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Antimicrobial susceptibility, serotype and genotype distribution of meningococci in Portugal, 2001–2002

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SUMMARY

One hundred and eighteen *Neisseria meningitidis* isolates were recovered from patients with invasive meningococcal disease in Portugal, over one year. Our study was undertaken to evaluate antimicrobial susceptibility, serogroup, serotype and genotype of isolates. One quarter (24·6%) of the isolates showed moderate resistance to penicillin and 47·4% were resistant to sulphadiazine. The two most common serosubtypes were C:2b:P1.5,2 (31·3%) and B:4:P1.15 (3·4%). Half (53·6%) of the isolates with moderate resistance to penicillin were phenotype C:2b:P1.5,2 ($n=14$), C:2b:P1.2 ($n=1$) or C:2b:NST ($n=1$); Pulsed-field gel electrophoresis (PFGE) showed that all these isolates were genetically related. Multilocus sequence typing (MLST) analysis of representative clones from each PFGE pattern showed the predominance of the ST-8 complex/cluster A4 among *N. meningitidis* with moderate resistance to penicillin. This clonal complex has been principally found in Southern Europe. The apparent emergence and dissemination of the hypervirulent ST-8 complex/cluster A4 among serogroup C strains increases the need for a continued surveillance of antimicrobial susceptibility of meningococci and of genotypic markers in Portugal.

INTRODUCTION

Neisseria meningitidis is a major cause of bacterial meningitis and septicaemia, and remains a serious public health problem, particularly among young children and adolescents. In Portugal this bacterium is the major agent of meningitis in children [1].

Serogroups A, B, C, Y and W135 are responsible for most meningococcal disease in Europe [2]. Penicillin was the antibiotic of choice in the treatment of meningococcal disease, but in recent years, the emergence of *N. meningitidis* isolates with moderate resistance to penicillin [minimum inhibitory concentration (MIC) 0·12–1 μg/ml] have been described in Portugal [3] and other countries [4–6]. This moderate resistance to penicillin is due to alterations on the structure of one penicillin-binding protein (PBP2), encoded by the *penA* gene. These alterations are the result of horizontal DNA transfer from commensal *Neisseria*, probably by transformation [7].

The genetic diversity within meningococci populations needs the use of genotypic approaches to identify clonal groups. Pulsed-field gel electrophoresis (PFGE) and a recent gold standard method, multilocus sequence typing (MLST), allow us detailed characterization of circulating *N. meningitidis* and the

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knowledge of the epidemiology of meningococcal disease [8].

AIMS

The purpose of this study was to determine the serogroup, serosubtype and the susceptibility to antimicrobial agents of a collection of invasive *N. meningitidis* strains isolated over one year between September 2001 and August 2002 from a representative population of cases (approximately 7 598 000 residents, 73% of the Portuguese population) of culture-confirmed invasive meningococcal disease (IMD) in Portugal.

The patients attended 24 Portuguese hospitals. A case of IMD was defined as disease in which a *N. meningitidis* had been isolated by culture from the two normally sterile sites (blood and cerebrospinal fluid) in a resident of the surveillance area. Genotype characterization of *N. meningitidis* with moderate resistance to penicillin was also studied. The study was carried out during the first period of voluntary vaccination (since the third quarter of 2001) with conjugate C vaccine in Portugal and before the inclusion of this vaccine in the National Vaccination Plan (January 2006).

METHODS

The serogroup and MIC of penicillin from 118 isolates were determined, and serotype, serosubtype and MIC of ampicillin, cefotaxime, ceftriaxone, ciprofloxacin, rifampicin and sulphadiazine from 116 available isolates were determined, as previously described [9]. Moderate resistance was considered when isolates had an intermediate level of susceptibility. Pearson's χ^2 test with Yates correction in the 2×2 tables was used to establish association between serogroups B and C and the penicillin susceptibility. Significance level was established at 5% ($P = 0{\cdot}05$).

Sixteen available serogroup C isolates with moderate resistance to penicillin (14 available isolates of serosubtype C:2b:P1.5,2, one of serosubtype C:2b:P1.2 and one of serosubtype C:2b:NST) were investigated by PFGE with the restriction enzyme *Bgl*II (New England Biolabs, Beverly, MA, USA) as previously described [10]. Computer analysis of PFGE fingerprints patterns were analysed as previously described [11]. MLST was used to characterize seven isolates randomly selected, but representing each of the PFGE patterns. The amplification and sequencing were as described on the MLST website (http://neisseria.mlst.net) with a new set of oligonucleotides [12].

RESULTS AND DISCUSSION

In Portugal 125 cases of IMD were reported to the Compulsory Notifiable Diseases system during the period of this laboratory surveillance study [13]. We report 118 isolates with a percentage of serogroup C (51·7%) slightly higher than that of serogroup B (43·2%). A surveillance study in Portugal between 1995 and 1999 showed that 41·8% of 55 *N. meningitidis* isolates were serogroup B and 43·7% were serogroup C [9]. Our continuous surveillance between July 2000 and August 2001 confirmed the emergence of serogroup C in Portugal [9]. In recent years, other countries in Europe, including the Czech Republic, Iceland and Switzerland, also have experienced an increase of serogroup C [14].

Twenty-nine (24·6%) isolates had moderate resistance to penicillin and 35 (30·2%) had moderate resistance to ampicillin; most of isolates had MICs of ampicillin that were double those of penicillin, as already observed in Spain [6]. Fifty-five isolates (47·4%) were resistant to sulphadiazine, a phenotype which is known to be a virulent marker [15]. All isolates were fully susceptible to cefotaxime, ceftriaxone, ciprofloxacin and rifampicin; rifampicin has been used in Portugal for chemoprophylaxis. Overall, moderate resistance to penicillin was mostly associated with serogroup C ($P = 0{\cdot}04$) (Table). Twenty of the 61 isolates of serogroup C (32·8%) showed moderate resistance to penicillin, as did 14·3% of serogroup B (7/49) and 33·3% of W135 (1/3) isolates. Overall, 28 isolates with moderate resistance to penicillin were available for serotyping: 71·4% (20/28) were of serogroup C, 25·0% (7/28) of serogroup B and 3·6% (1/28) of serogroup W135. The emergence of serogroup C being associated with a large percentage of isolates with moderate resistance to penicillin has already been reported [3, 4, 6].

The distribution of serogroup, serotype and serosubtype of the 116 isolates is shown in the Table. Isolates of serogroup B included 15 different phenotypes. Serotype 4 was the most frequent (16/49, 32·7%), followed by serotype 1 (6/49, 6·1%). In other countries, such as Canada (1994–1996) serotype 4 was also prevalent [16]. In Scotland (1994–1999) serotype

Table. *Serogroup:serotype:serosubtype and susceptibility to penicillin of 116 available* N. meningitidis *isolates*

Serogroup: serotype: serosubtype*	No. (%) of isolates with MICs to penicillin (μg/ml) of†			Total no. (%)
	⩽0·06	0·125	0·25	
Serogroup B				
B:4:P1.4	3 (6·1)			3 (6·1)
B:4:P1.15	3 (6·1)	1 (2·0)		4 (8·2)
B:4:P1.14	2 (4·1)	1 (2·0)		3 (6·1)
B:15:P1.7,16	2 (4·1)			2 (4·1)
B:1:P1.13	1 (2·0)			1 (2·0)
B:1:P1.6	1 (2·0)			1 (2·0)
B:1:P1.7,1	1 (2·0)			1 (2·0)
B:2b:P1.5,2	1 (2·0)			1 (2·0)
B:4;P1.5,2	1 (2·0)			1 (2·0)
B:4:P1.7,1			1 (2·0)	1 (2·0)
B:4:P1.7,16	1 (2·0)			1 (2·0)
B:4:P1.9	1 (2·0)			1 (2·0)
B:4:P1.13	1 (2·0)			1 (2·0)
B:4:P1.16	1 (2·0)			1 (2·0)
B:14:P1.7,1	1 (2·0)			1 (2·0)
NT	22 (44·9)	3 (6·1)	1 (2·0)	26 (53·0)
Subtotal	42 (85·7)	5 (10·2)	2 (4·0)	49 (100)
Serogroup C				
C:2b:P1.5,2	22 (36·1)	12 (19·7)	3 (4·9)	37 (60·7)
C:2b:P1.2	4 (6·6)	1 (1·6)		5 (8·2)
C:2a:P1.5	4 (6·6)			4 (6·6)
C:4:P1.2	2 (3·3)			2 (3·3)
C:4:P1.1	1 (1·6)			1 (1·6)
C:4:P1.5	1 (1·6)			1 (1·6)
C:2a:P1.5,2	1 (1·6)			1 (1·6)
NT	6 (9·8)	1 (1·6)	3 (5·0)	10 (16·4)
Subtotal	41 (67·2)	14 (23·0)	6 (9·8)	61 (100)
Serogroup W135				
W135:2a:P1.5,2	1 (33·3)			1 (33·3)
NT	1 (33·3)	1 (33·3)		2 (66·7)
Subtotal	2 (66·7)	1 (33·3)		3 (100)
Serogroup Y				
Y:14:P1.5,2	1 (33·3)			1 (33·3)
Y:14:P1.5	1 (33·3)			1 (33·3)
NT	1 (33·3)			1 (33·3)
Subtotal	3 (100)			3 (100)

MIC, Minimum inhibitory concentration; NT, non-typable.

* By order of frequency.

† MIC breakpoints used to penicillin: susceptible, MIC ⩽0·06 μg/ml; moderate resistance, MIC 0·125–1 μg/ml.

4 (23·3%) was also predominant, with serotype 1 (9·0%) the next most common [17]. Of the 10 serosubtypes were identified, P1.15 (4/49, 8·2%) was the most common. The phenotype B:4:P1.15. (4/49, 8·2%) was prevalent among isolates of serogroup B; to our knowledge, this phenotype has only previously been reported in Spain (1993–1996) [18], and more recently in the Czech Republic, Slovak Republic, Finland and Malta [14]. Among the serogroup B isolates, 53·0% were non-typable (NT). Serogroup C isolates included seven different phenotypes, with serotype 2b (42/61, 68·8%) and serotype 2a (5/61, 8·2%) the most prevalent. Only 16·4% of isolates were NT. The serosubtype P1.5,2 was the most prevalent (38/61, 62·3%). The phenotype C:2b:P1.5,2 (37/61, 60·7%) was predominant among isolates of serogroup C. This phenotype was also predominant among serogroup C isolates studied in Belgium between 1996 and 1998 [19]. In Spain, C:2b:P1-5,2 accounted for 80·7% of serogroup C isolates between 1992 and 1999 [20]. This serosubtype was already predominant in Scotland in the 1980s [21]. Currently, C:2b:P1-5,2 can be considered the most prevalent phenotype in Southern Europe [4, 19]. This study demonstrated a continuing predominance of isolates with moderate resistance to penicillin of phenotype C:2b:P1.5,2 (15/28, 53·6%) (Table) in Portugal [22].

Four different PFGE patterns were identified among the 16 isolates analysed (designated A1–A4). The C:2b:P1.5,2 isolates exhibited the PFGE pattern A1 (five isolates), A2 (seven isolates), A3 (one isolate) and A4 (one isolate). The two other phenotypes (C:2b:P1.2 and C:2b:NST) showed PFGE pattern A1. PFGE pattern A1 formed cluster I, pattern A2 cluster II and patterns A3 and A4 formed cluster III. MLST of seven serogroup C isolates, with moderate resistance to penicillin, identified two sequence types (ST): ST-8 (one isolate from cluster II) and ST-2289 (six isolates distributed by clusters I, II and III). Both sequence types belonged to the clonal complex ST-8/cluster A4.

This is the first report of ST-2289, a single locus (*aroE* locus) variant (SLV) of ST-8. The ST-2289 clone did not exist in Portugal before 1999 (M. Caniça, personal communication). The appearance of this clone may be responsible for the increase in penicillin resistance associated with serogroup C observed in recent years in Portugal [3, 9]. The slight PFGE pattern variations of the disseminated clones with moderate resistance to penicillin from serogroup C, may explain that representative clones all belonged to the ST-8 complex/cluster A4. This complex is usually associated with phenotypes C:2b:P1.5,2 and

C:2b:P1,2 and has been described widely in Southern European countries [4, 19, 20].

In conclusion, we believe that it is important to continue the phenotyping and genotyping based studies on *N. meningitidis* in Portugal, first correlated in this study, as it seems that particular clones are circulating in the country. The dissemination of the hypervirulent ST-8 complex/cluster A4, among moderate-penicillin-resistant serogroup C isolates in Portugal increases this need.

The study covered the first period of voluntary vaccination with conjugate C vaccine in Portugal, making possible the future linking of the pre-vaccine period to the pos-vaccine period. Thus, these results will help to explain dynamic clonal changes, even between other countries, since prevalence of meningococci could change rapidly. Monitoring changing patterns of the susceptibility to antibiotics, antigenic characteristics, and genotyping will provide the basis for understanding the epidemiological trends in a European perspective and thus for preventive and therapeutic policies.

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DECLARATION OF INTEREST

None.

REFERENCES

1. **Cunha F, Carvalho L.** Meningitis in children. In: *Patologia Infecciosa, Temas de pediatria*, vol. II. Monografia Beechman. Lisboa: Beechman Portuguesa Lda, 1993, pp. 169–183.
2. **Noah N, Henderson B.** Surveillance of bacterial meningitis in Europe 1999/2000. Communicable Disease Surveillance Centre, England, PHLS, 2001.
3. **Ferreira E, Caniça M.** Invasive meningococci with reduced susceptibility to penicillin in Portugal. *Journal of Antimicrobial Chemotherapy* 2002; **49**: 424–425.
4. **Stefanelli P, *et al.*** Emergence in Italy of a *Neisseria meningitidis* clone with decreased susceptibility to penicillin. *Antimicrobial Agents and Chemotherapy* 2004; **48**: 3103–3106.
5. **Sutcliffe EM, *et al.*** Penicillin-insensitivity meningococci in the UK. *Lancet* 1998; **i**: 657–658.
6. **Arreaza L, De La Fuente L, Vásquez JA.** Antibiotic susceptibility patterns of *Neisseria meningitidis* isolates from patients and asymptomatic carriers. *Antimicrobial Agents and Chemotherapy* 2000; **44**: 1705–1707.
7. **Spratt BG, *et al.*** Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. *Journal of Molecular Evolution* 1992; **34**: 115–125.
8. **Maiden MCJ.** Population genetics of a transformable bacterium: the influence of horizontal genetic exchange on the biology of *Neisseria meningitidis*. *FEMS Microbiology Letters* 1993; **112**: 143–150.
9. **Caniça M, *et al.*** Invasive culture-confirmed *Neisseria meningitidis* in Portugal: evaluation of serogroups in relation to different variables and antimicrobial susceptibility (2000–2001). *Journal of Medical Microbiology* 2004; **53**: 921–925.
10. **Mayer L, *et al.*** Outbreak of W135 meningococcal disease in 2000: not emergence of a new W135 strain but clonal expansion within the electophoretic type-37 complex. *Journal of Infectious Diseases* 2002; **185**: 1596–1605.
11. **Caniça M, *et al.*** Two major Spanish clones of penicillin-resistant *Streptococcus penumoniae* in Portuguese isolates of clinical origin. *Journal of Antimicrobial Chemotherapy* 2003; **51**: 409–414.
12. **Taha M-K, *et al.*** Continuing diversification of *Neisseria meningitidis* W135 as a primary cause of meningococcal disease after emergence of the serogroup in 2000. *Journal of Clinical Microbiology* 2004; **42**: 4158–4163.
13. **Direcção-Geral da Saúde.** Statistics. Compulsory notifiable diseases 1998/2000: tables of disease, region, subregion, sex and age, month, 2003, 17–18 (http://www2.dgsaude.pt/upload/membro.id/ficheiros/i005838.pdf). Accessed 20 September 2005.
14. **European Union Invasive Bacterial Infections Surveillance Network** (EU–IBIS). Invasive *Neisseria*

meningitidis in Europe – 2002. Communicable Disease Surveillance Centre, England, PHLS, 2003.

15. **Holten E, Froholm LO, Gaustad P.** Virulence markers in patient and carrier strains of *Neisseria meningitidis*. *Scandinavian Journal of Infectious Diseases* 1984; **16**: 267–270.
16. **Fraser EA, Caugant DA.** The panmitic nature of *Neisseria meningitidis* serogroup B during a period of endemic disease in Canada. *Canadian Journal of Microbiology* 2001; **4**: 283–289.
17. **Kyaw MH, *et al.*** Invasive meningococcal disease in Scotland, 1994 to 1999, with emphasis on group B meningococcal disease. *Journal of Clinical Microbiology* 2002; **40**: 1834–1837.
18. **Connoly M, Noah N.** Is group C meningococcal disease increasing in Europe? A report of surveillance of meningococcal infection in Europe 1993–1996. *Epidemiology and Infection* 1999; **122**: 41–49.
19. **Van Looveran M, *et al.*** Interpreting the rising incidence of meningococcal disease in Belgium: the contribution of molecular typing. *Journal of Medical Microbiology* 2001; **50**: 986–990.
20. **Alcalá B, *et al.*** The epidemic wave of meningococcal disease in Spain in 1996–1997: probable a consequence of strain displacement. *Journal of Medical Microbiology* 2002; **51**: 1102–1106.
21. **Hubert B, Caugant DA.** Recent changes in meningococcal disease in Europe. *Eurosurveillance* 1997; **2**: 69–71.
22. **Caniça M, *et al.*** *Neisseria meningitidis* C:2b:P1.2,5 with intermediate resistance to penicillin, Portugal. *Emerging Infectious Diseases* 2004; **10**: 526–529.