Phenotypic and genetic characterization of the 2008 WHO Neisseria gonorrhoeae reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes

Magnus Unemo1*, Oskar Fasth1, Hans Fredlund1, Athena Limnios2 and John Tapsall2

1National Reference Laboratory for Pathogenic Neisseria, Department of Clinical Microbiology, Örebro University Hospital, Örebro, Sweden; 2WHO Collaborating Centre for STD, Microbiology Department, The Prince of Wales Hospital, Randwick, Sydney, Australia

Received 22 December 2008; returned 11 February 2009; revised 24 February 2009; accepted 26 February 2009

Objectives: Emergence and spread of antimicrobial resistance (AMR) in Neisseria gonorrhoeae remain a major global problem and expanded, but valid, AMR surveillance is crucial for public health purposes. The World Health Organization (WHO) Collaborating Centre in Sydney, Australia, continually evaluates N. gonorrhoeae strains used in quality control and assurance aspects of the national, WHO regional and international programmes for AMR surveillance it conducts. Here we phenotypically and genetically characterized the 2008 WHO N. gonorrhoeae reference panel, widely used under existing WHO AMR surveillance protocols.

Materials and methods: The eight N. gonorrhoeae WHO reference strains were phenotypically characterized by antibiogram, auxotype, serovar and prolyliminopeptidase screening; and genetically with regard to resistance plasmid types, polymorphisms in divergent genetic resistance-mediating loci (n=9), porB sequencing and N. gonorrhoeae multi-antigen sequence typing.

Results: The 2008 WHO reference strains represented all the important susceptible and resistant phenotypes, including corresponding resistance genotypes, and the range of resistances currently seen for relevant antimicrobials. Several pertinent additional phenotypic and genotypic markers, for example, epidemiological markers, were also determined.

Conclusions: The 2008 WHO N. gonorrhoeae reference strain panel was extensively characterized, which is crucial for the expansion of gonococcal AMR surveillance nationally and internationally. The panel is available through WHO sources for quality assurance and quality control aspects of current phenotypic testing protocols, to allow valid comparison of AMR data derived by divergent methods, and also for the control of present and future molecular assays for AMR detection. Additional WHO reference strains can be included as required by the emergence of additional resistant phenotypes and/or genotypes.

Keywords: antimicrobial resistance testing and surveillance, 2008 World Health Organization reference strains, genotypic characterization, quality assurance and quality control

Introduction

The impact of antimicrobial resistance (AMR) in Neisseria gonorrhoeae on the effective treatment and thus the control of gonorrhoea is of long-standing concern. Recognition of clinical treatment failures of penicillins in widely dispersed jurisdictions shortly after their introduction prompted the World Health Organization (WHO) to develop systems for surveillance of in vitro AMR in N. gonorrhoeae. These surveillance data were used to prepare, and modify as required, standard regimens for the treatment of gonorrhoea, and in epidemiological studies and inter-laboratory data comparisons. An international collaborative
Characterization of the 2008 WHO N. gonorrhoeae strains

This present study characterized the 2008 WHO N. gonorrhoeae reference panel phenotypically [antibiograms, auxotypes, serovars and presence of prolyliminopeptidase (PIP) for epidemiological purposes] and genotypically [resistance plasmid types, polymorphisms in divergent genetic resistance-mediating loci (n=9) and full-length porB sequencing and N. gonorrhoeae multi-antigen sequence typing (NG-MAST) for molecular epidemiological purposes]. The molecular characterization of resistance mechanisms is becoming increasingly relevant for surveillance of resistance to antimicrobials such as fluoroquinolones, spectinomycin, azithromycin and especially for the expanded-spectrum third-generation cephalosporins, despite current test criteria remain based on in vitro MIC susceptibility determinations. This current panel of eight WHO N. gonorrhoeae control strains is intended for the internal and external quality assurance and quality control components of gonococcal AMR testing protocols, and is a prerequisite for any global WHO AMR surveillance programme for N. gonorrhoeae. It is also suggested that laboratories using the now superseded WHO A–E reference strains should update these to the current panel. The further rationale and applications for, and uses of, the WHO N. gonorrhoeae control strains and panels are provided in WHO documents elsewhere.

Materials and methods

Bacterial strains

The strains of N. gonorrhoeae examined (n=8) comprise the 2008 WHO reference strain panel for global quality assurance and quality control of gonococcal AMR testing. The strains, designated as WHO F (origin: Canada, 1991), WHO G (Thailand, 1997), WHO K (Japan, courtesy of Dr T. Muratani, University of Occupational and Environmental Health, Japan, 2003), WHO L (Asia, 1996), WHO M (Philippines, 1992), WHO N (Australia, 2001), WHO O (Canada, 1991) and WHO P (USA, courtesy of Dr J. S. Knapp, Centers for Disease Control and Prevention, USA), were cultivated and preserved as described previously.

Auxotype determination

Auxotyping was performed as described previously.

Serovar determination

Serovar determination using PhaeBact GC Monoclonal Serovar Test (Bactus AB, Stockholm, Sweden) was performed as described previously.

Detection of PIP

PIP production was detected as described previously.

Antimicrobial susceptibility testing

β-Lactamase production was detected using nitrocefin discs, and MICs (mg/L) of 14 antimicrobials were determined using the Etest method (AB Biodisk, Solna, Sweden) as described previously. For the purposes of their phenotypic characterization only, the susceptible, intermediate susceptibility and resistance (S, I and R) categorization, based on the interpretative criteria published by the Swedish
Reference Group on Antibiotics (SRGA; www.srga.com) using this method,\textsuperscript{25} was also determined. For the antimicrobials where SRGA does not describe any breakpoints (ertapenem, kanamycin, gentamicin and rifampicin), breakpoints from other sources were used (www.bsac.org.uk).\textsuperscript{26,27} In order to reduce the effect of inter-assay variations, for all strains and antimicrobials each determination was performed three times using new bacterial suspensions and the consensus MIC was reported.

**Isolation of bacterial DNA**

Genomic DNA was isolated using the MagNA Pure LC system (Roche Diagnostics GmbH, Mannheim, Germany), as described previously.\textsuperscript{17} Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. Isolated DNA was stored at 4°C prior to PCR amplification.

**PCR amplification of genes associated with AMR and molecular epidemiology**

The primers, instruments, PCR programmes and reaction mixtures used in the amplification of each gene are summarized in Table 1. All PCR products were stored at 4°C prior to purification.

**PCR product purification**

All PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH) in accordance with the instructions of the manufacturer.

**DNA sequencing and sequence analysis**

All cycle sequencing PCRs were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a GeneAmp 2720 Thermal Cycler (Applied Biosystems), according to the manufacturer’s instructions. The primers used are described in Table 2. The purification of the cycle sequencing extension products and the sequence determination on an ABI PRISM\textsuperscript{TM} 3100 Genetic Analyzer (Applied Biosystems) were performed as documented previously.\textsuperscript{23,36}

The resulting chromatograms were corrected and assembled using the ChromasPro (version 1.34) software (Technelysium Pty Ltd, Tewantin, Australia). Sequence alignments were performed using BioEdit Sequence Alignment Editor (version 7.0.9.0) software (Ibis Biosciences, Carlsbad, CA, USA). Determination of NG-MAST\textsuperscript{24} sequence types (STs) was performed using partial tcpB and porB sequences as described previously.\textsuperscript{25}

**Results**

The results of the phenotypic and genetic characterization are summarized in Tables 3 and 4.

**Phenotypic characterization**

Four (50%) of the strains were of proline-requiring auxotype and the remaining four were non-requiring. The strains represented both serogroups WI (PorB1a; \(n=3\)) and WII/III (PorB1b; \(n=5\)), and in total, five different serovars [Arst (\(n=3\)), Brpyust (\(n=2\)) and one strain each of serovar Brpyust, Boys and Bopt]. Two of the strains (WHO G and N) were PIP-negative (Table 3). \(\beta\)-Lactamase production was detected in three of the strains (WHO M, N and O). For the purposes of their phenotypic characterization only, the SIR categorization and consensus MICs for each antimicrobial and strain, as well as the range of detected MICs for all strains, as determined by the single method used, are presented in Table 4, and are also referred to further on.

**Genetic characterization**

Of the \(\beta\)-lactamase (TEM-1)-producing strains (\(n=3\)), two (WHO M and O; penicillin G, MIC 8 and >32 mg/L) carried plasmids of the African type and one (WHO N; penicillin G, MIC 8 mg/L) of the Asian type.\textsuperscript{29} The tet(M)-carrying conjuga-
tive plasmids, which encode a ribosome-protecting protein, identified in two of the strains (WHO G and N; tetracycline, MICs 16 and 32 mg/L), were of the Dutch type.\textsuperscript{28} Regarding penA, one of the strains (WHO F) contained a wild-type allele (penicillin G, MIC 0.032 mg/L), one a mosaic allele (WHO K; penicillin G, cefixime and ceftriaxone MICs 2, 0.5 and 0.064 mg/L, respectively) and six displayed the insertion of one codon causing a D345A alteration in penicillin-binding protein 2 (PBP 2), in conjunction with four to six characteristic additional amino acid alterations in the C-terminal transpeptidase region of the protein. WHO L also contained an A501V mutation in penA. Three strains (WHO F, L and N) contained a wild-type mtrR promoter region sequence. The remaining strains (\(n=5\)) displayed a deletion of a single nucleotide (A; \(n=4\)) or an A→C substitution (\(n=1\)) in the 13 bp inverted repeat of the promoter sequence. Three of the strains displayed wild-type mtrR coding region sequences. However, three strains (WHO K, L and M) displayed a G45→D alteration and the remaining two strains (WHO N and P) an insertion or deletion of a single nucleotide causing a frame-shift, subsequent premature stop codon and truncated peptide. Concerning the penB alterations, all the PorB1b strains (\(n=5\)) displayed the A121→D alteration and four of them contained also the G120→K alteration, which mediate decreased permeability of the porin PorB1b. Two of the strains (WHO F and P) had wild-type penA sequences and the remaining six carried mutations (penA1 allele), causing the L421→P alteration in PBP 1. With respect to fluoroquinolones, three of the strains (ciprofloxacin, MIC 0.004–0.008 mg/L) contained wild-type gyrA, parC and parE quinolone resistance-determining region (QRDR) sequences. Regarding gyrA and parC, one strain displayed only a GyrA S91→F mutation (WHO G; ciprofloxacin, MIC 0.125 mg/L), one a GyrA S91→F and a GyrA D95→G mutation (WHO M; ciprofloxacin, MIC 2 mg/L) and the remaining ciprofloxacin-resistant strains (WHO K, L and N) contained a GyrA S91→F mutation, a GyrA D95→G/N mutation and one or two amino acid alterations in ParC codons 86–88 (ciprofloxacin, MIC 4 to >32 mg/L). In addition, two strains (WHO G and N) displayed a G410→V substitution in parE. One strain (WHO O) displayed a C1192→T nucleotide transition in the 16S rRNA gene (C1192→U in the transcribed 16S rRNA; spectinomycin, MIC >1024 mg/L). Concerning rpoB, five strains had a wild-type allele and the three remaining (WHO M, N and P) had an H552→N mutation (rifampicin, MIC >32 mg/L) (Table 4). The strains displayed eight different porB alleles as well as eight divergent NG-MAST STs, of which three have not previously been described (Table 3).
<table>
<thead>
<tr>
<th>Gene/primers</th>
<th>Instrument</th>
<th>PCR programme (reference)</th>
<th>Reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>tet(M)</td>
<td>Roche LightCycler 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25 cycles of 95°C 10 s, 47°C 10 s, 72°C 31 s&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>β-Lactamase (TEM-1) plasmid</td>
<td>Roche LightCycler 1.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(29)</td>
<td>1</td>
</tr>
<tr>
<td>penA</td>
<td>PTC-100&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(7)</td>
<td>2</td>
</tr>
<tr>
<td>mtrR</td>
<td>Roche LightCycler 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(7)</td>
<td>1</td>
</tr>
<tr>
<td>ponA</td>
<td>Roche LightCycler 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(7)</td>
<td>1</td>
</tr>
<tr>
<td>gyrA</td>
<td>Roche LightCycler 2.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>45 cycles of 95°C 10 s, 60°C 10 s, 72°C 36 s&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>parC</td>
<td>Roche LightCycler 2.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>40 cycles of 95°C 10 s, 60°C 10 s, 72°C 36 s&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>parE</td>
<td>Roche LightCycler 2.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>45 cycles of 95°C 10 s, 60°C 10 s, 72°C 20 s&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>rpoB</td>
<td>Roche LightCycler 2.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>35 cycles of 95°C 10 s, 60°C 10 s, 72°C 36 s&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>ABI GeneAmp 2720&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(33)</td>
<td>3</td>
</tr>
<tr>
<td>porB</td>
<td>Roche LightCycler 1.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(23)</td>
<td>1</td>
</tr>
<tr>
<td>tbpB</td>
<td>Roche LightCycler 1.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>(23)</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Synthesized by Scandinavian Gene Synthesis AB, Köping, Sweden.
<sup>b</sup>Contents of reaction mixtures:

(i) 20 μL reaction mixture: 3 mM MgCl₂, 2 μL LightCycler FastStart DNA Master SYBR Green I, 0.5 μM of each primer, 2 μL DNA template.
(ii) 50 μL reaction mixture: 2.5 mM MgCl₂, 5 μL AmpliTaq Gold Buffer, 800 μM dNTPs, 1 μM PA2 primer, 1 μM B1 primer, 1.25 U AmpliTaq Gold enzyme, 1 μL DNA template.
(iii) 50 μL reaction mixture: 2.5 mM MgCl₂, 0.04 μg bovine serum albumin, 7.5% glycerol, 5 μL AmpliTaq Gold Buffer, 800 μM dNTPs, 0.6 μM ru8 primer, 1.2 μM fD1 primer, 2.5 U AmpliTaq Gold enzyme, 1 μL DNA template.

<sup>c</sup>Roche Diagnostics GmbH. Real-time PCR system.
<sup>d</sup>All reaction mixtures were activated at 95°C for 10 min prior to amplification. Melting curve analysis was performed according to the manufacturer’s instructions.
<sup>e</sup>MJ Research, Watertown, MA, USA. Conventional PCR system.
<sup>f</sup>Lower case letters denote adaptors corresponding to the SekvR/SekvF primers for sequencing (Table 2).
<sup>g</sup>Applied Biosystems. Conventional PCR system.

Characterization of the 2008 WHO N. gonorrhoeae strains
Discussion

This study describes the relevant phenotypic and genetic characteristics of the eight WHO *N. gonorrhoeae* strains comprising the 2008 WHO reference panel. These strains fulfil the necessary prerequisites for use in global quality assurance and quality control aspects of gonococcal AMR testing under existing WHO protocols. Details of the basis of this WHO approach and the rationale and applications for, and use of, these strains are set out elsewhere.\textsuperscript{16,20} The 2008 WHO panel strains represent the important susceptible and resistant phenotypes and the range of resistances currently seen for the antimicrobials recommended in different guidelines and/or used in the gonorrhoea treatment globally. It is emphasized that the consensus MIC values shown (Table 4) were determined using one specific method\textsuperscript{25} only and were included for illustrative purposes. The MIC values for these strains may be different in other test systems based on other methodologies;\textsuperscript{16} however, the resistance phenotypes displayed should be consistent between test methods. When these methods are reliably performed, they can be applied in AMR surveillance for public health purposes on the basis of the resistance phenotype determination only.\textsuperscript{16,20} If there are inconsistencies between the nominated resistance phenotypes and that obtained using a particular method, close attention should first be paid to the quality control procedures applicable to the test method concerned. Further information, if required, may be obtained from the WHO AMR Surveillance Standards.\textsuperscript{20} The exact MICs obtained for the 2008 WHO strains for kanamycin, gentamicin, cefixime, ceftriaxone and ertapenem are also shown in Table 4. However, for these antimicrobials, no nominated resistance phenotypes (or genotypes) were included because, at present, sufficient and reliable clinical/laboratory correlates are not available. Nevertheless, kanamycin and gentamicin have been used extensively to treat
Table 4. Antimicrobial susceptibility/resistance phenotypic and genetic characteristics present in the 2008 WHO N. gonorrhoeae reference strain panel, intended for global quality assurance and quality control of AMR testing and surveillance

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>F</th>
<th>G</th>
<th>K</th>
<th>L</th>
<th>M</th>
<th>N</th>
<th>O</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Lactamase production (PPNG)</strong></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Penicillin G (0.032 to &gt;2.32)</td>
<td>S (0.032)</td>
<td>I (0.5)</td>
<td>CMRNG&lt;sup&gt;a&lt;/sup&gt; (2)</td>
<td>CMRNG&lt;sup&gt;a&lt;/sup&gt; (2)</td>
<td>PPNG&lt;sup&gt;a&lt;/sup&gt; (8)</td>
<td>PPNG&lt;sup&gt;a&lt;/sup&gt; (8)</td>
<td>PPNG&lt;sup&gt;a&lt;/sup&gt; (&gt;32)</td>
<td>I (0.25)</td>
</tr>
<tr>
<td>Ampicillin (0.032–0.24)</td>
<td>S (0.032)</td>
<td>I (0.25)</td>
<td>I (2)</td>
<td>I (2)</td>
<td>PPNG&lt;sup&gt;a&lt;/sup&gt; (8)</td>
<td>PPNG&lt;sup&gt;a&lt;/sup&gt; (8)</td>
<td>PPNG&lt;sup&gt;a&lt;/sup&gt; (24)</td>
<td>S (0.064)</td>
</tr>
<tr>
<td>Cefuroxime (0.064–1.25)</td>
<td>S (0.064)</td>
<td>I (0.5)</td>
<td>R (12)</td>
<td>R (8)</td>
<td>I (0.5)</td>
<td>I (0.25)</td>
<td>I (1)</td>
<td>I (0.125)</td>
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<tr>
<td>Ceftriaxone (&lt;0.016–0.5)</td>
<td>S (&lt;0.016)</td>
<td>S (&lt;0.016)</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt; (0.5)</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt; (0.25)</td>
<td>S (&lt;0.016)</td>
<td>S (&lt;0.016)</td>
<td>S (0.016)</td>
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<td>Ertapenem (&lt;0.004–0.125)</td>
<td>S (&lt;0.004)</td>
<td>S (0.008)</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt; (0.125)</td>
<td>NS&lt;sup&gt;a&lt;/sup&gt; (0.064)</td>
<td>S (0.012)</td>
<td>S (0.004)</td>
<td>S (0.032)</td>
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<tr>
<td>Erythromycin (0.5–4)</td>
<td>S (0.5)</td>
<td>I (1)</td>
<td>I (1)</td>
<td>I (2)</td>
<td>I (1)</td>
<td>S (0.5)</td>
<td>I (1)</td>
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<tr>
<td>Azithromycin (0.125–2)</td>
<td>S (0.125)</td>
<td>S (0.25)</td>
<td>S (0.25)</td>
<td>I (0.5)</td>
<td>S (0.25)</td>
<td>S (0.125)</td>
<td>S (0.25)</td>
<td>R (2)</td>
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<tr>
<td>Ciprofloxacin (0.004 to &gt;32)</td>
<td>S (0.004)</td>
<td>LLR&lt;sup&gt;e&lt;/sup&gt; (0.125)</td>
<td>HLR&lt;sup&gt;e&lt;/sup&gt; (&gt;32)</td>
<td>HLR&lt;sup&gt;e&lt;/sup&gt; (&gt;32)</td>
<td>R (2)</td>
<td>R (4)</td>
<td>S (0.008)</td>
<td>S (0.004)</td>
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<tr>
<td>Tetracycline (0.25–32)</td>
<td>I (0.25)</td>
<td>TRNG&lt;sup&gt;f&lt;/sup&gt; (32)</td>
<td>R (2)</td>
<td>R (4)</td>
<td>I (1)</td>
<td>TRNG&lt;sup&gt;f&lt;/sup&gt; (16)</td>
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<td>I (0.5)</td>
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<tr>
<td>Rifampicin&lt;sup&gt;d&lt;/sup&gt; (0.125 to &gt;32)</td>
<td>S (0.125)</td>
<td>S (0.5)</td>
<td>S (0.5)</td>
<td>R (&gt;32)</td>
<td>R (&gt;32)</td>
<td>S (0.25)</td>
<td>R (&gt;32)</td>
<td></td>
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<tr>
<td>penA mosaic allele</td>
<td>—</td>
<td>—</td>
<td>yes&lt;sup&gt;g&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
<td>penA I312M, V316T, G545S&lt;sup&gt;h&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
<td>yes&lt;sup&gt;g&lt;/sup&gt;</td>
<td>—</td>
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<tr>
<td>penA AS01V&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>—</td>
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<td>—</td>
<td>yes</td>
<td>yes</td>
<td>—</td>
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<tr>
<td>mtrR promoter region 13 bp</td>
<td>WT&lt;sup&gt;i&lt;/sup&gt;</td>
<td>deletion of A</td>
<td>deletion of A</td>
<td>WT&lt;sup&gt;i&lt;/sup&gt;</td>
<td>deletion of A</td>
<td>WT&lt;sup&gt;i&lt;/sup&gt;</td>
<td>—</td>
<td>deletion of A at bp 158&lt;sup&gt;k&lt;/sup&gt;</td>
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<tr>
<td>mtrR Gly 45&lt;sup&gt;j&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;i&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;i&lt;/sup&gt;</td>
<td>G&lt;sup&gt;45→D&lt;/sup&gt;</td>
<td>G&lt;sup&gt;45→D&lt;/sup&gt;</td>
<td>G&lt;sup&gt;45→D&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>—</td>
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<tr>
<td>mtrR coding region frame-shift mutation</td>
<td>—</td>
<td>—</td>
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<tr>
<td>porB&lt;sup&gt;1b&lt;/sup&gt; codon 120</td>
<td>NA&lt;sup&gt;j&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;j&lt;/sup&gt;</td>
<td>G&lt;sup&gt;120→K&lt;/sup&gt;</td>
<td>G&lt;sup&gt;120→K&lt;/sup&gt;</td>
<td>G&lt;sup&gt;120→K&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;j&lt;/sup&gt;</td>
<td>G&lt;sup&gt;120→K&lt;/sup&gt;</td>
<td>WT&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>NA&lt;sup&gt;j&lt;/sup&gt;</td>
<td>A&lt;sup&gt;121→D&lt;/sup&gt;</td>
<td>A&lt;sup&gt;121→D&lt;/sup&gt;</td>
<td>A&lt;sup&gt;121→D&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;j&lt;/sup&gt;</td>
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<td>A&lt;sup&gt;121→D&lt;/sup&gt;</td>
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<tr>
<td>penA&lt;sup&gt;i&lt;/sup&gt;, i.e. causing L421→P in PBP 1</td>
<td>—</td>
<td>—</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
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Table 4. Continued

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Note: 
- **WHO reference panel**
- **TTRNG**, plasmid-mediated tetracycline-resistant *N. gonorrhoeae*
- **NG-MAST**: New gonococcal AMR testing in the immediate future.
- **PLR**, low-level resistance; **HLR**, high-level resistance.
- **NS**, non-susceptible containing genetic resistance markers, but clinical/laboratory correlates are insufficient to allow resistance phenotype designation at present.
- **BBP**, β-lactamase producing.
- **TTRNG**, plasmid-mediated tetracycline-resistant *N. gonorrhoeae*

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... gonorrhoea in some settings, for example, Indonesia and Malawi, and gentamicin, in single use or in combination therapy, and ertapenem have been proposed for possible use in this disease as further resistance to established antimicrobial agents increases or availability of others reduces. Rifampicin has been used in, for example, Indonesia and resistance has been emerging. A spectrum of relevant additional phenotypic markers (auxotypes, serovars and presence of PIP for epidemiological purposes) was also determined.

The presently known genetic alterations, acting singly or collaboratively, that mediate the AMR patterns and genotypic epidemiological markers (full-length *porB* sequencing and NG-MAST) present in the different resistant phenotypes were also comprehensively detailed (Table 4). The documentation of the genetic determinants mediating the AMR to most antimicrobials in the 2008 WHO panel strains is of increasing importance. Determination of AMR phenotypes by isolate-based methods will remain a mainstay of AMR surveillance programmes for gonococcal AMR testing in the immediate future. However, already a combination of isolate-based examination for resistance and molecular-based determination of the mechanisms of resistance has been proposed for the detection of resistance to the oral third-generation cephalosporins. Further, the feasibility of using molecular methods for detecting particular molecular resistance determinants in non-viable gonococci has been proposed (F. Vernel-Pauliac, T. S. Hogan, J. W. Tapsall, M. S. Goorant, unpublished results) to assist in AMR surveillance in situations in which maintaining gonococci for AMR testing is difficult. Additionally, the combined introduction and increasing use of nucleic acid amplification assays in developed countries and that of syndromic, as opposed to aetiologically, based diagnosis in resource-poor settings have resulted in a sharp decline in the number of isolates available for conventional AMR testing and the need for exploration of molecular-based approaches to AMR surveillance. Although there are significant problems regarding the application of current molecular assays to AMR surveillance, it is reasonable to clearly document the currently known genetic changes associated with the resistances present in the WHO reference strains in the expectation that they may be of help in new approaches to AMR surveillance.

The genetic determinants mediating the AMR in the WHO strains were wide ranging, in keeping with the requirements for a reference panel. The resistance to penicillins was plasmid-mediated in three strains (WHO M, N and O). In four strains, however, the intermediate susceptibility (WHO G and P) or resistance (WHO K and L) was chromosomally mediated due to the presence of the *penA* mosaic allele (WHO K) or the *penA* allele with D345a insertion (WHO G, L and P), the initial and most important mechanism for chromosomally mediated resistance to penicillins. Other genetic AMR determinants that function in a stepwise, specific order when present were also determined. For example, mutations in the *mtrR* promoter or coding sequence, and *penB* and *ponA1* substitutions, which cumulatively increase the penicillin and tetracycline (*mtrR* and *penB*) MICs, were detected. *mtrR* mutations can increase the expression of the MtrCDE efflux pump and consequently confer resistance to many hydrophobic agents, for example, macrolides, as well as some hydrophilic antimicrobials, e.g. penicillins and tetracyclines. Four of the WHO strains (WHO G, K, M and O) displayed the previously described deletion of a single nucleotide (A) in the 13 bp inverted repeat in the promoter region of
Characterization of the 2008 WHO *N. gonorrhoeae* strains

*mitR*\(^{7,30,31,47}\) and three strains displayed the G45→D mutation that is important mainly if the dominant ‘A deletion’ in the promoter sequence is not present (WHO L).\(^{7,47}\) The *mitR* coding and promoter region mutations in WHO P (Table 3) seem to be rare but have been previously described in azithromycin-resistant *N. gonorrhoeae*.\(^{38}\)

The *penA* mosaic allele most notably, but also some of the *penA* D345a alleles, together with at least *mitR* and *penB* have been previously shown to be associated with reduced susceptibility to expanded-spectrum third-generation cephalosporins, for example, cefixime and cefetromox, that are currently the recommended first-line treatment of gonorrhoea in most countries.\(^{7,30,38,41,49}\) WHO K (*penA* mosaic allele, variant X\(^{38}\)) also contained the I312M, V316T and G545S mutations proposed to be important for reduced susceptibility to this group of antimicrobials.\(^{39}\) WHO L (*penA* D345a allele, variant VII\(^{38}\)) contained the A501V mutation suggested by some to also contribute to reduced susceptibility to this group of antimicrobials.\(^{40,41}\)

All the specific *gyrA* and *parC* QRDR mutations identified in the present study (Table 4) have been previously described as being of relevance to the accumulation of sequential QRDR mutations, resulting in increased MICs of fluoroquinolones in *N. gonorrhoeae*.\(^{57},23,30,50–52\) These QRDR changes initially involve specific *gyrA* mutations encoding alterations in subunit A of DNA gyrase, followed by specific *parC* mutations encoding alterations in subunit C of topoisomerase IV. The importance of the, to our knowledge, not previously described ParE G410→V alteration (WHO G and N strains), as well as overall ParE (encoding subunit E of topoisomerase IV) alterations for increased MICs of fluoroquinolones in clinical isolates, is most likely of limited microbiological and clinical relevance.\(^{31,52}\)

All the three rifampicin-resistant (MIC >32 mg/L) reference strains (WHO M, N and P) contained an H552→N mutation in *rpoB* that encodes RNA polymerase subunit B. This RpoB alteration has been previously correlated to rifampicin resistance in *Neisseria meningitidis*\(^{53}\) and most probably is also a main mechanism for rifampicin resistance in *N. gonorrhoeae*.\(^{54}\)

The single spectinomycin-resistant (MIC >1024 mg/L) strain (WHO O) displayed a C1192→T nucleotide transition in the 16S rRNA gene (C1192→U in the transcribed 16S rRNA). This single nucleotide polymorphism has been previously associated with spectinomycin resistance in *N. gonorrhoeae*, as well as in other bacterial species.\(^{54}\)

Inevitably, a few previously identified genetic AMR markers were not examined, such as mutations in chromosomal *rpsL*-encoding ribosomal protein S10 that, such as *mitR* mutations and *penB*, can increase the MICs of tetracyclines,\(^{55}\) or 23S rRNA gene mutations,\(^{56}\) the macrolide–linosamide–streptogramin B resistance *erm* genes encoding 23S rRNA methylases\(^{57}\) and *mef(A)* efflux\(^{57}\) that, such as *mitR* mutations, can cause resistance to macrolides. This was mainly because this was not considered essential and these antimicrobials, as well as, for example, chloramphenicol that was also not examined, are no longer recommended for the treatment of gonorrhoea.

In conclusion, in the present study, the 2008 WHO *N. gonorrhoeae* reference strain panel was extensively characterized both phenotypically and genetically. This characterization was crucial to anticipate the proposed expansion of AMR surveillance in *N. gonorrhoeae* in national and international programmes. Not only will this panel be widely available through WHO sources for quality assurance and quality control aspects of current testing protocols, it will allow valid comparison of AMR data derived by divergent methods. The panel will also form a basis for controls for possible future molecular assays for AMR detection. Procedures are also in place to allow timely inclusion of additional WHO reference strains if additional resistant phenotypes and/or genotypes emerge or the need for additional studies on these or other WHO strains arises. The emerging resistance to oral cephalosporins\(^{6,9}\) and also to azithromycin together with the different doses and drug formulations proposed for this agent suggests that the in vitro criteria for ‘resistance’ for these two groups of drugs may be candidates for revision in the near future and these processes are currently in train.

**Funding**

This study was supported by grants from the Örebro County Council Research Committee and the Foundation for Medical Research at Örebro University Hospital, Sweden.

**Transparency declarations**

None to declare.

**References**


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