MOLECULAR EPIDEMIOLOGY

Interpreting the rising incidence of meningococcal disease in Belgium: the contribution of molecular typing

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During a period of increasing meningococcal disease incidence in Belgium, all 538 serogroup B and all 87 serogroup C strains isolated between 1996 and 1998 were investigated by PCR with the arbitrary primer D8635, which is able to identify lineage III strains. In all, 399 strains (64%) were attributed to lineage III on the basis of PCR-based typing. Since their introduction in the Belgian population in the early 1990s, lineage III strains have become increasingly variable in phenotype. Currently, they are represented by strains belonging to 38 different phenotypes, of which 25 were not found in the period 1990–1995. The 87 serogroup C strains were further investigated by pulsed-field gel electrophoresis (PFGE), and a subset of 30 strains was also investigated by multilocus sequence typing (MLST). Strains of phenotype C:2b:P1.5,2, which currently constitute the majority of the serogroup C strains, were demonstrated to belong to cluster A4. Comparison of the discriminatory ability of D8635-primed PCR, PFGE and MLST revealed that D8635-primed PCR was the least discriminatory method and PFGE the most discriminatory method. However, the MLST data were more readily interpreted than the PFGE fingerprint patterns and can be compared easily with data obtained in other studies. In conclusion, the ongoing increase of meningococcal disease in Belgium could be attributed not only to the further expansion of lineage III, but also to the introduction of C:2b:P1.5,2 strains of cluster A4 in to the Belgian population.

Introduction

In Belgium the incidence of meningococcal disease increased from 0.8 cases per 10⁵ inhabitants in 1990 to 2.24 per 10⁵ inhabitants in 1998. In particular, the incidence in the age group 15–19 years increased to 7.2 per 10⁵ inhabitants in 1998. Investigation of a set of 420 clinical strains isolated during the period 1990–1995 revealed that this increase was due to the introduction of strains of lineage III in the susceptible Belgian population since the early 1990s [1]. Strains of lineage III were first encountered in the Netherlands and have caused disease there since the early 1980s [2]. The epidemic clone could be identified by oligonucleotide D8635-primed PCR, and encompassed mainly the

phenotypes B:4:P1.4 and B:non-typable (NT):P1.4, although strains of several other phenotypes were also encountered [1, 3]. Strains with phenotypes B:4:P1.4 and B:NT:P1.4 currently cause 39.7% of the cases of meningococcal disease in Belgium.

From 1997 onwards, the proportion of serogroup B strains decreased, while that of serogroup C strains increased in Belgium. In 1996, serogroup C constituted 7.0% of the strains and in 1998 it represented 20.8%. The subtype P1.5,2 sharply increased from 7% in 1996 to 48% in 1998. Strains of phenotype C:2b:P1.5,2 currently constitute the majority of the serogroup C strains (28%), followed by C:2a:P1.5,2 (15%).

The aim of the present study was to investigate the molecular epidemiology of meningococcal disease in Belgium from 1996 to 1998 by the use of several genotyping methods, including arbitrarily primed PCR, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). A secondary aim was to

Received 15 Jan. 2001; revised manuscript accepted 11 April 2001.

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compare the discriminatory ability of the different genotyping methods for serogroup C meningococci.

Materials and methods

Bacterial strains

Between Jan. 1996 and Dec. 1998, 685 strains of *Neisseria meningitidis* isolated from patients with meningococcal disease were received at the Belgian Meningococcal Reference Centre. The serogroup was determined, and serotyping and subtyping were performed as described previously [3].

PCR-based typing

A subset of 625 strains, comprising all 538 serogroup B strains and all 87 serogroup C strains, was investigated by D8635-primed PCR. DNA extraction and PCR with the arbitrary primer D8635 were performed as described previously [1].

PFGE-based typing

All 87 serogroup C strains were further investigated by PFGE with the restriction enzyme *SpeI*. PFGE was performed as described previously [1].

MLST

The MLST procedure was applied to a subset of 30 serogroup C strains, representing the different PCR subtypes. The sequences of fragments from the *abcZ* (putative ABC transporter), *adk* (adenylate kinase), *aroE* (shikimate dehydrogenase), *fumC* (fumarate hydratase), *gdh* (glucose-6-phosphate dehydrogenase), *pdhC* (pyruvate dehydrogenase subunit) and *pgm* (phosphoglucosyltransferase) genes were determined as described on the MLST website (<http://mlst.zoo.ox.ac.uk>) and compared to the sequences of known alleles. A novel allele at the *aroE* locus was assigned the allelic designation *aroE120*. New combinations of the seven alleles were reported to the MLST website and were assigned the designations ST1025–ST1035.

Results

PCR-based typing

A total of 399 strains (64%) exhibited the epidemic pattern 2723 or a very similar pattern (Table 1), associated with lineage III. A previous study [1] demonstrated that the epidemic clone of lineage III consisted of strains belonging to 16 different phenotypes (B:4:P1.4; B:NT:P1.4; B:4:P1.13; B:NT:NST; B:1:P1.4; B:NT:P1.5,2; B:4:P1.1,7; B:14:P1.4; B:14:P1.15; B:4:P1.6; B:4:P1.7; B:NT:P1.7; B:4:NST; C:4:P1.4 and NG:NT:NST). In the present investigation, all these phenotypes except three (B:4:P1.6; B:NT:P1.7; and NG:NT:NST) were found again.

However, the present study also found another 25 serotype:serosubtype combinations with the epidemic pattern 2723. All these additional combinations were of serogroup B (Table 1). Serotypes B:4:P1.4 and B:NT:P1.4 constituted 72.7% of the pattern 2723 strains. The remaining 27.3% of the pattern 2723 strains had other phenotypes.

Amongst the 87 serogroup C strains, 14 different pattern types (A–N) could be distinguished, of which two (B and C) comprised the majority of the strains. Nine pattern types (F–N) were represented by a single strain. All C:2b:P1.5,2 strains belonged to pattern type C (Table 2).

PFGE-based typing

Among the serogroup C strains the same clusters were found as with PCR-based typing, but PFGE was more discriminatory. The 38 strains of PCR pattern B showed 25 PFGE patterns, each represented by one-to-three strains. Among the 25 isolates with PCR pattern C, 8 PFGE patterns were seen. All strains with phenotype C:2b:P1.7,1 had an indistinguishable PFGE pattern and 13 (81%) of the 16 strains with phenotype C:2b:P1.5,2 also had an indistinguishable PFGE pattern. The remaining three C:2b:P1.5,2 strains were, respectively, closely related (two strains) and possibly related (one) [4] to the other 13 C:2b:P1.5,2 strains. The single C:2b:P1.2 strain of PCR pattern C had the same PFGE pattern as the C:2b:P1.5,2 strains. For PCR pattern D, six of eight C:NT:NST strains and the one C:4:P1.7 strain also showed an indistinguishable pattern. The three PCR pattern E strains each showed a different PFGE pattern. The strains belonging to PCR pattern A and PCR patterns F–N all had different PFGE patterns.

MLST

The 30 strains were assigned to 21 sequence types (ST), of which 11 were new (designated ST1025–ST1035) (Table 3). The C:4:P1.4 strain of PCR pattern A (Table 2) belonged to ST42, which represents lineage III. The other PCR pattern A strain had a new ST (ST1028) which differed from ST42 at the *abcZ* and *fumC* loci. The C:2a:NST; C:2b:P1.5; C:2b:NST and C:NT:P1.2,5 strains belonging to PCR pattern B were all ST11 (Table 2), the ST most commonly associated with strains of the ET-37 complex. PFGE of the four ST11 strains revealed three different PFGE patterns. The C:2a:NST; C:2a:P1.5 and C:2a:P1.5,2 strains of PCR pattern B belonged to three novel STs labelled ST1025, ST1026 and ST1027, each differing from ST11 at a single locus (*fumC* or *pgm*). The pattern C strains belonged to ST153 or ST66, both representing the A4 cluster. The two ST153 strains showed a different PFGE pattern. Among the three ST66 strains, two PFGE patterns could be distinguished.

Table 1. Distribution of pattern 2723, as determined by D8635-PCR, among 625 *N. meningitidis* strains isolated in Belgium between 1996 and 1998

Strain and phenotype*	Number of strains with D8635-primed PCR DNA amplification pattern				
	pattern 2723				
	pattern 2723	absence of one band	presence of an additional band	difference in density of one band	other patterns
B:1:P1.4	4	0	1	1	0
B:1:P1.9	1	0	0	0	0
B:4:P1.1	2	0	0	0	2
B:4:P1.1,7	8	2	0	0	3
B:4:P1.2,5	2	0	0	0	12
B:4:P1.4	237	5	4	23	3
B:4:P1.4,7	6	0	0	1	1
B:4:P1.5	1	0	0	0	0
B:4:P1.7	4	0	1	0	2
B:4:P1.7,14	1	0	0	0	0
B:4:P1.9	1	0	0	0	0
B:4:P1.12,13	1	0	0	0	0
B:4:P1.13	11	1	2	1	2
B:4:P1.15	4	0	0	0	20
B:4:P1.16	2	0	0	0	1
B:4,21:P1.4	0	0	0	1	0
B:4:NST	9	1	2	0	2
B:14:P1.1,7	1	2	0	0	1
B:14:P1.2,5	1	0	0	0	0
B:14:P1.4	1	0	0	0	3
B:14:P1.5	3	0	0	0	1
B:14:P1.13	1	1	0	0	2
B:14:P1.15	4	0	1	0	0
B:14:P1.16	1	0	0	0	0
B:14:NST	1	0	0	0	0
B:15:P1.4	3	0	0	0	0
B:15:P1.4,7	1	0	0	0	0
B:21:P1.4	0	0	0	1	0
B:NT:P1.1	2	0	0	0	0
B:NT:P1.1,7	2	0	0	0	2
B:NT:P1.2,5	2	0	0	0	0
B:NT:P1.4	18	0	0	3	2
B:NT:P1.4,7	1	0	0	0	0
B:NT:P1.6	0	0	0	1	2
B:NT:P1.9	0	0	0	1	2
B:NT:P1.13	1	0	0	0	0
B:NT:NST	4	0	1	0	4
C:4:P1.4	1	0	0	0	1
Others	0	0	0	0	158
Total	342	12	12	33	226

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

Strains of PCR pattern D belonged to two novel STs labelled ST1031 and ST1032, differing from one another solely at the *aroE* locus. The three ST1031 isolates presented two different PFGE patterns. One of the ST1031 isolates (C:NT:NST) showed the same PFGE pattern as the ST1032 isolate (C:NT:NST).

Discussion

Since 1990, the incidence of meningococcal disease in Belgium has increased almost three-fold. This increase is mainly due to genotypically related serogroup B isolates, first identified in the Netherlands [2] and designated lineage III strains [1]. Recently, lineage III strains have been isolated in many other western European countries, including Iceland, Finland, Norway, the UK, Greece and Austria. However, they are

not restricted to Europe. Recently, a strain belonging to lineage III was found in Chile, and in New Zealand lineage III strains are causing an ongoing epidemic [5]. As lineage III strains are particularly associated with an increased incidence of disease, this lineage is termed a 'hyper-virulent lineage' [6].

Lineage III encompasses mainly the phenotypes B:4:P1.4 and B:NT:P1.4. The proportion of these phenotypes further increased from 36.0% in 1995 [3] to 39.7% in 1998. However, lineage III is not confined to these phenotypes only, as was demonstrated in an earlier study [1]. In the present investigation B:4:P1.4 and B:NT:P1.4 strains constituted 72.7% of the pattern 2723 strains. The remaining 27.3% belonged to 36 different phenotypes. Of these, 25 phenotypes were not encountered in the previous years [1]. Thus, lineage III strains show rapid evolutionary changes in their

Table 2. D8635-primed PCR for all 87 serogroup C strains isolated in Belgium during 1996–1998, and MLST of a selection of 30 serogroup C strains representing the different PCR subtypes

Type D8635-primed PCR (number of strains)	Phenotype (n)	Sequence type (n)
A* (2)	C:4:P1.4 (1)	42 (1)
	C:NT:NST (1)	1028 (1)
B (38)	C:2a:NST (7)	1025 (1) and 11 (1)
	C:2a:P1.5 (14)	1027 (1)
	C:2a:P1.5,2 (11)	1026 (1)
	C:2b:P1.5 (3)	11 (1)
	C:2b:NST (1)	11 (1)
	C:NT:P1.2,5 (2)	11 (1)
C (25)	C:2b:NST (1)	153 (1)
	C:2b:P1.7,1 (6)	66 (1)
	C:2b:P1.2(1)	66 (1)
	C:2b:P1.5,2 (16)	66 (1)
	C:2b:P1.10 (1)	153 (1)
D (10)	C:4:P1.4 (1)	
	C:4:P1.7(1)	1031 (1)
	C:NT:NST (8)	1031 (2) and 1032 (1)
E (3)	C:14:P1.7,1 (1)	1034 (1)
	C:14:P1.7(1)	1034 (1)
	C:2a:P1.5,2 (1)	1033 (1)
F (1)	C:4:P1.1 (1)	206 (1)
G (1)	C:NT:P1.15 (1)	1030 (1)
H (1)	C:21:P1.1 (1)	1029 (1)
I (1)	C:NT:P1.5,2 (1)	60 (1)
J (1)	C:21:P1.15 (1)	467 (1)
K (1)	C:NT:P1.1 (1)	103 (1)
L (1)	C:4:NST (1)	461 (1)
M (1)	C:4,21:P1.5 (1)	1035 (1)
N (1)	C:15:P1.7,16 (1)	32 (1)

*Corresponds with pattern 2723

Table 3. Novel STs found among Belgian serogroup C strains

Phenotype	ST number	MLST allele number						
		<i>abcZ</i>	<i>adk</i>	<i>aroE</i>	<i>fumC</i>	<i>gdh</i>	<i>pdhC</i>	<i>pgm</i>
C:2a:NST	1025	2	3	4	3	8	4	2
C:2a:P1.5,2	1026	2	3	4	3	8	4	7
C:2a:P1.5	1027	2	3	4	55	8	4	6
C:NT:NST	1028	3	6	9	2	9	6	9
C:21:P1.1	1029	6	6	9	21	9	73	9
C:NT:P1.15	1030	8	5	12	21	3	22	7
C:NT:NST; C:4:P1.7	1031	13	5	15	9	3	8	18
C:NT:NST	1032	13	5	27	9	3	8	18
C:2a:P1.5,2	1033	14	2	120	9	3	8	18
C:14:P1.7,1; C:14:P1.7	1034	14	5	120	9	3	8	18
C:4,21:P1.5	1035	25	5	4	17	21	13	9

serotype and sero-subtype, as well as in their genotypes. This is further demonstrated by the fact that the lineage III strains isolated in the Netherlands in the early 1980s were B:4:P1.4 and belonged to the same clone, ET-24. However, after 1984 other clones appeared within the lineage, also with different serotypes and sero-subtypes [7]. In the 1990s seven new ETs of lineage III were found in Belgium, that had not previously been encountered in the Netherlands [1]. In the present study these clones were found to be still further diversified, as can be deduced from the PCR-typing data. Thus, the longer clones are prevalent in a population, the more heterogeneous they become in

phenotype and genotype. Finally, their relationships become so obscured that they may no longer be recognisable as belonging to the ancestral clone.

From 1997 a clonal replacement started to take place in Belgium. The number of serogroup B strains gradually decreased, which was associated with an increase in the number of serogroup C strains. Their prevalence rose from 7.0% in 1996 to 20.8% in 1998. *N. meningitidis* of serogroup C are associated with a higher case fatality rate than cases of serogroup B infection. The most prevalent serotype was C:2b:P1.5,2, accounting for 28% of the serogroup C strains. It is noteworthy

that C:2b:P1.5,2 strains are not new in Europe and predominated in Scotland in the 1980s [8]. Currently, strains of this phenotype are most prevalent in the southern part of Europe. In 1993, they caused an increase in the incidence of meningococcal disease in Spain, and in 1996, they accounted for 65% of the serogroup C strains in Spain [9]. Strains of phenotype C:2b:P1.5,2 have often been shown to belong to meningococcal cluster A4, as did the majority of the C:2b isolates analysed here. This same clone complex was predominant in Greece in the early 1990s, but was associated with serogroup B polysaccharide [10], as it was in several European countries in the 1970s [5]. The 87 serogroup C strains were all investigated by D8635-primed PCR and PFGE, and a selection of 30 strains was also investigated by MLST. These three genotyping techniques provide estimates of similarity between isolates by sampling many unlinked genetic regions of the chromosome and are thus more reliable than typing methods that target single genes. The MLST data confirmed that the C:2b:P1.5,2 strains indeed belonged to cluster A4. Strains of phenotype C:2a:P1.5,2, which was the second most important phenotype, were related to the globally distributed ET-37 complex.

When the discriminatory abilities of the different genotyping methods were compared, it was found that D8635-primed PCR was the least and PFGE the most discriminatory method. Indeed, strains with PCR-patterns A–E could be further subdivided by MLST. The STs could be further subdivided by PFGE. There was one exception where two strains with the same PFGE pattern showed differing STs. In comparison with PFGE fingerprint data, the MLST data were more readily interpreted and can easily be compared with data obtained in other studies. Consequently, MLST is becoming the reference method for *N. meningitidis* typing.

In conclusion, the incidence of meningococcal disease

is still increasing in Belgium. This could be attributed to both the further expansion of the epidemic clone of lineage III and to the introduction of phenotype C:2b:P1.2,5 strains of cluster A4 in the Belgian population. Further, this study illustrates the added value of genotyping in interpreting the epidemiology of meningococcal disease.

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