Antibiotic resistant meningococci in Europe: Any need to act?

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Abstract

Meningococcal disease is a serious and rapidly progressing illness. It is therefore very important to monitor changes in the level of antibiotic susceptibility among clinical isolates. Different aspects such as interpretation of laboratory results, determination of breakpoints predicting treatment failure as well as definition of susceptibility levels in clinical samples using molecular methods are critical points for the surveillance of antibiotic resistance in Neisseria meningitidis. Within the strategic framework outlined by the EU.MenNet project, several objectives were identified to analyze 'The spread of antibiotic resistant meningococci in Europe', including the extent of antimicrobial resistance, its association with particular meningococcal lineages and geographical areas, as well as molecular characterization of the mechanisms involved, particularly in penicillin resistance. A heterogeneous figure for the frequency of intermediate resistance to penicillin appears across Europe. This heterogeneity may reflect different clonal lineages and/or uneven access to antibiotics in each country. In addition, the use of different criteria for the methodology used for definition cannot be avoided. The description of five specific changes associated with intermediate resistance to penicillin also allows the design of PCR-based tools to objectify results and for application in clinical samples.

Introduction

Neisseria meningitidis do not appear to be particularly efficient in developing resistance to antimicrobial agents. Apart from resistance to sulphonamides, which is currently present in more than 25% isolates, meningococci remain susceptible to the antibiotics classically used for treatment and chemoprophylaxis (Vázquez, 2001).

However, the history of the resistance of N. meningitidis to antimicrobial agents shows an important inflexion point when some strains with reduced penicillin susceptibility caused by alterations in the penicillin binding proteins (PBPs) (Sáez-Nieto et al., 1992) were described in the middle of the 1980s, after a long period of widespread use. Since then, the frequency of such strains with 'intermediate' resistance to penicillin (PenI) has been increasing in many countries (Richter et al., 2001; Tapsall et al., 2001; Vázquez, 2001). Nevertheless the degree of resistance seems to be quite stable, with most of the PenI isolates showing minimal inhibitory concentrations (MICs) of 0.12 or 0.25 mg L⁻¹. MICs of 1 mg L⁻¹ or higher are still the exception. In this context, beta-lactamase-producing strains remain an anecdotal success (Vázquez, 2001).

Third generation cephalosporins are increasingly used, especially when susceptibility to antibiotics is unknown. Cross-resistance to third generation cephalosporins has not been described and the correlation between diminished susceptibility to penicillin G and broad spectrum cephalosporins (cefotaxime and ceftriaxone) has not been well established (Sáez-Nieto et al., 1992). However, continuous surveillance of trends in susceptibility to this group of beta-lactam drugs is of clinical relevance.

Susceptibility to quinolones appears to be developing in a similar manner. The classical susceptibility of meningococci to this group of antibiotics has been altering in the last few years (Corso et al., 2005). Once again, the change appears to evolve very slowly, with a spread of the intermediate resistance that is not reflected by an increase in the MICs found.

Resistance to rifampicin is only occasionally observed; this can be identified following chemoprophylaxis (Cuevas & Hart, 1993).

It seems that the biological cost of altering specific structures in N. meningitidis may be too high for the bacteria, as has been proposed after in vitro experiments (Miller & Bohnhoff, 1947).
Because meningococcal disease is such a serious and rapidly progressing illness, it is very important to monitor changes in the degree of antibiotic susceptibility among clinical isolates of meningococci. Several aspects such as interpretation of laboratory results, determination of breakpoints predicting treatment failure as well as definition of susceptibility levels in clinical samples using molecular methods are critical for the surveillance of antibiotic resistance in *N. meningitidis*.

In this context, as part of the EU.MenNet project, several objectives were identified for the Work Package in charge of analyzing "The spread of antibiotic resistant meningococci in Europe":

1. description of extent of antimicrobial resistance;
2. defining the association of antimicrobial resistance with particular meningococcal lineages and geographical areas;
3. description of the mutations responsible for antimicrobial resistance;
4. design of a PCR-based tool using the *penA* sequence data.

In this article the data generated and the objectives achieved will be presented.

**MIC determination**

**Methods and standardization**

To determine the real extent of antimicrobial resistance and be able to compare data between countries (laboratories) monitoring resistance trends, it is crucial that standardized protocols for the determination of susceptibility to antibiotics are used. With the purpose of identifying what methods are being used for MIC determination in different countries, a questionnaire compiled by the European Monitoring Group of Meningococci (EMGM) was sent to 26 national and regional reference laboratories, including not only European centres but also laboratories from Australia, Israel and the US (Block, 2001). The results of the questionnaire highlighted the heterogeneity of methods not only with regard to the media but also with regard to the breakpoints used for definition of the resistance level. This lack of standardization makes it very difficult to compare susceptibility data between different countries.

The Clinical and Laboratory Standards Institute (CLSI/NCCLS) recommend a specific methodology to determine the MIC of antibiotics against meningococci (CLSI/NCCLS, 2005), including microdilution in cation-adjusted Mueller–Hinton broth supplemented with lysed horse blood or agar dilution with Mueller–Hinton agar supplemented with sheep blood. However, according to the questionnaire already mentioned, the Etest method appears to be the method most widely used across Europe for MIC determination. The use of a disc diffusion test with 2 U penicillin or 1 μg mL⁻¹ oxacillin has been proposed for that purpose. Some countries were using this methodology when the results of the survey were analyzed, but there is some concern about the reliability of this methodology (Block *et al.*, 1998) and it should not be used for clinical laboratories in either clinical or epidemiological investigations.

The EMGM, during its fifth meeting in Greece (1999), concluded that it would be useful and instructive to standardize methods and media used for MIC determination. With this objective, a panel of 17 meningococcal strains was distributed to 14 different European laboratories (Vázquez *et al.*, 2003), which were asked to determine the MIC of the strains to different antimicrobial agents, including penicillin, rifampicin, ciprofloxacin, ofloxacin, cefotaxime and ceftriaxone. The strains were tested on Mueller–Hinton (MH) agar, MH agar supplemented with sheep blood (MH+B) and MH agar supplemented with heated ('chocolated') sheep blood (MH+CH). The laboratories used not only the same protocols but also the same culture media and antibiotic trademarks and lots. The MICs were determined by both agar dilution and Etest methods.

The study produced very interesting data and concluded that MH+B should be used as the culture medium for both agar dilution and Etest methods. Importantly, there was a high level of agreement as to the MICs with both methods. Even though there were some discrepancies between the methods as to the resistance or susceptibility of some strains, these would only affect epidemiological surveillance trends, not clinical information. This indicates that susceptibility data among laboratories using agar dilution or Etest can be compared.

**Analysis of the sequence of the *penA* gene**

The only well known mechanism involved in the development of intermediate resistance to penicillin is the expression of altered forms of PBP2 as a result of differences in the sequence of the *penA* coding gene. Microbiology should therefore be able to contribute to the definition of the breakpoints with information about the association between DNA or AA polymorphism and the level of susceptibility to penicillin. This information, together with clinical and pharmacokinetic data, will give a more accurate definition of the breakpoints to be used. Analysis of the sequence of the *penA* gene in both susceptible and intermediate strains then became an important task within the Eu-MenNet project.

Analysis of the DNA sequence (and also of the corresponding AA sequence deduced) would be a very important step for defining the breakpoints using solid genetic bases. With the generated data it might also be possible to design
PCR-based tools as a valid methodology for identifying intermediate meningococcal strains even in clinical samples.

As part of the EU-MenNet project, a 1.4-kb region of the penA gene (from codon 183–187 bp downstream of the coding region) was chosen for the analysis. The first fragment of this gene (~600 bp) is highly conserved in both susceptible and intermediate isolates, so it was not considered necessary to include it.

To identify the polymorphic sites that might differentiate PenS/PenI strains, a total of 84 (24 PenS and 60 PenI) meningococcal strains were analyzed by sequencing the complete penA gene. The conditions of amplification and sequence determination of the gene have been already described (Arreaza & Vázquez, 2001). DNA sequence alignment allowed us to define conserved and variable regions at the penA gene and finally to identify a total of five polymorphic sites among PenS/PenI strains at the transpeptidase encoding region of that gene (Table 1). Several other polymorphic sites were also present in the penA gene but because they were not always present in all PenI strains, they were not considered to be related to the intermediate resistance to penicillin. Although there has been some controversy about the number of polymorphic sites (Taha et al., 2006), there is now a general consensus that those five decisive positions are the main key to the definition of intermediate resistance to penicillin.

In addition, the study of the complete DNA sequence of the penA gene was extended to a total of 74 PenS and 132 PenI isolates to clarify how the variability of the gene evolves; 25 and 47 alleles were found, respectively, in both groups. All sequences associated with susceptibility to penicillin appear to be evolving in node 1, which is exclusive for this type of isolate. Twenty-five different alleles appear in this node, all of them the result of point mutations, probably not affecting to the affinity of the codified protein. Most of the alleles associated with intermediate resistance appear in node 2, in which there is a strong process of point mutations resulting in different sequences. It is very clear how the recombination events produce the penA alleles of the PenI isolates. Of course, point mutations are the most frequent genetic event but they do not influence the appearance of intermediate resistance (Fig. 1).

### Table 1. Amino acid sequence changes at the trans peptidase-encoding region of the penA gene

<table>
<thead>
<tr>
<th>Positions from the start codon of the penA gene</th>
<th>504</th>
<th>510</th>
<th>515</th>
<th>541</th>
<th>566</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible (wild type) strains</td>
<td>F</td>
<td>A</td>
<td>I</td>
<td>H</td>
<td>I</td>
</tr>
<tr>
<td>Intermediate resistance strains</td>
<td>L</td>
<td>V</td>
<td>V</td>
<td>N</td>
<td>V</td>
</tr>
</tbody>
</table>

Molecular tools for definition of penicillin susceptibility in *N. meningitidis*

The knowledge generated by the analysis of the penA sequence made it possible to design two oligonucleotides that target a complementary sequence of the gene in penicillin-susceptible strains, including some of the five polymorphisms. They amplify the region from nucleotides 1526 to 1714 (including both positions), corresponding to amino acids 509–572 in PenS strains. Successful amplification implies penicillin-susceptibility, and amplification failure implies intermediate resistance. This strategy was later compared with two additional rapid methods to detect penA polymorphisms (Taha et al., 2006) with the purpose of establishing gold standards for the molecular detection of alterations to penA. Clinical samples from patients with meningococcal disease and the corresponding strains were used for the comparison. The three rapid PCR-based methods showed a high level of agreement with each other and were completely correlated with sequences from cultured bacteria.

The PCR strategy developed under the EU-MenNet project has been now adapted to a Real Time PCR protocol, using allelic discrimination algorithms to define susceptibility or resistance.

In an archetypal model, these nonculture tests should not replace isolation of the strains by culture. However, with the aim of achieving an accurate interpretation and in the light of the difficulties concerning the definition of proper criteria, an objective method such as molecular tools might offer a good alternative in the future.

### Definition of breakpoints

This may be the most critical and also outstanding ‘hot spot’ for determining the real extent of antibiotic resistance in meningococcal strains. In some cases it is extremely difficult to distinguish organisms likely to respond to particular treatments from those not likely to respond. This is particularly important in meningococci where false resistance has been described for most of the antimicrobial agents. The CLSI/NCCLS only recently added breakpoint recommendations in its January 2005 document (CLSI/NCCLS, 2005). However, particularly for the definition of penicillin resistance (MIC ≥ 0.5 mg L⁻¹), there is some controversy about breakpoints. The main groups working in penicillin-resistance mechanisms in meningococcal strains have generally used MICs of > 1 mg L⁻¹ to determine full resistance to this antibiotic and most of the national laboratories use the same definition. The application of the CLSI/NCCLS recommendation may radically change the figures for penicillin resistance in many countries. Because of this, the problem of definition is still present and an important quantitative as
well as qualitative reform will be required if the CLSI breakpoints are to be widely adopted.

Perhaps one major issue is the real meaning of this intermediate level of penicillin resistance in *N. meningitidis*. Although many cases of meningococcal disease are caused by strains with intermediate resistance, only a few reports of failure cases, many of them not conclusive, have been published (Contoyiannis & Adamopoulos, 1974; Turner *et al*., 1990; Casado-Flores *et al*., 1997). This situation illustrates how difficult the definition of breakpoints is. However, the clinical implications of this problem of definition are limited because of the widespread use of third generation cephalosporins for initial treatment of meningitis in developed countries.

The uncertainty with regard to breakpoints is particularly a problem for definition of penicillin resistance when there is a general consensus about the cut-off points for other antimicrobial agents.

This field was also analyzed in the context of the EU-MenNet Consortium. In an attempt to define proper breakpoints for intermediate resistance to penicillin, particularly using the Etest method (the most widely used across Europe), 43 strains isolated from cases of invasive meningococcal disease in Spain were analyzed (Arreaza *et al*., 2004). Susceptibility to penicillin G was determined by Etest in Mueller–Hinton agar supplemented with 5% whole defibrinated sheep blood according to the manufacturer’s instructions. At the same time, the 1.4-kb DNA fragment of the penA gene was sequenced and analyzed as previously described (Arreaza & Vázquez, 2001). All the strains for which the MIC was \( \leq 0.047 \text{ mg L}^{-1} \) showed penA alleles related to PenS strains. Mosaic penA sequences were identified in all the strains for which the MIC was \( \geq 0.094 \text{ mg L}^{-1} \). Among those strains for which the MIC was 0.064 mg L\(^{-1}\) (\( n = 9 \)), two different situations were found: five isolates showed penA alleles related to Pen\(^5\) strains and four isolates showed mosaic penA alleles.

A very interesting finding was that the MIC for ampicillin was the best marker to define intermediate resistance to penicillin: MICs for ampicillin \( \geq 0.125 \text{ mg L}^{-1} \) were fully points for intermediate resistance to penicillin, particularly using the Etest method (the most widely used across Europe), 43 strains isolated from cases of invasive meningococcal disease in Spain were analyzed (Arreaza *et al*., 2004). Susceptibility to penicillin G was determined by Etest in Mueller–Hinton agar supplemented with 5% whole defibrinated sheep blood according to the manufacturer’s instructions. At the same time, the 1.4-kb DNA fragment of the penA gene was sequenced and analyzed as previously described (Arreaza & Vázquez, 2001). All the strains for which the MIC was \( \leq 0.047 \text{ mg L}^{-1} \) showed penA alleles related to PenS strains. Mosaic penA sequences were identified in all the strains for which the MIC was \( \geq 0.094 \text{ mg L}^{-1} \). Among those strains for which the MIC was 0.064 mg L\(^{-1}\) (\( n = 9 \)), two different situations were found: five isolates showed penA alleles related to Pen\(^5\) strains and four isolates showed mosaic penA alleles.

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associated with PenI strains, whereas MICs ≤ 0.094 mg L⁻¹ identified susceptible isolates. Testing ampicillin (in addition to penicillin G) should therefore be recommended to better define PenI isolates.

In line with these data, 0.094 mg L⁻¹ was suggested as the penicillin G intermediate breakpoint when the Etest method is used.

The breakpoint for full resistance has still not been resolved. According to the current study, there is no evidence of additional changes on the penA gene in strains with MICs of 0.25 or 0.5 mg L⁻¹. Furthermore, alterations in other PBPs have been associated with acquisition of the highest level of resistance (Antignac et al., 2003). Only with characterization of the mechanisms that increase the level of resistance will it be possible to obtain microbiological bases that provide more precise breakpoints at this level.

**Extent of resistance in Europe**

When the EU-MenNet project was ongoing, there was a strong heterogeneity as to the breakpoints used for resistance definition, reflected in the analysis of the data obtained from the questionnaire. However, most of the European laboratories were already using similar breakpoints to those applied for Neisseria gonorrhoeae: susceptibility with MICs ≤ 0.06 mg L⁻¹ PBPs, intermediate resistance with MICs > 0.12 mg L⁻¹ and < 2 mg L⁻¹, and resistance with MICs ≥ 2 mg L⁻¹. These criteria were applied when, in a co-ordinated effort between EU-MenNet and the European project on 'Invasive Bacterial Infections Surveillance' (EU-IBIS), the same definitions were used for the analysis of the European data collected. In addition to this initiative, and as part of the tasks of the EU-MenNet project, a second questionnaire was also sent to be able to determine a figure for the real extent of antimicrobial resistance, focused mainly on penicillin but also on rifampicin. As result of both analyses, the map of the intermediate resistance to penicillin (Fig. 2) appeared heterogeneous and the differences among countries difficult to explain and still poorly understood. Spain appears to lead this particular ranking, whereas several Eastern European countries have a low level of resistance. Differences found even between neighbouring countries might be reflecting the use of different methodologies to define resistance. It is well known that techniques for in vitro testing appear to depend on several, sometimes complex factors which affect the final results and their interpretation. However, the differences are more probably due to both different clonal lineages and/or uneven access to antibiotics in each country.

To analyze whether different alleles of the penA gene are present in different clonal lineages or geographical areas, we obtained 78 strains isolated in Austria, Czech Republic, Greece, Italy, Norway and Scotland. No additional alleles to those already found among Spanish strains appeared. An interesting finding was that the highest genetic heterogeneity of group B meningococci was also reflected at the penA gene level, with a high variety of alleles not particularly related to the clonal lineage determined by MLST. However, those PenI strains characterized as C:2b showed uniformly the allele number 20, including both those belonging to the

![Fig. 2. European percentage distribution of intermediate resistance to penicillin in meningococcal strains. The breakpoints used for the definition of this level of resistance were > 0.06 mg L⁻¹ and < 1 mg L⁻¹.](image)
ST11 (ET37) and the ST8 (A4) lineages. The strains belonging to the ST11 complex characterized as C:2a showed a different penA gene sequence (allele 5) than did the strains included in the ST11 genetic lineage that are characterized as C:2b (allele 4), which could be explained by horizontal genetic exchange with different commensal Neisseria species. In fact, allele 5 showed high homology with the penA gene sequence of a Pen^{1} strain (N. meningitidis 1072) isolated in Spain 14 years ago, which, according to Spratt et al. (1992), could have arisen by recombination with the penA gene of Neisseria cinerea. However, the mosaic structure found in allele 4 has been related to the penA gene of Neisseria flavescens (Spratt et al., 1992).

The horizontal transfer of specific penA alleles (e.g. allele 4) could suggest that these alleles show advantages (e.g. more stability and better enzymatic activity) compared with other mosaic penA genes. If this is true, specific penA alleles could be overrepresented in the Pen^{1} N. meningitidis population, and the spread of the Pen^{1} strains could be related not only to the expansion of particular clones but also to the horizontal exchange of those ‘successful’ alleles. More sequencing data are needed before this issue can be resolved.

The organization of a wide database including penA sequences from different isolates (Pen^{1} but also Pen^{5} strains) is attractive as a way to answer some of the questions related to the definition of antimicrobial resistance in meningococci. In this sense, as an initiative of the Working Group for antimicrobial resistance of the EMGM, an on-line database (something unusual but not rare) indicates that we have only just started down the road to being able to understand antimicrobial resistance in N. meningitidis.

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