Polymerase chain reaction-based screening for the ceftriaxone-resistant *Neisseria gonorrhoeae* F89 strain

N Goire[1,2], M M Lahra[3], M Ohnishi[4], T Hogan[3], A E Liminios[3], M D Nissen[1,2,5], T P Sloots[1,2,5], D M Whiley (d.whiley@uq.edu.au)[1,2]

1. Queensland Paediatric Infectious Diseases Laboratory, Queensland Children's Medical Research Institute, The University of Queensland, Queensland, Australia
2. Sir Albert Sakzewski Virus Research Centre, Children's Health Service, Queensland, Australia
3. WHO Collaborating Centre for STD, Microbiology Department, South Eastern Area Laboratory Services, The Prince of Wales Hospital, Sydney, New South Wales, Australia
4. National Institute of Infectious Diseases, Tokyo, Japan
5. Microbiology Division, Pathology Queensland Central Laboratory, Herston, Queensland, Australia

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Emergence and spread of *Neisseria gonorrhoeae* resistant to extended spectrum cephalosporins is a major problem threatening treatment of gonorrhoea and is further highlighted by the recent report of a second ceftriaxone-resistant *N. gonorrhoeae* strain (F89) in Europe, initially observed in France and subsequently identified in Spain. *N. gonorrhoeae* antimicrobial resistance (AMR) surveillance has acquired new importance and molecular tools have the potential to enhance bacterial culture-based methods. In this study, we established a polymerase chain reaction (PCR) protocol for direct detection of the F89 strain. A key component of this screening protocol was the development of a hybridisation probe-based melting curve analysis assay (mosaic501-hybPCR) to detect the presence of an A501P substitution on the *N. gonorrhoeae* mosaic penicillin binding protein 2 (PBP2) sequence, an important characteristic of the F89 strain. The mosaic501-hybPCR was evaluated using plasmid-derived positive controls (n=3) and characterised gonococcal (n=33) and non-gonococcal (n=58) isolates. The protocol was then applied to 159 clinical specimens from Sydney, Australia, collected during the first half of the year 2012 that were *N. gonorrhoeae* PCR-positive. Overall, the results indicate that the PCR-based protocol is suitable for direct detection of the *N. gonorrhoeae* F89 strain in non-cultured clinical samples. It therefore provides an additional tool to aid investigations into the potential spread of F89 strain throughout Europe and elsewhere.

Introduction

With the emergence of the ceftriaxone-resistant and extensively-drug resistant (XDR) *Neisseria gonorrhoeae* strains H041 in Japan [1] and F89 in France and Spain [2,3], there exists a real threat that such strains may emerge and spread worldwide. Molecular characterisation of these strains has implicated mutations in the penicillin binding protein 2 (PBP2) in conferring resistance to ceftriaxone [1,2]. Notably, the 'mosaic' variant of PBP2 has long been associated with reduced susceptibility to ceftriaxone and other extended spectrum cephalosporins (ESCs), and both the Japanese H041 and European F89 strains harboured a mosaic PBP2. In each of these cases however, additional mutations on the mosaic PBP2 were responsible for expression of resistant phenotypes displaying a raised ceftriaxone minimum inhibitory concentration (MIC). The Japanese H041 (ceftriaxone MIC=2 to 4 mg/L) featured several novel substitutions on the mosaic PBP2, of which two (A311V and T316S) have been experimentally shown to contribute to ESC resistance [1]. Similarly the European F89 strain (ceftriaxone MIC=1 to 2 mg/L) harboured an amino acid substitution at position 501 (A501P) of the mosaic PBP2, resulting in ESC resistance [2,3]. The contribution of other substitutions at position 501 of PBP2, especially A501V but also A501T, towards reduced susceptibility to ESCs has also been shown [4-7].

N. gonorrhoeae antimicrobial resistance (AMR) surveillance is a pivotal component of public health efforts to control gonorrhoea. Molecular methods for tracking gonococci of major public health importance have considerable potential to enhance *N. gonorrhoeae* AMR surveillance and to this effect we have previously described a rapid real-time polymerase chain reaction (PCR) method for direct detection of the H041 strain [8]. In this study we describe a testing algorithm for direct detection of the *N. gonorrhoeae* F89 strain. Briefly, the algorithm involved two steps. The first step screened *N. gonorrhoeae* nucleic acid amplification test (NAAT)-positive specimens for the presence of mosaic PBP2 using a previously described real-time PCR assay (mosaic-PCR; [9,10]). Samples testing positive by the mosaic-PCR were then subjected to a hybridisation probe-based melting curve analysis assay, developed and validated as part of this study, to characterise the 501 amino acid on the mosaic PBP2 sequence (mosaic501-hybPCR).

Table 1

Primers and probes used to analyse *Neisseria gonorrhoeae* mosaic penicillin binding protein 2 sequences

Designation	Sequence (5' to 3')[a]
Mosaic-PCR	
Mosaic-F	GTTGGATGCCCGTACTGGG
Mosaic-R	ACCGATTTTGTAAGGCAGGG
Mosaic-Probe	FAM-CGGCAAAGTGGATGCAACCGA-BHQ
Mosaic501-hybPCR	
Mosaic501-F	GGCGCAAAAACCGGTACG
Mosaic501-R	ATCACACGCGGATTTTTAGCC
Mosaic501-Probe1	CGCAAAAACCGGTACGCCG-fluorescein
Mosaic501-Probe2	Quasar 670-GTAAGTTGGTTAACGGTCGTTACGTCGATTACAAACAC-phosphate
Plasmid control development	
Control-A501P-F	GGCGCAAAAACCGGTACGCCG
Control-A501T-F	GGCGCAAAAACCGGTACGACG
Control-A501V-F	GGCGCAAAAACCGGTACGGTG
PenA-R	GCCCAAGATGTTCAGGCTGC

BHQ: black hole quencher dye; F: forward primer; PCR: polymerase chain reaction; R: reverse primer.

[a] When present, dye labels or 3' phosphates are also indicated.

Methods

Mosaic-PCR assay

The mosaic-PCR was performed using primers and TaqMan probe previously described by Ochiai et al. [10]. Briefly, the reaction mix consisted of 12.5 µl of QuantiTect Probe PCR Master Mix (QIAGEN, Doncaster, Australia), 10.0 pmoles each of forward and reverse primer (Mosaic-F and Mosaic-R; Table 1), 4.0 pmoles of probe (Mosaic-Probe; Table 1) and 2.5 µl of sample nucleic acid extract in a final reaction volume of 25.0 µl. Reactions were thermo-cycled on a Rotor-Gene 6000 real-time PCR instrument (QIAGEN, Doncaster, Australia) with the following parameters: initial hold at 95°C for 15 min followed by 45 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Results were analysed using the quantification analysis tool of the Rotor-Gene software and samples were considered positive if their amplification curves crossed above background fluorescence. We have previously shown this assay to be suitable for direct screening for the *N. gonorrhoeae* mosaic PBP2 sequence directly in non-cultured clinical samples [9].

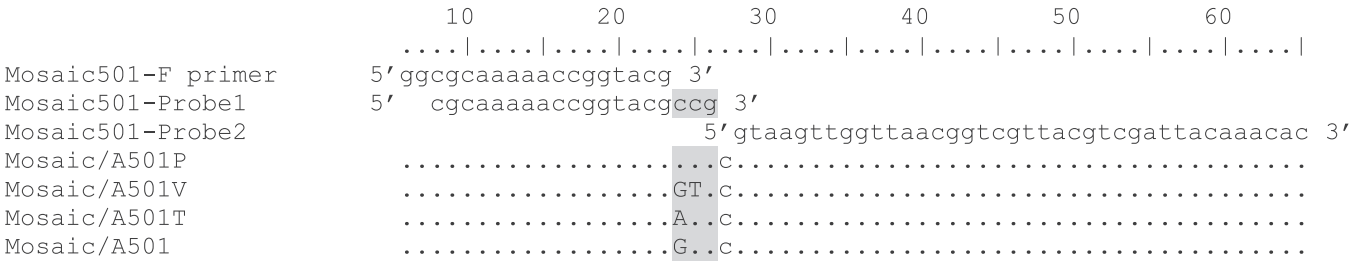
Mosaic501-hybPCR assay

The mosaic501-hybPCR was developed as per a modified hybridisation probe-based real-time PCR approach previously described by our laboratory [11]. Briefly, two primers (Mosaic501-F and Mosaic501-R; Table 1) were used for amplification of the target region and two probes (Mosaic501-Probe1 and Mosaic501-Probe2; Table 1) were used for detection, with Mosaic501-Probe1 being the sensor probe and Mosaic501-Probe2 being the anchor probe. In this modified hybridisation probe approach, the Mosaic501-F primer shares the same target sequence as the Mosaic501-Probe1 sensor probe except for the latter 3-base codon representing the 501 amino acid position (Figure). As previously described, this ensures the sensor probe has full homology with its target sequence with the exception of any variations, if present, in the 501 codon of interest [11].

The mosaic501-hybPCR assay was performed using the LightCycler FastStart DNA Master Hybridization Probes kit (Roche Diagnostics, Australia) on a LightCycler 2.0 real-time PCR instrument (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, each LightCycler capillary was loaded with 2 µl of 10 x kit Master reagent (Roche Diagnostics, Australia, reagent 1), 1.6 µl of $MgCl_2$ (25mM; Roche Diagnostics, Australia, reagent 2), 5.0 pmoles of Mosaic501-F primer (Table 1), 10.0 pmoles of Mosaic501-R primer (Table 1), 2.0 µl of nucleic acid extract or control and made up to a total reaction volume of 20 µl. Capillaries were then placed into LightCycler centrifuge adapters (Roche Molecular Biochemicals, Mannheim, Germany) and spun in a standard 24 x 1.5 ml microfuge at approximately 735 g for 10 seconds. A 2.0 µl suspension comprising a total of 4.0 pmoles of each probe (Table 1) was then added into the sample receptacle of each capillary. The capillaries were then capped but were not microfuged again at this point; this left the PCR reaction in the bottom of the capillary and separated from the probes in the upper receptacle. In this modified hybridisation probe format, the probes must remain separate from the reaction mix during PCR amplification otherwise amplification can be inhibited [11].

Figure

Alignment of the mosaic501-hybPCR forward primer and hybridisation probes with sequences of *Neisseria gonorrhoeae* strains harbouring mosaic penicillin binding protein 2 types differing at residue 501



PBP2: penicillin binding protein 2.

The nucleotide sequences of the mosaic PBP2 types are designated by 'mosaic' followed by details of the particular residue or mutation at position 501 of the protein sequence. Nucleotide sequence numbering is based on the first 60 nucleotides of the 107 base pair product obtained in the real-time polymerase chain reaction assay. The codon for the PBP2 '501' amino acid position is represented by nucleotides 19 to 21. Both probes match 100% with the mosaic/A501P sequence whereas Mosaic501-probe1 has mismatches with the other PBP2 types, enabling discrimination by melting curve analysis. Dots indicate sequence identity with the primer or probe sequences. Capitalised bases indicate mismatches with Mosaic501- Probe1.

Amplification was performed on the LightCycler with the following conditions: initial hold at 95°C for 10 minutes followed by 50 cycles at 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 20 seconds. No data collection was conducted during the PCR as the probes were not present in the mix. Following PCR, the capillaries were spun upside down (without the LightCycler centrifuge adapters) in a 24 x 1.5 ml microfuge at approximately 735 g for 10 seconds, and then placed back into the LightCycler centrifuge adapters and respun right side up as above. This double spinning was used to wash the probes out of the top of the capillary and bring the whole contents (probes and PCR mix) into the bottom of the capillary. Capillaries were then placed back into the LightCycler and melting curve analysis conducted using the following parameters; 95°C for 5 seconds, then the reactions were continuously monitored as they were heated at a rate of 0.2°C/second, starting at 40°C for 30 seconds to a final temperature of 85°C.

Assay reference controls

A mosaic PBP2-harbouring *N. gonorrhoeae* isolate from a previous study (sequence pattern X; [5]) was used as the positive control for the mosaic-PCR assay. This isolate lacked further alterations at amino acid position 501 (mosaic/A501) and so was also used as the reference control for the mosaic501-hybPCR assay. Given that we did not have access to the *N. gonorrhoeae* F89 strain, which has the sequence encoding the mosaic PBP2 protein with the A501P mutation (mosaic/A501P), we had a mosaic/A501P plasmid control (i.e. a mosaic *penA* sequence with a A501P codon; CCG) synthesised by the University of Queensland Protein Expression Facility (University of Queensland, Australia). The mosaic/A501 clinical isolate was amplified using primers Control-A501P-F and PenA-R (Table 1). Use of the Control-A501P-F primer, with 'CCG' at the 3'end, enabled the A501P codon to be incorporated into the mosaic PBP2 sequence in the resulting PCR product. The PCR product was then cloned using the pGEM-T Easy Vector system and the plasmid purified. Plasmid controls were also created for the partial sequences of the mosaic PBP2 harbouring the A501T (mosaic/A501T) and A501V (mosaic/A501V) mutations as described above, using primers Control-A501T-F and Control-A501V-F respectively (Table 1).

Mosaic501-hybPCR assay evaluation

The specificity of the mosaic501-hybPCR assay was investigated using a panel of *Neisseria* species, comprising both gonococcal (n=33) and non-gonococcal strains (n=58). The 33 *N. gonorrhoeae* isolates were of mainly Australian and Asian origin (years of isolation: 1988 to 2009) from a previous study [5], comprising 29 different *N. gonorrhoeae* multi-antigen sequence types (NG-MAST) and 23 different PBP2 types. Four isolates comprised mosaic PBP2 sequences and included the *N. gonorrhoeae* H041 strain [1]. Of these four mosaic-harbouring isolates, one isolate also possessed an A501V alteration (mosaic/A501V) and was the previously described cefixime-resistant NG0304 strain [7], kindly provided by Dr. Makoto Ohnishi, institute of infectious diseases, Japan. The 58 non-gonococcal strains comprised *Moraxella catarrhalis* (n=6), *M. osloensis* (n=2), *N. cinerea* (n=4), *N. elongata* (n=1), *N. lactamica* (n=8), *N. meningitidis* (n=21), *N. polysaccharea* (n=4), *N. sicca* (n=1), *N. subflava* (n=10) and *N. weaveri* (n=1), and were predominantly isolated in New South Wales, Australia during the years 2007 to 2009.

Application to clinical samples

Following evaluation of the mosaic501-hybPCR assay, 159 clinical specimens were screened for the presence of mosaic PBP2 sequences and alterations at amino acid 501. The samples comprised DNA extracts from 62 urine specimens and 13 endocervical, eight vaginal, 74 rectal and two throat swabs that were PCR-positive for *N. gonorrhoeae* at the South Eastern Area Laboratory Services in Sydney, New South Wales during the first half of 2012. Samples were initially tested using the mosaic-PCR. Samples providing positive results in the mosaic-PCR were then characterised using the mosaic501-hybPCR assay.

Detection limit

A ten-fold dilution series of DNA from the *N. gonorrhoeae* NG0304 strain [7] in water (10E-1 to 10E-9) was prepared. The dilutions were tested in duplicate in the mosaic501-hybPCR and mosaic-PCR assays above, as well as an *N. gonorrhoeae* duplex real-time PCR assay (NG-duplex) routinely used by our laboratory for detection of gonorrhoea and targeting the gonococcal *porA* pseudogene and multicopy *opa* genes [12]. The detection limit of each assay was determined as the lowest concentration returning positive results in both duplicates.

Results

Table 2 provides the melting temperatures for the controls (samples 1 to 4; Table 2), all isolates (samples 5 to 30; Table 2) and all clinical specimens (samples 31 to 40; Table 2) for which melting curves were obtained in the mosaic501-hybPCR assay.

Mosaic501-hybPCR assay evaluation

The results for the controls (samples 1 to 4; Table 2) showed that based on melting temperature, isolates with mosaic/A501P (68.0°C) or mosaic/A501V (56.5°C) could be distinguished from isolates with mosaic/A501 or mosaic/A501T (58.48°C and 58.50°C respectively). However, isolates with mosaic/A501 or mosaic/A501T could not be distinguished from each other given their similar melting temperatures. The latter was not considered a problem given that an isolate with a mosaic/A501T alteration has not been described to date.

The mosaic501-hybPCR assay results for the 33 *N. gonorrhoeae* clinical isolates were consistent with the results of DNA sequencing. The isolates with mosaic/A501 (n=3) and mosaic/A501V (n=1) were correctly characterised by the mosaic501-hybPCR assay (samples 5 to 8; Table 2). No melting curves were observed for the remaining 29 *N. gonorrhoeae* isolates and were consistent with all of these isolates lacking a mosaic PBP2 sequence.

Of the 58 non-gonococcal strains, 22 (samples 9 to 30; Table 2) provided melting curves in the mosaic501-hybPCR assay; *N. cinerea* (n=3), *N. elongata* (n=1), *N. lactamica* (n=7), *N. meningitidis* (n=3), *N. polysaccharea* (n=2) and *N. subflava* (n=6). Of these, 19 were characterised as mosaic/A501 whereas the remaining three isolates could not be characterised on the basis that their melting curves were not consistent with any of the reference controls.

Application to clinical samples

Of the 159 clinical specimens, 10 (6 rectal swabs and 4 urine samples) provided positive results in the mosaic-PCR assay. When tested by the mosaic501-hybPCR assay, all were characterised as mosaic/A501 strains (samples 31 to 40; Table 2).

Detection limit

The testing of ten-fold dilutions of DNA from the NG0304 strain showed that the mosaic501-hybPCR and mosaic-PCR assays had comparable detection limits, with both detecting to the 10E-7 dilution. However, both these methods were 10-fold less sensitive than the NG-duplex assay which detected to the 10E-8 dilution.

Discussion

The fact that the *N. gonorrhoeae* F89 strain has been found in both France and Spain suggests that this may indeed be the first high-level ceftriaxone-resistant gonococcal strain spreading internationally. Enhanced surveillance for this strain is therefore warranted, particularly in Europe. The main aim of this study was to develop a real-time PCR method that could distinguish *N. gonorrhoeae* strains of the mosaic/A501P PBP2 type so that it could be used for direct screening for the *N. gonorrhoeae* F89 strain. Overall, the approach appears suitable for this purpose, and in fact could potentially also be used to detect strains of the mosaic/A501V PBP2 type. When applied to 159 *N. gonorrhoeae* PCR-positive samples from our local Sydney (Australia) population, we found that 10/159 (6.3%) had a mosaic PBP2, but that none of these possessed the A501P or A501V alterations. These data are consistent with the fact that while isolates with ceftriaxone-reduced susceptibility are prevalent in the Australian population, no isolates exhibiting ceftriaxone MICs reflective of the H041 or F89 strains have yet been reported in the Australian population. Our ongoing concern is that with the speed with which *N. gonorrhoeae* isolates with the mosaic PBP2 have spread globally [2,13], importation of the F89 strain remains a very real threat.

One limitation of the approach was that there were high rates of cross-reaction observed with the commensal *Neisseria* species, with 22 of the 58 (38%) non-gonococcal species providing melting peaks in the mosaic501-hybPCR assay. While none of these strains were found to possess either the A501P or A501V alterations, the results nevertheless highlight the considerable sequence homology between gonococcal sequences associated with antimicrobial resistance and those of commensal *Neisseria* strains. In practical terms, this means that the method may not be suitable for use on pharyngeal specimens where commensal *Neisseria* species are prevalent, given that amplification of the *penA* of such species, together or instead

Table 2

Melting temperatures for controls, isolates and clinical samples obtained in the mosaic501-hybPCR assay, which was developed to detect nucleotide substitutions leading to mosaic penicillin binding protein 2 types differing at residue 501

Sample typeand number	Description	*penA*/PBP2 type[a]	Melting temperature (call)
Controls			
1.	*Neisseria gonorrhoeae* isolate	Mosaic/A501	58.48°C (mosaic/A501[b])
2.	Plasmid control	Mosaic/A501T	58.50°C
3.	Plasmid control	Mosaic/A501V	56.50°C
4.	Plasmid control	Mosaic/A501P	68.00°C
Neisseria isolate panel			
5.	*N. gonorrhoeae*	Mosaic/A501	58.51°C (mosaic/A501[b])
6.	*N. gonorrhoeae*	Mosaic/A501	58.54°C (mosaic/A501[b])
7.	*N. gonorrhoeae* (H041)	Mosaic/A501	58.63°C (mosaic/A501[b])
8.	*N. gonorrhoeae* (NG0304)	Mosaic/A501V	56.15°C (mosaic/A501V)
9.	*N. cinerea*	NA	58.49°C (mosaic/A501[b])
10.	*N. cinerea*	NA	58.67°C (mosaic/A501[b])
11.	*N. cinerea*	NA	58.02°C (mosaic/A501[b])
12.	*N. elongata*	NA	59.20°C (mosaic/A501[b])
13.	*N. lactamica*	NA	58.41°C (mosaic/A501[b])
14.	*N. lactamica*	NA	58.45°C (mosaic/A501[b])
15.	*N. lactamica*	NA	58.49°C (mosaic/A501[b])
16.	*N. lactamica*	NA	58.49°C (mosaic/A501[b])
17.	*N. lactamica*	NA	58.68°C (mosaic/A501[b])
18.	*N. lactamica*	NA	58.71°C (mosaic/A501[b])
19.	*N. lactamica*	NA	58.98°C (mosaic/A501[b])
20.	*N. meningitidis*	NA	58.96°C (mosaic/A501[b])
21.	*N. meningitidis*	NA	58.68°C (mosaic/A501[b])
22.	*N. meningitidis*	NA	53.23°C (NT)
23.	*N. polysaccharea*e	NA	58.38°C (mosaic/A501[b])
24.	*N. polysaccharea*e	NA	58.60°C (mosaic/A501[b])
25.	*N. subflava*	NA	45.75°C (NT)
26.	*N. subflava*	NA	46.98°C (NT)
27.	*N. subflava*	NA	58.40°C (mosaic/A501[b])
28.	*N. subflava*	NA	58.51°C (mosaic/A501[b])
29.	*N. subflava*	NA	58.55°C (mosaic/A501[b])
30.	*N. subflava*	NA	58.92°C (mosaic/A501[b])
PCR-positive clinical samples[c]			
31.	Rectal swab	NA	58.57°C (mosaic/A501[b])
32.	Rectal swab	NA	58.68°C (mosaic/A501[b])
33.	Rectal swab	NA	58.78°C (mosaic/A501[b])
34.	Rectal swab	NA	58.83°C (mosaic/A501[b])
35.	Rectal swab	NA	58.84°C (mosaic/A501[b])
36.	Rectal swab	NA	59.13°C (mosaic/A501[b])
37.	Urine sample	NA	58.57°C (mosaic/A501[b])
38.	Urine sample	NA	58.60°C (mosaic/A501[b])
39.	Urine sample	NA	58.61°C (mosaic/A501[b])
40.	Urine sample	NA	58.63°C (mosaic/A501[b])

NA: not available; NT: not typed, as melting temperature was not consistent with any of the reference controls; PBP2: penicillin binding protein 2; PCR: polymerase chain reaction.

[a] Based on DNA sequencing. The mosaic PBP2 types are designated by 'mosaic' followed by details of the particular residue or mutation at position 501 of the protein sequence.

[b] It should be noted that the assay cannot distinguish between mosaic/A501 and mosaic/A501T.

[c] These samples were positive by *Neisseria gonorrhoeae* PCR as well as the mosaic-PCR assay.

of *penA* of *N. gonorrhoeae*, could potentially interfere with characterisation by melting curve analysis. A second limitation of the mosaic501-hybPCR and mosaic-PCR assays, as shown by the detection limit studies, is that they are less sensitive than diagnostic *N. gonorrhoeae* NAAT methods, such as the NG-duplex method tested in this study. Hence, the approach will not be able to characterise all *N. gonorrhoeae* NAAT, particularly samples with very low *N. gonorrhoeae* DNA loads. It should also be noted that in addition to a 'CCG' codon, an A501P alteration may also arise via 'CCT', 'CCC' or 'CCA' and would therefore not be identified by the current method. We do not however consider this a major limitation in the context of screening for the F89 strain, which is known to possess the 'CCG' codon.

In summary, our results show that the mosaic501-hyb-PCR assay can be readily used to detect the presence of the mosaic/A501P PBP2 alteration, and therefore, when used in combination with mosaic-PCR, could be used for direct detection of the *N. gonorrhoeae* F89 strain in non-cultured clinical samples. Use of the assay could considerably strengthen bacterial culture-based investigations into the broader prevalence, if any, of the F89 strain in Europe. Likewise, for settings where AMR surveillance gaps exist because of increased use of NAAT for gonorrhoea diagnosis or where the scope for bacterial culture is limited, such methods also demonstrate considerable potential of molecular technology to enhance *N. gonorrhoeae* antimicrobial resistance surveillance.

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