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NOTES

Emergence in Italy of a *Neisseria meningitidis* Clone with Decreased Susceptibility to Penicillin

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A rise in invasive diseases due to *Neisseria meningitidis* C:2b:P1.5 with decreased penicillin susceptibility occurred in Italy during the last 2 years. Real-time PCR identified the Pen^i phenotype, and the *penA* sequence revealed the mosaicism of the gene. Molecular analyses assigned the isolates to a single emergent clone of the hypervirulent A4 cluster.

In Italy, the incidence of meningococcal disease has been consistently low (about 0.3 to 0.4 per 100,000 inhabitants per year). Throughout the 1990s, *Neisseria meningitidis* serogroup C strains represented fewer than 30% of all isolated meningococci. In 2002 to 2003, we observed an increase in serogroup C meningococcal disease, which became responsible for 42.5% of culture-confirmed cases. Interestingly, most of these isolates have the antigenic phenotype C:2b:P1.5 and show intermediate susceptibility to penicillin (Pen^i) (0.06 μg/ml > MIC < 1 μg/ml), due to sequence changes in the penicillin binding protein 2 *penA* gene. Pen^i meningococci have been reported and are being monitored in several countries (3, 5, 8). The attention is driven by the concept that strains with decreased susceptibility may still be evolving and in the process of acquiring further alterations in the *penA* gene, anticipating the appearance of resistant strains, as observed for *Streptococcus pneumoniae* and *Neisseria gonorrhoeae* (9, 12). In Italy, before 2002 Pen^i meningococci accounted for 7.5% of the isolates, but since then the percentage has risen significantly (to 27.4%). All Pen^i strains showed a mosaic structure in the transpeptidase region of the *penA* gene (1), and a recently validated real-time PCR assay (10) has been used for rapid confirmation of this phenotype. In this study, all C:2b:P1.5 Pen^i meningococci isolated in Italy were analyzed by *penA* sequencing, multilocus sequence typing, and pulsed-field gel electrophoresis (PFGE) to determine whether the recent increase in the number of cases due to this phenotype correlates with the emergence of a single clone and whether it circulates in other European countries.

A total of 214 invasive *N. meningitidis* strains were received at the reference laboratory of the National Surveillance of Meningococcal Meningitis between January 2002 and December 2003. All isolates were typed (7), and of 214 *N. meningitidis* isolates received, 91 were serogroup C and 38 of these (42.2%) belonged to phenotype C:2b:P1.5. Among the remaining serogroup C strains, 21% had the phenotype C:2a:P1.5, which up to then was the most frequent phenotype among C strains, and the others showed a variety of different sero- and subtypes.

The recently described real-time PCR (10) was used to discriminate between penicillin-susceptible (Pen^s) and Pen^i strains. Two hybridization probes were used to distinguish the wild-type *penA* gene in the Pen^s meningococci from the mutated gene at codon 566 in Pen^i strains. Thermal analysis of probe hybridization revealed melting temperatures of 45.5 and 55°C for the Pen^s and Pen^i strains, respectively, as shown in Fig. 1A. MICs of penicillin were also assessed by use of the E-test (AB Biodisk) on Mueller-Hinton agar (Oxoid) supplemented with 5% sheep blood, and the majority of C:2b:P1.5 strains (78.4%) showed MICs of ≥0.094 μg/ml, whereas a few serogroup C strains (24%) with other sero- or subtypes were Pen^i.

The Pen^i phenotype was finally confirmed by sequencing the *penA* PCR products in the transpeptidase domain of the encoding gene. Analysis of the sequences was performed with the Accelrys Wisconsin Genetics Computer Group package.

The *penA* sequence analysis showed a short DNA region, between nucleotides 1364 and 1545, with 98% identity to the sequence derived from *Neisseria perflava*/*sicca* (accession number X76422) and 100% homology with all *penA* sequences from Spanish C:2b:P1.5,2 Pen^i strains deposited in the National Center for Biotechnology Information databank (http://www.ncbi.nlm.nih.gov). Conversely, the *penA* genes of Pen^i meningococci with other serotypes or serosubtypes appear to have acquired larger blocks of DNA, often from more than one commensal *Neisseria* species along all of the transpeptidase domain (Fig. 1B). The replacement of the short region between nucleotides 1364 and 1545 in the C:2b:P1.5 strains seems to be sufficient to produce a form of the protein with a lower affinity to penicillin.

The spread of Pen^i C:2b:P1.5 meningococci over the country was more remarkable in the first 6 months of 2003, when twice as many were isolated as in the previous year. This sharp increase led us to carry out a molecular analysis of these strains to confirm the circulation of a clone. According to multilocus sequence typing results, obtained by the methodology described by Maiden et al. (6) (http://neisseria.org/nm/typing

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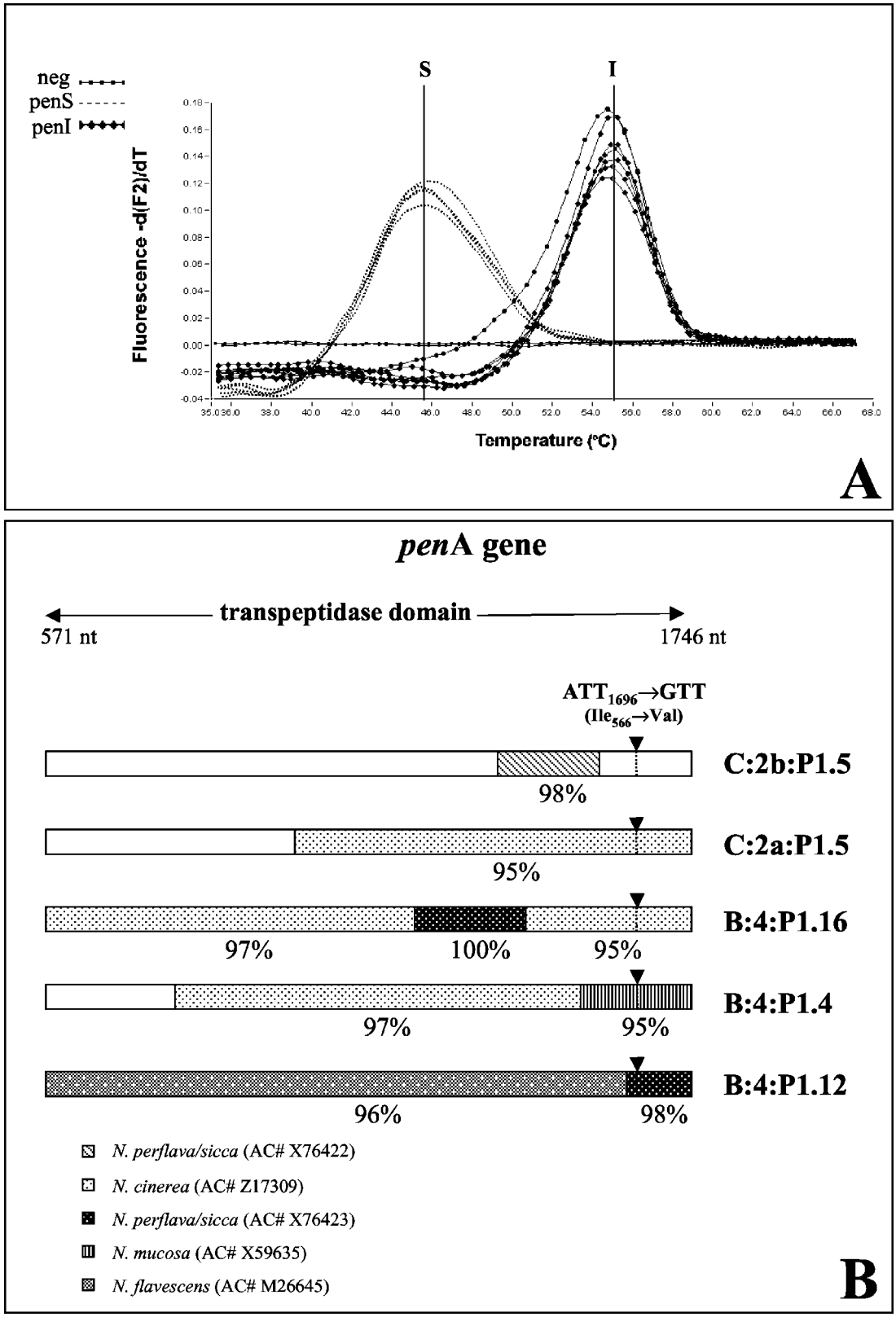

FIG. 1. (A) Examples of T_m curves for Pen^s and Pen^i *N. meningitidis* phenotypes obtained by real-time PCR assay with the mutated 566 codon in the *penA* gene. (B) Mosaic *penA* genes of Pen^i *N. meningitidis* strains. Each line indicates the proposed origin of the exogenous DNA blocks in the transpeptidase domain of the gene. The arrowheads show the position of the mutated 566 codon used as a marker of *penA* translocation in the real-time PCR assay. nt, nucleotides; AC#, accession number.

TABLE 1. Characteristics of *N. meningitidis* C:2b:P1.5 strains belonging to the ST8/A4 cluster isolated between January 2002 and December 2003

No. of strains	Yr of isolation	Patient age(s) (yr)	MIC of penicillin (μg/ml)	Pulse type
1	2002	1	0.094	PTA
4	2002	3, 4, 17, 19	0.125	PTA
1	2002	18	0.125	PTA1
2	2002	4, 7	0.19	PTA
6	2003	<1, 4, 15, 24, 49, NK[a]	0.094	PTA
1	2003	<1	0.094	PTA2
5	2003	4, 11, 12, 17, 35	0.125	PTA
1	2003	<1	0.125	PTA2
2	2003	23, 46	0.125	PTA1
4	2003	8, 30, 60, 62	0.19	PTA
2	2003	21, NK	0.25	PTA
1	2003	18	0.38	PTA

[a] NK, not known.

/mlst/), all of the C:2b:P1.5 Pen^i meningococci were assigned to the ST8/A4 cluster (Table 1), which is one of the two hypervirulent lineages responsible for most of serogroup C disease worldwide (the other is ST11). DNA macrorestriction fragments generated with the NheI restriction enzyme and analyzed by PFGE, as previously described (4), showed one main pulse type (PTA) and two subclones with two or three minor differences attributable to point mutations, named PTA1 and PTA2 (Fig. 2). When BglII was used, all of the strains showed the same pulse type (data not shown). One Pen^i strain with phenotype C:2b:nst, isolated in the first months of 2003, belonged to the same clone. The lack of identification of a serosubtype in this strain was due to the presence of *IS*1301 in the *porA* gene and its subsequent inactivation, as demonstrated by sequencing (data not shown).

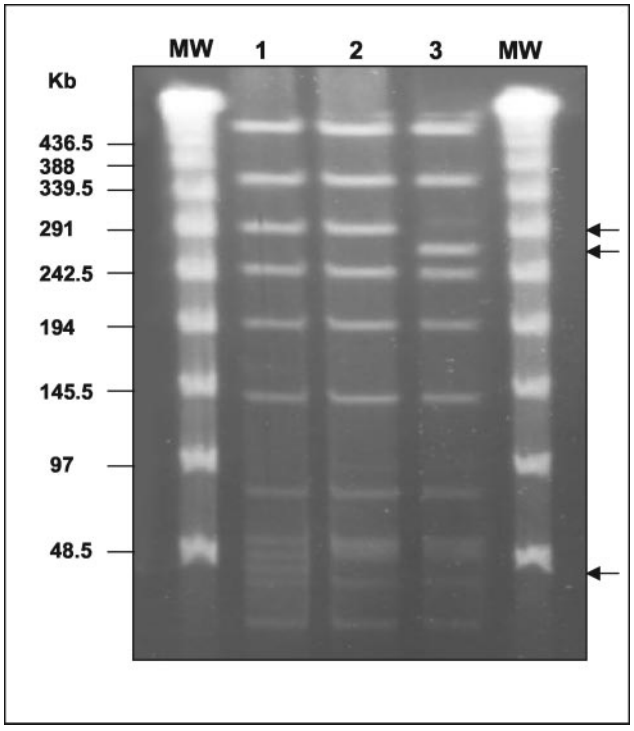

FIG. 2. PFGE profiles of genomic DNAs from *N. meningitidis* C:2b:P1.5 strains after digestion with endonuclease NheI. The lambda ladder DNA marker (New England Biolabs) (lane MW) was used as a molecular size standard (48.5 kb). Lane 1, pulse type PTA; lane 2, PTA1; lane 3, PTA2. The arrows identify the band differences.

Interestingly, the pulse type PTA found in most of the examined C:2b:P1.5 strains is identical to fingerprint pattern 2 (PT2) described by Arreaza et al. (2) for C:2b:P1.5,2 epidemic Spanish strains, just as our PTA1 resembles PT1 described in the same paper. Unlike the Spanish PT1 strains, the PTA1 strains are able to cause disease in all age groups and not predominantly in children under 2 years of age. In contrast, the subclone PTA2 is a unique profile found only in two strains, both of which were responsible for meningitis in infants.

All of these findings seem to confirm that the recent increase of meningococcal disease caused by *N. meningitidis* C:2b:P1.5 in Italy is due to the spread of a single emergent clone with decreased penicillin susceptibility and belonging to the hypervirulent cluster A4. Although the first strain C:2b:P1.5, ST8/A4 cluster appeared in Italy in 1998, in the following 3 years all meningococci with phenotype C:2b:P1.5 belonged to ST1860, a new ST of the ET37 complex detected in Italy (11). None of these strains were Pen^i, in contrast to most of the serogroup C ET37 strains isolated in other countries. In 2002, Pen^i meningococci of phenotype C:2b:P1.5, ST8/A4 cluster suddenly reappeared and rapidly spread all over the country.

We speculate that the Spanish clone might have been imported in the second half of the 1990s. In fact, one Pen^i strain with phenotype C:2b:P1.5,2 ST8/A4 and pulse type PTA1 was isolated in Italy in 1996, and it showed the same molecular characteristics as the Spanish strains. After this episode, for reasons difficult to understand and linked to the evolution and dynamics of the meningococcal population, it took some years to settle in, with a minor modification in the outer membrane proteins encoded by the *porA* gene. However, once established, the phenotype C:2b:P1.5 has become so fit as to spread very rapidly and to successfully compete with the other phenotypes. The acquisition of a very small exogenous DNA fragment might have provided an advantage in terms of fitness compared to the other Pen^i phenotypes. It is important to underline that there is a direct relationship between the increase in serogroup C strains causing meningococcal disease in Italy and the eightfold increase of meningococci with decreased susceptibility to penicillin as a result of the spread of this virulent clone. It will be of extreme importance to closely monitor the endemic circulation of this phenotype in order to plan specific vaccination programs in a timely fashion.

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