A national quality assurance survey of *Neisseria gonorrhoeae* testing

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The aims of this study were to (1) conduct a national survey of *Neisseria gonorrhoeae* identification by National Neisseria Network (NNN) reference laboratories contributing data to the Australian Gonococcal Surveillance Programme and (2) determine the prevalence in Australia of strains of *N. gonorrhoeae* lacking gene sequences commonly targeted by in-house PCR assays for confirmation of gonococcal nucleic acid amplification tests. Gonococcal clinical isolates referred to NNN laboratories for the first half of 2012 were screened using in-house real-time PCR assays targeting multicopy *opa*, *porA* pseudogene and *cppB* genes. There were 2455 clinical gonococcal isolates received in the study period; 98.6 % (2420/2455) of isolates harboured all three gene targets, 0.12 % (3/2455) were *porA*-negative, 0.04 % (1/2455) *opa*-negative and 1.14 % (28/2455) *cppB*-negative by PCR. Notably, no isolates were simultaneously negative for two targets. However, three isolates failed to be amplified by all three PCR methods, one isolate of which was shown to be a commensal *Neisseria* strain by 16S rRNA sequencing.

Using PCR as the reference standard the results showed that (1) identification of *N. gonorrhoeae* isolates by NNN laboratories was highly specific (99.96 %) and (2) strains of *N. gonorrhoeae* lacking gene sequences commonly targeted by in-house PCR assays are present but not widespread throughout Australia at this point in time.

INTRODUCTION

Effective clinical and public health management of gonorrhea is highly dependent on the ability of diagnostic test systems to detect and identify *Neisseria gonorrhoeae* as well as the availability of reliable local data on antimicrobial

Abbreviations: AGSP, Australia Gonococcal Surveillance Programme; AMR, antimicrobial resistance; NAAT, nucleic acid amplification test; NNN, National Neisseria Network; QPID, Queensland Paediatric Infectious Diseases; NSW, New South Wales; NT, Northern Territory; Qld, Queensland; SA, South Australia; Vic, Victoria; WA, Western Australia.
resistance (AMR) rates (Tapsall, 2001). Limitations with either sensitivity or specificity of diagnostic test methods have the potential to seriously undermine these activities. Notably, both phenotypic identification of \textit{N. gonorrhoeae} isolates and molecular detection of \textit{N. gonorrhoeae} nucleic acids in clinical samples can be problematic as this bacterium readily changes its phenotypic and genotypic properties (Whiley \textit{et al.}, 2006; Bennett \textit{et al.}, 2007; Unemo \textit{et al.}, 2007).

A series of provisional and confirmatory tests for identification of \textit{N. gonorrhoeae} isolates is required to provide valid and precise identification of this pathogenic \textit{Neisseria} species for both public health considerations and also those of the individual patient. A number of species other than \textit{N. gonorrhoeae} and \textit{Neisseria meningitidis} may occasionally be isolated on the selective agars developed for the pathogenic \textit{Neisseria} species (Janda & Knapp, 2003). In addition, atypical isolates of \textit{N. gonorrhoeae} may occasionally appear which compromise identification of this organism and the problem is enhanced when these isolates become prevalent in a population group. For example, confirmation of \textit{N. gonorrhoeae} identification is commonly performed using commercial biochemical kits that rely entirely or in part on the activity of the enzyme prolylarginipeptidase, which was previously considered to be universally present in the gonococcus. However, strains lacking the expression of this enzyme have been identified (Alexander & Ison, 2005; Feldsøe-Nielsen \textit{et al.}, 2005), potentially leading to incorrect species identification. Similarly, strains with mutations in target gene sequences can be problematic when utilizing nucleic acid amplification tests (NAATs) for direct detection of gonococcal nucleic acids in clinical samples (Whiley \textit{et al.}, 2006). Examples have been observed for several of the sequence targets in common use for in-house real-time PCR assays, and have been particularly problematic for countries such as Australia where, due to local \textit{N. gonorrhoeae} NAAT guidelines, in-house real-time PCR assays are used extensively for confirmatory testing of \textit{N. gonorrhoeae}-positive results from commercial NAAT systems (Smith \textit{et al.}, 2005). Notably, gonococci lacking the \textit{cppB} gene have been found in several regions (Bruisten \textit{et al.}, 2004; Lum \textit{et al.}, 2005). As a consequence \textit{cppB} as a target gene is no longer recommended for use in Australia, and elsewhere (Smith \textit{et al.}, 2005). Strains exhibiting variation in the multicopy \textit{opa} gene (Geraats-Peters \textit{et al.}, 2005) and the \textit{porA} pseudogene (Whiley \textit{et al.}, 2011; Eastick \textit{et al.}, 2012; Golparian \textit{et al.}, 2012; Ison \textit{et al.}, 2013) have also been reported.

Australia has a long established collaborative network of reference laboratories (National Neisseria Network; NNN) that receive and test clinical isolates of gonococci for the purposes of AMR surveillance as part of the Australia Gonococcal Surveillance Programme (AGSP). This study was designed to achieve two independent aims: (1) to evaluate the accuracy of identification of \textit{N. gonorrhoeae} isolates by NNN reference laboratories contributing data to the AGSP; (2) concurrently, to determine the percentage of \textit{N. gonorrhoeae} isolates lacking gene sequences commonly targeted by gonococcal in-house real-time PCR methods and widely used in Australia and elsewhere.

\textbf{METHODS}

\textit{Neisseria gonorrhoeae} isolates. The NNN was established in Australia in 1979 to provide nationally comparable and quality assured gonococcal AMR data. In this study, NNN laboratories from Victoria (Vic), New South Wales (NSW), Queensland (Qld), Northern Territory (NT), Western Australia (WA) and South Australia (SA) were asked to provide clinical gonococcal isolates for the period of 1 January 2012 to 30 June 2012. A total of 2455 \textit{N. gonorrhoeae} clinical isolates were included in the study (Table 1), and were estimated to comprise 98\% of all gonococci isolated, and 34.6\% of the total 7089 cases (including NAAT-positive samples) of all gonorrhoea notified in Australia in this time period (Australian Government, Department of Health and Ageing, 2012); 688 isolates were received from Vic, 823 from NSW, 397 from Qld, 114 from NT, 271 from WA and 162 from SA.

\textit{N. gonorrhoeae} identification methods. Isolates were identified as \textit{N. gonorrhoeae} on the basis of Gram-negative diplococci on Gram stain and positive test for oxidase. Additional methods included Superoxol reaction (NSW, NT and WA), growth on nutrient agar (SA, Qld and Vic), growth on gonococcal selective agar (SA, NSW, NT, Vic and WA) and carbohydrate utilization (glucose, lactose, maltose and sucrose; SA, NSW, NT, Qld and Vic). Identification was confirmed by a number of commercial systems: RapID NH API (bioMérieux; NT and WA), VITEK 2 NH (bioMérieux; SA, NT and WA), Phadebact Monoclonal GC Test reagents (Boule; WA, NSW and NT), BD BBL Crystal Neisseria/Haemophilus ID kit (Becton Dickinson; WA), Microflex LT benchtop MALDI-TOF mass spectrometer (Bruker Daltonik; SA, NT, WA and NT). Several laboratories (SA, Vic, NT, Qld and WA) also reported selective use of gonococcal NAAT methods to confirm organism identification. Further characterization of \textit{N. gonorrhoeae} isolates (all laboratories) was provided by antibiogram using antibiotic susceptibility categories obtained from the minimum inhibitory concentrations (MICs) determined by the agar plate dilution method developed, agreed and introduced into participating laboratories at the establishment of the NNN. All laboratories also take part in a comprehensive quality assurance programme that was established for the AGSP (Tapsall, 1990).

\textbf{Isolate preparation for PCR.} For each region, excluding SA, isolates were prepared at the respective NNN laboratory for PCR analysis using the heat denaturation method previously described by our laboratory (Kugelman \textit{et al.}, 2009); three to six colonies from a 24 h single colony subculture of each isolate were suspended in 1.0 ml distilled water in a 1.5 ml tube, and heated at 100 °C for 8 min (Kugelman \textit{et al.}, 2009). Isolates were sent on dry ice to the Queensland Paediatric Infectious Diseases (QPID) Laboratory for PCR testing. For isolates from SA, colonies were initially sent to the QPID Laboratory on swabs where they were then processed as above. Isolates were then tested at the QPID Laboratory by real-time PCR methods targeting the multicopy \textit{opa} genes, \textit{porA} pseudogene and \textit{cppB} gene.

\textbf{Detection of \textit{opa} and \textit{porA} by duplex PCR.} Detection of \textit{opa} and \textit{porA} was based on a previously described duplex real-time PCR method (Goire \textit{et al.}, 2008). Each reaction mix contained 10.0 μl 2× Sensimix II probe (Bioline) reaction mix, 10.0 pmol \textit{opa} and \textit{porA} forward and reverse primers, 2.0 pmol \textit{porA} and \textit{opa} probes and...
2.0 µl heat-denatured isolate, and was made up to a total volume of 20.0 µl with DNase-free water. Cycling was performed on RotorGene (Corbett Life Science) and LightCycler 480 (Roche) real-time PCR instruments with the following cycling conditions: an initial hold at 95 °C for 15 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s; with fluorescence signal read on FAM channel for porA and JOE channel for opa detection.

Detection of cppB by PCR. Detection of cppB was performed using previously described primers (Ho et al., 1992) in a SYBR-green real-time PCR format. Each reaction mix contained 10.0 µl 2 × SYBR green (Qiagen), 10.0 pmol forward and reverse primers and 2.0 µl heat-denatured isolate, and was made up to a total volume of 20.0 µl with DNase-free water. Cycling was performed on RotorGene and LightCycler 480 instruments with the following cycling conditions: an initial hold at 95 °C for 15 min, followed by 45 cycles of 95 °C for 10 s, 50 °C for 15 s and 72 °C for 30 s; with fluorescence signal read on green at 72 °C following extension. Melting curve analysis was performed following PCR amplification; reaction mixes were continuously analysed from 60 °C to 95 °C, with temperature increments of 1 °C s⁻¹. Positive results were indicated by the presence of amplification curves and where a melting temperature of approximately 85 °C was observed.

Control specimens. The World Health Organization (WHO) reference strain (WHO-M) (Unemo et al., 2009) was used as a positive control for all three PCR assay targets. Each PCR run included at least two positive and two negative (no DNA) controls.

16S DNA sequencing. Isolates found negative for all three PCR target genes were further analysed by 16S rRNA PCR and DNA sequencing. Briefly, isolates were amplified by 16S rRNA PCR using previously described primers (Wang et al., 2007) and submitted for automated sequencing at the Australian Genome Research Facility (AGRF). 16S rRNA sequences were then subject to GenBankBLAST searches.

RESULTS

Table 1 provides a summary of results, including individual results for each state. Of the total 2455 isolates, 2420 (98.6 %) were positive and 3 (0.12 %) were negative by PCR for all three targets – multicopy opa, porA pseudogene and cppB. A further 32 (1.3 %) isolates were each negative for one of the three targets – multicopy opa (n=1), porA pseudogene (n=3) and cppB (n=28) (Table 1).

Three isolates negative for all three diagnostic targets were subjected to 16S rRNA PCR, DNA sequencing and GenBank BLAST analysis. GenBank BLAST analysis indicated one of the isolates was a commensal Neisseria species. The remaining two isolates did not produce reliable 16S rRNA sequence data and so genus or species could not be determined. This failure to obtain reliable 16S rRNA sequence data from these two isolate suspensions was consistent with low levels of DNA, most likely as a result of incorrect sample preparation for the purposes of this study.

Using the combined results of the real-time PCR assays and 16S rRNA sequencing as the reference standard, 2454/2455 clinical isolates were considered to be correctly identified as N. gonorrhoeae, providing 99.96 % specificity for identification of N. gonorrhoeae by Australian NNN laboratories. Note that the two isolates for which there were
concerns over sample preparation were excluded from this analysis.

**DISCUSSION**

The NNN Australia is an extensive national network of laboratories that has collaborated continuously for 32 years to provide Australia’s long-standing comprehensive surveillance of gonococcal AMR using a systematic approach, standardized methodology and a specific quality assurance programme. Currently the NNN tests 35 % of all notified cases of gonorrhoea in Australia annually (2012). Overall, the results of the real-time methods showed that identification of *N. gonorrhoeae* isolates was highly specific (99.96 %). These results are evidence of the contribution to quality outputs of a nationally coordinated approach such as that of the Australian NNN.

The results of the real-time PCR testing also, for the first time to our knowledge, showed the prevalence of gonococci lacking certain in-house PCR targets across the Australian continent. In total, 2420/2455 (98.6 %) of isolates harboured all three genes: *cppB*, multicity *opa* and *porA* pseudogene; 0.12 % (3/2455) were *porA*-negative, 0.04 % *opa*-negative (1/2455) and 1.14 % (28/2455) *cppB*-negative by PCR. Importantly, none of the isolates tested was lacking two diagnostic targets simultaneously. On a state-by-state basis, WA had a significantly higher proportion of isolates negative for *cppB* (19/271; 7.0 %) compared with the rest of the country (*P*<0.001; chi-squared test), with the majority of *cppB*-negative strains found in WA and Qld. In previous studies the prevalence of *cppB*-negative strains was high (9.8 %) in the NT (Lum et al., 2005) but not previously reported in Qld (Whiley et al., 2008). The finding of *cppB*-negative strains from Qld in this study supports the changing distribution of gonococcal subtypes demographically over time observed by others (Mavroidi et al., 2011; Tapsall et al., 2010). Higher rates of *cppB*-negative isolates from WA and Qld compared with the rest of the country also illustrate the propensity for gonococcal subtypes to become established in some communities and not others. This is also observed with gonococci and AMR: in Australia, higher levels of AMR were reported in urban/metropolitan population groups in contrast to the lower levels of AMR occurring in population groups in remote regions such as the NT (Australian Gonococcal Surveillance Programme, 2012).

There are sampling biases, however, with this study that may have impacted upon the PCR sequence target findings. Notably, in this study we only investigated isolates and not *N. gonorrhoeae* NAAT-positive samples. Isolates were less available from some states and some regions of Australia, particularly geographically isolated areas such as the NT, where prolonged transport times and subsequent impacts upon organism viability effectively prohibit the use of culture-based diagnosis. In these areas gonococcal disease testing is now done almost exclusively by NAAT methods. For example, isolates from the NT represented just 13.1 % of the gonorrhoea notifications for the NT for this time period – substantially lower than for all other regions (Table 1). Thus, our isolate-based sampling could potentially miss clusters of *cppB*-negative strains in some areas. This sampling bias should also be considered when interpreting the *opa* and *porA* data. For these targets, we only observed one *opa*-negative isolate, in NSW, and three *porA*-negative isolates (one each in Qld, Vic and NSW). While these numbers are indeed low, they likely indicate the presence of sexual networks in which such strains may be more prevalent, but not readily captured by bacterial culture-based screening. Overall, these data highlight a need for ongoing surveillance of NAAT sequence targets amongst isolates of *N. gonorrhoeae* to ensure that targets utilized in NAAT detection systems remain sensitive and specific for disease diagnosis. In particular, laboratories using gonococcal NAAT methods need to remain vigilant to the possibility of target sequence variants. In our opinion, the use of two distinct sequence targets is optimal for PCR-based detection of *N. gonorrhoeae* in order to avoid false-negative results. In this context, it should also be noted that we have only investigated common in-house real-time PCR methodology here, and not the more widely used commercial systems. The main reason for this focus on in-house methodology was the fact that the particular methods investigated here have been used extensively in Australia owing to local *N. gonorrhoeae* NAAT guidelines requiring the use of supplementary testing (Smith et al., 2005). Nevertheless, we believe there may be some benefit in conducting similar studies for the commercial systems, particularly for those using single sequence targets.

To our knowledge, this study represents the largest national assessment of the accuracy of identification of *N. gonorrhoeae* isolates by laboratories contributing to gonococcal antimicrobial surveillance data. It is also the largest study looking at the dissemination of strains of *N. gonorrhoeae* exhibiting PCR gene target sequence variations. In summary, the results show (1) highly accurate identification of *N. gonorrhoeae* by Australian laboratories contributing to the AGSP, and (2) that for this period of time in Australia, less than 1.4 % of isolates of *N. gonorrhoeae* lacked gene sequences commonly targeted by in-house PCR assays for confirmation of gonococcal NAAT.

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