Non-culture Neisseria gonorrhoeae molecular penicillinase production surveillance demonstrates the long-term success of empirical dual therapy and informs gonorrhoea management guidelines in a highly endemic setting

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Objectives: Unlike most of the world, penicillin resistance in Neisseria gonorrhoeae from remote regions of Western Australia (WA) with high gonorrhoea notification rates has not increased despite many years of empirical oral therapy. With the advent of non-culture molecular diagnosis of gonorrhoea and the consequent decline in culture-based susceptibility, it is imperative to ensure the ongoing reliability of combination oral azithromycin, amoxicillin and probenecid for uncomplicated gonorrhoea in this setting. PCR-based non-culture N. gonorrhoeae antimicrobial resistance surveillance for penicillinase production was therefore employed.

Methods: Genital and non-genital specimens that were PCR-positive for N. gonorrhoeae were assessed for penicillinase production by detection of the N. gonorrhoeae TEM-1 plasmid using specific real-time PCR.

Results: In remote regions of WA where gonorrhoea is highly endemic, <5% of N. gonorrhoeae isolates were penicillinase-producing. This contrasts with rates of up to 20% observed in the more densely populated metropolitan and rural regions.

Conclusions: In the era of molecular diagnosis of gonorrhoea, non-culture-based antimicrobial resistance surveillance proved useful when developing evidence-based guidelines for the clinical management of locally acquired gonorrhoea in highly endemic regions in WA. The continued efficacy of combination oral amoxicillin, probenecid and azithromycin therapy despite many years of use in a setting highly endemic for gonorrhoea may explain the low rate of penicillin resistance in these remote regions and supports the concept of adding azithromycin to β-lactam antibiotics to help delay the emergence of multiresistant N. gonorrhoeae.

Keywords: antimicrobial resistance surveillance, plasmids, public health

Introduction

Neisseria gonorrhoeae has shown a remarkable capacity to become resistant to the antimicrobial agents employed to control it. The recent detection of ceftriaxone-resistant N. gonorrhoeae in Japan,¹ France² and Spain³ has forced the recommendation of a combination of ceftriaxone and azithromycin for the treatment of uncomplicated anogenital gonococcal infections.⁴,⁵ in the hope that this strategy will delay the spread of multiresistant N. gonorrhoeae.⁶ However, the efficacy of an empirical combination of macrolide and β-lactam therapy to prevent the development of resistance in gonorrhoea has yet to be proved.

There are few places in the world where penicillins remain the first-line treatment for gonorrhoea, but one such region is remote Western Australia (WA).⁷ Despite gonorrhoea being highly endemic in the remote Kimberley, Pilbara, Midwest and Goldfields regions of WA (Figure 1), oral combinations of amoxicillin, probenecid and azithromycin have been employed for many years for the syndromic management of gonorrhoea, based on the relatively high co-occurrence of N. gonorrhoeae and Chlamydia trachomatis infection and the relative susceptibility of N. gonorrhoeae isolates to penicillin.⁸ Antimicrobial susceptibility testing of gonococci from the remote regions of WA has since 1998 shown a low rate of penicillin resistance in contrast to the more populated WA regions.⁹,¹⁰
in WA occur in the sparsely populated remote regions, but only 7426 bp multicopy TEM-1 plasmid, which can be detected by healthcare and delayed specimen transport. PPNG possess a viable cultures. We applied this method to the molecular detection real-time PCR using nucleic acid extracts, thereby not requiring susceptibility testing. Sixty percent of gonorrhoea notifications representativeness of culture-based gonococcal phenotypic susceptibility testing. Non-culture-based molecular gonococcal diagnosis threatens the resistance in N. gonorrhoeae for routine genital, urine, rectal and throat specimens forwarded for specific when validated against cultures and commercial PCR methods in-house multiplex nested real-time PCR, which was 100% sensitive and 100% specific when validated against cultures and commercial PCR methods.

**Figure 1.** Regions of WA.

In 2012, penicillin resistance was found in 7 of 193 (4%) isolates from the WA remote regions, with all but one due to penicillinase-producing N. gonorrhoeae (PPNG). Therefore almost all penicillin resistance in N. gonorrhoeae isolates in the remote regions of WA is due to PPNG.

One of the primary aims of any microbiological surveillance programme is to inform clinical guidelines, but the popularity of non-culture-based molecular gonococcal diagnosis threatens the representativeness of culture-based gonococcal phenotypic susceptibility testing. Sixty percent of gonorrhoea notifications in WA occur in the sparsely populated remote regions, but only 17% of these notifications are culture based due to a limited access to healthcare and delayed specimen transport. PPNG possess a 7426 bp multicopy TEM-1 plasmid, which can be detected by real-time PCR using nucleic acid extracts, thereby not requiring viable cultures. We applied this method to the molecular detection of N. gonorrhoeae in WA over a 12 month period to help guide public health antimicrobial recommendations.

**Materials and methods**

Specimens for the molecular detection of N. gonorrhoeae were collected using either flocked wire-shafted dry swabs or sterile urine containers and were maintained at room temperature during transport to our laboratory. The molecular detection of N. gonorrhoeae was performed using in-house multiplex nested real-time PCR, which was 100% sensitive and specific when validated against cultures and commercial PCR methods for routine genital, urine, rectal and throat specimens forwarded for N. gonorrhoeae testing.

**Table 1.** Primers and probes used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence (′5′−3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMT-F</td>
<td>TGG CGA AAA CGG CTT A</td>
<td>this study</td>
</tr>
<tr>
<td>CMT-R</td>
<td>TTC ATT ATT ACT GGC ATT TTG TG</td>
<td>this study</td>
</tr>
<tr>
<td>CMT-probe</td>
<td>FAM—ATA GCA CTG TCA AGC CT—MGBNFQ</td>
<td>this study</td>
</tr>
<tr>
<td>PORA-F</td>
<td>CGA TTC CCC CGG ATT TTC</td>
<td>11</td>
</tr>
<tr>
<td>PORA-F</td>
<td>ATA GGC GGA CTG CCT GTG TTG</td>
<td>11</td>
</tr>
<tr>
<td>PORA-probe</td>
<td>FAM—AGC AGG TCA GGC CAT ACA—MGBNFQ</td>
<td>11</td>
</tr>
<tr>
<td>PPNG-F</td>
<td>AGC TGT TCG TTT TTT ACT ACC AAT CA</td>
<td>7</td>
</tr>
<tr>
<td>PPNG-R</td>
<td>TGA TTT AGT CGT TGA GGT TGA ACA</td>
<td>7</td>
</tr>
<tr>
<td>PPNG-probe</td>
<td>FAM—AAT GTA AAG AGT GAA TAG TAC GCC CAC</td>
<td>7</td>
</tr>
</tbody>
</table>

*Detects positions 937–1024 on the Asian gonococcal plasmid (GenBank accession number U20374).

Samples were extracted using either Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) or QIAGEN columns (QIAGEN, Doncaster, Australia). Step 1 of the nested PCR involved enrichment of the amplicons generated by primers directed at the cytosine methylase gene and the porA pseudogene (CMT-F/CMT-R and PORA-F/PORA-R; Table 1) in 20 amplification cycles. Each reaction mixture for the first round consisted of 12 μL of PCR mixture containing 0.2 μM each of the forward and reverse primers, 0.75 U of DNA polymerase (Applied Biosystems), 4 mM magnesium chloride (Invitrogen, USA), 1 U of 10× PCR buffer (Applied Biosystems), 0.01% BSA (Sigma-Aldrich, USA), 0.2 μM dNTP pool (Fisher Biotech, Australia), 2.5 μM 5-carboxy-X-rhodamine succinimidyl ester (ROX) dye (Biotium, USA) and 8 μL of purified nucleic acid. Reaction plates were cycled on ABI 2700 thermal cyclers (Applied Biosystems) under the following conditions: 95°C for 10 min followed by 20 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 45 s, and then 7 min at 72°C.

Following this, 0.75 μL of Step 1 PCR product was added to a real-time amplification cycle with TaqMan internal primers and probes (CMT-F/CMT-R and PORA-F/PORA-R; Table 1) in 20 amplification cycles. Each reaction mixture for the second round consisted of a similar reaction mixture to Step 1 except for the use of 0.5 μL of DNA polymerase and the exclusion of ROX dye. The Step 2 reactions were either performed on Rotor-Gene real-time PCR instruments (QIAGEN) at 95°C for 10 min followed by 40 cycles of 94°C for 10 s, 56°C for 11 s, 60°C for 90 s and then a 68°C temperature increase before three cycles of 30°C for 20 s, or on Viia7 real-time PCR instruments (Applied Biosystems) at 95°C for 10 min followed by 40 cycles of 94°C for 10 s and then 60°C for 90 s. Samples in which both targets were detected with a cycle threshold (CT) of <35 were reported as being positive for N. gonorrhoeae. The multiplex tandem assay included checks for extraction efficiency and removal of the PCR inhibitor, and was validated according to the criteria established by the National Pathology Accreditation Advisory Committee and approved by the National Association of Testing Authorities.

The nucleic acid extract from N. gonorrhoeae DNA-positive cases was then used for the detection of PPNG by targeting the conserved region at positions 937–1024 on the Asian gonococcal plasmid (GenBank accession number U20374). Briefly, each reaction mixture consisted of 1 U of 10×PCR Buffer II (Applied Biosystems, USA), 0.01% BSA (Sigma Aldrich, USA), 4 mM magnesium chloride (Invitrogen, USA), 0.2 mM dNTP pool (Fisher Biotech, Australia), 0.2 μM each of the forward and reverse primers (PPNG-F and PPNG-R; Table 1) and 0.1 μM probe (PPNG-probe; Table 1). An aliquot of 8 μL of purified sample nucleic acid was added to 12 μL of the PPNG reaction mixture and cycled on Rotor-Gene real-time PCR instruments (QIAGEN) under the following conditions: 95°C for 10 min followed by 50 cycles of 94°C for 12 s, 55°C for 15 s and 72°C for 20 s.
The PPNG real-time PCR was 100% concordant for 10 PPNG and 10 non-PPNG strains. An analytical sensitivity study of serial 10-fold dilutions of genital swab, rectal swab, throat swab and urine DNA extracts from PPNG-positive cases was run in parallel with the multiplex tandem diagnostic PCR. The PPNG assay was 10-fold more sensitive compared with the tandem PCR assay for all specimen types and for both extraction methods (data not shown), which is likely to be due to the multicycle presence of the TEM-1 plasmid in PPNG. From here on, PPNG refers to the molecular detection of the conserved region of the gonococcal plasmid containing TEM-1.

Notification data for gonorrhoea were obtained from the WA Notifiable Infectious Diseases Database, maintained by the WA Health Department. Statistical analysis was performed using GraphPad Prism software, version 5.02 (GraphPad Software, Inc., San Diego, CA, USA).

This research was conducted in accordance with the Declaration of Helsinki and national and institutional standards.

## Results

The dichotomy of gonorrhoea notification rates between the remote and the populated regions of WA for the period 1 August 2011 to 31 July 2012 is shown in Table 2. Over the 12 month period, 47715 specimens were received for N. gonorrhoeae testing for WA (Table 2). The exception was the Goldfields region (Figure 1), where specimens from the remote indigenous desert communities near the South Australian and Northern Territory borders were referred interstate. Of the specimens received, N. gonorrhoeae DNA was detected from 2368 specimens collected from 1472 cases. There was sufficient DNA extract available from 1235 (84%) of these cases to perform PPNG PCR testing. Of these, 1171 were genital specimens, 27 were rectal swabs, 32 were throat swabs and 5 specimens were collected from other non-genital sites (skin, eye, blood and joint fluid).

The results of the PPNG PCR assay are shown in Table 2. Although the proportion of gonorrhoea notifications confirmed in our laboratory from the more densely populated regions of WA was relatively low (24%–53%), 90%–99% of notifications from the remote Pilbara, Midwest and Kimberley regions were confirmed in our laboratory. Fifteen PPNG detections were observed among 915 N. gonorrhoeae DNA-positive specimens from the remote regions. In the Kimberley and Midwest regions, the proportion of PPNG-positive specimens was <1%, with 4% prevalence in the Pilbara region. The 10% prevalence found in the Goldfields area was based on a total of only 39 cases. All PPNG-positive specimens from the Goldfields and Pilbara regions were diagnosed in the large regional towns.

### Discussion

As the world comes to terms with the emergence of extensively drug-resistant gonorrhoea, it is widely recognized that antimicrobial surveillance has a crucial role to play in guiding anti-gonococcal therapy. The list of antibiotics that can be successfully used for gonorrhoea is being progressively eroded and newer strategies for controlling the spread of resistance are urgently required. With this global backdrop, it is critical to delay the introduction of multiresistant N. gonorrhoeae strains into the indigenous population of remote WA, with its high rate of gonorrhoea and limited access to healthcare. The gonorrhoea notification rate for the remote regions of WA in general has shown no increase since 2001, but the incursion of ceftriaxone-resistant gonococci into these communities would create an enormous public health challenge. Moreover, there is a large external workforce that commutes into several of these remote regions of WA, mandating surveillance for the introduction of multiresistant N. gonorrhoeae strains acquired elsewhere into these local communities.

The WHO recommends that only drugs with an expected efficacy as over 90% of gonorrhoea notifications in the WA remote regions are based on a molecular diagnosis (Table 2). The molecular detection of PPNG was considered to be an appropriate tool to investigate whether combination oral azithromycin, amoxicillin and probenecid continues to meet the WHO criteria for effective treatment of gonorrhoea.

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### Table 2. Molecular detection of PPNG in WA from 1 August 2011 to 31 July 2012

<table>
<thead>
<tr>
<th>Government region</th>
<th>N. gonorrhoeae notification rate (per 100000)</th>
<th>PWLM PCR-positive N. gonorrhoeae cases</th>
<th>Proportion of all N. gonorrhoeae notifications by PWLM PCR (%)</th>
<th>PPNG cases detected by PCR, n (%), 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remote regions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kimberley</td>
<td>1776</td>
<td>592</td>
<td>99</td>
<td>4 (0.7, 0.2 – 1.8)</td>
</tr>
<tr>
<td>Pilbara</td>
<td>310</td>
<td>177</td>
<td>90</td>
<td>7 (4.0, 1.8 – 8.1)</td>
</tr>
<tr>
<td>Midwest</td>
<td>142</td>
<td>107</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td>Goldfields</td>
<td>199</td>
<td>39</td>
<td>49</td>
<td>4 (10.3, 3.5 – 24)</td>
</tr>
<tr>
<td>Populous regions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perth</td>
<td>51</td>
<td>267</td>
<td>24</td>
<td>31 (11.6, 8.0 – 16)</td>
</tr>
<tr>
<td>Wheatbelt</td>
<td>32</td>
<td>17</td>
<td>44</td>
<td>2 (11.8, 2.0 – 36)</td>
</tr>
<tr>
<td>Great Southern</td>
<td>18</td>
<td>14</td>
<td>53</td>
<td>1 (7.1, 0.0 – 33)</td>
</tr>
<tr>
<td>South West</td>
<td>13</td>
<td>5</td>
<td>32</td>
<td>1 (20.0, 2.0 – 64)</td>
</tr>
</tbody>
</table>

PWLM, PathWest Laboratory Medicine Queen Elizabeth II Medical Centre Laboratory.
The majority of WA gonococcal isolate penicillin resistance is due to PPNG, with only an additional 4%–19% of penicillin resistance encoded by chromosomal genes.5 As expected, the rate of PPNG detection from the more densely populated regions of WA was high. However, even combining the chromosomally mediated penicillin resistance rate found in WA isolates with the PPNG rates from this study, amoxicillin and probenecid would maintain at least 95% efficacy in most of remote WA. This, together with the lack of azithromycin resistance in N. gonorrhoeae from WA, suggests that the use of a combination of azithromycin, amoxicillin and probenecid remains effective as oral therapy for locally acquired gonorrhoea in the indigenous communities in these remote regions. The analytical sensitivity of the PPNG assay was found to be greater than our diagnostic N. gonorrhoeae PCR, reducing the risk of false-negative PPNG results. Goire et al.7 reported a false-positive rate for the PPNG assay of up to 1.3%, which could be identified by high CT values. No adjustment for this was made in this study as our diagnostic N. gonorrhoeae tandem PCR format resulted in lower CT values compared with those of the single-round PCR used by Goire et al.,7 thus negating the ability to perform a CT delta calculation. Moreover, as a decision tool for the efficacy of empirical therapy, a minor over-reporting of PPNG resistance rates does not invalidate the recommendations.

Previous WA gonorrhoea treatment guidelines8 recommended ceftriaxone monotherapy for the empirical treatment of gonorrhoea in the populated WA regions, whereas locally acquired gonorrhoea in the remote WA regions has been managed syndromically with a combination of oral azithromycin, amoxicillin and probenecid. Similar to the gonorrhoea treatment recommendations in the USA,4 the WA guidelines have now been revised to use combination ceftriaxone and azithromycin therapy for the more populous regions of WA. However, the results of this study support the recommendation to continue combination oral azithromycin, amoxicillin and probenecid for locally acquired gonorrhoea in the remote WA regions.

Interestingly, unlike the trend seen in most of the world, there has been no increase in gonorrhoea notification rates or penicillin resistance rates from the remote and highly endemic WA communities despite a myriad of sociocultural issues and the use of combination oral azithromycin, amoxicillin and probenecid for over a decade.10,11 It is possible that the empirical addition of azithromycin to amoxicillin and probenecid has protected against the development of β-lactam antimicrobial resistance, as suggested by Whiley et al.,8 although a combination of β-lactam and azithromycin therapy is now recommended in the USA4 and the UK5 to potentially protect against the development of ceftriaxone resistance, data demonstrating the efficacy of this strategy are lacking. Our study supports the notion that empirical dual therapy may delay the escalation of antimicrobial resistance in N. gonorrhoeae.

To our knowledge, this is the first time that non-culture molecular-based antimicrobial resistance surveillance has been used to inform clinical guidelines for the empirical management of gonorrhoea. The continued efficacy of a combination of oral azithromycin, amoxicillin and probenecid limits the exposure of remote WA region N. gonorrhoeae strains to third-generation cephalosporins, potentially delaying the establishment of multidrug-resistant N. gonorrhoeae strains. However, ongoing molecular surveillance for PPNG is essential for the early detection of strain replacement by penicillin-resistant N. gonorrhoeae acquired elsewhere to allow a prompt public health response. In addition, it is imperative that this surveillance strategy is continually evaluated against phenotypic susceptibility test results and trends in disease incidence to ensure that any increase in chromosomally mediated penicillin resistance can be detected in a timely manner.

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Transparency declarations
None to declare.

References
Molecular antimicrobial surveillance informs gonorrhoea guidelines


