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Association between asymptomatic carriage and sporadic (endemic) meningococcal disease in an open community

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SUMMARY

We analysed a strain collection representative of the overall *Neisseria meningitidis* population circulating in an open community (46000 inhabitants, Spain) during an endemic period (30 isolates from patients and 191 from throat cultures of healthy individuals) by both phenotypic and molecular techniques. Almost all patient isolates were assigned to three hyper-virulent lineages (ET-5 complex, ET-37 complex and cluster A4) by both multilocus enzyme electrophoresis (MEE) and pulsed-field gel electrophoresis (PFGE). In contrast, MEE and PFGE assigned 20% and 15% respectively of carrier isolates to the hyper-virulent clones (4% for both methods together). There was also a higher correlation between PFGE and phenotypes associated with virulent clones. These notable differences between the two molecular methods were further observed in more than half the carrier isolates, suggesting that the associations between these strains were distorted by recombination events. However, almost one-third of total endemic strains from symptom-free carriers and almost all patient strains belonged to clones defined by MEE and PFGE, with no known epidemiological connection. These data indicate low transmission and a weak clonal structure for *N. meningitidis*.

INTRODUCTION

Infections due to *Neisseria meningitidis* are still a public health concern. Meningococci colonize the upper respiratory tract of 10–15% of the human population in open communities [1, 2], occasionally invading the blood stream or spinal fluid causing disease. In Europe, most cases are sporadic (or endemic) [3] and the main causal agents are strains of serogroup B, followed by serogroup C [4, 5]. Although the prevalence of serogroup C has increased in Spain, serogroup B still prevails especially in the Mediterranean area [6].

Exposure to pathogenic meningococcal strains, together with environmental and host factors are assumed to contribute to the development of invasive disease [7]. Typing of meningococcal strains by efficient markers may improve both the understanding and the control of meningococcal disease (MD). Various methods have been used to characterize meningococci, such as serotyping [8], ribotyping [9, 10], pulsed-field

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gel electrophoresis (PFGE) [11–13], multilocus enzyme electrophoresis (MEE) [14–17], multilocus sequence typing (MLST) [18, 19], random amplified polymorphism analysis (RAP–PCR) [20] and PCR-restriction fragment length polymorphism analysis [9, 21].

Before the development of MLST, a method based on DNA sequencing, MEE was the method of choice for analysing bacterial population structures and global epidemiology [14, 22]. Most serogroup B or C isolates associated with invasive meningococcal disease in the developed world have been assigned to a small number of hyper-virulent lineages, referred to as ET-5 complex, ET-37 complex, and cluster A4 [15, 16]. Recently, these major lineages have also been identified by MLST [18]. In addition, several studies have shown that methods such as PFGE, which are very sensitive to microevolution, are useful for short-term epidemiology [11, 23].

Although transmission of virulent clones through human populations during endemic periods has been inferred from a disease-typing study [24], the whole *N. meningitidis* population from an endemic area, i.e. strains from both healthy carriers and patients, has not been characterized. In the context of a period of hyperendemic disease in Norway, Caugant et al. [25] used MEE to compare isolates from healthy carriers in a localized community (Trømso) and patients with MD from all over the country and found a low frequency of carriage of hyper-virulent clones (ET-5 and ET-37 complexes). However, when isolates from healthy military recruits were examined, ET-5 complex prevailed amongst both carriers and cases [26].

To characterize the overall *N. meningitidis* population accurately, both a suitable typing method and a representative strain collection have to be selected. Most studies have focused on a small number of lineages that are responsible for most cases of the disease, and so have been performed with highly biased samples, in which these lineages are over-represented [18, 27]. This is likely to underestimate the diversity of the population as a whole and hinder our understanding of both the dynamics of meningococcal carriage and the connection between such carriage and invasive meningococcal disease.

Here, we report an analysis of the phenotypic and genotypic composition of a representative strain collection of the overall *N. meningitidis* population recovered from a localized open community during an endemic period. Using serology, MEE and PFGE, we characterized a meningococcal strain collection obtained from all cases of MD reported between 1987 and 1993 [11] and from a cross-sectional seasonal carriage survey carried out between March 1992 and January 1993 in a Spanish town (Cerdanyola, 46000 inhabitants) [2].

MATERIALS AND METHODS

Population and data collection

Between 1987 and 1993, when no localized outbreaks were reported, 36 cases of MD were notified in Cerdanyola, Barcelona, Spain (population 46000). *N. meningitidis* was isolated from 30 of these 36 patients. Meningococci were not obtained by culture from the remaining six [11].

Throat samples were collected from 1500 individuals between March 1992 and January 1993 [2]. In order to include different age and social groups, the sampling survey was carried out in day-care centres, schools, colleges, and cultural and working centres in various areas (residential, central and peripheral) in Cerdanyola in four campaigns (March, June, September and January). *N. meningitidis* was identified in 191 of these throat cultures and one colony from each carrier was kept at −80 °C. None of these individuals had had contact with a MD case. The overall meningococcal carriage rate was 10·5 % [2].

Serogrouping, serotyping and subtyping

Serogrouping was performed by standard slide agglutination. Serotypes and subtypes were determined by a whole-cell ELISA. Antigens were prepared as described by Abdillahi and Poolman [28]. Monoclonal antibodies with serotype specificities 1, 2a, 2b, 2c, 4, 14 and 15 and subtype specificities P1.1, P1.2, P1.3, P1.4, P1.6, P1.7, P1.9, P1.10, P1.12, P1.13, P.14, P1.15 and P1.16 were supplied by Dr J. T. Poolman (RIVM, Bilthoven, the Netherlands).

Enzyme electrophoresis

Enzyme extraction, polyacrylamide gel electrophoresis and selective enzyme staining were performed as described elsewhere [29]. Relative enzyme mobilities for each of the eight enzymes assayed (alleles) were numbered in order of decreasing anodal mobility and each unique set of alleles was defined as an electrophoretic type (ET). At a maximal genetic distance of

Table 1. *Characteristics of 221 isolates of* N. meningitidis *from carriers and MD cases*

Isolate no.		Allele at the following enzyme locus										Serogroup: serotype: subtype	*Cluster* no.*
Patient	Carrier	G6P	ME	ADH	GD1	GD2	IDH	ALP	MDH	ET	PT		
	108	5	1	2	4	1	1	4	2	1	A_1	NG:NT:P1.15	1
	1378	5	1	2	3	2	1	4	2	2	AV_1	NG:NT:NST	
	1457	5	1	2	3	2	1	4	2		AV_2	NG:NT:NST	
	109	2	2	2	3	2	4	1	2	3	AB_1	B:4:P1.14	2
	241	2	2	2	3	2	2	1	2	4	C_1	B:1:P1.14	
	95	2	2	2	3	2	3	1	2	5	A_1	NG:4:P1.14,16	
	697	2	2	2	3	2	3	4	2	6	AX_3	B:1:NST	
	700	2	2	2	3	2	3	4	2		AX_3	B:1:NST	
	267	1	2	2	3	2	2	1	2	7	V_1	B:4:NST	
	1128	1	2	2	3	2	4	1	2	8	V_2	B:NT:P1.14	
	306	1	2	2	3	2	3	1	2	9	C_1	B:1:P1.14	
	325	1	2	2	3	2	3	1	2		C_2	B:4:P1.14	
	268	1	2	2	3	2	3	1	2		C_4	NG:4:P1.14	
	490	1	2	2	3	2	5	4	2	10	AX_5	B:NT:NST	
	444	2	1	2	2	2	1	4	2	11	AH_1	B:4:P1.4	3
	486	2	1	2	2	2	1	4	2		AH_1	B:4:P1.4	
	528	2	1	2	2	2	1	4	2		AH_1	B:4:P:1.1,2	
	470	2	1	2	2	2	1	4	2		AH_2	B:4:P1.1,2	
	473	2	1	2	2	2	1	4	2		AH_2	B:4:P1.1,2	
	1340	2	1	2	2	2	3	4	2	12	AH_4	NG:4:P1.2	
	594	2	1	2	3	2	4	4	2	13	H	B:4:P1.6	
	508	2	2	2	2	2	2	4	2	14	AH_1	B:4:P1.1,2	
	162	2	2	2	2	2	1	1	2	15	P_1	B:NT:P1.16	4
	1233	3	2	2	2	2	1	1	2	16	W_1	B:14:P1.2	
	808	5	2	2	2	2	3	1	2	17	C_3	B:4:P1.14	
	242	3	2	2	2	2	2	3	2	18	C_1	NG:4:P1.9	
	1220	3	2	2	2	2	2	2	2	19	W_2	B:14:NST	
	836	3	2	2	2	2	2	2	2		W_3	B:14:NST	
	244	3	2	2	2	2	2	2	2		AS_1	NG:15:P1.6	
	636	3	2	2	2	2	2	2	2		AS_4	B:4:P1.6	
	758	3	2	2	2	2	2	2	2		AS_5	B:4:NST	
	1277	3	2	2	2	2	2	2	2		AW_1	B:4:NST	
	1358	3	2	2	2	2	2	2	2		AW_1	B:4:P1.6	
	495	3	2	2	2	2	2	2	2		AJ_1	B:14:NST	
	1054	3	2	2	2	2	2	5	2	20	AF_7	B:1:P1.6	
	271	3	2	2	2	2	2	5	2		AK_2	B:4:P1.9	
	1390	3	2	2	2	2	2	5	2		AP_1	B:1:NST	
	243	3	2	2	2	2	2	5	2		AP_3	NG:1:P1.6	
	161	3	2	2	2	2	4	3	2	21	S_1	NG:4:P1.7,9	
	464	3	2	2	2	2	4	3	2		S_2	B:4:P1.9	
	50	3	2	2	2	2	4	2	2	22	F_1	B:1:P1.6	
	54	3	2	2	2	2	4	2	2		F_1	B:1:NST	
	1546	3	2	2	2	2	4	2	2		AJ_1	NG:1:P1.10	
889638		3	2	2	2	3	4	2	2	23	Y_1	B:NT:NST	
	827	3	2	2	2	3	4	2	2		AP_1	B:1:NST	
	1152	2	2	2	2	2	4	2	2	24	AF_3	B:NT:P1.16	
	344	3	2	2	2	2	3	4	2	25	N_1	C:2b:NST	
	40	3	2	2	2	2	3	2	2	26	AC	B:1:NST	
	990	3	2	2	2	2	3	2	2		AP_1	B:1:NST	
	655	4	2	2	2	2	3	2	2	27	I_5	C:NT:P1.6	
	840	4	2	2	2	2	3	2	2		T	NG.NT.P1.6	
	568	5	1	2	2	2	4	2	2	28	M	B:4:NST	5
	1370	3	1	2	2	2	4	2	2	29	AG	B:1:P1.15	

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Table 1 (*cont.*)

Isolate no.		Allele at the following enzyme locus										Serogroup: serotype: subtype	*Cluster* no.*
Patient	Carrier	G6P	ME	ADH	GD1	GD2	IDH	ALP	MDH	ET	PT		
	680	5	3	2	2	2	3	2	2	30	AY_1	B:NT:NST	
	204	2	2	2	3	2	2	2	2	31	AT_2	B:1:P1.6	6
	536	3	2	2	3	2	2	2	2	32	AS_6	B:4:P1.6	
	319	3	2	2	3	2	2	2	2		AT_1	B:NT:P1.6	
	721	3	2	2	3	2	2	2	2		AU_2	B:4:P1.6	
	1294	3	2	2	3	2	2	2	2		E	B:NT:NST	
	356	3	1	2	3	2	2	2	2	33	AF_2	C:NT:P1.1,2	
	388	3	2	2	3	2	1	2	2	34	AU_3	B:4:P1.6	
	326	3	2	2	3	2	3	2	2	35	AK_5	B:4:P1.6	
	694	3	2	2	3	2	3	2	2		AS_5	B:4:NST	
	947	3	2	2	3	2	3	2	2		AT_1	B:4:P1.6	
	565	3	2	2	3	2	3	2	2		AV_1	B:4:P1.6	
	843	3	2	2	3	2	3	2	2		AW_2	B:4:P1.6	
	276	4	2	2	3	2	4	2	2	36	R_1	NG:NT:NST	
	1164	4	1	2	3	2	4	2	2	37	AF_1	B:4:P1.6	
	266	3	2	2	3	2	5	2	2	38	AK_6	NG:15:P1.6	7
	348	3	2	2	3	2	5	2	2		AL_1	B:NT:NST	
	28	3	2	2	3	2	5	1	2	39	K	B:4:NST	
	36	3	2	2	4	2	5	1	2	40	K	B:4:NST	
	462	3	2	2	4	2	2	4	2	41	N_1	NG:2a:NST	
	639	3	2	2	4	2	2	4	2		N_1	C:2b:NST	
	454	3	2	2	4	2	2	4	2		N_3	NG:2a:NST	
	250	3	2	2	4	2	2	4	2		N_4	C:2b:NST	
	1613	3	2	2	3	2	2	4	2	42	N_1	NG:2b:NST	
	185	3	2	2	3	2	2	4	2		N_1	NG:2b:NST	
	654	3	2	2	3	2	2	4	2		N_2	C:2b:NST	
	1290	3	2	2	3	2	2	4	2		AO_1	B:2b:P1.10	
9304432†		**3**	**2**	**2**	**3**	**2**	**4**	**4**	**2**	**43**	**AO_1**	**B:2b:P1.10**	
	498	3	2	2	3	2	4	4	2		AO_2	C:2b:P1.1,2	
9315144		**3**	**2**	**2**	**3**	**2**	**4**	**4**	**2**		**AO_3**	**C:2b:P1.2**	
	59	3	2	2	4	2	3	4	2	44	N_1	C:2b:NST	
	665	3	1	2	1	2	3	2	2	45	I_4	Y:NT:P1.6	8
	132	3	2	2	1	2	3	2	2	46	I_1	NG:NT:NST	
	262	3	2	2	1	2	3	2	2		I_1	B:1:P1.6	
	1392	3	2	2	1	2	2	2	2	47	I_2	NG:NT:NST	
	434	3	2	2	1	2	2	2	2		I_2	NG:NT:P1.6	
	659	3	2	2	1	2	2	2	2		AY_1	NG:NT:P1.6	
	667	3	2	2	1	2	4	2	2	48	I_2	NG:NT:P1.6	
	1478	3	2	2	1	2	4	2	2		J	B:4:P1.4	
	1053	3	2	2	1	2	1	1	2	49	L_1	B:NT:P1.6	
	1346	3	2	2	1	2	2	1	2	50	I_1	NG:4:P1.6	
	658	2	1	2	1	2	5	2	2	51	Q_1	NG:4:P1.16	—
	118	5	2	2	2	3	3	2	2	52	U_1	NG:4:NST	9
	871	4	2	2	2	3	3	2	2	53	U_1	NG:4:NST	
	638	4	2	2	2	3	3	2	2		Y_2	Y:14:NST	
	482	4	2	2	3	3	3	2	2	54	U_1	B:4:NST	
	810	3	3	2	2	3	2	2	2	55	AY_2	B:NT:NST	—
	110	5	1	4	2	3	4	1	4	56	A_1	B:4:P1.15	10
	99	3	1	4	2	3	4	1	4	57	A_1	NG:4:P1.15	
	92	4	1	4	3	3	3	5	4	58	A_1	NG:NT:P1.15	—
	390	2	1	2	2	3	4	1	2	59	A_5	B:4:P1.15	11
	119	2	1	2	2	3	4	1	2		D	B:4:P1.1,7	

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Table 1 (*cont.*)

Isolate no.		Allele at the following enzyme locus										Serogroup: serotype: subtype	*Cluster* no.*
Patient	Carrier	G6P	ME	ADH	GD1	GD2	IDH	ALP	MDH	ET	PT		
	642	1	1	2	2	3	4	1	2	60	A_6	B:4:P1.15	
	1375	1	1	2	2	3	4	1	2		A_8	B:15:P1.7,16	
	263	1	1	2	3	3	4	1	2	61	A_5	NG:4:P1.15	
	293	1	1	2	2	3	4	1	1	62	A_{14}	B:15:P1.7,16	
	452	6	1	2	2	3	4	5	2	63	A_8	B:15:P1.7,16	—
	240	5	1	3	3	3	4	3	3	64	O_1	NG:15:P1.6	12
	690	5	4	3	3	3	4	3	3	65	O_1	B:15:P1.6	
	720	3	1	3	3	3	1	3	3	66	X_1	B:15:P1.6	
	1005	3	1	3	3	3	1	3	3		X_2	B:15:P1.6	
	1531	5	1	3	2	3	5	3	3	67	O_1	B:15:P1.6	13
	1218	5	1	3	2	3	5	3	3		O_2	B:15:P1.6	
	296	5	4	3	2	3	5	3	3	68	P_2	B:15:P1.6	
	236	2	2	4	2	2	4	2	4	69	AK_1	B:15:P1.15	—
	578	5	3	1	2	2	3	2	1	70	AY_2	NG:NT:NST	14
	753	5	3	1	2	2	3	2	1		AY_2	NG:NT:NST	
	1436	5	3	1	2	2	3	2	1		AY_1	B:4:NST	
	1518	5	3	1	2	2	2	2	1	71	AY_2	B:4:P1.4	
	1363	5	3	1	1	2	2	2	1	72	AY_1	B:4:NST	
	509	5	3	3	2	2	2	2	3	73	AY_1	NG:NT:P1.15	—
	717	5	3	3	2	2	2	2	3		AY_1	NG:NT:NST	—
	496	4	2	3	1	2	2	2	3	74	F_2	B:4:P1.6	—
	1522	3	2	3	3	2	5	2	3	75	L_2	B:4:P1.1,7	—
	1210	1	2	3	2	2	3	1	3	76	V_1	B:4:P1.14	—
	1572	4	2	3	2	2	4	3	3	77	I_8	B:NT:NST	—
	443	6	2	3	2	2	2	4	3	78	AE	B:1:NST	—
	290	3	2	1	3	3	4	2	1	79	U_2	B:4:NST	15
	1560	3	2	1	3	2	4	2	1	80	I_2	NG:NT:P1.6	
	1356	3	2	1	2	2	4	3	1	81	S_1	B:4:P1.9	
	1398	3	2	1	2	2	4	2	1	82	AW_3	B:4:P1.6	
	79	3	2	1	2	2	4	2	1		AP_1	B:1:NST	
	1412	3	2	1	2	2	4	2	1		AP_1	B:1:P1.2	
9120240		3	2	1	2	2	4	2	1		AP_1	B:1:P1.6	
	544	3	2	1	2	2	4	2	1		AP_2	B:1:P1.10	
	1451	3	1	1	2	2	4	2	1	83	AF_2	B:NT:P1.2	
	1420	2	2	1	2	2	5	2	1	84	AF_4	B:NT:P1.6	
	902	2	2	1	2	2	5	2	1		AK_3	B:NT:P1.9	
	1073	3	2	1	2	2	5	2	1	85	AL_3	B:NT:P1.9	
	652	3	2	1	2	2	5	2	1		AR_1	B:NT:NST	
	1547	3	2	1	2	2	5	2	1		AS_2	B:4:P1.15	
	1468	3	2	1	2	2	5	3	1	86	S_1	B:4:P1.9	
	974	4	2	1	2	2	3	2	1	87	AK_7	C:NT:P1.6	
	1601	3	2	1	2	2	3	2	1	88	AK_1	B:15:P1.15	
	65	3	2	1	1	2	3	2	1	89	I_2	NG:NT:P1.6	
	661	3	2	1	1	2	3	2	1		I_2	NG:NT:P1.6	
	1049	3	2	1	1	2	3	2	1		I_6	B:NT:NST	
	1341	3	2	1	1	2	2	2	1	90	I_2	NG:4:P1.6	
	1395	3	2	1	2	2	2	2	1	91	AW_1	B:4:P1.6	
	1593	3	2	1	2	2	1	2	1	92	AS_3	B:4:P1.6	
	1402	3	2	1	4	2	1	2	1	93	Q_2	NG:4:P1.15	
	1432	3	1	1	3	2	3	2	1	94	AF_2	B:NT:P1.2	—
	1433	3	1	1	3	2	3	2	1		AF_2	B:NT:NST	—

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Table 1 (*cont.*)

Isolate no.		Allele at the following enzyme locus										Serogroup: serotype: subtype	*Cluster* no.*
Patient	Carrier	G6P	ME	ADH	GD1	GD2	IDH	ALP	MDH	ET	PT		
	647	3	3	1	2	3	5	2	1	95	AF_5	B:4:P1.4	16
	979	3	3	1	3	2	5	2	1	96	AL_2	B:4:P1.4	
	402	2	2	1	3	2	3	1	1	97	C_4	B:4:P1.14	17
	552	2	2	1	3	2	3	2	1	98	AN	NG:2a:P1.1,7	
	770	2	2	1	3	2	3	2	1		I_3	NG:2b:NST	
	768	2	2	1	3	2	3	4	1	99	AX_6	NG:1:NST	
	491	2	2	1	3	2	1	2	1	100	AS_3	B:4:P1.6	
	561	1	2	1	3	2	4	1	1	101	C_5	NG:NT:P1.14	
	748	2	2	1	3	2	4	1	1	102	AY_2	B:4:P1.14	
	849	2	2	1	3	2	4	4	1	103	AO_1	B:NT:P1.10	
	1534	6	2	1	3	2	4	4	1	104	AX_4	B:1:P1.13	
	645	1	2	1	3	2	5	4	1	105	AX_2	B:1:NST	
	1427	1	2	1	3	2	5	4	1		AX_2	B:1:NST	
	504	1	2	1	3	2	3	4	1	106	AX_1	B:1:NST	
	656	3	2	1	3	2	3	4	1	107	N_3	C:2b:NST	
878736‡		**3**	**2**	**1**	**3**	**2**	**2**	**4**	**1**	**108**	**N_1**	**C:2b:NST**	
918077‡		**3**	**2**	**1**	**3**	**2**	**2**	**4**	**1**		**N_1**	**C:2b:NST**	
9121019‡		**3**	**2**	**1**	**3**	**2**	**2**	**4**	**1**		**N_1**	**C:2b:NST**	
913369‡		**3**	**2**	**1**	**3**	**2**	**2**	**4**	**1**		**N_2**	**C:2b:NST**	
	1434	3	2	1	3	2	2	4	1		N_5	C:2b:NST	
	886	5	2	1	3	2	5	2	1	109	AI	B:1:P1.6	
	1325	5	2	1	3	2	5	2	1		AI	B:1:P1.6	
	25	3	2	1	3	2	5	1	1	110	K	B:4:NST	
	526	3	2	1	2	2	3	1	1	111	G	B:15:P1.12	18
9208301		6	2	1	2	2	2	1	1	112	AB_2	B:4:P1.14	
	559	4	2	1	3	2	2	2	1	113	AJ_2	B:NT:P1.7	19
	891	4	1	1	2	2	2	2	1	114	AM	B:4:P1.15	
	903	4	1	1	1	2	4	2	1	115	R_2	B:NT:NST	20
	861	4	1	1	1	2	4	1	1	116	Z	B:NT:NST	
	1076	4	2	1	1	2	5	2	1	117	I_7	B:15:P1.14	
	1449	4	1	1	1	2	5	2	1	118	AR_2	B:NT:NST	
	1589	4	2	1	2	3	4	2	1	119	U_1	Y:4:P1.15	—
	829	1	1	1	2	2	3	3	1	120	B_2	B:4:P1.2	21
891529§		**1**	**1**	**1**	**2**	**2**	**4**	**1**	**1**	**121**	**A_{10}**	**B:4:P1.15**	
	1407	2	1	1	2	3	4	1	1	122	A_2	B:4:P1·15	
	1409	2	1	1	2	3	4	1	1		A_2	B:4:P1·10	
912465§		**6**	**1**	**1**	**2**	**3**	**4**	**1**	**1**	**123**	**A_5**	**B:4:P1.15**	
914324§		**6**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_5**	**B:4:P1.15**	
9205370§		**6**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_5**	**B:4:P1.15**	
87595§		**6**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_8**	**B:15:P1.7,16**	
	572	6	1	1	2	3	4	1	1		A_9	B:4:P1·1,2	
903943§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**	**124**	**A_4**	**B:NT:P1.1,7**	
881601§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_5**	**B:4:P1.15**	
902342§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_5**	**B:4:P1.15**	
913128§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_7**	**B:4:P1.15**	
905544§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_8**	**B:15:P1.7,16**	
911643§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_8**	**B:15:P1.7,16**	
908567§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_{10}**	**B:4:P1.15**	
897147§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_{10}**	**B:4:P1.14**	
888402§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_{10}**	**B:4:P1.15**	
904775§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_{11}**	**B:4:P1.15**	
9327251§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_{13}**	**B:4:P1.15**	
912396§		**4**	**1**	**1**	**2**	**3**	**4**	**1**	**1**	**125**	**A_3**	**B:4:P1.15**	

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Table 1 (*cont.*)

Isolate no.		Allele at the following enzyme locus										Serogroup: serotype: subtype	*Cluster* no.*
Patient	Carrier	G6P	ME	ADH	GD1	GD2	IDH	ALP	MDH	ET	PT		
9016914§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**AQ**	**B:NT:P1.1,7**	
90595§		**1**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_8**	**B:15:P1.7,16**	
871760§		**4**	**1**	**1**	**2**	**3**	**4**	**1**	**1**		**A_3**	**B:4:P1.15**	
	1521	4	1	1	2	3	5	1	1	126	B_1	B:4:P1.15	
	1018	4	1	1	2	3	5	1	1		AF_6	B:4:P1.12	
	950	4	1	1	2	3	5	1	1		AK_4	B:4:P1.12	
937833§		**1**	**1**	**1**	**2**	**3**	**5**	**1**	**1**	**127**	**A_5**	**B:4:P1.15**	
	494	1	1	1	2	3	3	1	1	128	A_{12}	B:15:P1.16	
	837	2	1	1	2	2	2	4	1	129	AH_3	B:NT:P1.2	22
	1336	3	1	1	2	2	1	4	1	130	AH_1	B:4:P1.2	
	863	2	1	5	3	2	2	4	5	131	AD	B:4:NST	—
	1332	4	2	5	3	3	2	2	5	132	AL_4	B:NT:P1.14	—

ET, electrophoretic type; PT, PFGE type or pulsetype; NG, non-groupable; NT, non-serotypable; NST, non-subtypable. Enzyme abbreviations: G6P, glucose 6-phosphate; ME, malic enzyme; ADH, alcohol dehydrogenase; GD1, nicotinamide adenine dinucleotide (NAD) phosphate-linked glutamate dehydrogenase; GD2, NAD-linked glutamate dehydrogenase; IDH, isocitrate dehydrogenase; ALP, alkaline phosphatase; MDH, malate dehydrogenase

* The dash indicates an unrelated ET.

Disease-associated strains correlated with the cluster A4†, ET-37 complex‡, and ET-5 complex§ [11] are indicated in **bold** type.

0·34, all members of the ET-5 complex [11] were included (criteria for interpretation of closely related ETs).

Pulsed-field gel electrophoresis

DNA for PFGE analysis was prepared as described elsewhere [11]. The inserts were subjected to digestion with *Nhe*I (Amersham Pharmacia Biotech, Uppsala, Sweden) [11]. PFGE was performed by orthogonal field-alternation electrophoresis (Gene Navigator, Pharmacia LKB Biotechnology, Uppsala, Sweden) for 18 h at a constant 250 V and 12 °C; the pulse time was 20, 10 and 2 sec for 9, 7 and 2 h, respectively. Bacteriophage lambda concatemers (New England BioLabs Inc., Beverly, MA, USA) were used as size standards.

The analysis of PFGE data was facilitated by a computerized image analysis system (Bioimage, Ann Arbor, MI, USA). A dendrogram was constructed using the unweighted pair group with mathematical averaging (UPGMA) method [30].

PFGE results were classified as proposed by Tenover et al. [31]. Each unrelated pattern or pulsetype (PT) (differing by $\geqslant 7$ bands) was assigned a letter code (A, B, C, etc), in alphabetical order according to the position of the PTs in the dendrogram, whereas the groups of related patterns (differing by $\leqslant 6$ bands) were given a single common letter code subdivided by a number code (D_1, D_2, D_3, etc). Within a subdivision (e.g. D_1) all the strains shared a given PT.

RESULTS

Serogroup/serotype/subtype, MEE and PFGE data from 191 carriers and 30 disease-associated *N. meningitidis* strains are listed in Table 1.

Phenotypic characteristics of isolates

Of the 191 strains isolated from carriers, 44 (23%) were non-groupable (NG) and the remaining were assigned to one of the following serogroups: B (n = 133, 69·6%), C (n = 11, 5·8%) or Y (n = 3, 1·6%). The 30 strains from patients were assigned to either serogroup B (n = 25, 83%) or C (n = 5, 17%).

Six serotypes were represented among 142 (74%) serotypable carrier isolates; serotype 4 predominated among both serogroup B (62/133, 47%) and NG isolates (13/44, 29·5%) and serotype 2b prevailed among serogroup C (8/11, 73%). Serotypes 4 (53%) and 2b (20%) were also the most frequent of the four serotypes represented in the patient isolates.

Sixteen subtypes were identified among 128 (67%) subtypable carrier isolates; subtype P1.6 (48/191;

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Table 2. *Phenotypes of* Neisseria meningitidis *strains recovered from carriers* (n = *191*) *and from patients* (n = *30*) *in Cerdañola, Spain*

	No. of strains (%)			No. of strains (%)	
Phenotypes*	Carriers	Patients	Phenotypes*	Carriers	Patients
B:4:P1.6	16 (8·3)		B:NT:P1.9	2 (1·0)	
B:4:P1.15	7 (3·6)	14 (46.6)	B:NT:P1·1,7		2 (6·6)
B:4:P1.14	6 (3·1)	2 (6.6)	B:NT:P1·14	2 (1·0)	
B:4:P1.9	4 (2·0)		B:NT:P1.7	1 (0·5)	
B:4:P1.4	5 (2·6)		B:NT:NST	12 (6·2)	1 (3·3)
B:4:P1.1,7	1 (0·5)		C:2b:P1.2		1 (3·3)
B:4:P1.1,2	5 (2·6)		C:2b:P1.1,2	1 (0·5)	
B:4:P1.2	2 (1·0)		C:2b:NST	7 (3·6)	4 (13·3)
B:4:P1.12	2 (1·0)		C:NT:P1.1,2	1 (0·5)	
B:4:P1.10	1 (0·5)		C:NT:P1.6	2 (1·0)	
B:4:NST	13 (6·8)		Y:14:P1.15	1 (0·5)	
B:1:P1.6	6 (3·1)	1 (3·3)	Y:4:P1.6	1 (0·5)	
B:1.P1.14	2 (1·0)		Y:NT:P1.6	1 (0·5)	
B:1:P1.10	1 (0·5)		NG:4:P1.14,16	1 (0·5)	
B:1:P1.15	1 (0·5)		NG:4:P1.15	3 (1·5)	
B:1:P1.2	1 (0·5)		NG:4:P1.6	2 (1·0)	
B:1:P1.13	1 (0·5)		NG:4:P1.1,6	1 (0·5)	
B:1:NST	12 (6·2)		NG:4:P1.2	1 (0·5)	
B:14:P1.1,7	1(0·5)		NG:4:P1.9	1 (0·5)	
B:14:P1.2	1(0·5)		NG:4:P1.7,9	1 (0·5)	
B:14:P1.4	1(0·5)		NG:4:P1.14	1 (0·5)	
B:14:NST	3 (1·5)		NG:4:NST	2 (1·0)	
B:15:P1.15	2 (1·0)		NG:1:P1.6	1 (0·5)	
B:15:P1.7,16	3 (1·5)	4 (13·3)	NG:1:P1·10	1 (0·5)	
B:15:P1.6	6 (3·1)		NG:1:NST	1 (0·5)	
B:15:P1.16	1 (0·5)		NG:15:P1.6	3 (1·5)	
B:15:P1.12	1 (0·5)		NG:2a:P1.1,7	1 (0·5)	
B:15:P1.14	1 (0·5)		NG:2a:NST	2 (1·0)	
B:2b:P1.10	1 (0·5)	1 (3·3)	NG:2b:NST	3 (1·5)	
B:NT:P1.16	2 (1·0)		NG:NT:P1.6	7 (3·6)	
B:NT:P1.6	3 (1·5)		NG:NT:P1.15	3 (1·5)	
B:NT:P1.2	3 (1·5)		NG:NT:P1.14	1 (0·5)	
B:NT:P1.10	1 (0·5)		NG:NT:NST	8 (4·1)	

* NG, non-groupable; NT, non-serotypable; NST, non-subtypable.

25%) was common. Seven subtypes were observed among patient isolates, with a low frequency of subtype P1.6 (1/30, 3%) and a high frequency of subtype P1.15 (14/30, 47%).

Among 191 carrier strains, 64 antigenic combinations or phenotypes were detected, none of which accounted for more than 9% of the isolates. In contrast, 22 (73%) of 30 patient strains belonged to phenotypes B:4:P1.15 or B:15:P1.7, 16 ($n = 14$ and 4 respectively), and C:2b:NST ($n = 4$), which were exclusively associated with the hyper-virulent lineages ET-5 complex and ET-37 complex [11]. In contrast, these pathogenic phenotypes amounted to 17 (9%) carrier strains (Table 2).

Multilocus genotype analysis

All of the eight enzyme loci were polymorphic for 3 (GD2)–6 (GD6) alleles, with a mean of 4·6 alleles per locus. MEE revealed 132 ETs: 127 ETs among the 191 carrier isolates and 10 ETs among the 30 isolates from patients. The isolate/ET ratio was higher in patients (3·0) than in carriers (1·5). Fifty percent (5/10) and 28% (36/127) of ETs from patients and carriers were represented by multiple isolates, containing 83% (25/30) and 53% (101/191) of all strains, respectively. Of these ETs, only five (ETs 23, 43, 82, 108 and 123) were recovered from both patients and carriers.

The genetic relationships revealed by MEE within the overall *N. meningitidis* population are shown in

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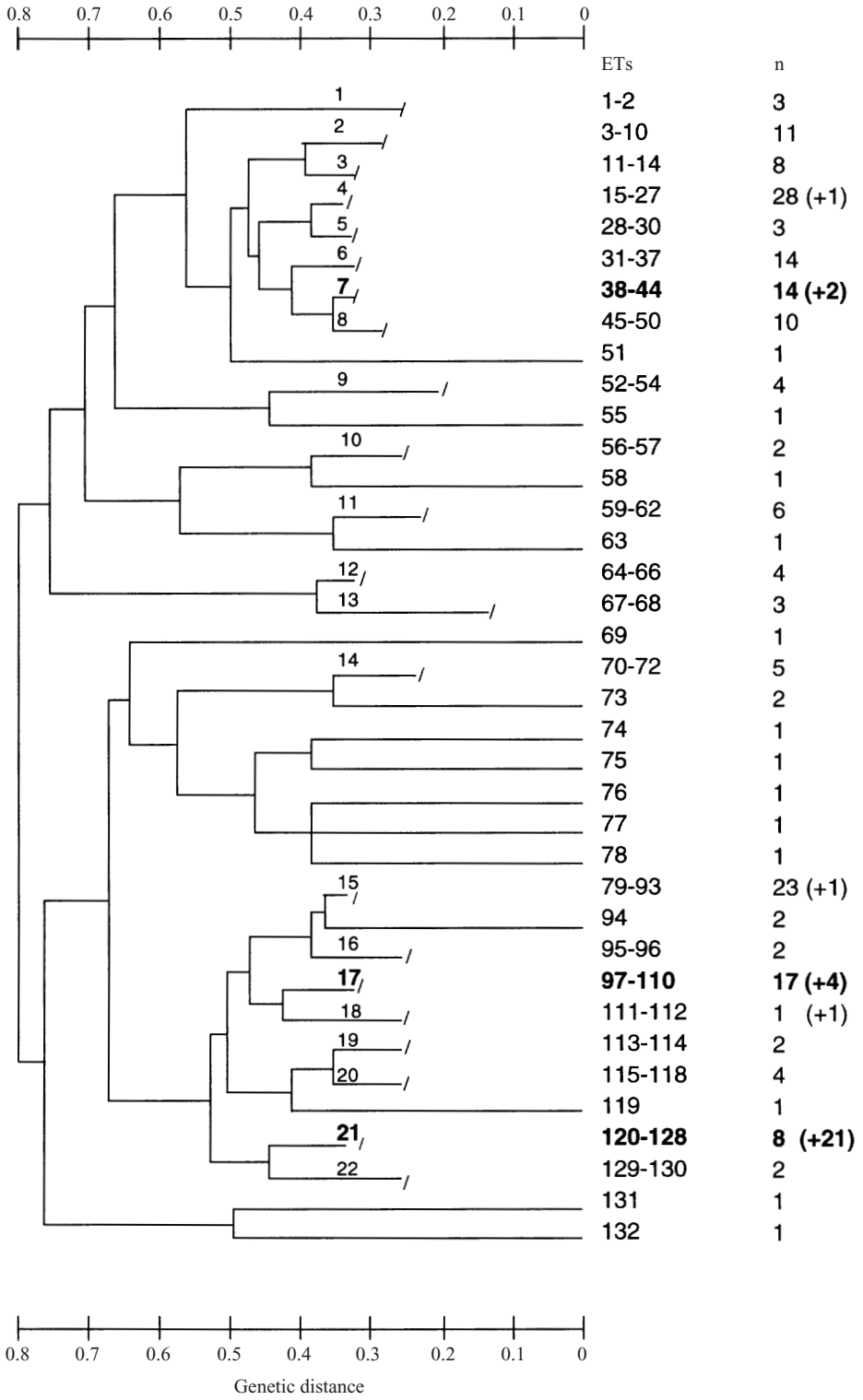

Fig. 1. Dendrogram showing genetic relationships among 132 electrophoretic types (ETs) of *Neisseria meningitidis* strains isolated from asymptomatic carriers and MD cases. ETs are sequentially numbered from top to bottom in the order of their listing in Table 1. Clusters represented by multiple ETs diverging at a genetic distance of $\leqslant 0{\cdot}34$ were truncated. There were 22 clusters (1–22). ET numbers and number of carrier and patient isolates (n) in each cluster are indicated (the number of patient isolates are shown in parentheses). Clones of the ET-5 complex, ET-37 complex and cluster A4 form cluster 21, 17 and 7, respectively.

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Table 3. *Number* (%) *of carrier and patient isolates associated with hyper-virulent lineages by multilocus enzyme electrophoresis* (*MEE*), *pulsed-field gel electrophoresis* (*PFGE*) *and by both methods together, classified by phenotypes**

	Hyper-virulent lineages†								
	ET-5 complex			ET-37 complex			Cluster A4		
Source	MEE	PFGE	MEE/PFGE	MEE	PFGE	MEE/PFGE	MEE	PFGE	MEE/PFGE
Carriers									
B/NG:4:P1.15	2	6	1	—	—	—	—	—	—
B:15:P1.7,16	—	3	—	—	—	—	—	—	—
C/NG:2b:NST	—	—	—	3	9	2	6	—	—
Others	6	6	3	14	2	—	8	3	2
Total ($n = 191$)	8 (4%)	15 (8%)	4 (2%)	17 (9%)	11 (6%)	2 (1%)	14 (7%)	3 (1·6%)	2 (1%)
Patients									
B:4:P1.15	14	14	14	—	—	—	—	—	—
B:15:P1.7,16	4	4	4	—	—	—	—	—	—
C:2b:NST	—	—	—	4	4	4	—	—	—
Others	3	2	2	—	—	—	2	2	2
Total ($n = 30$)	21 (70%)	20 (67%)	20 (67%)	4 (13%)	4 (13%)	4 (13%)	2 (7%)	2 (7%)	2 (7%)

* Phenotypes B:4:P1.15/B:15:P1.7,16 and C:2b:NST were characteristic of the ET-5 complex and ET-37 complex in our area, respectively [11]. Non-groupable (NG) carrier isolates showing these serotype:subtype combinations are included.
† ET-5 complex corresponds to: ETs 120–128; PTs A_1–A_{14}. ET-37 complex corresponds to: ETs 97–110; PTs N_1–N_5. Cluster A4 corresponds to: ETs 38–44; PTs AO_1–AO_3 (see Table 1 and Figs 1 and 2).

Figure 1. At a genetic distance 0·34, all patient strains and 178 (93%) of 191 carrier isolates were identical or related to another. Of these 178 carrier strains, 4 were classified into 2 unrelated ETs with > 1 isolate (ETs 73 and 94) and the remaining 174 into 22 clusters of related ETs (range 2–15 ETs, average 5 ETs), with at most 28 (15%) carrier strains per cluster. Eight (4%) carrier isolates belonged to the ET-5 complex (ETs 120–128), accounting for 21 (70%) of 30 sporadic MD cases in the study population [11]. Of these 8, only 2 (25%) were B:4:P1.15. Seventeen (9%) and 14 (7%) carrier isolates belonged to the ET-37 complex (ETs 97-110) and cluster A4 (ETs 38–44), which accounted for 4 (13%) and 2 (7%) of the disease cases, respectively. Only 3 (18%) of these 17 carrier strains related to ET-37 complex were C/NG:2b:NST and 8 of the 14 (57%) strains that belonged to cluster A4 were serotype 2b, which is characteristic of this lineage (Table 3) [16].

Macrorestriction fingerprint of isolates

When PFGE was performed using the endonuclease *Nhe*I 6–13 fragments were detected per isolate. PFGE distinguished 134 PTs; 124 PTs among the 191 carrier isolates and 16 PTs among the 30 isolates from clinical cases. The isolate/PT ratio of patients (1·88) was almost as low as that of carriers (1·54). Only five PTs (PTs A_5, A_8, N_2, AO_1 and AP_1) included both carrier and patient isolates. At a coefficient of similarity of $\geqslant 70\%$ (i.e. $\leqslant 6$ different bands) (Fig. 2), all but 1 patient strain and 177 (92·6%) of 191 carrier isolates were identical or related to another. Of these 177 carrier strains, 5 were distributed into 2 unrelated PTs with > 1 isolate (PTs K, AI) and the remaining 172 into 33 clusters of related PTs (range: 2–14 PTs, average: 3.5 PTs), with at most 15 carrier strains per cluster. Specifically, these 15 (8%) carrier strains were associated with those patient strains that belonged to ET-5 complex (PTs A_1–A_{14}), of which 9 (73%) were either B/NG:4:P1.15 ($n = 6$) or B:15:P1.7,16 ($n = 3$). Eleven (6%) carrier isolates were associated with those patient strains related to ET-37 complex (PTs N_1–N_5), of which 9 (82%) were C/NG:2b:NST. Of the 3 (1·6%) carrier strains associated with cluster A4 (PTs AO_1–AO_3), 2 were serotype 2b. Only 8 (4%) were assigned to the 3 pathogenic lineages by both PFGE and MEE (Table 3).

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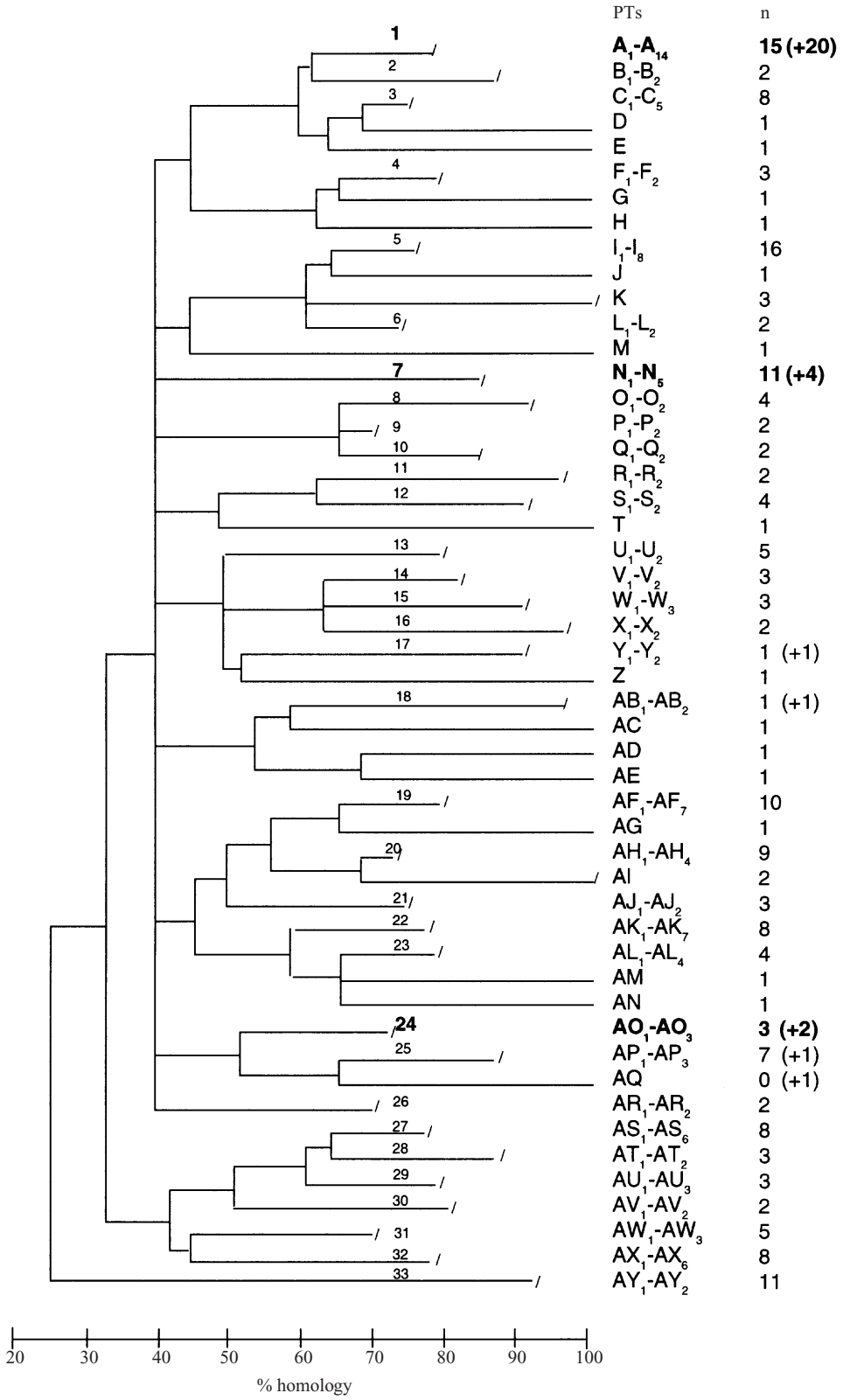

Fig. 2. Dendrogram showing the relationships between the Dice coefficients for the 221 *N. meningitidis* strains as estimated by PFGE analysis. The dendrogram was generated by the UPGMA method of clustering. Numbers 1–33 indicate clusters of pulse types (PTs) that diverge at coefficient of similarity $\geqslant 70\,\%$. Clones of the ET-5 complex, ET-37 complex and cluster A4 form cluster 1, 7 and 24, respectively. PT designations and numbers of carrier and patient isolates (*n*) in each cluster are indicated (the numbers of patient isolates are shown in parentheses).

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Table 4. *Electrophoretic types (ETs) represented by multiple isolates from carriers and patients associated with identical or related PFGE types**

Source	ET no.†	No. of ETs	No. of isolates (%)	
			Total	With identical phenotype
Carriers	2, 6, 9, 11, 19, 20, 21, 22, 41, 42, 46, 47, 60, 66, 67, 70, 73, 82, 89, 94, 105, 109, 122	23	57 (30)	27 (14)
Patients	43, 108, 123, 124, 125	5	24 (80)	19 (63)

* PFGE types within each ET, see Table 1.
† ET numbers are as set out in Table 1

Comparison of typing systems for both carrier and sporadic meningococcal strains

Twenty-three ETs (23/36) recovered from more than 1 carrier and all 5 ETs causing $\geqslant 2$ sporadic cases also showed identical or related PFGE types, accounting for 57 (30%) of 191 carrier strains and 24 (80%) of 30 patient strains, respectively. These numbers decreased to 27 (14%) and 19 (63%) respectively when an identical phenotype was considered (Table 4). Of those 57 healthy carriers, an epidemiological connection (defined as isolation in the same centre) was detected in 21 (37%), which harboured isolates distributed into 9 of those 23 ET type–PFGE type combinations. Conversely, 2 of these 9 combinations and the remaining 14 were identified in more than 1 centre.

The remaining 44 (23%; 44/191) carrier strains belonging to multiple ETs and 1 (3%; 1/30) patient strain, i.e. an ET-5 complex strain, had PFGE types that differed by $\geqslant 7$ bands. Conversely, 59 (31%; 59/191) carrier isolates with distinct ET types were indistinguishable by PFGE. Of the 175 PFGE clustered carrier isolates, only 88 (46%; 88/191) were also related as determined by MEE (taking a genetic distance $\leqslant 0{\cdot}34$ by MEE and coefficient of similarity $\geqslant 70\%$ by PFGE as indicators of relatedness).

DISCUSSION

During the 7-year survey, when no localized outbreaks or case clusters were reported, the vast majority of isolates causing sporadic MD (70%) belonged to the ET-5 complex by both MEE and PFGE and ET-37 complex and cluster A4 were responsible for 13% and 6·6% respectively of sporadic MD cases. Thus, most endemic cases of MD were caused by strains of a limited number of genetically defined clonal groups [11] that were also responsible for MD during hyperendemic periods [15] or epidemic outbreaks [16, 19]. These data are consistent with a gradual evolution and a slow spread of virulent meningococcal clones in geographically defined human populations over years [24, 32]. This concept is supported by our finding of a limited number of these three hypervirulent clonal groups in the nasopharynx of symptom-free carriers (less than 20% and similar to the 16% reported by Caugant et al. [25]).

Although MEE and PFGE gave almost identical identification of virulent clones in patient isolates, concordance in carriers was less close. MEE and PFGE assigned 20% and 15% of carrier isolates to the hyper-virulent lineages respectively but only 4% were assigned by both methods. In an attempt to resolve this discrepancy, we compared phenotypes. A correlation has been established between phenotypes and pathogenic clones in limited geographical areas [32–34]. In our area, B:4:P1.15/B:15:P1.7,16 and C:2b:NST characterized the ET-5 complex and ET-37 complex, respectively. These phenotypes were more prevalent among carriers associated with these pathogenic clones by PFGE (69%) than by MEE (20%). Thuse, PFGE may be the more reliable marker to determine the dissemination of virulent clones in open communities.

Although MEE and PFGE give varying results on the genetic clustering of the overall *N. meningitidis* population, approximately 90% of this population was distributed into 22 and 33 clusters of related ETs and PTs, respectively. Except for one patient who had

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a unique unrelated PT, all patient strains were identical or related (by both techniques) to other isolates from carriers, who had had no known contact with MD patients. Thus, neither technique split patient and carrier isolates into two separate subpopulations. However, patient strains were phenotypically and genotypically more homogeneous than carrier strains.

Almost 75% of invasive strains belonged to the three phenotypes characteristic of the ET-5 complex and ET-37 complex in our area (as mentioned above). In contrast, these phenotypes constituted only 9·1% of carrier strains, amongst which no phenotype had a prevalence higher than 9%. This increased antigenic diversity among carrier isolates was mainly due to subtype rather than serogroup : serotype. These results are in agreement with the hypothesis that the pathogenic meningococcal population is structured in discrete subtype combinations by the action of the human immune system against the polymorphic epitopes of the outer membrane protein PorA [35]. Moreover, the proportion of ETs with more than one isolate per ET was higher among patients than carriers, as reflected by the twofold increase in the isolate/ET ratio.

PFGE confirmed all the related patient strains defined by MEE but one. In addition, PFGE identified subgroups of related PTs within the vast majority of multiple ETs, suggesting that it can resolve the microvariation within *N. meningitidis* hyper-virulent lineages circulating within a geographic area [23, 24]. Conversely, PFGE corroborated the related strains defined by MEE in only almost half of the carrier strains included in multiple ETs. Thus, by two different typing systems, 30% of carrier isolates were assigned to clonal groups including identical genotypes by MEE and identical or closely related by PFGE. This percentage was increased to 46% when carrier isolates related at a genetic distance $\leqslant 0{\cdot}34$ by MEE were included.

Since recombination did not prevent the identification of the hyper-virulent clones among patients (previously recovered from other countries since the 1980s) by both typing techniques, our data provide evidence for a considerable stability of the clones associated with disease. In contrast, despite the limited number of clonal groups (by both techniques) among the carrier population, more than half of the nasopharyngeal isolates from healthy carriers showed inconsistent results by PFGE and MEE, i.e. some isolates with indistinguishable PFGE patterns were unrelated by MEE and vice versa. These data support the occurrence of frequent recombination in meningococci [36–38], making it difficult to reconstruct the network of relationships between carrier isolates and those that cause disease, as mentioned above. Therefore, phylogenetic inferences from the general meningococcal population using MEE should be interpreted cautiously.

Although virulent clonal groups arise, diversify and decay throughout the years through genetic recombination, immune selection and mutation [15, 18, 24, 39, 40], the dynamics of meningococcal carriage remains shrouded. Our data show that non-pathogenic clusters of genetically related carrier isolates were represented by no more than 15% of strains and pathogenic clusters (i.e. clusters associated with hypervirulent lineages) by no more than 9% of carrier strains, and that transmission is infrequent in the overall population of meningococci. Moreover, we failed to detect any epidemiological connection between 63% of carriers of strains with the same ET and PFGE patterns, suggesting that these clones are commonly found among carriers, and supporting the concept of low transmission rates. Similarly, in a randomly sampled carrier population, Caugant et al. [41] were unable to identify connections between carriers included in multiple ETs and reported that some ETs were repeatedly recovered in several surveys performed in Norway [25]. Our data suggested stability not only of virulent meningococcal clones, but also of carrier clones as defined by two molecular typing methods (i.e. nearly one-third of all endemic strains recovered from symptom-free carriers in our geographically defined human population). This hints at a weakly clonal structure for the *N. meningitidis* population as a whole. However, the lack of data on the rates of recombinational exchanges makes it impossible to assess the persistence of carrier clonal groups in the population.

We have provided evidence that the use of multiple markers and, especially, the analysis of overall populations of strains, provide a better understanding of the dynamics of meningococcal carriage and the association between such carriage and sporadic meningococcal disease. Accurate strain characterization will inform future studies on both meningococcal population structure and bacterial virulence, and will be augmented by the recently developed technique of MLST and the publication of the complete genome sequence of *N. meningitidis* strain MC58.

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