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Multitarget PCR Assay for Direct Detection of Penicillinase-Producing *Neisseria gonorrhoeae* for Enhanced Surveillance of Gonococcal Antimicrobial Resistance

Cameron Buckley,[a,b] Ella Trembizki,[a,b] Robert W. Baird,[c] Marcus Chen,[d,e] Basil Donovan,[f,g] Kevin Freeman,[c] Namraj Goire,[h,i] Rebecca Guy,[f] Monica M. Lahra,[h] David Regan,[f] David M. Whiley,[a,b] on behalf of the GRAND Study Investigators

Queensland Paediatric Infectious Diseases Laboratory, Queensland Children's Medical Research Institute, Queensland, Australia[a]; The University of Queensland, Brisbane, Queensland, Australia[b]; Microbiology Laboratory, Pathology Department, Royal Darwin Hospital, Darwin, Northern Territory, Australia[c]; Melbourne Sexual Health Centre, Alfred Health, Carlton Victoria, Australia[d]; Central Clinical School, Monash University, Victoria, Australia[e]; Kirby Institute, University of New South Wales, Sydney, NSW, Australia[f]; Sydney Sexual Health Centre, Sydney Hospital, Sydney, NSW, Australia[g]; WHO Collaborating Centre for STD, Microbiology Department, South Eastern Area Laboratory Services, Prince of Wales Hospital, Sydney, New South Wales, Australia[h]; Nepean Clinical School, University of Sydney, Kingswood, New South Wales, Australia[i]

A multitarget PCR was developed for the direct detection of penicillinase-producing *Neisseria gonorrhoeae* (PPNG). The assay was validated by testing 342 PPNG isolates and 415 clinical samples. The method is suitable for routine detection of PPNG strains. Its multitarget approach reduces the potential for false-negative results caused by sequence variations.

Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae*, the causative agent of the disease gonorrhea, is a major public issue and is now recognized by the U.S. Centers for Disease Control and Prevention (CDC) as one of the top three urgent AMR threats (1). Action plans to control the impact and spread of *N. gonorrhoeae* AMR have been released by the World Health Organization (WHO) and CDC (2, 3), and prominent among the listed recommendations is the need to improve AMR surveillance capabilities. Conventional *N. gonorrhoeae* AMR surveillance is performed by bacterial culture. However, bacterial culture has several major limitations, including the need for stringent sample handling and transport systems to maintain viable organisms, which are problems in remote and resource-limited settings. In addition, nucleic acid amplification tests (NAATs) are increasingly commonplace in the diagnosis of gonococcal infections in both remote and urban settings, which in turn are seeing further reductions in the use of bacterial culture and widening gaps in AMR data. For these reasons, there has been an increasing need for the development of molecular methods to enhance AMR surveillance activity (4). We recently described a real-time PCR method to detect penicillinase-producing *N. gonorrhoeae* (PPNG) directly within clinical samples (5). The method acts as an indirect marker for PPNG by targeting a region of DNA on the gonococcal plasmids carrying the penicillinase gene but not the penicillinase gene itself. The original evaluations of this PPNG-PCR showed 100% sensitivity and 98.7% specificity compared to bacterial culture for detecting PPNG strains in clinical specimens (5). However, a more recent evaluation of the assay revealed a variant plasmid type, now called the Australian plasmid, which was negative by PPNG-PCR (6). In brief, we tested 342 PPNG isolates from throughout Australia from the year 2012 and found one isolate that was negative by PPNG-PCR. DNA sequencing revealed a novel 1,885-bp deletion (corresponding to nucleotides 502 to 2385 of the Asian plasmid type, GenBank accession no. U20374), which included the region targeted by the PPNG-PCR (937 to 1024 bp) and hence accounted for the false-negative result. Several different gonococcal plasmids with various different deletions and insertions have now been described and named according to the geographical location of their initial discovery; these include the Asian (7,426 bp), African (5,599 bp), Rio de Janeiro/Toronto (5,154 bp), Nimes (6,798 bp), New Zealand (9,309 bp), Johannesburg (4,865 bp), and now the Australian (3,629 bp) plasmids (6–8).

While our initial data suggest that the Australian plasmid may be rare in local gonococci, we sought to rectify the PPNG-PCR to account for this plasmid, particularly given that the PPNG-PCR is now used as a routine tool to inform gonorrhea treatment guidelines in remote parts of Australia where penicillin-based treatments are still used (9). In doing so, we aimed to limit the potential for false-negative results caused by further unrecognized deletions or insertions by adopting a multitarget approach. To this effect, the modified PPNG-PCR method (PPNG-triplex-PCR) is a multiplex PCR method and comprises the original PPNG-PCR oligonucleotides (PPNG target 1, Table 1), a second set of newly designed PPNG oligonucleotides (PPNG target 2, Table 1), and oligonucleotides targeting the gonococcal *opa* genes to serve as an internal control (opa gene target, Table 1). The new PPNG target 2 oligonucleotides target a different region from that of the original PPNG-PCR method (corresponding to nucleotides 6160 to 6313 of the Asian plasmid type, GenBank accession no. U20374). This particular region, which flanks the start of transposon Tn*2* (nucleotide 6235; GenBank accession no. U20374), was predicted to be conserved across all recognized gonococcal plasmid types based on previous descriptions of these plasmids (6–8) and anal-

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Address correspondence to David M. Whiley, d.whiley@uq.edu.au.

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TABLE 1 Primer and probe information

Primer/probe	Sequence (5′ to 3′)[a]	Target (positions)[b]	Reference or source	Supplier
PPNG target 1 (original)				
PPNG-t1-F	AGCTGTTCGTTTTTTACTACCAATCA	PPNG plasmid (937–962)	5	GeneWorks
PPNG-t1-R	TGATTTAGTCGTTGAGGTTGAACAA	PPNG plasmid (1024–1000)	5	GeneWorks
PPNG-t1-Probe	670-AAGAGTGAATAGTACGCCCACGCTTGA-BHQ2[c]	PPNG plasmid (972–998)	5	Biosearch Technologies
PPNG target 2 (new)				
PPNG-t2-F	TTGATTGCCGTAAATGTCCGTA	PPNG plasmid (6160–6181)	Current study	GeneWorks
PPNG-t2-R	CTCCACCTTCATCCTCAGCAA	PPNG plasmid (6313–6293)	Current study	GeneWorks
PPNG-t2-Probe	FAM-CAGCCCTTCCCGGTCTGTTGAAC-BHQ1	PPNG plasmid (6289–6267)	Current study	GeneWorks
opa gene target (internal control target)				
GCopa-F	TTGAAACACCGCCCGGAA	*opa* genes	10	GeneWorks
GCopa-R	TTTCGGCTCCTTATTCGGTTTAA	*opa* genes	10	GeneWorks
GCopa-Probe	Yak-CCGATATAATC+CGTC+CTTCAA+CATCAG-BHQ1	*opa* genes	10	Tib Molbiol

[a] 670, Quasar 670; BHQ, black hole quencher; FAM, 6-carboxyfluorescein; Yak, Yakima yellow; +, locked nucleic acid (LNA) bases.
[b] Positions based on Asian plasmid (GenBank accession no. U20374).
[c] Sequence is a shortened version of the original PPNG-PCR2 assay (4) with a different fluorophore.

ysis of available sequences from the GenBank database. The *opa*-based internal control PCR was included to show that amplifiable gonococcal DNA was present when testing nucleic acids extracted from clinical samples.

The PPNG-triplex-PCR assay consisted of 10.0 μl of Quanti-Tect probe PCR master mix (Qiagen, Doncaster, Australia), 0.125 μM PPNG target 1 primers (PPNG-t1-F and PPNG-t1-2, Table 1), 0.5 μM PPNG target 2 primers (PPNG-t2-F and PPNG-t2-2, Table 1), 0.25 μM *opa* gene primers (GCopa-F and GCopa-R, Table 1), 0.2 μM each probe (PPNG-t1-probe, PPNG-t2-probe, and GCopa-Probe, Table 1), and 2.0 μl of sample extract or isolate DNA and was made up to 20.0 μl using DNase-free water. The differing primer concentrations were used to limit competition between the three targets (data not shown). Real-time PCR was carried out using the Rotor-Gene Q and Rotor-Gene 6000 instruments (Qiagen), with the following cycling parameters: hold at 95°C for 15 min, followed by 45 cycles at 95°C for 15 s and then 60°C for 60 s. Only samples providing cycle threshold (C_T) values of <35 cycles by either of the PPNG-PCR methods were considered to be true PPNG positives, as previously described (5).

The PPNG-triplex-PCR assay was initially evaluated by retesting the above-mentioned 342 Australian PPNG isolates, which included representatives of the Asian, African, and Rio de Janeiro/Toronto plasmids in addition to the Australian plasmid. All isolates provided positive results in both PPNG targets, with the exception of the isolate harboring the Australian variant, which was positive by PPNG target 2 and, as expected, was negative by the PPNG target 1 PCR. To examine clinical performance, the PPNG-triplex-PCR was then applied to DNA extracts of 415 clinical samples (Table 2), providing positive results for *N. gonorrhoeae* by the Versant CT/GC DNA NAAT (Siemens Healthcare Diagnostics) and by supplemental in-house PCR methods. These 415 samples comprised 94 vaginal (22.7% of samples), 40 urethral (9.6%), 29 cervical (7.0%), 14 throat (3.4%), 11 anal/rectal (2.7%), 2 penile swabs (0.5%), and 1 eye swab (0.2%) samples, as well as 198 urine samples (47.7%), 6 joint fluid samples (1.4%), and 20 samples (4.8%) for which the site was not specified. The extracts were kindly provided by the Royal Darwin Hospital Laboratory, Northern Territory, and comprised all available *N. gonorrhoeae* NAAT-positive samples tested by the hospital in the first half of 2014. Based on the gonorrheal notification data, these were estimated to comprise approximately 35% of all notified gonorrhea cases in the Northern Territory during this period. A subset of these samples (n = 193) also had corresponding bacterial culture results. Of these, 85 were negative by culture (i.e., no gonococci were isolated), 96 were penicillin β-lactamase negative, and the remaining 12 were identified as PPNG via phenotypic methods. The low yield of culture reflects the challenges of transporting clinical samples across great distances in the Northern Territory.

The results of the PPNG-triplex-PCR testing for the 415 samples are summarized in Table 2. In brief, 21 (5.1%) samples were PPNG positive, and 311 (74.9%) were non-PPNG by the PPNG-triplex-PCR method. A further 83 (20%) samples were negative by both PPNG-PCR methods and the *opa* internal control PCR and were considered to be no-call results by the PPNG-triplex-PCR. Compared to the available culture results, the PPNG-triplex-PCR assay successfully identified 11 of 12 samples that were PPNG positive by culture (sensitivity, 91.7%; 95% confidence interval [CI], 64.6% to 98.5%) and gave complete agreement for the non-PPNG samples (84/84 samples; specificity, 100%; 95% CI, 95.6% to 100%). However, the PPNG-triplex-PCR assay missed one sample that was recorded as PPNG positive by culture. This sample provided a C_T value of 30 cycles in the *opa* gene PCR, indicating that a moderate load of amplifiable gonococcal DNA was present. At such a load, we would have expected the PPNG-PCR assays to have readily amplified their plasmid targets. The isolate from this sample was not available for repeat testing. Additional testing was carried out on the sample using a previously described multiplex PCR to differentiate β-lactamase plasmids of *N. gonorrhoeae* (7), and negative results were again obtained (data not shown). We therefore do not believe this reflects a false-negative result by the PPNG-triplex-PCR assay but rather is suggestive of a sample mixup or data entry error.

Overall, the results show that the PPNG-triplex-PCR is suitable

TABLE 2 Summary of results for the 415 clinical sample extracts

Bacterial culture result (where available)[a]	No. of samples	PPNG-triplex-PCR results (C_T range; mean)			
		Overall result[b]	PPNG target 1[b]	PPNG target 2[b]	*opa* gene (internal control)[c]
PPNG	11	PPNG	Positive (24–32; 26)	Positive (23–32; 27)	Positive (24–44; 30)
	1	Non-PPNG	Negative	Negative	Positive (30)
Non-PPNG	84	Non-PPNG	Negative	Negative	Positive (20–40; 29)
	12	NC	Negative	Negative	Negative
No growth	2	Non-PPNG	Positive (39–42; 40)	Negative	Positive (32–38; 35)
	1	Non-PPNG	Negative	Positive (39)	Positive (38)
	62	Non-PPNG	Negative	Negative	Positive (27–45; 33)
	20	NC	Negative	Negative	Negative
NP	10	PPNG	Positive (23–35; 30)[d]	Positive (23–34; 30)	Positive (29–40; 34)
	160	Non-PPNG	Negative	Negative	Positive (24–43; 32)
	1	Non-PPNG	Negative	Positive (38)	Positive (35)
	51	NC	Negative	Negative	Negative

[a] No growth, bacterial culture was attempted but gonococci were not isolated; NP, not performed, bacterial culture was not attempted on these samples.
[b] Only samples providing C_T value of <35 cycles by either of the PPNG-PCR were considered to be true PPNG positives by the PPNG-triplex-PCR. NC, no call; these samples gave negative results in both PPNG-PCR methods and the *opa* internal control PCR and therefore did not provide a valid result call by the PPNG-triplex-PCR.
[c] A C_T value cutoff was not used for the *opa* gene PCR assay, and so all samples providing an amplification curve in the *opa* assay were considered positive.
[d] One sample provided a C_T value of 35 cycles in target 1 but 34 cycles for target 2 and thus was PPNG positive on the basis of target 2.

for routine detection of PPNG strains directly in clinical samples so as to enhance bacterial culture-based surveillance. Moreover, its multitarget approach limits the potential for sequence variation to cause false-negative results.

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REFERENCES

1. **Centers for Disease Control and Prevention.** 2013. Antibiotic resistance threats in the United States, 2013. U.S. Department of Health and Human Services, Atlanta, GA. http://www.cdc.gov/drugresistance/threat-report-2013/.
2. **World Health Organization.** 2012. Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*. World Health Organization, Geneva, Switzerland. http://whqlibdoc.who.int/publications/2012/9789241503501_eng.pdf.
3. **Centers for Disease Control and Prevention.** 2012. Cephalosporin-resistant *Neisseria gonorrhoeae* public health response plan. Division of STD Prevention, National Center for HIV, STD, and TB Prevention, Atlanta, GA.
4. **Goire N, Lahra MM, Chen M, Donovan B, Fairley CK, Guy R, Kaldor J, Regan D, Ward J, Nissen MD, Sloots TP, Whiley DM.** 2014. Molecular approaches to enhance surveillance of gonococcal antimicrobial resistance. Nat Rev Microbiol **12:**223–229. http://dx.doi.org/10.1038/nrmicro3217.
5. **Goire N, Freeman K, Tapsall JW, Lambert SB, Nissen MD, Sloots TP, Whiley DM.** 2011. Enhancing gonococcal antimicrobial resistance surveillance: a real-time PCR assay for detection of penicillinase-producing *Neisseria gonorrhoeae* by use of noncultured clinical samples. J Clin Microbiol **49:**513–518. http://dx.doi.org/10.1128/JCM.02024-10.
6. **Trembizki E, Buckley C, Lawrence A, Lahra M, Whiley D, GRAND Study Investigators.** 2014. Characterization of a novel *Neisseria gonorrhoeae* penicillinase-producing plasmid isolated in Australia in 2012. Antimicrob Agents Chemother **58:**4984–4985. http://dx.doi.org/10.1128/AAC.02993-14.
7. **Palmer HM, Leeming JP, Turner A.** 2000. A multiplex polymerase chain reaction to differentiate β-lactamase plasmids of *Neisseria gonorrhoeae*. J Antimicrob Chemother **45:**777–782. http://dx.doi.org/10.1093/jac/45.6.777.
8. **Muller EE, Fayemiwo SA, Lewis DA.** 2011. Characterization of a novel β-lactamase-producing plasmid in *Neisseria gonorrhoeae*: sequence analysis and molecular typing of host gonococci. J Antimicrob Chemother **66:**1514–1517. http://dx.doi.org/10.1093/jac/dkr162.
9. **Speers DJ, Fisk RE, Goire N, Mak DB.** 2014. 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. J Antimicrob Chemother **69:**1243–1247. http://dx.doi.org/10.1093/jac/dkt501.
10. **Tabrizi SN, Chen S, Tapsall J, Garland SM.** 2005. Evaluation of *opa*-based real-time PCR for detection of *Neisseria gonorrhoeae*. Sex Transm Dis **32:**199–202. http://dx.doi.org/10.1097/01.olq.0000154495.24519.bf.