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Genotypes and antimicrobial-resistant phenotypes of *Neisseria gonorrhoeae* in Portugal (2004–2009)

Carlos Florindo,¹ Rui Pereira,¹ Márcia Boura,¹ Baltazar Nunes,² Albertina Paulino,¹ João P Gomes,¹ Maria J Borrego¹

ABSTRACT

Objectives To determine the antibiotic phenotype and MAST-genotype distribution of *Neisseria gonorrhoeae* isolates in Portugal between 2004 and 2009, and to evaluate specific associations between MAST-genotypes and sexual orientation, age and antibiotic resistance.

Methods A total of 236 *N gonorrhoeae* isolates were typed through *N gonorrhoeae* multiantigen sequence typing (NG-MAST). The degree of polymorphism and the phylogenetic relatedness among NG-MAST sequence types (STs) were evaluated with MEGA4 software on concatenated sequences of *por* and *tbpB* alleles. Etest was used to determine the susceptibility to ceftriaxone, ciprofloxacin, penicillin and spectinomycin.

Results No isolates displayed resistance to spectinomycin and ceftriaxone, whereas 79.1% and 37.4% were resistant to penicillin and ciprofloxacin, respectively. A total of 104 different STs (one per 2.3 isolates) were found; the most common were ST210 (8.1%) and ST225 (7.6%). STs formed two major groups separated by 159.8 (SE 8.9) nucleotide differences, yielding several subgroups, one of them including the worldwide-prevalent ST225. The probability of ciprofloxacin resistance among isolates within this subgroup was 73.5-fold higher than for the remaining isolates. Indeed, for the genetically closest subgroup, which includes the most prevalent ST (ST210), only 8.0% of isolates were resistant to ciprofloxacin. There was a non-homogenous distribution per year for ST225 (p<0.001), ST210 (p=0.011) and ST2 (p=0.007).

Conclusions The heterogeneous ST scenario may represent the ‘tip of the iceberg’, reflecting a high number of undiagnosed and unreported gonorrhoea cases. A laboratory-based national surveillance of *N gonorrhoeae* infections is necessary to provide a broader spectrum of isolates that will allow the sexual network situation in Portugal to be established.

INTRODUCTION

*Neisseria gonorrhoeae*, the aetiological agent of gonorrhoea, remains one of the most common bacterial sexually transmitted infections (STIs), as more than 60 million new cases are reported annually worldwide.¹ ² Recent trends in gonorrhoea have shown a progressive increase in several countries in Western Europe (eg, Sweden and Switzerland), resulting in the resurgence of an old public health problem.² ³ These alarming data may indicate a return to high-risk sexual behaviours, contributing to onward transmission of STIs, such as HIV, which highlights the need for routine surveillance, prevention and control measures.⁵

Methods *N gonorrhoeae* isolates

Between 2006 and 2009, more than 100 laboratories throughout the country were asked to send *N gonorrhoeae* isolates to the national bacterial STI laboratory located in the Portuguese NIH. The national collection was sent yearly to the European Surveillance of Sexually Transmitted Infections network. Twenty-five laboratories (including the NIH reference laboratory) from the Lisbon area (n=15), Porto area (n=6), Leiria (n=2) and Algarve (n=2) participated, leading to the collection of 274 *N gonorrhoeae* isolates. Participating laboratories provided the following data: date of isolation,
specimen anatomical site, and gender, age and sexual orientation of the infected individual. All data were anonymous, to avoid any ethical conflict.

A total of 236 of the total (274) \textit{N. gonorrhoeae} isolate collection were fully typed through NG-MAST. The infected people were aged from under 1 year to 58 years, median age 28. Of these, 26 were isolated from women and 210 from men during the years 2004 (n=17), 2006 (n=29), 2007 (n=75), 2008 (n=50) and 2009 (n=65). Considering the sexual orientation, 74 were heterosexual (18 women and 56 men), 70 were men who had sex with men, and for 92 no information was provided.

\textbf{Antimicrobial susceptibility testing}

The isolates were subcultured once in chocolate agar (BioMérieux, Marcy l’Etoile, France) before susceptibility testing was performed. Suspensions of cultures aged 24 h were prepared equivalent to McFarland’s standard 0.5. The ceftriaxone, ciprofloxacin, penicillin, and spectinomycin minimum inhibitory concentration (MIC) of isolates was determined on GC agar (Oxoid, Basingstoke, UK) with 1% Isovitalex (Becton, Dickinson and Company, Sparks, Maryland, USA) using Etests (AB bioMérieux, Solna, Sweden) according to the manufacturer’s instructions. Plates were allowed to incubate for 24 h at 36°C in 5% CO\textsubscript{2}.

Isolates were categorised according to the following definitions: ciprofloxacin resistant (MICs $\geq$ 1 mg/l), spectinomycin resistant (MICs $\geq$ 128 mg/l), ceftriaxone resistant (MICs $\geq$ 0.5 mg/l), decreased susceptibility to ceftriaxone (0.125 $\leq$ MICs $< 0.25$ mg/l), and penicillin resistant (MICs $\geq$ 2 mg/l). All penicillin intermediate or resistant isolates were tested for penicillinase production using the chromogenic reagent, Nitrocefin (Oxoid), according to the manufacturer’s instructions.

\textbf{NG-MAST}

The molecular genotyping of all \textit{N. gonorrhoeae} isolates was performed using the NG-MAST method, as previously described.\textsuperscript{14} Briefly, \textit{N. gonorrhoeae} DNA was extracted with QIAamp DNA mini kit (Qiagen, Valencia, California, USA), according to the manufacturer’s instructions. Two highly polymorphic loci, \textit{por} and \textit{tbpB}, were subjected to PCR amplification, and both strands of internal fragments were sequenced (490 and 390 bp, respectively) by using Big Dye V.1.1 chemistry on an ABI3700 capillary sequencer (Applied BioSystems, Foster City, California, USA). Sequences were aligned by using Lasergen software (DNASTAR, Madison, Wisconsin, USA). Alleles and sequence types (STs) were assigned from the international database of the NG-MAST website (http://www.ng-mast.net/).

\textbf{Genetic analysis}

To evaluate the degree of polymorphism and the phylogenetic relatedness among NG-MAST STs, we created concatenated sequences of \textit{por} and \textit{tbpB} alleles in a head-to-tail fashion, as previously described for other pathogens.\textsuperscript{15} Subsequently, a neighbour-joining phylogenetic tree of all concatenated sequences was generated with MEGA4 software using Kimura two-parameter and p-distance models.\textsuperscript{15–18} We also generated matrices of pairwise comparisons to estimate the total number of variable sites, the overall mean genetic distance among all STs, and the genetic relatedness between putative ST clusters. The pairwise-deletion option was chosen to remove all sites containing missing data or alignment gaps for all genetic distance estimations. No analyses were performed at the protein level because the \textit{tbpB} allele becomes out-of-frame within the concatenated sequence.

\textbf{Statistical analysis}

$\chi^2$, Fisher exact and linear-by-linear tests were used to evaluate the associations between STs and antibiotic resistance, year of isolation and population characteristics (sexual orientation, age and gender). Observed differences were considered significant at $p<0.05$. All statistical results were obtained with SPSS V.15.0.

\textbf{RESULTS}

\textbf{Antimicrobial susceptibility phenotypes}

Of the 236 \textit{N. gonorrhoeae} isolates, 187 were subjected to antimicrobial susceptibility testing. No antibiotic resistance to spectinomycin and ceftriaxone was observed. Furthermore, only four isolates showed reduced susceptibility to ceftriaxone, which occurred in 2007. In contrast, 148 (79.1%) and 70 (37.4%) isolates displayed resistance to penicillin and ciprofloxacin, respectively, where 15.5% of the former were penicillinase producers. Multiresistance was detected in 35.8% of isolates considering that 95.7% of ciprofloxacin-resistant isolates were also resistant to penicillin.

\textbf{Genetic diversity of \textit{N. gonorrhoeae} isolates}

According to the NG-MAST method, 236 \textit{N. gonorrhoeae} isolates were distributed among 104 different STs, 60 (57.7%) of which had not been reported before to the international database. The majority of STs (66.3%, 69 of 104) were represented by a single isolate, whereas the remaining STs included between two and 19 isolates. This high genetic diversity arose from the allelic combination of 86 \textit{por} and 59 \textit{tbpB} alleles, which is supported by the fully branched phylogenetic tree based on concatenated sequences of these alleles (figure 1). The overall mean genetic distance was 97.3 (SE 5.1) nucleotide differences, where a maximum distance of 205 (SE 12.5) nucleotides was observed between ST4524 and ST4334. Interestingly, the 104 STs formed two major groups (figure 1) separated by 159.8 (SE 8.9) nucleotide differences, yielding several subgroups of genetically close STs, such as the one that includes the worldwide-prevalent ST225 (subgroup A2 in figure 1), which shows a genetic distance within the same-group strains of only 5.5 (SE 1.1).

The most common STs were ST210 (n=19, 8.1%) and ST225 (n=18, 7.6%), which, together with the other eight STs (table 1), represent 42% (100/236) of all isolates. There was a significant non-homogeneous distribution over the 5 years for ST225 (p<0.001), ST210 (p=0.011) and ST2 (p=0.007), with a decreasing tendency for the first two (p=0.016 and p=0.002, respectively) and an increasing tendency for the latter (p=0.005).

Evaluation of STs from nine pairs of isolates recovered from known sexual partners showed 88.9% concordance. These are represented by a diverse set of STs and are dispersed throughout the study period. The single pair of isolates that showed non-identical STs involved two novel STs (ST3628 and ST3630), which share the \textit{por} 2171 allele.

\textbf{STs versus sociodemographic data}

There were substantial differences in ST distribution by age and sexual orientation. Indeed, ST2 and ST783 were predominantly found in patients under 25 years of age (p=0.02 and p=0.009, respectively) (table 1). On the other hand, one of the most common STs, ST225, was identified predominantly in patients above 25 years (p=0.028). Regarding sexual orientation, there was a significant association between ST225 and men who have sex with men (p=0.001), whereas ST783 and ST1518 were associated with heterosexual people (p=0.014). No statistical association between geographic origin of isolates and ST was found.

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Figure 1  Phylogenetic reconstruction of concatenated sequences of *por* and *tbpB* alleles for all sequence types (STs) identified in Portugal by neighbour-joining tree topology based on distance estimates using the ‘number of nucleotide differences’ model. Two major groups of STs are marked (group A and B), which are genetically separated by 158.8 (SE 8.9) nucleotides. For group A, two subgroups are identified, A1 and A2, including ST210 and ST225, respectively. Branch lengths are proportional to nucleotide distances between STs. Bootstrap analysis with 500 replications was applied. Phylogenetic reconstruction based on the Kimura two-parameter model for substitutions events yielded a similar topology.

**STs versus antibiotic resistance**

There was a significant association between resistance to ciprofloxacin and isolates with ST1479 (100%, p<0.001), ST3615 (100%, p=0.007), ST225 (94.4%, p<0.001) and ST1407 (88.9%, p=0.002). For all the other antibiotics, no association was found.

**DISCUSSION**

In Portugal it is assumed that only a small fraction of *N gonorrhoeae* infections are notified to health authorities, which may be the reason for the apparently stable incidence rates, contradicting reports from other countries of evidence of increasing rates. It is expected that the implementation of laboratory and clinical notification (according to European Centre for Disease Prevention and Control guidelines) will provide more accurate epidemiological data on this STI. In view of the lack of data in Portugal and the recent development of the NG-MAST technique, we aimed to determine the antibiotic phenotype and NG-MAST-genotype distribution of *N gonorrhoeae* in Portugal since 2004.

The results from the antibiotic susceptibility profiles matched those reported for other countries. In fact, no isolates displayed resistance to spectinomycin and ceftriaxone, whereas 79.1% and 37.4% were resistant to penicillin and ciprofloxacin, respectively, which supports the currently recommended treatment strategy.

However, in contrast with what has been described in some countries, we observed no increased tendency for reduced susceptibility to ceftixime. Nevertheless, continuous monitoring of profiles of susceptibility to this antibiotic is advisable. Our results also support previous reports with respect to the association between antibiotic susceptibility phenotypes and STs. Indeed, ST225, which was the second most common ST in our study, and the only one disseminated worldwide, was mostly found in ciprofloxacin-resistant *N gonorrhoeae* isolates (p<0.001). We also found a similar association for ST1459 (p<0.001), so far only described in France, and this comprised solely isolates resistant to this antibiotic. Curiously, the rate of ciprofloxacin resistance within a subgroup of isolates that includes ST225 was 96.4%. Indeed, the probability of ciprofloxacin resistance among these isolates was 73.5-fold higher than for the remaining isolates with other STs. Interestingly, the genetically and phylogenetically closest subgroup (subgroup A1 in figure 1), which includes the most prevalent ST (ST210), showed only 8.0% resistance to ciprofloxacin. Thus, on the basis of our results and the literature, we can speculate that ciprofloxacin resistance is associated with ST225 and genetically closely related STs. However, the opposite cannot be stated, as isolates resistant to ciprofloxacin can be found phylogenetically distant from ST225, such as for ST1479 (in group B in figure 1), which has 148 (SE 11)
nucleotide differences from ST225. Further studies including more isolates and STs are essential to understand this phenomenon.

The phylogenetic tree showed a large strain diversity, which could be expected because of the high polymorphism of the two alleles involved in the NG-MAST method, and classified STs into two major groups (figure 1) with a genetic distance of 159.8 (SE 8.9) nucleotide differences. However, a large number of STs are represented by a single isolate, and no predominant ST exists, which may be due to the local emergence of new STs, the recent introduction of foreign STs, or incomplete/insufficient epidemiological surveillance. In Portugal, although our data do not represent the whole country, this heterogeneous ST situation may represent the ‘tip of the iceberg’, reflecting a large number of undiagnosed and unreported gonorrhoea cases. Thus, such diversity of STs in the studied population did not allow identification of the sexual networks that spread this infection, in contrast with what has been achieved in the UK. 23 However, eight of the nine sexual partners included in this study were infected by isolates with the same ST, which reinforces the interest in NG-MAST as a tool for the construction of sexual networks. Nonetheless, the high rate of polymorphism of the two genes could lead to slight differences in the resulting combined genotype. In fact, the discordant couple (ST3628 and ST3630) had the same combined genotype. In fact, the discordant couple (ST3628 and ST3630) had the same combined genotype.

<table>
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<th>n</th>
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<th>HS</th>
<th>p Value</th>
<th>Sexual orientation</th>
<th>Age</th>
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Values are number; (%), percentage determined from known information.

HS, heterosexual; I, intermediate; MSM, men who have sex with men; R, resistant; S, susceptible.

Key messages

- Sequence types (STs) formed two major groups separated by 159.8 (SE 8.9) nucleotide differences, yielding several subgroups.
- Ciprofloxacin resistance seems to be associated with ST225 and genetically closely related STs.
- The multiplicity of STs (one per 2.3 isolates) may reflect a high number of undiagnosed and unreported gonorrhoea cases.

References


