ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2004, p. 358–359 Vol. 48, No. 1
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.1.358–359.2004

Sequencing of *Neisseria meningitidis penA* Gene: the Key to Success in Defining Penicillin G Breakpoints

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Received 17 June 2003/Returned for modification 18 August 2003/Accepted 26 September 2003

Testing of susceptibility to penicillin G by E-test and sequencing of an internal fragment of the *penA* gene were done for 43 meningococcal strains. Those strains for which the MIC was ≥0.094 μg/ml showed mosaic alleles, so 0.094 μg/ml is suggested as the penicillin G intermediate breakpoint when E-test is used.

Methods recommended for the susceptibility testing of *Neisseria meningitidis* include broth microdilution and agar dilution (AD) (4). However, over the last few decades E-test has been frequently used for meningococcal susceptibility analysis, because it is simple to use and is applicable for single-isolate susceptibility testing (3). E-test uses a continuous antibiotic concentration gradient, and as a result, the MICs obtained might be more precise than conventional MICs, based on discontinuous twofold serial dilutions.

Penicillin-resistant meningococcal strains are extremely rare and are related with β-lactamase production. However, those defined as intermediate (Pen^i) have been widely described in different countries (7), although their clinical significance is still not clear. The Pen^i strains show altered forms of the penicillin-binding protein 2. This fact is due to genetic events at the *penA* gene, which encodes that protein. So, while the *penA* gene of penicillin-susceptible strains (Pen^s strains) appears uniform in sequence, those from Pen^i strains are quite diverse, showing mosaic structures (6).

So far, 0.12 μg/ml has been used as a cutoff point for Pen^i definition for *N. meningitidis* when the susceptibility analysis is done by AD or broth macrodilution (2, 5), so strains for which the penicillin G MIC is ≤0.06 μg/ml are defined as susceptible and those for which MICs range between 0.12 and 1.0 μg/ml are considered intermediate. Strains for which MICs are between 0.06 and 0.12 μg/ml when E-test is used can be found, but so far they cannot be properly categorized. Although a MIC of 0.094 as determined by E-test is rounded up to fit the classical dilution scheme, it is not known if these isolates are properly assigned to the intermediate resistance category or not.

Because the molecular basis of Pen^i in *N. meningitidis* is based on detection of mosaic structures at the *penA* gene, the aim of this study was to establish the Pen^i breakpoint, when the susceptibility analysis is done by E-test, by *penA* gene sequencing.

Forty-three strains (13 serogroup B, 28 serogroup C, 1 serogroup 29E, and 1 serogroup W135 strain) isolated from cases of invasive meningococcal disease in Spain were included.

Susceptibility to penicillin G was determined by E-test in Mueller-Hinton agar supplemented with 5% whole defibrinated sheep blood. E-test was carried out according to the manufacturer's instructions by using CO_2 incubation. The MICs were determined twice for each strain by two people independently reading the values on the E-test strips.

A 1.4-kb DNA fragment of the *penA* gene, encoding the transpeptidase domain, was amplified from the chromosomal DNA of *N. meningitidis* by PCR, purified and sequenced, as previously described (1).

All the strains for which the MIC was ≤0.047 μg/ml as determined by E-test possessed *penA* alleles related to Pen^s strains. Mosaic *penA* alleles were identified in all the strains for which the MIC was ≥0.094 μg/ml. Among those strains for which the MIC was 0.064 μg/ml (n = 9), two groups were defined according to the *penA* gene sequence: five isolates showed *penA* alleles related with Pen^s strains and four isolates possessed mosaic *penA* alleles. The results are summarized in Table 1, where the MICs determined by AD, which is routinely done in our laboratory, are also included.

A similar correlation can be found between AD and *penA* polymorphism (Table 1). All the strains for which the MIC was <0.06 μg/ml showed *penA* alleles of Pen^s meningococci, while mosaic alleles were identified in those for which the MIC was >0.06. Once again there was a less clear correlation with the isolates for which the MIC was 0.06 μg/ml, with three of eight strains showing mosaic *penA* alleles. On the other hand, it is important that 23 strains for which the MIC was 0.12 μg/ml as determined by AD were used in this study (Table 1). For 13 of them the MIC was 0.094 μg/ml by E-test, so the frequency of meningococcal strains for which the MIC is that value, as determined by E-test, might be very high.

Because in our laboratory the meningococcal isolates are routinely tested against other β-lactam antibiotics (ampicillin, cefotaxime, and ceftriaxone), for those strains for which the penicillin G MIC was 0.064 μg/ml, the MICs of other β-lactams were studied. The most relevant finding was that, for all the strains showing mosaic structures at the *penA* gene, ampicillin MICs determined by E-test were ≥0.125 μg/ml. However, for those strains possessing *penA* alleles related to sus-

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TABLE 1. Relationship between penicillin G MIC determined by E-test and *penA* gene sequence

No. of isolates	MIC (μg/ml) determined by:		No. of isolates possessing:	
	E-test	AD	Pen^s *penA* alleles	Mosaic *penA* alleles
7	0.023	0.03	7	0
4	0.032	0.03	4	0
1	0.047	0.03	1	0
6	0.064	0.06	5	1
3	0.064	0.12	0	3
2	0.094	0.06	0	2
13	0.094	0.12	0	13
7	0.125	0.12	0	7

ceptible strains, ampicillin MICs were lower (≤0.094 μg/ml) (Table 2).

According to these results, 0.094 μg/ml should be used as the Pen^i breakpoint when E-test is used as the susceptibility testing method. The heterogeneous situation found among strains for which the MIC was 0.064 μg/ml as determined by E-test determines that isolates for which the MIC is at this level should be defined as susceptible in order to avoid an overestimation of the Pen^i meningococcal population. The evaluation of different inocula and/or media in the E-test susceptibility testing method could be important for clarifying the confusing situation found for those isolates for which the MIC was 0.064 μg/ml. However, the level of susceptibility to ampicillin might be used in order to decide properly if those isolates for which the penicillin G MIC determined by E-test was 0.064 μg/ml should be included in the Pen^s or Pen^i group.

TABLE 2. Relationship between penicillin G and ampicillin MICs determined by E-test and mosaic *penA* alleles

Group no.	MIC (μg/ml) determined by E-test of:		Possession of mosaic *penA* allele
	Penicillin	Ampicillin	
1	0.064	≤0.094	No
2	0.064	≥0.125	Yes

Although we have found a good correlation between determination of the MIC with E-test and sequence variation in the *penA* gene, the possibility of other mechanisms that cause resistance should not be discarded.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this study were deposited in the GenBank database under accession numbers AF519582, AF519584, AF519585, AF519586, AF519588, AF519590, AF519579, AF519581, AF519591, AF519587, AF519577, AF519575, AF519576, AF519589, AY292990 to AY293001, AY294547 to AY294556, AF519596, AF519603, AF519607, AF519609, AF519612, AF519616, and AF519617.

This study was supported by grants 01/18 and QLK2-CT-2001-01436 from Instituto de Salud Carlos III and the European Commission, respectively. C. Salcedo and R. Enríquez worked with a predoctoral fellowship from Wyeth España. M. J. Uría and R. Abad were supported by grants from FIS and ISCIII, respectively. A postdoctoral fellowship from Instituto de Salud Carlos III supported B. Alcalá.

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