

Evolutionary Changes in Antimicrobial Resistance of Invasive *Neisseria meningitidis* Isolates in Belgium from 2000 to 2010: Increasing Prevalence of Penicillin Nonsusceptibility

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This study was conducted to evaluate the evolution of the antimicrobial susceptibility of *Neisseria meningitidis* causing invasive diseases in Belgium in the period of January 2000 to December 2010. A total of 1,933 cases of *N. meningitidis* from invasive infections were analyzed by antimicrobial susceptibility testing at the Belgian Meningococcal Reference Centre. The majority of strains were susceptible to antibiotics that are currently used for the treatment and prophylaxis of meningococcal disease, but the prevalence of clinical isolates with reduced susceptibility to penicillin increased over the years. The phenotyping, genotyping, and determination of MICs of penicillin G were performed. The systematic shift of the curves toward higher penicillin MICs in the susceptible population indicated that this population became less sensitive to penicillin in this period. A 402-bp DNA fragment in the 3' end of *penA* was sequenced for the 296 nonsusceptible meningococcal strains isolated between 2000 and 2010 to examine the genetic diversity and evolution of their *penA* gene. In conclusion, the data obtained in our study support the statement that the position of penicillin G as a first choice in the treatment of invasive meningococcal diseases in Belgium should be reexamined. Despite an important number of isolates displaying a reduced susceptibility to penicillin, at present the expanded-spectrum cephalosporins, such as ceftriaxone, are not affected. The follow-up of the evolutionary changes in antimicrobial resistance has also proved to be essential for the recommendation of an appropriate antimicrobial treatment for invasive meningococcal diseases.

Neisseria meningitidis, a human pathogen, colonizes the nasopharynx of individuals and can cross the epithelial barrier of the nasopharynx, causing septicemia and/or meningitis (8, 9, 12). Despite appropriate treatment, the case fatality rate varies from 5 to 15% (8, 9) due to fulminant septicemia, frequently resulting in sequelae.

In Europe and North America, the overall incidence of meningococcal disease is 1 to 3 per 100,000 inhabitants, and the infections are mostly sporadic cases among infants (8, 9).

Serogroup B has always been the main cause of meningococcal disease in Belgium (24, 25), but in the late 1990s the incidence of serogroup C disease increased considerably and peaked in 2001 (10). The greatest burden of serogroup C meningococcal disease was observed in young children and in teenagers. Health authorities decided to finance a mass immunization campaign targeting children aged 1 to 5 years and using a conjugate vaccine against serogroup C (10). Vaccination against serogroup C meningococcal infection was incorporated into the childhood immunization program from 2002 onwards, with a single dose of vaccine at the age of 12 to 15 months (10).

Prompt treatment is critical in the management of invasive meningococcal diseases. Penicillin G remains, in several countries, the drug of choice, particularly when a bacteriological diagnosis has been established.

The degree of susceptibility of *N. meningitidis* to penicillin has decreased during the past 2 decades, and meningococcal isolates showing intermediate resistance to penicillin (Penⁱ) have been widely described in several countries (11).

Decreased susceptibility to penicillin G is linked to alterations in the structure of a penicillin-binding protein (PBP2) encoded by the *penA* gene (2, 3, 22). The *penA* gene from penicillin-susceptible

meningococcal strains (Pen^s) appears to be highly conserved. However, those from nonsusceptible strains (Pen^{ns}) are variable, showing mosaic structures. These variations have been suggested to be due to genetic exchange through transformation between *N. meningitidis* and nonpathogenic commensal *Neisseria* species (2, 22, 23). The modification of the PBP2 resulted in a decreased affinity of PBP2 to penicillin G, as well as in modifications in the peptidoglycan structure of the bacterial cell wall (4).

The aim of this study was to evaluate the evolution of the antimicrobial susceptibility of *Neisseria meningitidis* causing invasive diseases in Belgium in the period of 2000 to 2010 and to examine the genetic diversity and the evolution of their *penA* genes.

MATERIALS AND METHODS

Bacterial strains and phenotypic characterization. Meningococcal strains from patients with invasive meningococcal disease are referred to the Belgian Reference Centre for *N. meningitidis* by clinical laboratories for the confirmation and determination of serogroup, serotype, subtype, and antimicrobial susceptibility pattern. Serogrouping was performed by slide agglutination using commercial antisera (Remel Europe Ltd., Dartford, United Kingdom). Serotypes and subtypes were determined by whole-cell enzyme-linked immunosorbent assay (ELISA) using monoclo-

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TABLE 1 Evolution of MIC₅₀ and geometric mean MIC (expressed in µg/ml) in Pen^s and Pen^{ns} isolates of *N. meningitidis*

Year	MIC ₅₀ ^a values and no. of isolates at indicated concn according to resistance category											Geometric mean MIC			
	Pen ^s						Pen ^{ns}					Total	Pen ^s	Pen ^{ns}	
	≤0.012	0.016	0.023	0.032	0.047	0.064	Pen ⁱ	Pen ^r							
						0.094	0.125	0.19	0.25	0.38	0.5				
2000	33	22	49	72	41	23	3	4	1	4			0.029	0.027	0.152
2001	46	65	96	78	24	20	7	15	4	3			0.027	0.023	0.133
2002	12	59	77	46	17	6	8	10	7	3			0.029	0.023	0.138
2003	11	23	61	58	21	6	4	12	5	2	1		0.032	0.026	0.144
2004	3	8	31	37	26	14	7	7	5	3	1	1	0.042	0.032	0.152
2005	8	13	48	43	13	6	10	10	7	3		1	0.037	0.027	0.140
2006	1	4	26	32	24	9	3	17	6	2	4	1	0.050	0.033	0.164
2007	5	8	22	57	22	18	2	6	7	3	2		0.041	0.033	0.174
2008	1	3	7	17	21	16	8	8	10	8	3		0.067	0.040	0.167
2009	1	3	10	21	22	19	2	7	3	8	2		0.055	0.039	0.184
2010	0	0	6	9	22	15	10	7	3	9	7		0.078	0.044	0.177

^a MIC₅₀, MIC at which 50% of the tested isolates are inhibited. MIC₅₀ values are shaded.

nal antibodies, including monoclonal antibodies for the detection of P3.1, P2.2a, P2.2b, P3.4, P3.15, and P3.21 antigens (NIBSC, Pottery Bar, United Kingdom) (1).

Antimicrobial susceptibility. The MICs of penicillin G, cefotaxime, chloramphenicol, rifampin, and ciprofloxacin were determined by Etest (AB Biodisk, Solna, Sweden). Etest was carried out according to the manufacturer's instructions, and the EUCAST breakpoint definitions (www.eucast.org) were used for the interpretation of antimicrobial susceptibility test results.

Molecular analysis. Multilocus sequence typing (MLST) was carried out using the method of Taha et al. (20), and isolates were assigned to clonal complexes according to the *Neisseria* MLST website (<http://pubmlst.org/neisseria/>) (14).

The amplification and sequencing of the *porA* and *penA* genes was carried out as described in Birtles et al. (6) and Taha et al. (22), respectively.

The amplification and sequencing of the *fetA* gene was performed by following the protocol mentioned on the Neisseria.org website (<http://neisseria.org/nm/typing/>). The *porA* variable regions, VR1 and VR2, and *porB*, *penA*, and *fetA* variable regions were assigned using the databases of the Neisseria.org website.

Statistical analysis. The chi-square test was used to evaluate differences in proportions. Other evaluations (populations and mean MICs) were done by using the Mann-Whitney test and/or the Student's *t* test. Significance was set at 0.05.

RESULTS

Meningococcal surveillance: 2000 to 2010. In the period of January 2000 to December 2010, a total of 2,025 cases of invasive meningococcal diseases were reported, with an average annual incidence of 1.8 cases per 100,000 inhabitants (ranging from 0.89 in 2010 to 3.7 in 2001). Overall, 93.2% (1,933/2,025) of the isolates remained viable and were included for analysis in the study.

During this 11-year period, antimicrobial susceptibility testing was performed by Etest on 1,933 *N. meningitidis* strains. All isolates were found to be susceptible to cefotaxime, chloramphenicol, and ciprofloxacin. Only one strain, isolated in 2007, presented resistance against rifampin (MIC, 8 µg/ml). The large majority of isolates (1,637/1,933; 84.7%) were fully susceptible to penicillin (MICs of ≤0.064 µg/ml), 273 strains (14.1%) had reduced susceptibility (Penⁱ), with MICs between 0.125 and 0.25 µg/ml, and 23 isolates presented full resistance to penicillin (MICs of ≥0.5

µg/ml) (Pen^r). One hundred four of these 296 Pen^{ns} isolates belonged to serogroup C, 159 to serogroup B, 28 to serogroup W135, and 4 to serogroup Y, while one isolate could not be classified in a particular group. The prevalence of Pen^{ns} isolates was higher in serogroup W135 (56.3%) and in serogroup C (21.7%) than in serogroup B (11.7%).

The proportion of Penⁱ isolates fluctuated between 1.2 and 31.8% in serogroup B, between 10.8 and 75% in serogroup C, and between 4.8 and 33.0% in the total population of isolates. All Pen^{ns} isolates were tested for ampicillin susceptibility. The MICs of ampicillin ranged from 0.032 to 1 µg/ml, and 26.0% of the isolates presented a reduced susceptibility to ampicillin (MIC, ≥0.12 µg/ml).

Table 1 shows the evolution of the MICs at which 50% of the tested isolates are inhibited (MIC₅₀) and the geometric mean MICs (expressed in µg/ml) of Pen^s and Pen^{ns} isolates.

Between 2000 and 2003, there was no significant change in mean penicillin MICs for the total population. In 2004, a significantly higher mean MIC was found compared to the period of 2000 to 2003 ($P < 0.001$). In the period of 2004 to 2007, the mean MIC remained stable, albeit at a significantly higher value than that of the period of 2000 to 2003. From 2007 to 2010, a significant increase was noted again ($0.01 > P > 0.001$). The increase in mean MIC in the Pen^s population showed a comparable evolution, while such a systematic evolution was not found in the Pen^{ns} population (Table 1).

During the period of 2000 to 2010, a significant increase in the rates of nonsusceptibility to penicillin was observed (from $0.01 > P > 0.001$ to $P < 0.001$). The nonsusceptibility rate increased from 4.8% in 2000 to 16.8% in 2004 and 25.6% in 2006. In 2007, this rate dropped to 13.1% but increased again to 36.3% in 2008. In 2009, we noticed again a drop to 20.4%. Finally, the 2010 rate was 40.9%. For the Pen^r isolates, the first significant increase was seen in 2006 (3.9%; it was 0% in 2000; $0.01 > P > 0.001$), and no further significant increase was seen between 2006 and 2010 (7.9%). Overall, analyses for linear trends in proportions showed that there was a significant increase in Pen^{ns} and Pen^r isolates during the period of 2000 to 2010 ($P < 0.001$). The prevalence of the Pen^{ns} isolates increased significantly over the years in all of the serogroups.

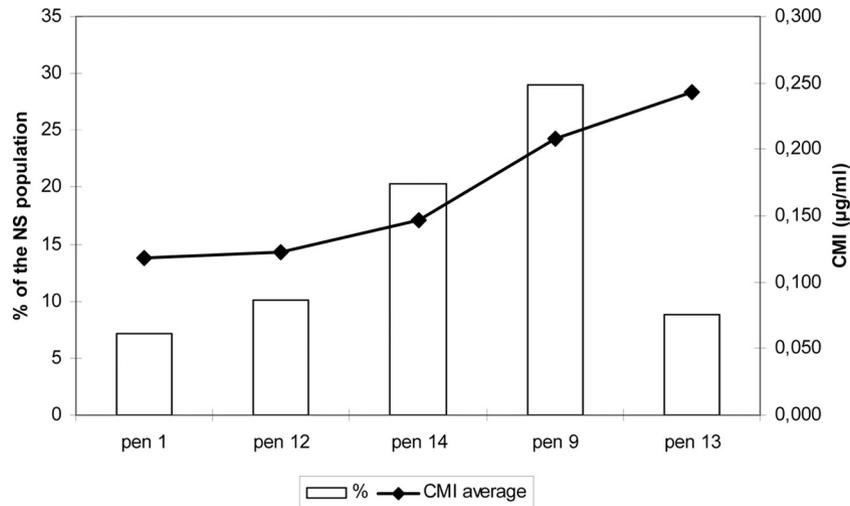


FIG 1 Distribution of the *penA* allele among the nonsusceptible population and relationship between the *penA* allele and the average MIC value.

Characterization of *penA*: correlation between *penA* allele and MIC and allelic evolution from 2000 to 2010. The sequence of a 402-bp DNA fragment of the 3' part of *penA* was obtained for 56 randomly selected Pen^s isolates and for the 296 Pen^{ns} isolates. Fifty-four of 56 Pen^s isolates harbored the *penA1* allele or an allele with an identical deduced amino acid sequence (only silent, synonymous DNA polymorphisms for the corresponding 402-bp fragment). The *penA12* allele was found in only 2 susceptible isolates (with a MIC of 0.064).

Forty-one different alleles of *penA* were identified among the 296 Pen^{ns} isolates. The most frequent alleles in the Pen^{ns} population were *penA9* (29.1%), *penA14* (20.3%), *penA12* (10.1%), *penA13* (8.8%), and *penA1* (7.1%) (see Fig. 2). Most of these alleles (21 out of 41) had an identical deduced amino acid sequence. The alleles *penA14*, *penA13*, and *penA9* were most frequent among isolates, presenting a high mean MIC of 0.139, 0.189, and 0.231, respectively. These mean MIC values differed significantly from each other ($0.05 > P > 0.02$ for *penA9* versus *penA13* and $P < 0.001$ for *penA14* versus *penA9* and *penA1*). On the other hand,

penA1 and *penA12* were most frequent in isolates presenting a lower MIC (mean MIC of 0.115 and 0.118, respectively). There was no significant difference between the mean MIC values of *penA1* and *penA12*, while their values were significantly lower than those for the other three types ($0.05 > P > 0.02$ compared to *penA14* and $P < 0.001$ for *penA9* and *penA13*) (Fig. 1).

The prevalence of *penA14* increased systematically beginning in 2003 and reached a prevalence of 42.2% in the Pen^{ns} isolates in 2006 ($P < 0.001$). The *penA13* allele was detected for the first time in 2003, and its prevalence remained relatively stable over the years (between 5.6 and 16.7% of the nonsusceptible population). The prevalence of the *penA9* allele fluctuated over the years. The lowest prevalences were found in 2006 (12.0%) and 2010 (8.3%). In the other years its prevalence varied from 21.6% (2008) to 46.4% (2002) (Fig. 2). The proportion of *penA1* allele increased significantly from 2007 (0%) to 2009 (31.8%) and 2010 (19.4%) ($0.02 > P > 0.01$). The prevalence of *penA12* decreased drastically during the period of 2001 (31.0%) to 2008 (2.7%) ($0.01 > P > 0.001$), while it was not present in 2009 and 2010.

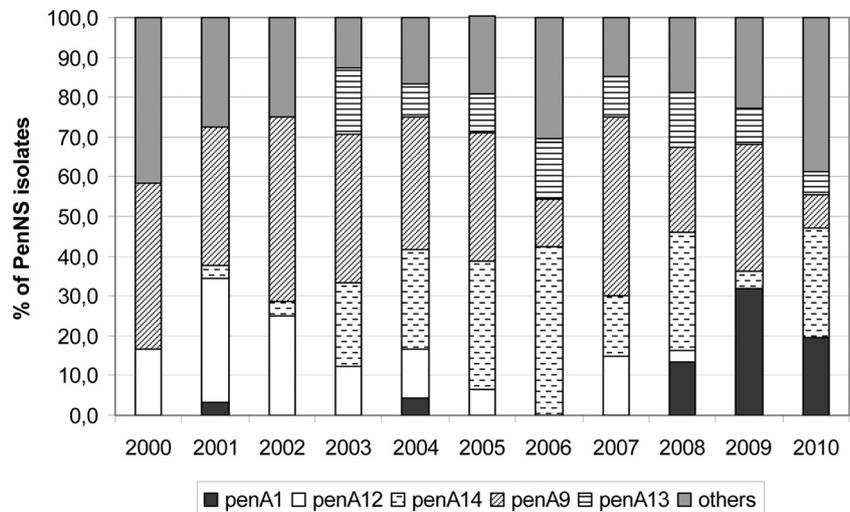


FIG 2 Distribution of the most frequent *penA* alleles (*penA1*, *penA12*, *penA9*, *penA14*, and *penA13*) in the Pen^{ns} isolates between 2000 and 2010.

Distribution of *penA* alleles in the different serogroups. The association between serogroups and the alteration of PBP2 was analyzed for the 296 Pen^{ns} isolates. A significant association was observed between isolates of serogroup B and the *pen13* allele and between serogroup C and the *pen12* allele. Indeed, 24 isolates out of 26 harboring *penA13* belonged to serogroup B, with the majority ($n = 23$) of them belonging to serotype 4 and presenting the phenotype B:P1.4:F1-5:cc-41/44 ($P < 0.001$). Twenty-six out of 30 isolates harboring *penA12* belonged to serogroup C. In this group, the majority ($n = 24$) of the isolates belonged to serotype 2b with the phenotype C:P1.1,7:F5-8:cc-8 or C:P1.2,5:F5-8:cc-8 ($P < 0.001$). Among the isolates presenting the *pen14* allele, 37 belonged to the B serogroup (the majority belonged to the phenotype B:P1.4:F1-5:cc-41/44 complex/Lineage 3) and 24 to serogroup W135 (the majority belonged to the phenotype W135:P1.3,6:F4-1:cc-22).

DISCUSSION

Since the emergence of resistance is correlated with the outcome of treatment and the successful prophylaxis of close contacts, the surveillance of antimicrobial resistance is particularly important for *Neisseria meningitidis* (18). Due to the high risks of morbidity and death, immediate treatment with effective antibiotics is essential when a patient is suspected of having meningococcal diseases. In Belgium, as in other countries, rifampin and ciprofloxacin are recommended to eliminate the pathogen from the nasopharynx and are used to avoid transmission to close contacts (15).

Our data show that meningococcal strains isolated in Belgium during the last 11 years remained highly susceptible to the antibiotics currently used to treat patients with meningococcal diseases and their close contacts. Only one case presenting resistance to rifampin was detected in 1,933 tested strains. The resistance was due to a point mutation of the *rpoB* gene (*rpoB* allele 36) (21). As previously described, resistance to rifampin is only occasionally observed and can occur following chemoprophylaxis (18, 19).

No resistance or intermediate resistance to fluoroquinolones was observed on the 1,933 Belgian isolates. To date, reduced susceptibility or resistance to fluoroquinolones in *Neisseria meningitidis* remains rare (13). Nevertheless, sporadic cases have been reported in Europe, India, and North and South America (16, 17, 26). In Belgium, the use of quinolones to treat meningococcal diseases is limited because quinolones are not approved for routine pediatric use (15).

Another important issue shown in the present study is the systematic and significant shift of the curves toward higher penicillin MICs. This shift can be attributed to a shift in the susceptible population, indicating that the susceptible population became less sensitive to penicillin in this period.

As described in other European countries, our study shows that the degree of susceptibility of *N. meningitidis* to penicillin has decreased in the last decade. In the last 3 years, the meningococcal population displayed a high degree of reduced susceptibility to penicillin, and this has led clinicians to use expanded-spectrum cephalosporins for initial treatment. Interestingly, in our study, none of the isolates presented resistance or a reduced susceptibility to expanded-spectrum cephalosporins.

The proportion of Penⁱ isolates increased from 2.2% in 1997 to 13.1% in 2002 and was higher in serogroup C than in serogroup B. However, this percentage was lower than the nonsusceptibility rate reported in France (31.2%) in 1999 to 2002 (26). In Belgium,

serogroup:serotype C:2a (especially C:2a:P1.5 isolates) was frequently associated with reduced susceptibility to penicillin in 2001 and 2002. The increase of meningococcal diseases caused by C:2a strains contributed to the overall increase in the proportion of Penⁱ isolates in Belgium during this period. As has been described in Spain (5) and in Portugal (7), we also found a strong association between reduced susceptibility to penicillin and serogroup:serotype C:2b and, more specifically, with the phenotypes C:P1.1,7:F5-8:cc-8 and C:P1.2,5:F5-8:cc-8. Moreover, these strains harbored the *penA12* allele. Interestingly, in 2006, 2009, and 2010, no cases of invasive meningococcal diseases were caused by a *Neisseria* isolate belonging to serotype C2b, and the *penA12* allele was absent in these years, suggesting a strong association between the C2b serotype and this *penA* allele.

In the present study, the most common alleles in the nonsusceptible population were *penA9* and *penA14*, representing 29.1 and 20.3% of the isolates, respectively. This is in agreement with an extensive study presenting data on the sequencing of the *penA* gene of *N. meningitidis* isolates ($n = 1,670$) from 22 mainly European countries (22). In that study, *penA9* and *penA14* were found in 5 and 6% of the total isolates, respectively, but were more frequently isolated in France and Denmark (22). As it was shown in that particular study, the *penA9* allele was present in isolates with different phenotypes and genotypes, whereas *penA14* was found primarily in ST-22 isolates. In our study, the *penA14* allele was detected in both ST-22 and ST-41/44.

In conclusion, our data indicate that *N. meningitidis* isolates from Belgian patients remained highly susceptible to the antibiotics used for treatment and prophylaxis, with the exception of penicillin. However, the increasing prevalence of Pen^{ns} strains urges the reexamination of the position of penicillin as a first choice in the treatment of invasive meningococcal diseases in Belgium. The follow-up of the evolutionary changes in antimicrobial resistance has also proved to be essential for the recommendation of an appropriate antimicrobial treatment for invasive meningococcal diseases.

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