Nonculture Prediction of Neisseria meningitidis Susceptibility to Penicillin

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We developed a nonculture method to predict the susceptibility of Neisseria meningitidis to penicillin G. The penA gene was amplified and submitted to restriction fragment length polymorphism analysis. This approach was first validated with a collection of 75 meningococcal strains of known phenotypes. It was next successfully applied to 29 clinical samples.

Treatment of meningococcal infections is a medical emergency and requires a rapid diagnosis by the isolation of bacteria from cerebrospinal fluid (CSF), blood, or other body fluids (synovial, pericardial, etc.). However, the isolation of Neisseria meningitidis by culture is frequently hindered by early antibiotic treatment, which is highly recommended whenever meningococcal infection is suspected (6, 21). Several methods for nonculture diagnosis of N. meningitidis have been recently reported. These methods rapidly (1 to 2 h) identify N. meningitidis by amplifying one target gene, such as that encoding 16S rRNA (12, 14), IS1106 (17), porB (29), dhpS (13), ctra (9), or crgA (25). The serogroup, which reflects the capsular immunospecificity, is subsequently predicted by PCR amplification of serogroup-specific allele of the siaD gene for sialic acid-containing serogroups (B, C, Y, and W135) and by PCR amplification of the mynB gene for the mannoseamine-containing serogroup A (3, 4, 8, 19, 24, 25). These nonculture methods provide essential data for assessing the etiological diagnosis of the disease, but they do not provide information about antibiotic susceptibility. Penicillin G is still effective for the treatment of meningococcal infections. However, meningococcal strains with reduced susceptibility to penicillin (Pen'), for which MICs range from 0.125 to 1 µg/ml (11), have been increasingly reported in several countries (18). Penicillin-resistant strains (Pen') for which MICs are ≥1 µg/ml are now emerging (Table 1). This phenotype is thought to be due to a reduction in the affinity of penicillin-binding protein 2 (PBPs), encoded by an altered penA gene, for penicillin (16, 22). Mosaic structures in penA result from horizontal DNA exchange by transformation between commensal Neisseria species and N. meningitidis (5, 22, 23). We have previously used restriction fragment length polymorphism (RFLP) and DNA sequencing to show that penicillin-susceptible strains (Pen') of different geographic origins, antigenic formulas, and genetic lineages have the same penA allele, whereas Pen' strains have a variety of different penA alleles (1). Moreover, penA sequences from Pen' strains are highly divergent, particularly in the transpeptidase-encoding region (nucleotides 718 to 1743). This approach was shown to be suitable for the analysis of genetic relatedness between different penA alleles. The aim of the present study was to develop a nonculture method to predict the susceptibility of N. meningitidis to penicillin G.

DNA sequence alignment revealed conserved regions in the transpeptidase-encoding region of the penA gene of both Pen' and Pen' strains (1). Two oligonucleotides were designed on the basis of these conserved sequences: AA-1 (5' -ATCGAACAGCCGACGTGTC-3'; nucleotides 1237 to 1256) and AA-2 (5'-AGGCGACGATGTC-3'; nucleotides 1728 to 1748). They amplify a 500-bp fragment of the 3' end of the penA gene. These oligonucleotides were first tested on a collection of 75 meningococcal strains for which there were different MICs of penicillin G (39 Pen' and 36 Pen' strains). Penicillin G susceptibility was tested by the agar dilution method on G medium with G supplement (Sanofi Diagnostic Pasteur, Marnes La Coquette, France). The strains were tested with inocula of 10^6 CFU/ml on plates containing penicillin G at the following concentrations: 0.06, 0.125, 0.25, 0.5, and 0.75 µg/ml. The MIC was defined as the lowest concentration of penicillin G that inhibited visible growth after 18 h of incubation in 5% CO2 at 37°C. Penicillin G susceptibility was also tested by the diffusion method on G medium (Etest; AB-Biodisk, Solna, Sweden). β-Lactamase activity was detected with a Cefinase disk (BioMérieux, Marcy l’Etoile, France).

PCR was performed as previously described (1, 25). Amplification products of the expected size (500 bp) were obtained for all of the strains (Fig. 1A). After digestion with TaqI and separation on a 4% agarose gel, six different profiles were obtained (Fig. 1B). An arbitrary number was assigned to each profile (patterns 1 to 6). The results of PCR-RFLP from 75 Pen' and Pen' N. meningitidis strains are shown in the Table 1. On the basis of the RFLPs, two classes of strains were identified. (i) All Pen' strains for which the MIC was <0.125 µg/ml had the same pattern (RFLP1). (ii) Pen' strains for which the MIC was 0.125 µg/ml had altered patterns (RFLP2 to -6) compared to Pen' strains. However, for five strains for which the MIC was 0.125 µg/ml, we found RFLP1 as for Pen' strains. MICs for these strains were controlled by Etest and the agar dilution method. These strains could have been misclassified as Pen' strains, as we have previously suggested for such strains for which the MIC of penicillin G is at the breakpoint level (1). The fact that these strains are susceptible to amoxicillin supports this interpretation (Table 1). This observation underlines the technical difficulties in determining meningococcal suscept-
tibility to penicillin by MIC tests. Indeed, 14 European Reference Laboratories using identical methods, media, and strains reported differences in MIC determinations. Only molecular approaches showed complete agreement in detection of Peni meningococcal strains (2). The PCR-RFLP results with the penA gene were highly consistent with susceptibility to penicillin. No correlation was observed between a particular RFLP pattern and MICs in Peni strains. Indeed, the MICs for strains with the same RFLP could be different (Table 1). Molecular approaches are therefore able to overcome technical problems of MIC determination and to detect meningococcal strains with reduced susceptibility to penicillin G, regardless of the MIC for them. Treatment could be immediately adapted.

Our PCR-RFLP assay was subsequently used to test 29 clinical samples from various biological fluids obtained from different patients with suspected meningococcal infection (Table 2). Samples were treated as previously described (25), and PCR was performed with 15 μl of each sample. PCR was first used to amplify the ergA gene as recently described (25) and all of the samples were found to contain meningococcal DNA. Culture methods also found that 10 of the samples were positive. This allowed the MICs of penicillin G for them to be determined (Table 2). We next amplified the penA gene. Amplification products of the expected size (500 bp) were obtained for all 29 samples. Analysis of restriction patterns after digestion with TaqI showed that 20 samples had the same RFLP as the PenS strains (RFLP1), whereas 9 samples had different RFLPs, as observed for PenI strains. A complete correlation was observed between the PCR-RFLP results for biological samples, the PCR-RFLP results from bacterial DNA extracts, and the MIC of penicillin G for the corresponding strains (Table 2).

Molecular detection of genes associated with bacterial resistance to antibiotics has been developed for several species. Methicillin resistance in Staphylococcus aureus and coagulase-negative staphylococci results from the synthesis of a novel PBP encoded by the mecA gene. The PCR-based detection of the mecA gene is becoming the standard method for the detection of methicillin resistance (27, 28). Resistance to vancomycin encoded by the van genes (vanA, vanB, vanC, vanD, vanE, and vanG) is now widespread in enterococci. Therefore, amplification assays for the detection of the van genes have been developed (7, 20). β-Lactamase-producing Haemophilus influenzae can be detected by PCR with specific primers for the blaTEM and blaROB β-lactamase genes (26).

Early antibiotic treatment is the major element in the immediate management of meningococcal infections. The use of rapid and specific methods of nonculture diagnosis and the development of a molecular approach for the prediction of meningococcal susceptibility to penicillin are needed because of the increasing number of culture-negative cases. The use of PCR to detect reduced susceptibility to penicillin was recently reported with seven different PCRs with the penA gene (15). A strain was considered to be PenI if it failed to produce at least one PCR product. However, this approach was not tested in clinical samples. Our approach only uses one PCR product that is produced in all PenS and PenI strains and is easily and directly applicable for clinical samples. Our data clearly sug-

**TABLE 1. Distribution of the 75 N. meningitidis strains tested in this study according to PCR-RFLP and susceptibility to penicillin (MICs)**

<table>
<thead>
<tr>
<th>PenS (n = 39): MIC &lt; 0.125</th>
<th>1 2 3 4 5 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PenI (n = 36): 0.125 ≤ MIC ≤ 1</td>
<td></td>
</tr>
<tr>
<td>0.125</td>
<td>5a</td>
</tr>
<tr>
<td>0.19</td>
<td>1</td>
</tr>
<tr>
<td>0.25</td>
<td>4</td>
</tr>
<tr>
<td>0.38</td>
<td>3</td>
</tr>
<tr>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>0.75</td>
<td>1</td>
</tr>
<tr>
<td>1b</td>
<td>2b</td>
</tr>
<tr>
<td>1.5</td>
<td>1</td>
</tr>
<tr>
<td>&gt;32c</td>
<td>1</td>
</tr>
</tbody>
</table>

*a Strains susceptible to amoxicillin (MIC = 0.125 or 0.19 μg/ml).


*c β-Lactamase-positive strain.

**FIG. 1. Agarose gel electrophoresis of PCR-amplified DNA fragment of penA gene (A) and corresponding restriction fragment length patterns after digestion by TaqI (B) of PenS and PenI N. meningitidis strains (one representative strain by RFLP).**
suggest that the detection of an altered penA allele is always correlated with reduced susceptibility to penicillin. Moreover, the combination of this molecular approach with the phenotypic antibiotic susceptibility tests to penicillin may enable us to clearly classify strains as Pen+ or Pen− and to overcome the difficulties encountered for strains for which the MIC of penicillin G is close to the breakpoint level of 0.125 μg/ml (1).

The acquisition of β-lactamases seems to be rare in N. meningitidis, and resistance to penicillin G is now evolving by alteration of PBP2. Pen+ strains (MIC > 1 μg/ml) may be expected in the near future, analogous to Streptococcus pneumoniae and as suggested by the recent characterization in our laboratory of meningococcal strains for which the MICs were 1 and 1.5 μg/ml (Table 1). The threshold of 1 μg/ml corresponds to the therapeutic concentration in the CSF obtained during treatment with penicillin G (10). The emergence of meningococcal strains for which the MIC was >1 μg/ml may provoke treatment failure. The development of a reliable molecular approach to surveillance is clinically relevant and is expected to anticipate and enhance detection of the resistant strains in order to administer an adequate antibiotic treatment. Moreover, this nonculture detection of meningococcal strains with altered susceptibility to penicillin G provides information that otherwise is inaccessible in culture-negative cases of meningococcal infections. It also complements our approach combining nonculture diagnosis and serogroup prediction of meningococcal infections (25).

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REFERENCES


