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Enhancing Gonococcal Antimicrobial Resistance Surveillance: a Real-Time PCR Assay for Detection of Penicillinase-Producing *Neisseria gonorrhoeae* by Use of Noncultured Clinical Samples[▽]

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With increasing concerns regarding diminishing treatment options for gonorrhea, maintaining the efficacy of currently used treatments and ensuring optimal *Neisseria gonorrhoeae* antimicrobial resistance surveillance are of the utmost importance. Penicillin is still used to treat gonorrhea in some parts of the world. In this study, we developed and validated a real-time PCR assay for the detection of penicillinase-producing *N. gonorrhoeae* (PPNG) in noncultured clinical samples with the aim of enhancing penicillin resistance surveillance. The assay (PPNG-PCR2) was designed to be an indirect marker of penicillinase activity, by targeting a region of sequence predicted to be conserved across all *N. gonorrhoeae* plasmid types harboring the beta-lactamase gene while not specifically targeting the actual beta-lactamase-encoding sequence. The assay was evaluated by using a total of 118 *N. gonorrhoeae* clinical isolates and 1,194 clinical specimens, including 239 *N. gonorrhoeae*-positive clinical samples from which *N. gonorrhoeae* cells were isolated and for which phenotypic penicillinase results are available. Overall, the PPNG-PCR2 assay provided 100% sensitivity and 98.7% specificity compared to bacterial culture results for the detection of PPNG in clinical specimens. PPNG-PCR2 false-positive results, presumably due to cross-reactions with unrelated bacterial species, were observed for up to 1.3% of clinical samples but could be distinguished on the basis of high cycle threshold values. In tandem with phenotypic surveillance, the PPNG-PCR2 assay has the potential to provide enhanced epidemiological surveillance of *N. gonorrhoeae* penicillin resistance and is of particular relevance to regions where penicillin is still used to treat gonorrhea.

The sexually transmitted disease gonorrhea remains a significant public health challenge. *Neisseria gonorrhoeae*, the etiological agent of gonorrhea, has developed resistance to multiple classes of antibiotics, including penicillins, tetracyclines, macrolides, and quinolones, and the extended-spectrum cephalosporins are now the mainstay of treatment in most settings (10). However, there are still certain population "pockets" where, owing to factors ranging from the limited use of antimicrobials to geographical and microbial isolation from populations harboring resistant strains, antimicrobial resistance (AMR) is low and antibiotics such as penicillin can still be used successfully in accordance with WHO guidelines (10). An example of this is Australia's Northern Territory, where, unlike the other Australian mainland states, *in vitro* resistance to penicillin is observed for <5% of the gonococci isolated from the local population (9). Similarly, penicillin is still used to treat gonorrhea in the French Overseas Territory of New Caledonia (13). Much as this comes as a welcome respite for local public health officials, the history of AMR in *N. gonorrhoeae* suggests that penicillin resistance will eventually emerge in these populations unless there is a concerted application of appropriate public health strategies, including the optimal surveillance of AMR in gonococci.

In remote parts of the world, including regions of the Australian Northern Territory, AMR surveillance poses significant challenges. First, there are difficulties in obtaining viable gonococcal isolates from remote populations. Second, the completeness and representativeness of AMR data are gradually being undermined by the increased use of nucleic acid amplification tests (NAATs) over bacterial culture techniques for the diagnosis of gonorrhea. The viability of the bacterium is not considered an issue when specimens are collected for NAATs, and so there is often a limited possibility of pursuing either phenotypic antibiotic susceptibility determinations or typing following a NAAT-positive result (20). For these reasons, there is now increased interest in the development of systems for the non-culture-based detection of gonococcal

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TABLE 1. Primers and probes used in the study

Primer or probe	Sequence (5′–3′)[a]	Positions (bp)[b]
PPNG-F1	AATTCATTTAAAAAATCAGATTTTGAGCCTA	671–701
PPNG-R1	ACGAAAGTTACCAATGAAGATTTGC	774–750
PPNG-TM1	FAM-ATCTATTGCTATCGTTACCCGCTAGAAATACCCAG-BHQ	708–742
PPNG-F2	AGCTGTTCGTTTTTTACTACCAATCA	937–962
PPNG-R2	TGATTTAGTCGTTGAGGTTGAACAA	1024–1000
PPNG-TM2	FAM-AATTTAAAGAGTGAATAGTACGCCCACGCTTGA-BHQ	966–998

[a] FAM, 6-carboxyfluorescein; BHQ, black hole quencher.
[b] Asian plasmid (GenBank accession number U20374).

AMR mechanisms. In this study, we develop and evaluate real-time PCR for the detection of penicillinase-producing *N. gonorrhoeae* (PPNG) using noncultured clinical samples.

MATERIALS AND METHODS

PPNG real-time PCR assays. The production of penicillinase in *N. gonorrhoeae* is encoded by plasmid-mediated beta-lactamase genes found in a number of related gonococcal plasmid types that vary in size and are named on the basis of the geographical location where they were first isolated. The three most common plasmids are the Asian plasmid (7,426 bp), which is considered the ancestral plasmid; the African plasmid (5,599 bp); and the Rio de Janeiro/Toronto plasmid (5,154 bp). Other less common types are the Nimes (6,798 bp) and New Zealand (9,309 bp) plasmids (3, 7, 8). In this study, two real-time assays (PPNG-PCR1 and PPNG-PCR2) were designed by using Primer Express software (Applied Biosystems Pty. Ltd., Australia), targeting sequence regions at positions 671 to 774 and 937 to 1024, respectively, on the Asian plasmid (GenBank accession number U20374). These regions fell within a larger region of the sequence (nucleotides 605 to 1880 [Asian plasmid]) predicted to be conserved across all the above-described plasmid types (8). Furthermore, these sequences fell outside the TEM-1 beta-lactamase-encoding sequence (nucleotides 6235 to 470 [Asian plasmid]) that is conserved across a diverse range of bacterial species and the reported deletions of all other plasmid types. Given that the gonococcal plasmids were originally thought to be acquired from *Haemophilus* species (7), the GenBank Blast tool was used to aid primer and probe selections to limit the sequence homology of the oligonucleotide targets with sequences of unrelated species.

The reaction mix for each assay consisted of 12.5 μl of QuantiTect Probe PCR master mix (Qiagen, Doncaster, Australia), 10.0 pmol each forward and reverse primer (PPNG-F1 and PPNG-R1 for PPNG-PCR1 and PPNG-F2 and PPNG-R2 for PPNG-PCR2) (Table 1), 4.0 pmol probe (PPNG-TM1 for PPNG-PCR1 and PPNG-TM2 for PPNG-PCR2) (Table 1), and 2.5 μl of sample nucleic acid extract in a final reaction mixture volume of 25.0 μl. Reaction mixtures were cycled on a Rotorgene 6000 real-time PCR instrument (Qiagen, Doncaster, Australia) under the following parameters: an initial hold at 95°C for 15 min followed by 45 cycles at 95°C for 15 s and 60°C for 60 s.

Isolates and clinical samples. The performances of the methods were assessed in three stages. In stage 1, the sequence conservation and specificity of the PPNG-PCR1 and PPNG-PCR2 assay targets were investigated by using a panel of 118 *N. gonorrhoeae* isolates comprising both PPNG and non-PPNG strains. In stage 2, specificity was further examined by using 652 specimens submitted for sexual health screening that tested negative for *N. gonorrhoeae* by PCR. This testing was performed to investigate cross-reactions with nongonococcal bacterial species that may be present in these specimens. In stage 3, clinical sensitivity and specificity for detecting PPNG were then investigated by using 542 specimens submitted for sexual health screening that tested positive for *N. gonorrhoeae* by PCR. A subset of these samples ($n = 239$) had also been tested by bacterial culture, and the presence of penicillinase was assessed by standard phenotypic methods. A summary of isolates and clinical samples used in this study is provided in Table 2.

***N. gonorrhoeae* isolates.** The 118 *N. gonorrhoeae* clinical isolates included 105 genetically diverse gonococci described in a previous study (19), comprising 58 *N. gonorrhoeae* multiantigen sequence types (NG-MAST) (6) of mainly Australian and Asian origins (years of isolation, 1988 to 2005), of which 10 isolates were phenotypically identified as being PPNG isolates (isolates 1 to 10) (Table 3). The remaining 13 gonococcal isolates were from Queensland, Australia (years of isolation, 2001 to 2004), and were selected on the basis that they were PPNG isolates (isolates 11 to 23) (Table 3) and included representatives of each of the three main plasmid types, the Asian plasmid ($n = 8$), the African plasmid ($n = 4$), and the Rio de Janeiro/Toronto plasmid ($n = 1$), as determined by using a previously described multiplex PCR for the differentiation of beta-lactamase plasmids of *N. gonorrhoeae* (8).

***N. gonorrhoeae*-negative clinical specimens.** Two panels of *N. gonorrhoeae*-negative clinical samples were used in the study.

The first panel was comprised of DNA extracts of 192 specimens providing *N. gonorrhoeae*-negative results using four different PCR assays as part of a previous study (4). These specimens were submitted to Pathology Queensland (Path-QLD) for *N. gonorrhoeae* screening in 2006 to 2007 and included 142 urogenital specimens (42 urine specimens, 86 cervical swabs, and 14 vaginal swabs) and 50 throat swabs and had been stored at −70°C.

The second *N. gonorrhoeae*-negative specimen panel included 460 DNA extracts from Pathology, Royal Darwin Hospital (Path-RDH), Northern Territory, Australia. These extracts provided negative results by an *N. gonorrhoeae porA* pseudogene PCR method at Path-RDH and were derived from clinical samples submitted to Path-RDH for *N. gonorrhoeae* testing during a 1-year period from 2008 to 2009. Samples from Path-RDH were extracted by using a MagNAPure

TABLE 2. Summary of isolates, specimens, and PPNG-PCR2 assay results

Specimen type and no. of specimens	Penicillinase phenotype[a]	Results of PPNG-PCR2 assay
N. gonorrhoeae isolates (total = 118)		
23	PPNG	Positive
95	Non-PPNG	Negative
N. gonorrhoeae-negative clinical samples (total = 652)		
Queensland ($n = 192$)		
2	NA	Positive
190	NA	Negative
Northern Territory ($n = 460$)		
6	NA	Positive
454	NA	Negative
N. gonorrhoeae-positive clinical samples (total = 542)		
Queensland ($n = 119$)		
3	PPNG	Positive
22	Non-PPNG	Negative
6	NA	Positive
88	NA	Negative
Northern Territory ($n = 423$)		
8	PPNG	Positive
3	Non-PPNG	Positive
203	Non-PPNG	Negative
8	NA	Positive
201	NA	Negative

[a] The presence of penicillinase was assessed by phenotypic methods. NA, not available.

TABLE 3. All specimens providing positive results in the PPNG-PCR2 assay[d]

Specimen (type; yr of isolation)	Phenotype[a]	PPNG-PCR2 result (C_T value)	*porA* PCR result (C_T value)	ΔC_T value	NG-MAST	Plasmid type[b]
N. gonorrhoeae						
1 (Iso; 2004)	PPNG	Positive (21)	Positive (25)	+4	211	
2 (Iso; 2004)	PPNG	Positive (25)	Positive (29)	+4	211	
3 (Iso; 2004)	PPNG	Positive (26)	Positive (28)	+2	211	
4 (Iso; 2005)	PPNG	Positive (31)	Positive (34)	+3	421	
5 (Iso; 2005)	PPNG	Positive (26)	Positive (30)	+4	1496	
6 (Iso; 2005)	PPNG	Positive (34)	Positive (38)	+4	1797	
7 (Iso; 2005)	PPNG	Positive (24)	Positive (27)	+3	1919	
8 (Iso; 2005)	PPNG	Positive (24)	Positive (27)	+3	1902	
9 (Iso; 2005)	PPNG	Positive (31)	Positive (34)	+3	1921	
10 (Iso; 2005)	PPNG	Positive (32)	Positive (34)	+2	1922	
11 (Iso; 2002)	PPNG	Positive (13)	Positive (17)	+4	NP	African
12 (Iso; 2004)	PPNG	Positive (14)	Positive (18)	+4	NP	African
13 (Iso; 2004)	PPNG	Positive (15)	Positive (18)	+3	NP	African
14 (Iso; 2004)	PPNG	Positive (15)	Positive (19)	+4	NP	African
15 (Iso; 2001)	PPNG	Positive (12)	Positive (15)	+3	NP	Asian
16 (Iso; 2002)	PPNG	Positive (13)	Positive (17)	+4	NP	Asian
17 (Iso; 2002)	PPNG	Positive (14)	Positive (16)	+2	NP	Asian
18 (Iso; 2004)	PPNG	Positive (14)	Positive (17)	+3	NP	Asian
19 (Iso; 2004)	PPNG	Positive (14)	Positive (17)	+3	NP	Asian
20 (Iso; 2002)	PPNG	Positive (15)	Positive (16)	+1	NP	Asian
21 (Iso; 2001)	PPNG	Positive (15)	Positive (18)	+3	NP	Asian
22 (Iso; 2004)	PPNG	Positive (18)	Positive (20)	+2	NP	Asian
23 (Iso; 2004)	PPNG	Positive (13)	Positive (17)	+4	NP	Rio/Toronto
N. gonorrhoeae-negative clinical samples from Queensland (Path-QLD)						
24 (urine; 2007)	NA	Positive (33)	Negative	Not applicable		
25 (urine; 2007)	NA	Positive (39)	Negative	Not applicable		
N. gonorrhoeae-negative clinical samples from the Northern Territory (Path-RDH)						
26 (NA; 2008-2009)	NA	Positive (35)	Negative	Not applicable		
27 (NA; 2008-2009)	NA	Positive (38)	Negative	Not applicable		
28 (NA; 2008-2009)	NA	Positive (39)	Negative	Not applicable		
29 (NA; 2008-2009)	NA	Positive (39)	Negative	Not applicable		
30 (NA; 2008-2009)	NA	Positive (41)	Negative	Not applicable		
31 (NA; 2008-2009)	NA	Positive (43)	Negative	Not applicable		
N. gonorrhoeae-positive clinical samples from Queensland (Path-QLD)						
32 (UrSw; 2004)	PPNG	Positive (17)	Positive (21)	+4	798	
33 (UrSw; 2004)	PPNG	Positive (19)	Positive (23)	+4	New	
34 (urine; 2003)	NA	Positive (22)	Positive (25)	+3	NP	
35 (urine; 2003)	NA	Positive (22)	Positive (26)	+4	NP	
36 (PnSw; 2003)	NA	Positive (22)	Positive (26)	+4	NP	
37 (CxSw; 2003)	PPNG	Positive (24)	Positive (30)	+6	NP	
38 (urine; 2006)	NA	Positive (25)	Positive (29)	+4	1797	
39 (urine; 2003)	NA	Positive (28)	Positive (33)	+5	NP	
40 (urine; 2003)	NA	Positive (43)	Positive (29)	−14	NP	
N. gonorrhoeae-positive clinical samples from the Northern Territory (Path-RDH)						
41 (NA; 2008-2009)	PPNG	Positive (31)	Positive (35)	+4	758	
42 (NA; 2008-2009)	PPNG	Positive (26)	Positive (30)	+4	1405	
43 (NA; 2008-2009)	NA	Positive (32)	Positive (37)	+5	1405	
44 (NA; 2008-2009)	PPNG	Positive (26)	Positive (29)	+3	1554	
45 (NA; 2008-2009)	PPNG	Positive (21)	Positive (26)	+5	1691	
46 (NA; 2008-2009)	NA	Positive (26)	Positive (30)	+4	1691	
47 (NA; 2008-2009)	NA	Positive (25)	Positive (29)	+4	2996	

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TABLE 3—*Continued*

Specimen (type; yr of isolation)	Phenotype[a]	PPNG-PCR2 result (C_T value)	*porA* PCR result (C_T value)	ΔC_T value	NG-MAST	Plasmid type[b]
48 (NA; 2008-2009)	NA	Positive (29)	Positive (32)	+4	2996	
49 (NA; 2008-2009)	NA	Positive (22)	Positive (24)	+2	3782	
50 (NA; 2008-2009)	PPNG	Positive (26)	Positive (30)	+4	3782	
51 (NA; 2008-2009)	PPNG	Positive (22)	Positive (26)	+4	New	
52 (NA; 2008-2009)	PPNG	Positive (22)	Positive (26)	+4	New	
53 (NA; 2008-2009)	PPNG	Positive (25)	Positive (31)	+6	New	
54 (NA; 2008-2009)	NA	Positive (28)	Positive (31)	+3	NA[c]	
55 (NA; 2008-2009)	NA	Positive (31)	Positive (36)	+5	NA[c]	
56 (NA; 2008-2009)	Non-PPNG	Positive (39)	Positive (31)	−8	NA[c]	
57 (NA; 2008-2009)	Non-PPNG	Positive (40)	Positive (31)	−9	NA[c]	
58 (NA; 2008-2009)	Non-PPNG	Positive (40)	Positive (32)	−8	NA[c]	
59 (NA; 2008-2009)	NA	Positive (43)	Positive (32)	−11	NA[c]	

[a] The presence of penicillinase was assessed by phenotypic methods.
[b] The plasmid type was available for the 13 Queensland isolates only.
[c] Amplification of the NG-MAST *porB* sequence, *tbpB* sequence, or both sequences was unsuccessful for these specimens.
[d] CxSw, cervical swab; PnSw, penile swab; UrSw, urethral swab; Iso, isolate; PPNG, penicillinase-producing *N. gonorrhoeae*; NA, not available; NP, not performed; New, a matching sequence type could not be assigned by the NG-MAST database, and the isolate was considered to represent previously unidentified alleles or combinations of alleles.

instrument (Roche Diagnostics, Dee Why, Australia) according to the manufacturer's instructions and stored at −70°C. Path-RDH samples were de-identified, and further information, including sample site, was not available.

***N. gonorrhoeae*-positive clinical specimens.** Two panels of *N. gonorrhoeae*-positive clinical samples were used in the study.

The first panel was comprised of 119 DNA extracts from 100 urogenital specimens (59 urine samples, 14 cervical swabs, 5 penile swabs, 6 vaginal swabs, 14 urethral swabs, and 2 swabs for which the genital site was not specified), 12 anal swabs, and 7 throat swabs submitted to Path-QLD from 2003 to 2007 and which provided *N. gonorrhoeae*-positive PCR results in previous studies (4, 16, 18). Phenotypic penicillinase results were available for *N. gonorrhoeae* isolates from 25 samples (3 urine samples, 4 anal swabs, 4 cervical swabs, 2 penile swabs, 1 vaginal swab, 10 urethral swabs, and 1 throat swab) and included 3 PPNG strains and 22 isolates that did not produce penicillinase (non-PPNG). NG-MAST data were available for 39 of these 119 samples (18).

The second panel was comprised of 423 DNA extracts from specimens submitted to Path-RDH for *N. gonorrhoeae* testing during a 1-year period from 2008 to 2009 and which provided positive results by *N. gonorrhoeae porA* pseudogene PCR. *N. gonorrhoeae* strains were isolated from 214 of these samples by bacterial culture at Path-RDH, 8 of which were phenotypically PPNG and 206 of which were non-PPNG strains.

***N. gonorrhoeae porA* pseudogene PCR.** All isolates and clinical samples providing positive results by PPNG-PCR were retested by a real-time PCR targeting the *N. gonorrhoeae porA* pseudogene (*porA* monoplex) (4). Briefly, the *porA* PCR method was performed by using the same basic reaction mix and cycling conditions as those used for the PPNG-PCR methods except that the primers and probe targeted the gonococcal *porA* pseudogene. The cycle threshold (C_T) values obtained by using the *porA* PCR were compared with those obtained by using the PPNG-PCR methods. In addition, a 10-fold dilution series of DNA from a PPNG strain was tested in the *porA* PCR and PPNG-PCR assays, and the results were compared. The detection limit of each assay was determined as the lowest concentration returning a positive reaction.

NG-MAST analysis. NG-MAST genotyping was performed on selected *N. gonorrhoeae*-positive specimens as previously described (6, 18).

RESULTS

A summary of results for isolates and clinical samples is provided in Table 2.

***N. gonorrhoeae* isolates.** The PPNG-PCR1 and PPNG-PCR2 assays provided 100% agreement with the results of phenotypic penicillinase testing for all 118 *N. gonorrhoeae* isolates for which phenotypic penicillinase results were available: 95 isolates were negative, and 23 isolates were positive (Table 3). Lower C_T values were observed for all 23 PPNG isolates in the PPNG-PCR2 assay compared to those obtained using the *porA* PCR (range of ΔC_T values, +1 to +4 cycles; mean, +3 cycles) (Table 3). When 10-fold dilutions of PPNG DNA were tested, the PPNG-PCR2 assay reliably detected the 10^{-4} dilution, and the *porA* PCR reliably detected the 10^{-3} dilution, indicating that the PPNG-PCR2 assay was approximately 10-fold-more sensitive than *porA* PCR.

***N. gonorrhoeae*-negative clinical specimens.** Of the 192 *N. gonorrhoeae*-negative clinical samples from Path-QLD, 50 (26%) samples provided positive results in the PPNG-PCR1 assay (C_T values ranged from 30 to 40 cycles; mean, 38 cycles), 2 two (1%) urine samples (specimens 24 and 25) (Table 3) provided positive results in the PPNG-PCR2 assay (C_T values of 33 and 39 cycles). Evaluations of the PPNG-PCR1 assay were discontinued on the basis of these results. When applied to the 460 Path-RDW *N. gonorrhoeae*-negative clinical samples, 6 (1.3%) samples (specimens 26 to 31) (Table 3) provided positive results in the PPNG-PCR2 assay (C_T values ranged from 35 to 43 cycles; mean, 39 cycles).

All eight *N. gonorrhoeae*-negative clinical samples providing positive results in the PPNG-PCR2 assay were retested with the *porA* PCR method, and negative results were obtained. This confirms that these were false-positive results in the PPNG-PCR2 assay.

***N. gonorrhoeae*-positive clinical specimens. (i) PPNG-PCR2 assay versus bacterial culture.** Of the 239 *N. gonorrhoeae*-positive samples for which bacterial cultures with phenotypic penicillinase results were available (comprising samples from both Queensland and the Northern Territory), 225 (94.1%) samples were identified as being PPNG negative, and 11 (4.6%) samples were identified as being PPNG positive (specimens 32, 33, 37, 41, 42, 44, 45, 50, 51, 52, and 53) (Table 3) by both the PPNG-PCR2 assay and bacterial culture/phenotypic analysis. The latter 11 samples comprised all PPNG-positive clinical samples detected by bacterial culture used in this study. Lower C_T values were observed for these 11 specimens in the PPNG-PCR2 assay than those obtained by using *porA* PCR (range of ΔC_T values, +3 to +6 cycles; mean, +4 cycles)

(Table 3). A further three (1.3%) samples (specimens 56, 57, and 58) (Table 3) were positive by the PPNG-PCR2 assay, yet *N. gonorrhoeae* isolates from these samples were non-PPNG isolates. These three specimens provided C_T values that were among the highest observed for the PPNG-PCR2 assay (39 to 40 cycles) (Table 3) and that were considerably higher than those obtained by *porA* PCR (−8 to −9 cycles) (Table 3).

(ii) Queensland samples. Of the 119 total *N. gonorrhoeae*-positive clinical samples from Path-QLD, 110 (92.4%) were negative and 9 (7.6%) were positive (specimens 32 to 40) (Table 3) in the PPNG-PCR2 assay. The nine positive specimens included all three Path-QLD specimens known to contain PPNG strains on the basis of bacterial culture results. A fourth positive sample (specimen 38) (Table 3) provided a matching NG-MAST type with a PPNG strain (specimen 6) (Table 3) isolated the preceding year in New South Wales, Australia. Lower C_T values were observed for the PPNG-PCR2 assay than for the *porA* PCR for all but one of the nine Path-QLD-positive specimens: specimen 40 provided a C_T value in the PPNG-PCR2 assay that was 14 cycles higher than that of the *porA* PCR (Table 3).

(iii) Northern Territory samples. Of the 423 total *N. gonorrhoeae*-positive clinical samples from Path-RDH, 404 (95.5%) were negative and 19 (4.5%) were positive (specimens 41 to 59) (Table 3) in the PPNG-PCR2 assay. The positive specimens included all eight Path-RDH specimens known to contain PPNG strains. These 19 positive samples were further investigated by NG-MAST; 3 samples for which bacterial culture results were not available (specimens 43, 46, and 49) (Table 3) provided matching NG-MAST types with samples harboring PPNG strains (specimens 42, 45, and 50, respectively) (Table 3). Four of the 19 positive specimens provided higher C_T values in the PPNG-PCR2 assay than in the *porA* PCR (specimens 56 to 59) (Table 3). As discussed above, non-PPNG strains were isolated from three of these samples, whereas bacterial culture results were not available for the remaining specimen.

DISCUSSION

With renewed concerns regarding the control of gonorrhea arising from recent reports of multidrug-resistant gonococci and the decreased *in vitro* susceptibility to both oral and injectable extended-spectrum cephalosporins (2, 9, 10, 11, 12), maintaining the efficacy of currently used treatments for gonorrhea is of the utmost importance. One facet of this is ensuring optimal AMR surveillance, particularly in light of the increased use of *N. gonorrhoeae* NAATs over bacterial culture techniques and the decreasing availability of gonococcal isolates for phenotypic testing. In this study, we successfully developed and validated a PCR assay for the detection PPNG in noncultured clinical samples.

When designing the PPNG-PCR1 and PPNG-PCR2 assays, we encountered a number of challenges: there was a need to (i) identify sequence targets that were conserved across all variants of the gonococcal plasmids carrying the beta-lactamase gene and (ii) limit the sequence homology of our oligonucleotide targets with those of other species. For assay designs, the actual TEM-1 beta-lactamase gene would be an obvious choice in terms of sequence conservation and also for being a direct marker of the presence of the gene; however, this sequence is conserved across a diverse range of bacterial species and therefore would clearly not be an optimal target in terms of PCR specificity. For these reasons, we targeted conserved regions outside the beta-lactamase gene on the gonococcal plasmids to serve as indirect markers of penicillinase activity. Despite these efforts, specificity problems were still evident in the PPNG-PCR1 assay and, to a lesser extent, in the PPNG-PCR2 assay, presumably due to the presence of the sequence targets in unrelated bacterial species present in these samples. In particular, the PPNG-PCR1 assay provided poor specificity (74%) when applied to a panel of *N. gonorrhoeae* PCR-negative samples, and evaluations of the PPNG-PCR1 assay were discontinued on this basis. It should also be noted that both PPNG-PCR1 and PPNG-PCR2 oligonucleotide sets provided a 100% match with a *Neisseria meningitidis* plasmid sequence (GenBank accession number AF126482) that is nearly identical to the gonococcal African plasmid. These assays therefore have the potential to produce false-positive results for gonorrhea-positive samples that also contain penicillinase-producing *N. meningitidis* strains. However, this is not considered a limitation given that *N. meningitidis* strains are infrequently found in the human genital tract (18) and that *N. meningitdis* isolates harboring this plasmid are rare (1).

Overall, the PPNG-PCR2 assay proved effective in detecting PPNG in noncultured clinical samples, providing 100% sensitivity and 98.7% specificity compared to bacterial culture results for the 239 clinical specimens tested using both methods. Based on the results of the *N. gonorrhoeae*-negative samples and the samples tested in parallel with bacterial cultures, it is evident that the PPNG-PCR2 assay may provide false-positive results in 1.0% to 1.3% of samples. However, decisions regarding the treatment of gonorrhea are not made on a case-by-case basis but are predetermined on the basis of antimicrobial surveillance data to ensure a cure rate of 95% (21). Therefore, an overestimation error of up to 1.3% could be considered acceptable when analyzing surveillance data obtained by using the PPNG-PCR2 assay. On the other hand, the results suggest that the PPNG-PCR2 false-positive results could otherwise be omitted or lessened on the basis of cycle threshold (C_T) values, using either a set cutoff of approximately 35 cycles or a ΔC_T value with the *porA* PCR. The gonococcal beta-lactamase plasmids are multicopy plasmids, whereas the *porA* pseudogene is present as a single copy per gonococcal genome. Therefore, one would expect the PPNG-PCR2 assay to have performance characteristics at least comparable to those of the *porA* PCR for PPNG strains. This is demonstrated by the fact that PPNG-PCR2 had a 10-fold-better detection limit than did the *porA* PCR and also had lower C_T values than did the *porA* PCR for all PPNG isolates and clinical samples known to contain PPNG. It is for these reasons that the considerably delayed PPNG-PCR2 C_T values compared to those of the *porA* PCR for samples 40 and 56 to 59 (−8 to −14 cycles) (Table 3) represent an obvious anomaly and suggest that the gonococci in the samples were not PPNG. NG-MAST analysis provided further evidence that four *N. gonorrhoeae*-positive samples with positive ΔC_T values (and for which bacterial culture results were not available) did contain PPNG.

The use of either a cutoff of 35 cycles or positive ΔC_T values for the Northern Territory *N. gonorrhoeae*-positive samples

removes samples 56 to 59 (Table 3) from the positive results and provides an overall PPNG rate of 3.6% (15/423) in this cohort using the PPNG-PCR2 assay. This figure is consistent with the results for the 214 samples used in the study from which *N. gonorrhoeae* was isolated and tested phenotypically for penicillinase at Path-RDH: 3.7% (8/214). However, these data vary from previously reported phenotypic surveillance rates for the Northern Territory for this period: *N. gonorrhoeae* penicillin resistance rates were 3.9% and 4.2% in 2008 to 2009, with PPNG rates of 2.9% and 2.5%, respectively (9). The basis for this discrepancy is not clear and may be due to sampling; however, this clearly warrants further investigation given that the observed *N. gonorrhoeae* penicillin resistance rates in the Northern Territory are sitting uneasily just below the WHO threshold of 5.0%. In this context, it should also be noted that chromosomally mediated penicillin resistance (CMPR) may also occur and that reliance solely on the PPNG-PCR2 method may underestimate true resistance levels. Thus, the PPNG-PCR2 assay should be regarded as providing a baseline indication of penicillin resistance rather than the complete picture but could nevertheless be used to show whether the WHO threshold has been breached (such as what was shown for the Queensland samples in this study). It is also for these reasons that the PPNG-PCR2 assay should be considered a means of enhancing phenotypic resistance surveillance rather than replacing it. Molecular methods for the detection of CMPR were described elsewhere previously (5, 14) but have not been validated for use on noncultured clinical samples. Furthermore, CMPR is complex, involving the interaction of several mechanisms (increased efflux pump activity, reduced permeability, and modification of the penicillin binding protein targets) and, in our opinion, is not sufficiently elucidated to accurately predict phenotypes.

In conclusion, we have developed and evaluated a real-time PCR assay for the detection of PPNG. To our knowledge, the PPNG-PCR2 assay is the first assay of its kind to provide definitive *N. gonorrhoeae* penicillin resistance data without the use of cultured isolates. In tandem with phenotypic surveillance, this assay has the potential to provide enhanced epidemiological surveillance of *N. gonorrhoeae* penicillin resistance and is of particular relevance to regions such as the Northern Territory, Australia, where penicillin is still used to treat gonorrhea.

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REFERENCES

1. **Backman, A., P. Overlid, J. A. Vazquez, O. Skold, and P. Oclen.** 2000. Complete sequence of beta-lactamase-encoding plasmid in Neisseria meningitidis. Antimicrob. Agents Chemother. **44:**210–212.
2. **Barry, P. M., and J. D. Klausner.** 2009. The use of cephalosporins for gonorrhoea: the problem of impending resistance. Expert Opin. Pharmacother. **10:**555–577.
3. **Dillon, J. R., H. Lee, K. H. Yeung, and T. A. Aman.** 1999. A PCR assay for discriminating Neisseria gonorrhoeae beta-lactamase-producing plasmids. Mol. Cell. Probes **13:**89–92.
4. **Goire, N., M. D. Nissen, G. M. LeCornec, T. P. Sloots, and D. M. Whiley.** 2008. A duplex Neisseria gonorrhoeae real-time polymerase chain reaction assay targeting the gonococcal porA pseudogene and multicopy opa genes. Diagn. Microbiol. Infect. Dis. **61:**6–12.
5. **Kugelman, G., et al.** 2009. Simple, rapid, and inexpensive detection of Neisseria gonorrhoeae resistance mechanisms using heat-denatured isolates and SYBR green-based real-time PCR. Antimicrob. Agents Chemother. **53:**4211–4216.
6. **Martin, I. M., C. A. Ison, D. M. Aanensen, K. A. Fenton, and B. G. Spratt.** 2004. Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. J. Infect. Dis. **189:**1497–1505.
7. **Pagotto, F., and J. A. Dillon.** 2001. Multiple origins and replication proteins influence biological properties of beta-lactamase-producing plasmids from Neisseria gonorrhoeae. J. Bacteriol. **183:**5472–5481.
8. **Palmer, H. M., J. P. Leeming, and A. Turner.** 2000. A multiplex chain reaction to differentiate between beta-lactamase plasmids of Neisseria gonorrhoeae. J. Antimicrob. Chemother. **45:**777–782.
9. **Tapsall, J.** 2009. Annual report of Australian Gonococcal Surveillance Program. 2008. Commun. Dis. Intell. **33:**268–274.
10. **Tapsall, J.** 2009. Implications of current recommendations for third-generation cephalosporin use in the WHO Western Pacific region following the emergence of multi-resistant gonococci. Sex. Transm. Infect. **85:**256–258.
11. **Tapsall, J.** 2006. Antibiotic resistance in Neisseria gonorrhoeae is diminishing available treatment options for gonorrhea: some possible remedies. Expert Rev. Anti Infect. Ther. **4:**619–628.
12. **Tapsall, J. W.** 2005. Antibiotic resistance in Neisseria gonorrhoeae. Clin. Infect. Dis. **41:**S263–268.
13. **Vernel-Pauillac, F., V. Falcot, D. Whiley, and F. Merien.** 2006. Rapid detection of a chromosomally mediated penicillin resistance-associated ponA mutation in Neisseria gonorrhoeae using a real-time PCR assay. FEMS Microbiol. Lett. **255:**66–74.
14. **Vernel-Pauillac, F., S. Nandi, R. A. Nicholas, and C. Goarant.** 2008. Genotyping as a tool for antibiotic resistance surveillance of Neisseria gonorrhoeae in New Caledonia: evidence of a novel genotype associated with reduced penicillin susceptibility. Antimicrob. Agents Chemother. **52:**3293–3300.
15. Reference deleted.
16. **Whiley, D. M., et al.** 2004. A new confirmatory Neisseria gonorrhoeae real-time PCR assay targeting the porA pseudogene. Eur. J. Clin. Microbiol. Infect. Dis. **23:**705–710.
17. Reference deleted.
18. **Whiley, D. M., et al.** 2010. Neisseria gonorrhoeae multi-antigen sequence typing using non-cultured clinical specimens. Sex. Trasm. Infect. **86:**51–55.
19. **Whiley, D. M., E. A. Limnios, S. Ray, T. P. Sloots, and J. W. Tapsall.** 2007. Diversity of penA alterations and subtypes in Neisseria gonorrhoeae strains from Sydney, Australia, that are less susceptible to ceftriaxone. Antimicrob. Agents Chemother. **51:**3111–3116.
20. **Whiley, D. M., J. W. Tapsall, and T. P. Sloots.** 2006. Nucleic acid amplification testing for Neisseria gonorrhoeae: an ongoing challenge. J. Mol. Diagn. **8:**3–15.
21. **World Health Organization Gonococcal Antimicrobial Surveillance Programme.** 2008. Rationale and applications for the current (2008) WHO panel of Neisseria gonorrhoeae for antimicrobial resistance surveillance for public health purposes, and instructions for their use. Technical document D007-0408-11. WHO Collaborating Centre for STD, Sydney, Australia.