Direct real-time PCR-based detection of Neisseria gonorrhoeae 23S rRNA mutations associated with azithromycin resistance

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Objectives: Surveillance for Neisseria gonorrhoeae azithromycin resistance is of growing importance given increasing use of ceftriaxone and azithromycin dual therapy for gonorrhoea treatment. In this study, we developed two real-time PCR methods for direct detection of two key N. gonorrhoeae 23S rRNA mutations associated with azithromycin resistance.

Methods: The real-time PCR assays, 2611-PCR and 2059-PCR, targeted the gonococcal 23S rRNA C2611T and A2059G mutations, respectively. A major design challenge was that gonococcal 23S rRNA sequences have high sequence homology with those of commensal Neisseria species. To limit the potential for cross-reaction, ‘non-template’ bases were utilized in primer sequences. The performance of the methods was initially assessed using a panel of gonococcal (n = 70) and non-gonococcal (n = 28) Neisseria species. Analytical specificity was further assessed by testing N. gonorrhoeae nucleic acid amplification test (NAAT)-negative clinical samples (n = 90), before being applied to N. gonorrhoeae NAAT-positive clinical samples (n = 306).

Results: Cross-reactions with commensal Neisseria strains remained evident for both assays; however, cycle threshold (Ct) values were significantly delayed, indicating reduced sensitivity for non-gonococcal species. For the N. gonorrhoeae NAAT-negative clinical samples, 7/121 pharyngeal samples provided evidence of cross-reaction (Ct values >40 cycles); however, the remaining urogenital and rectal swab samples were negative. In total, the gonococcal 2611 and 2059 23S rRNA nucleotides were both successfully characterized in 266/306 (87%) of the N. gonorrhoeae NAAT-positive clinical specimens.

Conclusions: Real-time PCR detection of gonococcal 23S rRNA mutations directly from clinical samples is feasible and may enhance culture- and non-culture-based N. gonorrhoeae resistance surveillance.

Introduction

Neisseria gonorrhoeae antimicrobial resistance (AMR) is now a global problem and is designated by the US CDC as one of the top three urgent AMR threats.¹ N. gonorrhoeae has developed resistance to almost all antimicrobials used for its treatment and ceftriaxone, a third-generation cephalosporin, is now the mainstay of treatment for gonococcal infection. However, over the last decade there have been increasing reports of gonococci with increased ceftriaxone MICs, along with sporadic reports of ceftriaxone-resistant strains from Japan, Europe and, more recently, Australia.²–⁴ Dual therapy regimen, combining ceftriaxone and azithromycin, have been recommended in various regions in an attempt to limit further development and spread of ceftriaxone resistance.⁵

A recognized problem with using azithromycin for treatment of gonorrhoea is that resistance can arise relatively quickly in settings where it is used frequently⁶ and is typically attributable to...
one of two mutations at positions 2611 and 2059 in the gonococcal 23S rRNA genes. In fact, in recent years there has been increasing reports of azithromycin resistance, including high-level azithromycin resistance, from various regions.\(^7\)\(^\text{13}\) as well as evidence of azithromycin resistance coexisting with reduced susceptibility to cephalosporins in gonococci.\(^1\text{6}\) There is, therefore, a need to ensure that AMR surveillance is optimal so as to monitor such trends and maintain the effectiveness of treatment regimens. Current surveillance systems are principally based on gonorrhoea culture and whilst this remains the most definitive means of measuring resistance, there are several limitations affecting the overall utility of culture-based AMR surveillance. These have been reviewed elsewhere and include the need for stringent sample handling and transport conditions to maintain organism viability.\(^1\text{5}\) More recently, there has been growing interest in the use of molecular methods to enhance gonococcal AMR surveillance.\(^1\text{6}\) Molecular methods have the potential to significantly strengthen surveillance activities, as they can potentially be used in conjunction with diagnostic nucleic acid amplification tests (NAATs) that are now commonplace for gonorrhoea. To this effect, we have previously described a PCR method for direct detection of penicillinase-producing \textit{N. gonorrhoeae}\(^1\text{7}\) which is now being used to inform treatment guidelines in some places.\(^1\text{8}\) In this study, we developed and evaluated two real-time PCR assays for the detection of two key \textit{N. gonorrhoeae} 23S rRNA mutations associated with azithromycin resistance.

**Materials and methods**

Two real-time PCR assays, 2611-PCR and 2059-PCR, were developed targeting the gonococcal 23S rRNA C2611T and A2059G mutations, respectively. The methods were initially assessed using a panel of gonococcal \((n=70)\) and non-gonococcal \textit{Neisseria} species \((n=28)\). Their specificity was further assessed by testing \textit{N. gonorrhoeae} NAAT-negative clinical samples \((n=90)\), before being applied to \textit{N. gonorrhoeae} NAAT-positive clinical samples \((n=306)\).

**2611-PCR and 2059-PCR assay design**

Both the 2611-PCR and 2059-PCR assays were designed using a hybridization probe real-time PCR format using two sets of primers (NG-2611-F and -R and NG-2059-F and -R; Table 1) for the amplification and two sets of probes for the detection of each amplicon (NG-2611-P1 and -P2 and NG-2059-P1 and -P2; Table 1). Both sets of probes were designed to have 100% match with the WT gonococcal 23S rRNA sequence such that any alterations in the target region would result in a decrease in melting temperature that could be observed upon melting curve analysis. A novel approach was used for primer design. The key problem that needed to be addressed was that the gonococcal 23S rRNA genes have very high sequence homology with other commensal \textit{Neisseria} species and, to a lesser extent, a diverse range of other bacterial species. Thus, to improve assay specificity, all primers included one strategically placed ‘non-template’ base, i.e. a base that mismatched with gonococcal and other bacterial 23S rRNA gene sequences (these are indicated in lower case in Table 1 and are further illustrated in Figure S1, available as Supplementary data at JAC Online). It was expected that the inclusion of these single non-template bases would have limited impact upon amplification of gonococcal species. However, in combination with other recognized mismatches to commensal \textit{Neisseria} and other bacterial species, these non-template bases would be expected to limit the ability of the primers to efficiently amplify non-gonococcal species. To assess the effects of these primers upon real-time PCR cycle threshold (Ct) values, additional testing was performed using a consensus 23S real-time PCR (Con23S-PCR) that used primers that matched with both gonococcal and commensal \textit{Neisseria} species. These investigations are further described below and in Table S1.

**2611-PCR and 2059-PCR reaction mix and cycling conditions**

Both the 2611-PCR and 2059-PCR assays were performed using the LightCycler 480 Genotyping Master Mix (Roche Diagnostics, Australia). Briefly, each reaction mix consisted of 4.0 \( \mu \text{L} \) of Roche Genotyping mix, 5.0 pmol of forward primer (Table 1), 10.0 pmol of reverse primer (Table 1), 4.0 pmol of each probe (Table 1) and 2.0 \( \mu \text{L} \) of nucleic acid extract in a final reaction volume of 20.0 \( \mu \text{L} \). Amplification was performed on the Rotorgene real-time PCR instrument (Qiagen, Australia) with the following cycling conditions: initial hold at 95\(^\circ\)C for 10 min followed by 55 cycles at 95\(^\circ\)C for 10 s, 60\(^\circ\)C for 20 s and 72\(^\circ\)C for 30 s, with a fluorescent signal read at the end of the 60\(^\circ\)C step. Melting curve analysis was conducted following PCR amplification, using the following parameters: 40\(^\circ\)C hold for 90 s, then reactions were monitored as heated at a rate of 1\(^\circ\)/s to 95\(^\circ\)C. Results were analysed using the melting curve analysis tool of the LightCycler 480 software. To allow the signal from the hybridization probe format to be read, a specific channel was created using the Rotorgene software: a source of 470 nm and detection at 660 nm.

**Con23S-PCR assay**

The consensus assay (Con23S-PCR) was only used to test the commensal \textit{Neisseria} species isolate panel \((n=28)\), described below so as to provide reference Ct values against which the 2611-PCR and 2059-PCR assays could be

<table>
<thead>
<tr>
<th>Assay</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2611-PCR</td>
<td>NG-2611-F</td>
<td>CTGCAGACCGACAGCATGCG</td>
</tr>
<tr>
<td></td>
<td>NG-2611-R</td>
<td>GTCCTCGAACAGGCTTATGAC</td>
</tr>
<tr>
<td></td>
<td>NG-2611-P1</td>
<td>quasar670-TCTGCAGTGCGGCGTTGGAAGATTTT-phosphate</td>
</tr>
<tr>
<td></td>
<td>NG-2611-P2</td>
<td>GTCGTGACAGATTTTGGGCTCCT-C3-fluorescein</td>
</tr>
<tr>
<td>2059-PCR</td>
<td>NG-2059-F</td>
<td>GTCGCTGAGTTATAAGTGAAT</td>
</tr>
<tr>
<td></td>
<td>NG-2059-R</td>
<td>CAGGCTGAGTATTTCAAGAAC</td>
</tr>
<tr>
<td></td>
<td>NG-2059-P1</td>
<td>quasar670-CGCCGAACTTTCTAGGTAGCTTTGCAAT-G-phosphate</td>
</tr>
<tr>
<td></td>
<td>NG-2059-P2</td>
<td>CCCCCTGTAGACGGAAGA-C3-fluorescein</td>
</tr>
</tbody>
</table>

Lower-case bases in the primer sequences indicate the non-template bases.
compared. The Con23S-PCR was performed as per the 2611-PCR reaction and cycling conditions above except that consensus primers were used; F- GGTATGGCCTGTCGCCAATTAA and R- AATTAGTTAGGTTAGCTTCACGC. These primers were used to test the commensal Neisseria panel (n = 28) and N. gonorrhoeae control isolates (n = 70). The results were then compared with those obtained using the 2611-PCR and 2059-PCR assays.

### N. gonorrhoeae isolates

Characterized azithromycin-resistant isolates with the C2611T mutation (laboratory strain; MIC ≥ 2 mg/L) and A2059G mutation (MIC > 256 mg/L) were used as positive (mutant) reference controls. Susceptible strains lacking either mutation were used as the WT reference controls. A further 70 N. gonorrhoeae isolates collected from throughout Australia in the first half of 2012 via the National Neisseria Network and as part of an ongoing national study were tested by both assays. These 70 isolates comprised representatives of the most common genotypes in Australia for the year 2012. As part of this previous testing, their 23S rRNA 2611 and 2059 nucleotides had also been characterized using the Sequenom MassArray iPLEX technology (E. Trembizki, B. Donovan, M. Chen, R. Guy, J. Kaldor, M. M. Lahra, D. G. Regan and D. M. Whily, unpublished data). Susceptibility categories for azithromycin as defined by the Australian Gonococcal Surveillance Programme are as follows: susceptible (MIC <0.5 mg/L) or resistant (MIC ≥1 mg/L). The isolates included 67 azithromycin-susceptible strains (MICs=0.12–0.5 mg/L) and 3 resistant strains (MICs ≥2 mg/L). All 70 isolates were WT for the 2059 nucleotide. Briefly, 64/67 susceptible isolates (MICs=0.06–0.5 mg/L) were WT at nucleotide 2611 and the 3 resistant isolates (MICs ≥2 mg/L) possessed the C2611T mutation. A further 3/67 susceptible isolates (MICs=0.125–0.25 mg/L) had both WT and mutant alleles at the 2611 position. Note that gonococci have four copies of the 23S rRNA gene and previous studies have demonstrated that it is possible for gonococci to harbour mixtures of both WT and mutant alleles amongst these four copies.

### Commensal Neisseria (n = 28)

The commensal Neisseria species panel comprised Neisseria subflava (n = 11), Neisseria sicca (n = 3), Neisseria mucosa (n = 3), Neisseria lactamica (n = 4), Neisseria flavescens (n = 1), Neisseria cinerea (n = 3) and Neisseria meningitidis (n = 3).

### Negative clinical samples

A panel of N. gonorrhoeae NAAT-negative clinical samples (n = 90) was used to further assess assay specificity. These comprised DNA extracts from samples testing negative for N. gonorrhoeae by the Cobas 4800 CT/NG method (Roche Diagnostics, Australia) and were kindly provided by Pathology Queensland. These included cervical swabs (n = 34), rectal swabs (n = 16), pharyngeal swabs (n = 21), vaginal swabs (n = 15), urine (n = 1) and other samples for which the sample site was not specified (n = 3).

### N. gonorrhoeae NAAT-positive clinical specimens

A total of 306 N. gonorrhoeae NAAT-positive clinical samples were tested. These comprised samples submitted to Pathology Queensland for N. gonorrhoeae NAAT testing in the first 6 months of 2014. The specimen types included anal/rectal swabs (n = 44), cervical swabs (n = 21), joint fluids (n = 6), penile swabs (n = 32), vaginal swabs (n = 14) and urine (n = 189). Sixty-five of the above 306 samples had corresponding bacterial culture results; 63 isolates were azithromycin susceptible (MICs=0.12–0.5 mg/L) and 2 were azithromycin resistant (MICs=1 mg/L). N. gonorrhoeae NAAT-positive pharyngeal samples were excluded, as commensal Neisseria species are ubiquitous at this site and therefore may present potential for cross-reaction.

### Results

#### N. gonorrhoeae control and test isolates

The WT and mutant control isolates provided melting temperatures of 68 and 63°C, respectively, in the 2611-PCR and 65 and 60°C, respectively, in the 2059-PCR. For the test isolates (n = 70), all results by the PCR methods were consistent with the known characteristics of these isolates. The three isolates resistant to azithromycin provided melting peaks by the real-time 2611-PCR consistent with the C2611T mutation, all at 63.5°C. The three isolates with mixed alleles for the 2611 position all provided melting peaks at 63.5°C as well as between 67.8 and 68°C (mean = 67.93°C). The remaining 64 susceptible isolates were determined to be WT by the 2611-PCR assay (67.8–68.2°C; mean = 67.9°C). For the 2059-PCR, all test isolates were shown to be WT (64.8–65.3°C; mean = 65°C). When the 2611-PCR and 2059-PCR Ct values were compared with those of the Con23S-PCR, the mean delta Ct values were 0.06 and 1 cycle, respectively (Table S1). This suggests the non-template bases used in the 2611-PCR and 2059-PCR primers had little impact upon amplification of these gonococcal strains.

#### Commensal Neisseria (n = 28)

Detailed results for the commensal Neisseria species are provided in Table S1. Briefly, when screened with the 2611-PCR, all except two isolates (one N. flavescens and one N. mucosa) provided positive results with melting temperatures ranging from 64.8 to 66.9°C (mean = 65.5°C). However, Ct values were significantly delayed (mean delay = 15 cycles) when compared with the Con23S-PCR. Similarly, all commensal species were positive by the 2059-PCR (melting temperatures ranging from 65 to 66°C; mean = 65.1°C), but with significant Ct value delays (mean delay = 18.1 cycles) compared with the Con23S-PCR. Overall, these data indicate these assays cross-react with commensal Neisseria, but have reduced sensitivity for these species.

#### Negative clinical samples

When the 2611-PCR assay was applied to the N. gonorrhoeae NAAT-negative clinical samples (n = 90), 721 pharyngeal samples provided evidence of cross-reaction, amplifying at Ct values >40 (40.9 and 49.01 cycles; mean = 44.66). A melting curve was provided in 3/7 samples corresponding with WT control, ranging between 68.3 and 68.7°C (mean = 68.5°C). For the 2059-PCR assay, 6/21 pharyngeal samples provided cross-reaction, amplifying at Ct values >40 (45.02–52.26 cycles; mean = 47.48), with 2/6 providing a melting curve corresponding with the WT control at 65 and 65.2°C (mean = 65.1°C). The remaining clinical samples, including all urogenital and rectal samples, did not provide any observable amplification or melting curves in the assays.

#### N. gonorrhoeae NAAT-positive clinical specimens

The results for this section are summarized in Table 2. Based on the above testing of N. gonorrhoeae NAAT-negative samples, a Ct cut-off value of 40 cycles was applied for this testing. That is, only samples providing Ct values <40 and providing melting curves consistent with the reference controls were assigned a call of either ‘WT’ or ‘mutant’ in the 2611-PCR and 2059-PCR assays; samples with amplification greater than Ct 40 were
Table 2. Summary results for the 306 clinical samples tested by 2611-PCR and 2059-PCR assays

<table>
<thead>
<tr>
<th>Specimen type (n=306)</th>
<th>2611-PCR</th>
<th>2059-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>call</td>
<td>Ct (cycles), range (mean)</td>
</tr>
<tr>
<td><strong>Samples providing a valid call(^a) by both PCR methods (n=266)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anal/rectal (n=40)</td>
<td>WT</td>
<td>26.5–39.02 (31.39)</td>
</tr>
<tr>
<td>cervical swab (n=16)</td>
<td>WT</td>
<td>30.89–36.88 (33.65)</td>
</tr>
<tr>
<td>joint fluid (n=6)</td>
<td>WT</td>
<td>30.5–37.6 (34)</td>
</tr>
<tr>
<td>penile swab (n=29)</td>
<td>WT</td>
<td>26.93–38.55 (31.54)</td>
</tr>
<tr>
<td>vaginal swab (n=12)</td>
<td>WT</td>
<td>29.87–37.98 (32.95)</td>
</tr>
<tr>
<td>urine (n=162)</td>
<td>WT</td>
<td>23.01–39.2 (31.65)</td>
</tr>
<tr>
<td>urine (n=1)</td>
<td>C2611T(^b)</td>
<td>25.98</td>
</tr>
<tr>
<td><strong>Samples not characterized by one or both methods (n=40)</strong></td>
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</tr>
<tr>
<td>anal/rectal (n=1)</td>
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<td>38.17</td>
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<td>40.56</td>
</tr>
<tr>
<td></td>
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<td>41.02–42.96 (41.99)</td>
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<tr>
<td>cervical swab (n=4)</td>
<td>WT</td>
<td>39.39–39.71 (39.5)</td>
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<td>41.64</td>
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</tr>
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<td></td>
<td>urine (n=4)</td>
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<td>no call</td>
<td>40.28–42.65 (41.25)</td>
</tr>
<tr>
<td></td>
<td>no call</td>
<td>40.17–42.62 (41.38)</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

\(^a\)Ct value <40 cycles and melting curve consistent with either reference control.

\(^b\)Alteration on the 25S rRNA gene at position 2611.

Ct, cycle threshold; Tm, melting temperature; negative, samples that had no obvious amplification curve; no call, samples that gave amplification with Ct values >40 cycles.
assigned 'no call' and samples that did not amplify were assigned 'negative'. There were 276 samples determined to be WT for the 2611-PCR assay, with Ct values ranging between 23.01 and 39.88 and melting peaks ranging between 67.5 and 69°C. One clinical sample was determined to be a 2611 mutant, with a Ct value of 25.98 cycles and a melting peak of 63°C. There were 272/306 samples determined to be WT for the 2059-PCR assay, with Ct values ranging between 24.88 and 39.8 cycles and melting peaks ranging between 64.7 and 66.2°C. No 2059 mutants were observed. In total, 266 of the 306 (87%) samples tested were assigned a call by both assays and 40/306 (13%) were not characterized by one or both 235 assays. Of the 266 samples successfully characterized by both assays, there were 64 samples that had corresponding bacterial culture data. All were determined to be WT by the 2611-PCR and 2059-PCR methods and there was consistency with the azithromycin-susceptible culture results for 62/64 of the samples. However, azithromycin-resistant isolates (MICs = 1 mg/L) were cultured from two samples with WT calls in both assays.

Discussion

In this study, we describe the development and application of two real-time PCR methods targeting two N. gonorrhoeae 23S rRNA gene mutations associated with azithromycin resistance. Overall, the results indicate the assays are suitable for screening for these mutations directly in clinical samples providing that testing is limited to urogenital (and potentially rectal swab) samples and a Ct value cut-off is utilized.

One of the main challenges in the development and design of these methods was limiting the potential for cross-reaction with non-gonococcal species, particularly other Neisseria species. To help achieve this, we added one ‘non-template’ base to each primer. The theory behind this approach is that a single mismatch expected to have little impact upon amplification of gonococcal 23S rRNA sequences. However, single mismatches in combination with other recognized mismatches in commensal Neisseria species would negatively impact their amplification. In practice, this approach did effectively lead to substantial delays in observed Ct values of the non-gonococcal species when compared with Ct values obtained in the Con235-PCR and is indicative of reduced sensitivity of these assays for the non-gonococcal species.

When the assays were applied to the N. gonorrhoeae NAAT-negative clinical samples, only pharyngeal samples cross-reacted. This was expected, as commensal Neisseria species are ubiquitous in the pharynx and are often observed at high organism loads at this site. This was the basis of our decision not to apply the assays to any N. gonorrhoeae NAAT-positive pharyngeal samples, as it would not be possible to reliably discern gonococcal signal from that of commensal strains. It was, however, encouraging to see that the observed cross-reactions for the pharyngeal samples provided real-time PCR Ct values of >40 cycles. This suggests that the primer designs were successful in delaying amplification of commensal strains present in these samples, as was demonstrated when testing the commensal Neisseria species isolate panel. More importantly, the assays provided no positive results when used to test the remaining N. gonorrhoeae NAAT-negative clinical samples, including urogenital and rectal samples. The observation that molecular N. gonorrhoeae resistance assays may be more suitable for these sites where commensal Neisseria species are less frequently found, particularly urogenital sites, has previously been documented.16,22

Despite not finding any false-positive results in the above N. gonorrhoeae NAAT-negative urogenital and rectal samples, a Ct value cut-off of 40 cycles (chosen on the basis of the above pharyngeal sample results) was nevertheless utilized when testing the N. gonorrhoeae NAAT-positive samples to maximize specificity. Using this cut-off, we were able to successfully characterize both the gonococcal 2611 and 2059 rRNA nucleotides in 87% of all N. gonorrhoeae NAAT-positive clinical specimens tested. Of the 266 samples characterized by both PCR assays, there were no 2059 mutants observed; however, 1 sample (0.37%) was positive for the 2611 mutation. The failure to characterize the remaining 40 samples by both assays was likely due to these samples having relatively low gonococcal DNA loads as compared with the characterized samples, but this was not specifically investigated.

Overall, these data show that the C2611T and A2059G mutations are not widespread in our population and this provides further support for ongoing use of ceftriaxone/azithromycin dual therapy. Moreover, the fact that 87% of samples could be characterized by the assays further highlights the potential for molecular resistance methods to enhance culture-based testing. This may be particularly relevant for regions where NAATs are used almost exclusively for the diagnosis of gonorrhoea. A limitation of the study, however, was that we were unable to provide definitive sensitivity and specificity data for detection of the mutations directly in clinical samples, owing to the low prevalence (C2611T and absence (A2059G) of these alterations in the study population. These investigations are ongoing.

A further limitation of the 2611-PCR and 2059-PCR assays is that they may not detect all forms of gonococcal azithromycin resistance. This is because other less common mutations, particularly in the mtrR gene and promoter sequences,23,26 may also cause azithromycin resistance. The problem is highlighted in our current study by azithromycin-resistant isolates that were cultured from two of the N. gonorrhoeae NAAT-positive samples that provided WT calls by both the 2611-PCR and 2059-PCR assays. Both isolates exhibited low-level azithromycin resistance (MICs = 1 mg/L). While the genetic basis of the resistance was not further investigated, such MICs are consistent with mtrR variants previously observed in our population.23

In summary, the results of this study show that specific real-time PCR detection of gonococcal 23S rRNA mutations directly in clinical samples is feasible via the use of non-template bases in primers, a Ct value cut-off and by excluding samples sites, particularly pharyngeal specimens, where commensal Neisseria species are ubiquitous. Whilst the 2611-PCR and 2059-PCR assays cannot detect all forms of azithromycin resistance, they offer considerable potential to provide clinically relevant data to both enhance culture-based resistance testing and enable molecular-based surveillance for azithromycin resistance.

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**Transparency declarations**

None to declare.

**Supplementary data**

Figure S1 and Table S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**