False-negative results using *Neisseria gonorrhoeae porA* pseudogene PCR - a clinical gonococcal isolate with an *N. meningitidis porA* sequence, Australia, March 2011

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The gonococcal *porA* pseudogene is a popular target for in-house *Neisseria gonorrhoeae* PCR methods. With this study we present two novel findings: the first case of an *N. gonorrhoeae porA* pseudogene PCR false-negative result caused by sequence variation, and in the same organism, the first description of a clinical *N. gonorrhoeae* strain harbouring an *N. meningitidis porA* sequence.

In this report, we describe the first case of a *Neisseria gonorrhoeae* false-negative test result using an *N. gonorrhoeae porA* pseudogene PCR method, caused by sequence variation. Nucleic acid amplification tests (NAATs) are widely used for the detection of gonorrhoea, yet there are challenges for *N. gonorrhoeae* NAATs because of the considerable sequence variation and genetic exchange that is exhibited by the *Neisseria* genus. Many gonococcal NAATs are known to cross-react with commensal *Neisseria* strains necessitating the use of supplementary testing [1,2]. In addition, sequence-related false-negative results have also been reported for NAATs targeting certain gonococcal sequences. These include the *N. gonorrhoeae cppB* and *opa* genes [3,4].

In March 2011, a young man in his early 20s presented with anal pain to a sexual health clinic in Newcastle, New South Wales, Australia. The man reported having recently had numerous sexual contacts with men (MSM), some with overseas visitors, including from the United States, but reported no recent overseas travel. Pharyngeal and rectal swabs, as well as a urine sample were obtained and submitted for *N. gonorrhoeae* testing. The urine sample and rectal swab were tested by NAAT, and both swab samples were tested by bacterial culture. A summary of results is provided in the Table.

The rectal swab provided positive results for *N. gonorrhoeae* by Cobas4800 CT/NG testing (Roche Diagnostics, Australia) which targets a direct repeat

Table

Culture and NAAT results for *Neisseria gonorrhoeae* by anatomical site and type of sample, New South Wales, Australia, March 2011

Anatomical site / type of sample	*Neisseria gonorrhoeae* diagnostic methods			
	Culture	Cobas4800 CT/NG	LightCycler PCR (porA pseudogene)	TaqMan PCR (porA pseudogene)
Urine sample	NP	Negative	NP	NP
Rectal swab	Positive	Positive	Negative	Negative
Rectal isolate	NA	Positive	Negative	Negative
Pharyngeal swab	Positive	NP	NP	NP
Pharyngeal isolate	NA	Positive	Negative	Negative

NA: not applicable; NAAT: nucleic acid amplification test; NP: not performed.

region, DR-9, with a cycle threshold value of 27, and *N. gonorrhoeae* was subsequently isolated from both the pharyngeal and rectal swabs. The urine sample was negative by NAAT (Cobas4800). Following Australian public health laboratory network guidelines [2] which require supplementary testing for *N. gonorrhoeae* NAAT-positive samples, a DNA extract from the rectal sample was tested using a LightCycler-hybridisation probe-based PCR protocol targeting the gonococcal *porA* pseudogene [5]. The *porA* pseudogene is a target widely used for this purpose and has previously been shown to be highly conserved and specific to *N. gonorrhoeae* [5-8]. Negative results were obtained using the LightCycler method for both the DNA extract of the rectal swab as well as the clinical isolates cultured from the pharyngeal and rectal sites. When the rectal sample and the rectal and pharyngeal isolates were subsequently tested using a TaqMan-based *N. gonorrhoeae porA* pseudogene (*porA*-monoplex) assay, the results were also negative [8]. Testing of the clinical isolates using the Cobas4800 CT/NG assay provided positive results for *N. gonorrhoeae*, with cycle threshold values of 17 for both isolates.

The clinical isolates were further characterised phenotypically and genotypically. Both isolates were indistinguishable and were identified phenotypically [9] as *N. gonorrhoeae* by Gram stain, colonial morphology on modified New York City agar, oxidase, superoxol and rapid carbohydrate utilisation tests. The isolates were tested for prolyliminopeptidase (PIP) activity, auxotyped, serogrouped and the serovar determined by coagglutination reactions with 14 monoclonal reagents (Boule, Huddinge, Sweden). Both isolates tested positive for PIP, were prototrophs and belonged to a common serovar, 'Bropyst'. An identification of *N. gonorrhoeae* was also provided by the Bruker Biotyper matrix-assisted laser desorption ionisation time of

Figure

Sequence alignment of *porA* sequences of *Neisseria gonorrhoeae* FA1090 strain[a], *N. gonorhoeae porA*-variant[b] and *N. meningitidis* 278 strain[c]

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                                10        20        30        40        50        60        70        80        90
N.gonorrhoeae (FA1090)          TTGGCAGCTCGAGCAAGACGTATCCGTTGCCGGCGGCGGCGCGACCCGTTGGGGTAACAGGGAATCCTTTATCGGCTTGGCAGGCGAATT
N.gonorrhoeae (variant)         .........T.....................................AG.....C.....................................
N.meningitidis (278)            .........T.....................................AG.....C.....................................

                               100       110       120       130       140       150       160       170       180
N.gonorrhoeae (FA1090)          CGGCACGGCGCTCGCCGGTCGCGTTGCGAATCCGTTTGGCGATGCCAGCAAAGCCATTGATCCTTGGGACAGCAATAATAATGTGGCTTC
N.gonorrhoeae (variant)         ...T...CT..G.....................A.....A..........C.............................G..........
N.meningitidis (278)            ...T...CT..G.....................A.....A..........C.............................G..........

                               190       200       210       220       230       240       250       260       270
N.gonorrhoeae(FA1090)           GCAATTGGGTATTTTCAAACGCCACGACGGTATGCCGGTTTCCGTGCGTTACGATTCCCCCGGATTTTCCGGTTTCAGCGGCAGCATTCA
N.gonorrhoeae (variant)         ............................A..............A..C.....C..T..G.AC.....................G.C..
N.meningitidis (278)            ............................A..............A..C.....C..T..G.AC.....................G.C..

                               280       290       300       310       320       330       340       350       360
N.gonorrhoeae(FA1090)           ATTTGTTCCGAGTCAAAACAGCAAGTCCGCCTATACGCCTGCTACTTTCACGCTGGAAAGTAATCAGATGAAACCAGTTCCGGCTGTTGT
N.gonorrhoeae (variant)         ...C......GC.............................G...-----T.T.-....T.AGG.G...G..---T.TCA.G..........
N.meningitidis ( 278)           ...C......GC.............................G...-----T.T.-....TGAG..G...G..---T.TCA.G..........

                               370       380       390       400       410       420       430       440       450
N.gonorrhoeae (FA1090)          CGGCAAGCCGGGGTCGGATGTGTATTATGCCGGTCTGAATTACAAAAATGGCGGCTTTTTCGGAAATTATGCCCTTAAATATGCGAAACA
N.gonorrhoeae (variant)         .........C..A.............................................A...GC...G.GC......T.................
N.meningitidis (278)            .........C..A.............................................A...GC...G.GC......T.................

                               460       470       480       490       500       510       520       530       540
N.gonorrhoeae (FA1090)          CGCCAATGAGGGGCATGATGCTTTCTTTTTGTTCTTGCTCGGCAGAGCGAGTGAT------------ACCGATCCATTGAAAAACCATCA
N.gonorrhoeae (variant)         ..............G.............................C.G.......GAAGCCAAAGGT.........C..............
N.meningitidis (278)            ..............G.............................C.G.......GAAGCCAAAGGT.........C..............

                               550       560       570       580       590       600       610       620       630
N.gonorrhoeae (FA1090)          GGTACACCGCCTGACGGGCGGCTATGGGGAAGGCGGCTTGAATCTCGCCTTGGCGGCTCAGTTGGATTTGTCTGAAAATGCCGACAAAAC
N.gonorrhoeae (variant)         ........................A..........................................................................
N.meningitidis (278)            ........................A..........................................................................

                               640       650       660       670       680       690       700       710       720
N.gonorrhoeae (FA1090)          CAAAAACAGTACGACCGAAATTGCCGCCACTGCTTCCTACCGCTTCGGTAATACAGTCCCGCGCATCAGCTATGCCCATGGTTTCGACTT
N.gonorrhoeae (variant)         ..................................................G....T.....................................
N.meningitidis (278)            ..................................................G....T.....................................

                               730       740       750       760       770       780
N.gonorrhoeae (FA1090)          TGTCGAACGCAGTCAGAAACGCGAACATACCAGCTATGATCAAATCATCGCCGGTGTCGATT
N.gonorrhoeae (variant)         ..............................................................
N.meningitidis (278)            .A........G..A.A...G.....A..........C................C..T....
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[a] Genbank accession AJ223447.

[b] Pseudogene PCR negative strain from this study.

[c] Genbank accession GQ173789.

Forward and reverse primer targets of the TaqMan-based *Neisseria gonorhoeae porA* pseudogene PCR are represented by arrows at positions 262 to 284 and 324 to 250 respectively.

The probe target is represented by the box at position 298 to 323.

flight mass spectrophotometer (MALDI TOF MS) Maldi Biotyper (Bruker Biosciences Pty Ltd.). Antimicrobial resistance patterns for these isolates were characteristic of *N. gonorrhoeae*, as determined by the minimum inhibitory concentrations (MICs) using the agar plate incorporation method of the Australian Gonococcal Surveillance Programme and using the CDS Antibiotic Susceptibility criteria [10]. The clinical isolates had chromosomally-mediated penicillin resistance (MIC: 2.0 mg/L), quinolone resistance (ciprofloxacin MIC: 16.0 mg/L), decreased susceptibility to ceftriaxone (MIC: 0.03 mg/L) and sensitivity to azithromycin and spectinomycin. Both isolates were of multilocus sequence type (MLST) 1901 (*abcZ* 109, *adk* 39, *aroE* 170, *fumC* 111, *gdh* 148, *pdhC* 153, *pgm* 65). This is an *N. gonorrhoeae* MLST type previously observed in Australia and elsewhere [11,12] and representatives of this type have previously provided positive results by *porA*-pseudogene PCR in our laboratory (data not shown). Using *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST), both isolates harboured the previously described POR and tbpB types, 1297 and 983 respectively, which represented a novel NG-MAST type 5377. It should be noted that MLST and NG-MAST are not performed as a routine part of *N. gonorrhoeae* investigations in our laboratory, therefore isolates of these MLST and NG-MAST types may have previously been circulating in our population but not characterised.

To examine the basis of the *porA* pseudogene PCR false-negative results, a 773 base-pair fragment of the *porA* sequences of each isolate were amplified using primers CGGCTCGTTTATCGGCTT and GGTATTTCGTTTCAGCCAAGC and subjected to DNA sequencing. The *porA*-pseudogene PCR-negative *N. gonorrhoeae* strains from this study exhibited only 90% homology with the reference *N. gonorrhoeae* FA1090 strain (genbank accession number AJ223447) and had multiple mismatches and deletions evident in the primer and probe targets for the *porA* pseudogene Taqman-based-PCR (Figure) and for the LightCycler hybridisation probe-based method targeting the same region (data not shown).

Notably, genbank blast searching indicated that the *porA* sequence from the clinical isolates in this study were more similar to that of *N. meningitidis*, having 99% homology with *N. meningitidis* 278 strain (Genbank accession GQ173789). Only the last 60 bases of the 773 base *porA* sequence provided greater homology with *N. gonorrhoeae* than with *N. meningitidis*.

Conclusions

Overall the results show that the rectal and pharyngeal *N. gonorrhoeae* isolates from this patient were typical in terms of genotypic and phenotypic characteristics, except that they had acquired a meningococcal *porA* sequence presumably through horizontal genetic exchange and recombination. This is yet another example of the problems faced with molecular detection of *N. gonorrhoeae*, and with PCR-based diagnostics more generally. For *N. gonorrhoeae*, the problem is exacerbated by the fact that the species comprises numerous subtypes that exhibit considerable sequence diversity as well as propensity to mutate. Notably, the distribution of subtypes can vary geographically, temporally, and between patient groups. This has implications for the performance of *N. gonorrhoeae* NAATs: firstly, the performance may vary between patient populations because of the presence of different subtypes; but secondly, as in our case, the performance within a given population can suddenly change either due to the importation of new strains or mutation of currently circulating strains. In our opinion, the use of different methods, such as NAAT and bacterial culture in parallel, or multi-target NAAT assays provides the most suitable means of circumventing these problems, and to this extent we have previously described a duplex real-time PCR assay for detecting *N. gonorrhoeae* combining both the *porA* pseudogene and *opa* targets [8]. To date, we have not observed any other *N. gonorrhoeae* isolates with a meningococcal *porA* sequence in our laboratory and to our best knowledge this has also not been observed elsewhere. Given the propensity for gonococci to spread through populations, we consider it likely that this strain is more widespread. Further investigations including contact tracing and prospectively testing *N. gonorrhoeae* isolates by *porA* pseudogene PCR, are continuing.

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