ABSTRACT

Objectives: To share information and genetic characterization of the New Zealand culture collection Neisseria gonorrhoeae isolates. This information makes the isolates suitable as quality control material for diagnostic laboratories considering setting up molecular assays to predict susceptibility profiles of clinical N. gonorrhoeae where a culture isolate is not available.

Methods: DNA was extracted from cultured isolates on the Cobas 4800 CT/NG platform. Taqman PCR assays with specific primers and probes were used to detect a sequence in penicillinase producing Neisseria gonorrhoeae (PPNG) or associated with reduced susceptibility to the cephalosporins (mosaic penA) and run on the LightCycler 480. The gyrA assay utilised a melt curve analysis to detect the Ser91→Phe mutation which occurs in ciprofloxacin resistant N. gonorrhoeae.

Results: The three molecular assays used to predict resistance (or reduced susceptibility) produced valid results against each of the ESR culture collection strains examined, namely the WHO-K calibrator strain, the PPNG positive isolates (222, 3330 and 4543), the gyrA Ser→Phe positive isolates (4033 and 4543) and the mosaic penA positive isolate (4543).

Conclusions: The N. gonorrhoeae isolates held by the New Zealand culture collection have been validated and characterised as positive or negative for three molecular markers associated with resistance to penicillin (PPNG), ciprofloxacin (gyrA) and ceftriaxone (mosaic penA). This goes some way to resolving the ever present issue of a lack of suitable control material when diagnostic laboratories are setting up bespoke and new molecular assays.

Key words: Neisseria gonorrhoeae, antimicrobial susceptibility, genotyping, PCR, quality control.


INTRODUCTION

In the age of molecular diagnostics an ongoing challenge for any laboratory setting up new molecular testing is the availability of suitable quality control material (1–3). This study characterises the Neisseria gonorrhoeae culture collection strains available from Institute of Environmental Science and Research (ESR) in Kenepuru, New Zealand by three molecular assays designed to predict resistance or reduced susceptibility.

New Zealand sexual health treatment guidelines state that empiric treatment in New Zealand should be with 500 mg IM ceftriaxone plus 1 g azithromycin. The guidelines also state that ciprofloxacin is an alternative treatment option if the Neisseria gonorrhoeae has tested as susceptible. (http://www.nzshs.org/guidelines/Gonorrhoea-guideline.pdf). It has been suggested that in some regions penicillin may still be considered a treatment option in susceptible populations (4–6). Monitoring of the resistance plasmid also provides some epidemiological information which is useful to trace emerging resistance and multidrug resistance.

The New Zealand Reference Culture Collection is a member of the World Federation for Culture Collections and is held by ESR. It holds approximately 4000 strains and supplies reference cultures for quality control, teaching and research. These cultures include isolates which are the subject of formal publications, the first New Zealand isolate of a species, and strains with particular antimicrobial sensitivity patterns, plasmid profiles or other properties (http://www.esr.cri.nz/competencies/Health/Pages/nzrcc.aspx).

As part of an investigation into the molecular markers of antibiotic resistance in New Zealand isolates of Neisseria gonorrhoeae, four isolates from ESR were tested to form suitable controls for diagnostic testing. In addition to culture-based susceptibility testing, three molecular assays were carried out to detect the gyrA gene, (associated with ciprofloxacin resistance), the penicillin resistance plasmid found in PPNG, and the mosaic penA sequence (associated with reduced ceftriaxone susceptibility).

MATERIALS AND METHODS

Genotypes

DNA Extraction by the Cobas 4800 CTNG Test
A swab of each cultured isolate was taken into Cobas 4800 collection buffer and was tested in the Cobas 4800 system as previously described (7). Residual DNA remaining from the CTNG amplification test was utilised for the three antimicrobial resistance marker molecular assays.

Fluoroquinolone resistance and gyrA
Siedner et al stated that the mutation of importance in determining quinolone resistance is the Ser91 codon →Phe alteration, with over 99% of QRNG shown to have mutations at this site (8). They developed a real time PCR assay for analysis of mutations in the Ser91 region of the gyrA gene by amplification and melt curve analysis. PCR and melt curve analysis on DNA showed the presence or absence of the gyrA
mutation and ciprofloxacin susceptibility could then be predicted. This previously described assay’s primers and probes, with run conditions matched to other APL assays, were further developed and validated at APL and the four culture collection isolates were tested.

**gyrA-PCR**

Briefly, each gyrA-PCR mastermix contained 1x FastStart DNA Master (Roche), 0.5U UNG (Roche) 2.5 mM MgCl2, 0.2 mM of primers NGGRASER91-F and NG-GRASER91-R (IDT) and probes gyrA-ser-LC and gyrA-ser-Flu (TIB MolBiol) (8) as well as 5mL of cobas 4800 residual DNA or control material. Amplification was carried out using a LightCycler 480 (v1.0) with a 10 minute denaturation at 95°C followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 52°C for 5 s, and extension at 72°C for 10 s, with a ramp rate of 20°C/s. Melting curve analysis was performed using 95°C for 60 s, 40°C for 20s, and heating to 80°C with a ramp rate of 0.03°C/s with continuous fluorescence acquisition.

**Penicillin resistance and PPNP**

Penicillin resistance in *N. gonorrhoeae* may be due to either mutations in chromosomal genes encoding penicillin-binding proteins (PBPs) and/or affecting outer membrane permeability or by acquisition of plasmids encoding production of a beta lactamase (penicillinase) (12). Goire et al developed a real-time PCR for detection of penicillinase producing *N. gonorrhoeae* using non-cultured clinical samples (9). They selected conserved targets outside of the beta lactamase gene on the gonococcal plasmids to serve as indirect markers of penicillinase activity specific to *N. gonorrhoeae* (pPPNG). This previously described assay’s primers and probes, with run conditions matched to other APL assays, were further developed and validated and the four culture collection isolates were tested.

**pPPNG-PCR**

Briefly, each PPNP-PCR mastermix contained 1x FastStart DNA Master (Roche), 0.5U UNG (Roche), 2.0mM MgCl2, 0.4 mM of primers PPNP-F2 and PPNP-R2 and 0.2 μM of the PPNP TM2 probe (IDT), and 5mL of cobas 4800 residual DNA or control material. Amplification was carried out using a 10 minute denaturation at 95°C followed by 5 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 60s.

**Reduced susceptibility to ceftriaxone and mosaic penA**

Goire et al (10) and Unemo et al (11) observed that the emergence of extended cephalosporin resistance is preceded by a gradual rise in MICs. Studies have implicated alterations in penicillinase production. Detection of these plasmids is a useful epidemiological tool and can confirm an isolate as multi drug resistant if other markers are assayed as well. The detection of the gyrA mutation at codon 91, from serine to phenylalanine, as a key marker or principal ‘alteration of interest’ (14,15). Therefore the previously described Goire et al (10) assay was developed and validated with APL run conditions and the four culture collection isolates were tested.

**Mosaic penA-PCR**

Briefly, each mosaic penA-PCR mastermix contained 1x FastStart DNA Master (Roche), 0.5U UNG (Roche), 2.0 mM MgCl2, 0.4 mM of primers Mosaic F and Mosaic R and 0.16μM of Mosaic probe (IDT), and 5mL of cobas 4800 residual DNA or control material. Amplification was carried out using a 10 minute denaturation at 95°C followed by 5 cycles of denaturation at 95°C for 15s, annealing at 60°C for 10s and extension at 72°C for 12s.

The three molecular predictors of resistance (or reduced susceptibility) assays produced valid results against each of the three ESR culture collection strains. The WHO-K calibrator strain (4543) also gave expected results (11).

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**Table 1. The phenotypes and genotypes of four *N. gonorrhoeae* isolates from the ESR culture collection**

<table>
<thead>
<tr>
<th>ESR Culture collection number</th>
<th>Cobas NG/CT result</th>
<th>Beta lactamase (cassette disc)</th>
<th>PEN</th>
<th>pPPNG</th>
<th>CIP</th>
<th>gyrA Ser91Phe</th>
<th>CTX MIC (μg/mL)</th>
<th>Mosaic penA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2222</td>
<td>Pos</td>
<td>Yes</td>
<td>R</td>
<td>Pos</td>
<td>S</td>
<td>Neg</td>
<td>0.006</td>
<td>Neg</td>
</tr>
<tr>
<td>3330</td>
<td>Pos</td>
<td>Yes</td>
<td>R</td>
<td>Pos</td>
<td>S</td>
<td>Neg</td>
<td>0.004</td>
<td>Neg</td>
</tr>
<tr>
<td>4033</td>
<td>Pos</td>
<td>No</td>
<td>R</td>
<td>Pos</td>
<td>S</td>
<td>Neg</td>
<td>0.023</td>
<td>Neg</td>
</tr>
<tr>
<td>4543*</td>
<td>Pos</td>
<td>No</td>
<td>R</td>
<td>Pos</td>
<td>S</td>
<td>Yes</td>
<td>0.064</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Key: Pos = Positive, Neg = Negative, R = resistant, S = susceptible, CMRNG = Chromosomal mediated resistant *N. gonorrhoeae*, pPPNG = plasmid of Penicillin Producing *N. gonorrhoeae*, CTX = ceftriaxone, gyrA ser91Phe = gyrA mutation, PEN = penicillin; "*WHO-K calibrator strain."

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**DISCUSSION**

As emerging technologies have led to the widespread use of molecular methods for the diagnosis of gonorrhoea, isolates are frequently not available for traditional culture-based antimicrobial susceptibility testing. It is therefore likely that laboratories will employ molecular methods to predict susceptibility to antibiotics even though it must be acknowledged that, in contrast to phenotypic methods, genotypic assays will not detect novel, uncharacterised mechanisms of resistance to antimicrobial agents. This is a major limitation when developing possible molecular solutions to address a lack of antimicrobial susceptibility information. The detection of a single mutation will not usually predict the complex interactions of multiple mutations and there is a need for laboratory tests to be clearly defined and measurable in regard to antibiotic susceptibility outcomes. However, molecular methods remain important technology to further investigate and develop. It is also important to acknowledge the need for ongoing culture of circulating isolates of *N. gonorrhoeae* in New Zealand so that antimicrobial susceptibility testing can continue and new and emerging resistance phenotypes detected.

One of the most challenging obstacles for laboratories when setting up a molecular test is the availability of suitable control material. This study outlines the genotype and phenotype of four *N. gonorrhoeae* isolates available from the New Zealand culture collection with regard to three classes of antimicrobial agents. These isolates are readily available in New Zealand and may therefore be useful to laboratories as they consider how to address the issues around providing susceptibility data on *N. gonorrhoeae*.

The detection of pPPNG in DNA from *N. gonorrhoeae* predicts resistance to penicillin due to plasmid-encoded beta lactamase production. Detection of these plasmids is a useful epidemiological tool and can confirm an isolate as multi drug resistant if other markers are assayed as well. The detection of the gyrA mutation at codon 91, from serine to phenylalanine, has been repeatedly reported in the literature (8,16,17) to predict reduced susceptibility to ciprofloxacin so is useful as a test on *N. gonorrhoeae* DNA from patients who are unable to be treated with ceftriaxone, and for monitoring and epidemiological purposes. A lack of the mutation is a good indication of susceptibility to ciprofloxacin. The detection and monitoring of the mosaic penA in New Zealand is useful to map the spread of decreased susceptibility to ceftriaxone (18).
CONCLUSIONS

Four *N. gonorrhoeae* isolates available in the New Zealand culture collection have been characterised for three molecular markers associated with resistance to penicillin, ciprofloxacin and ceftriaxone, thereby making them suitable for use as controls in any new molecular assays being set up by diagnostic laboratories in response to the need for data on resistance to antimicrobial agents in the absence of cultured isolates.

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