1)$, where x_1 is the frequency of the ith allele and n is the

https://doi.org/10.1017/S0950268800056508 Published online by Cambridge University Press

number of ETs or isolates. Mean genetic diversity (H) is the arithmetic average of h values over all loci. Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which dissimilar alleles occurred (mismatches), and clustering was performed from a matrix of genetic distances by the average linkage method [14].

RESULTS

The serogroups, serotypes, and enzyme electromorph profiles (ETs) of the 57 isolates are shown in Table 1. Serogroups C, B and Y were represented by 46 (81%), 9 (16%), and 2 (3%) isolates, respectively. Forty-eight (84%) isolates were serotypable. Of the 46 serogroup C isolates, 38 (87%) were serotype 2a but none of the isolates of serogroups B and Y were that serotype. Five (55%) of the nine serogroup B strains were serotype 15. The remaining isolates were assigned to serotype 11 (1 isolate), 2b (1 isolate), 2c (1 isolate), and 4 (2 isolates). All isolates were sulphonamide resistant, with MICs higher than 640 mg/l, with the exception of two non-serotypable isolates, 506 and 511 (representing ET-2 and ET-7, respectively) that were sensitive.

In the collection of 57 isolates, three (FUM, IP1, and ADK) of the 14 enzyme loci were monomorphic and 11 were polymorphic for 2–8 alleles. The average number of alleles per locus was 3·4 (Table 2).

The 57 isolates were assigned to 23 distinctive multilocus genotypes, among which mean genetic diversity per locus was 0·453. There was less genetic diversity per locus among isolates ($H = 0{\cdot}318$), reflecting the circumstance that four closely related ETs were represented by multiple isolates (range 3–25).

The genetic relationship among the 23 ETs is shown in the dendrogram (Figure 1). At a genetic distance of 0·20, corresponding to the occurrence of dissimilar alleles at an average of 3 of the 14 enzyme loci, there were 11 lineages, 7 represented by a cluster of 2 or more ETs, and 4 represented by a single ET.

ETs 3, 4 and 5 (3 isolates, or 5% of the strain collection) represented clones of the ET-5 complex, which have been previously recorded in various parts of Europe and other continents [15]. Thirty-eight isolates (67%) were assigned to one of four very closely related ETs (ETs 17 through 20), which also represented genotypes of a previously identified group of clones, that had then been designated as the ET-37 complex [16, 17]. All these 38 strains were serogroup C, serotype 2a.

DISCUSSION

The incidence of meningococcal meningitis in Italy over the last 5 years has been decreasing. However several features evidenced by the national surveillance system suggest an epidemic pattern. Firstly an increased number of cases occurring in young adults (15–24 years), secondly the strong predominance of serogroup C which accounted for 90% of the cases seen in military recruits and a high proportion of strains resistant to sulphonamide. This is a new epidemiological pattern of meningococcal disease in Italy, where in previous years serogroup B had caused the majority of cases.

Finally the prevalence of a single serotype 2a frequently associated with outbreaks of serogroup B and C meningococcal meningitis in the world, suggests

Table 1. *Characteristics of 57* N. meningitidis *isolates from Italy*

ET	Isolate	Alleles at indicated enzyme locus*														Year	GP	Type
		ME	G6P	PEP	IDH	ACO	GD1	GD2	ADH	FUM	ALK	IP1	IP2	ADK	UDH			
1	573	3	4	7	3	4	1	3	1	1	6	2	3	2	2	1986	B	15
2	506	3	3	1	2·5	5	1	3	1	1	3	2	5	2	3	1984	B	NT
3	575	1	1	7	8	4	2	3	2	1	1	2	5	2	3	1986	C	4†
4	587	3	1	7	8	4	2	3	2	1	1	2	3	2	3	1987	B	15
5	566	1	1	7	8	4	2	3	2	1	1	2	3	2	3	1986	B	4†
6	563	1	3	5	7	4	1	3	2	1	3	2	3	2	3	1986	B	15
7	511	1	3	7	7	4	1	3	2	1	3	2	3	2	3	1985	C	NT
8	505	3	3	5	7	4	2	3	2	1	2	2	5	2	3	1984	B	15
9	582	3	3	7	7	4	2	3	2	1	2	2	5	2	3	1987	B	15
10	579	3	1	7	7	4	1	3	2	1	2	2	3	2	3	1987	C	2
11	544	2	3	5	2	0	1	2	2	1	2	2	3	2	3	1986	C	2
12	590	2	3	5	0·5	0	1	2	2	1	2	2	3	2	3	1987	B	NT
13	514	3	4	5	9	0	1	2	2	1	3	2	3	2	3	1985	Y	2c
14	517	3	4	5	9	4	1	2	2	1	3	2	3	2	3	1985	C	NT
15	528	3	3	4	8	4	1	2	2	1	3	2	3	2	3	1985	C	NT
16	525	3	3	4	9	1	1	2	2	1	3	2	3	2	3	1985	Y	NT
17	500	4	3	4	5	2	1	4	1	1	8	2	3	2	3	1984	C	2a
	510	4	3	4	5	2	1	4	1	1	8	2	3	2	3	1985	C	2a
	523	4	3	4	5	2	1	4	1	1	8	2	3	2	3	1985	C	2a
18	497	4	3	4	5	2	1	4	0	1	8	2	3	2	3	1984	C	2a
	548	4	3	4	5	2	1	4	0	1	8	2	3	2	3	1986	C	2a
	504	4	3	4	5	2	1	4	0	1	8	2	3	2	3	1984	C	2a
19	501	4	3	4	5	2	2	4	1	1	8	2	3	2	3	1984	C	2a
	vt1	4	3	4	5	2	2	4	1	1	8	2	3	2	3	1984	C	2a
	vt2	4	3	4	5	2	2	4	1	1	8	2	3	2	3	1984	C	2a
	524	4	3	4	5	2	2	4	1	1	8	2	3	2	3	1985	C	2a
	541	4	3	4	5	2	2	4	1	1	8	3	2	2	3	1985	C	2a
	571	4	3	4	5	2	2	4	1	1	8	2	3	2	3	1986	C	2a
	562	4	3	4	5	2	2	4	1	1	8	2	3	2	3	1986	C	2a
20	43	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1984	C	2a
	502	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1984	C	2a
	503	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1984	C	2a

507	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1984	C	2a
vt3	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1984	C	2a
496	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1985	C	2a
515	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1985	C	2a
520	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1985	C	2a
521	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1985	C	2a
522	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1985	C	2a
51	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1985	C	2a
527	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1985	C	2a
542	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1985	C	2a
572	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
557	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
559	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
550	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
558	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
543	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
569	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
554	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
560	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
556	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
547	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1986	C	2a
584	4	3	4	5	2	2	4	0	1	8	2	3	2	3	1987	C	2a
21	577	4	3	4	7	2	1	4	1	1	3	2	3	2	3	1986	B	2b
22	513	4	3	4	7	4	1	3	1	1	3	2	3	2	3	1985	C	NT
23	545	1	2	2	7	2	1	3	0	1	5	2	3	2	3	1986	C	NT

* ME, malic enzyme; G6P, glucose 6-phosphate dehydrogenase; PEP, peptidase; IDH, isocitrate dehydrogenase; ACO, aconitase; GD1 and GD2, glutamate dehydrogenases; ADH, alcohol dehydrogenase; FUM, fumarase; ALK, alkaline phosphatase; IP1 and IP2, indophenol oxidases; ADK, adenylate kinase; UDH, unknown dehydrogenase.

NT, non-serotypable.

† Serotyping of these two strains was performed in Oslo with monoclonal antibodies.

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Table 2. *Genetic diversity at 14 loci in isolates and ETs of* N. meningitidis *from Italy*

Locus	No. of alleles	Genetic diversity in	
		Isolates	ETs
ME	4	0·476	0·719
G6P	4	0·258	0·549
PEP	5	0·438	0·747
IDH	8	0·536	0·830
ACO	5	0·468	0·668
GD1	2	0·464	0·443
GD2	3	0·485	0·640
ADH	3	0·632	0·569
FUM	1	0·000	0·000
ALK	6	0·529	0·783
IP1	1	0·000	0·000
IP2	2	0·133	0·300
ADK	1	0·000	0·000
UDH	2	0·035	0·087
Mean	3·4	0·318	0·453

the circulation of highly virulent strains in Italy. The analysis of multilocus enzyme genotypes of the 57 strains identified 23 distinctive multilocus genotypes, of which 19 were represented by single isolates. The four remaining ETs with multiple isolates were very similar, differing from one another at only one or two enzyme loci, and together were responsible for about 70% of the disease in Italy. All serogroup C, serotype 2a isolates belonged to one of these four clones. One of these clones (ET-20) predominated and was recovered from 44% of the patients. ET-19 and ET-20 (which differed from one another solely by the absence of detectable activity of alcohol dehydrogenase in ET-20) represented clones of the ET-37 complex, that had not been identified previously in a collection of 94 serotype 2a strains from various geographical sources [17].

Although clones of the ET-5 complex were present in Italy, they did not appear as a major cause of disease during the period studied.

The clear predominance of a single complex of clones confirmed the homogeneous results obtained by serogrouping and serotyping and by the analysis of DNA restriction endonuclease patterns [18] and suggest an apparent spreading of a single clone during recent years, a feature characteristic of an epidemic situation, despite the decreased incidence of meningococcal disease in Italy.

ACKNOWLEDGEMENTS

We thank L. Oddvar Frøholm for reviewing the manuscript and C. Fleming for technical assistance. This work was supported in part by grant no. 13.48.07–026 from the Norwegian Council for Science and Humanities to D.A.C., a Public Health Service grant, AI24631, from the National Institutes of Health to R.K.S., and a collaborative research grant, 86/0721, from the North Atlantic Treaty Organization.

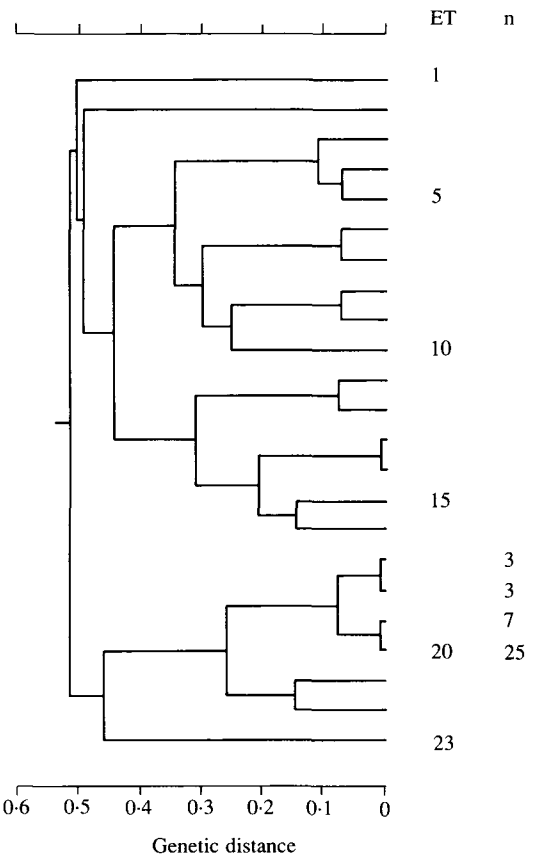

Fig. 1. Genetic relationships among 23 ETs of *N. meningitidis*. The dendrogram was generated by the average-linkage method of clustering from a matrix of coefficients of pairwise genetic distance, based on 14 enzyme loci. ETs are numbered sequentially from top to bottom in the order of their listing in Table 1. The number of isolates (n) in each ET represented by multiple isolates is indicated.

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