Molecular Assay for Detection of Genetic Markers Associated with Decreased Susceptibility to Cephalosporins in *Neisseria gonorrhoeae*

S. W. Peterson,^a I. Martin,^a W. Demczuk,^a A. Bharat,^a L. Hoang,^b J. Wylie,^c V. Allen,^d B. Lefebvre,^e G. Tyrrell,^f G. Horsman,^g D. Haldane,^h R. Garceau,ⁱ T. Wong,^j M. R. Mulvey^a

Bacteriology and Enteric Diseases Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada^a; British Columbia Centres for Disease Control Public Health Microbiology and Reference Laboratory, Vancouver, British Columbia, Canada^b; Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada^c; Public Health Ontario Laboratories, Toronto, Ontario, Canada^d; Laboratoire de Santé Publique du Québec, Ste-Anne-de-Bellevue, Québec, Canada^e; Provincial Laboratory for Public Health, Edmonton, Alberta, Canada^f; Saskatchewan Disease Control Laboratory, Regina, Saskatchewan, Canada^g; Queen Elizabeth II Health Sciences Centre, Halifax, Nova Scotia, Canada^h; Dr. G.L. Dumont Hospital, Moncton, New Brunswick, Canadaⁱ; Centre for Communicable Diseases and Infection Control, Public Health Agency of Canada, Ottawa, Ontario, Canada^j

The incidence of antimicrobial-resistant *Neisseria gonorrhoeae* continues to rise in Canada; however, antimicrobial resistance data are lacking for approximately 70% of gonorrhea infections that are diagnosed directly from clinical specimens by nucleic acid amplification tests (NAATs). We developed a molecular assay for surveillance use to detect mutations in genes associated with decreased susceptibility to cephalosporins that can be applied to both culture isolates and clinical samples. Real-time PCR assays were developed to detect single nucleotide polymorphisms (SNPs) in *ponA*, *mtrR*, *penA*, *porB*, and one *N. gonorrhoeae*-specific marker (*porA*). We tested the real-time PCR assay with 252 gonococcal isolates, 50 nongonococcal isolates, 24 *N. gonorrhoeae*-negative NAAT specimens, and 34 *N. gonorrhoeae*-positive NAAT specimens. Twenty-four of the *N. gonorrhoeae*-positive NAAT specimens had matched culture isolates. Assay results were confirmed by comparison with whole-genome sequencing data. For 252 *N. gonorrhoeae* strains, the agreement between the DNA sequence and real-time PCR was 100% for *porA*, *ponA*, and *penA*, 99.6% for *mtrR*, and 95.2% for *porB*. The presence of ≥ 2 SNPs correlated with decreased susceptibility to ceftriaxone (sensitivities of $>98\%$) and cefixime (sensitivities of $>96\%$). Of 24 NAAT specimens with matched cultures, the agreement between the DNA sequence and real-time PCR was 100% for *porB*, 95.8% for *ponA* and *mtrR*, and 91.7% for *penA*. We demonstrated the utility of a real-time PCR assay for sensitive detection of known markers for the decreased susceptibility to cephalosporins in *N. gonorrhoeae*. Preliminary results with clinical NAAT specimens were also promising, as they correlated well with bacterial culture results.

Neisseria gonorrhoeae, the causative agent of gonorrhea infection, has the second highest reported rate of bacterial sexually transmitted infections in Canada, with $>12,000$ reported cases in 2012 (36.18 cases per 100,000 population) (1). According to the latest World Health Organization (WHO) reports, worldwide gonococcal infections amount to 106 million cases per year (2). *N. gonorrhoeae* has acquired resistance to all of the antibiotics commonly used for treatment, including penicillin, tetracycline, spectinomycin, azithromycin, and ciprofloxacin, and reduced susceptibility to the third-generation cephalosporins has been reported (3). In recent years, the MICs to cefixime and ceftriaxone have been increasing, and there have been reports of cephalosporin treatment failures in Canada and around the world (4–8).

Canada has conducted antimicrobial susceptibility testing on *N. gonorrhoeae* cultures since the mid-1980s to monitor antimicrobial resistance trends and develop an understanding of the molecular subtypes circulating in the population. However, starting in the early 2000s, an increasing number of gonococcal infections have been diagnosed by nucleic acid amplification tests (NAATs) and a decreasing number of laboratories across Canada are culturing *N. gonorrhoeae*. This is of concern since *N. gonorrhoeae* cultures are required for antimicrobial susceptibility testing. In fact, $>70\%$ of gonococcal infections in Canada are now detected using NAATs, and hence antimicrobial susceptibility data are not available for these isolates (Public Health Agency of Canada, National Microbiology Laboratory, 2014, unpublished data).

Numerous molecular mechanisms for decreased susceptibility

(DS) to cephalosporins have been described in *N. gonorrhoeae*. Two classes of alterations of *penA*, which encodes penicillin-binding protein 2 (PBP2), have been described: the first is the *penA* mosaic allele, which contains segments of *penA* from nongonococcal neisserial species; the second is an alteration in the amino acids (A501, G542, P551) of PBP2 in nonmosaic *penA* alleles (3, 9, 10). Mutations in the promoter of the repressor gene *mtrR*, which cause overexpression of the MtrCDE efflux pump system, have been associated with cephalosporin DS (3, 11). Finally, *porB1b* gene mutations that alter amino acids G120 and A121 in the outer membrane PorB1b porin result in reduced permeability and thus further cephalosporin DS (3, 11). In addition, mutations in PBP1 (*ponA*) have been observed in *N. gonorrhoeae* strains with elevated

Received 20 February 2015 Returned for modification 27 March 2015

Accepted 9 April 2015

Accepted manuscript posted online 15 April 2015

Citation Peterson SW, Martin I, Demczuk W, Bharat A, Hoang L, Wylie J, Allen V, Lefebvre B, Tyrrell G, Horsman G, Haldane D, Garceau R, Wong T, Mulvey MR. 2015. Molecular assay for detection of genetic markers associated with decreased susceptibility to cephalosporins in *Neisseria gonorrhoeae*. J Clin Microbiol 53:2042–2048. doi:10.1128/JCM.00493-15.

Editor: N. A. Ledebore

Address correspondence to M. R. Mulvey, michael.mulvey@phac-aspc.gc.ca.

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doi:10.1128/JCM.00493-15

cephalosporin MICs, although this mutation has not been shown to cause resistance in transformation experiments (11).

In this study, real-time PCR assays were developed to detect single nucleotide polymorphisms (SNPs) in genes associated with DS to extended-spectrum cephalosporins (ESCs) for the purpose of providing surveillance data. Four targets associated with cephalosporin DS (*ponA*, *mtrR*, *penA*, and *porB*) and one *N. gonorrhoeae*-specific gene (*porA*) as an internal positive control were selected and evaluated using *N. gonorrhoeae* cultures. As proof of principle, all targets were also evaluated using clinical specimens tested by the Aptima CT/NG (*Chlamydia trachomatis*/Neisseria gonorrhoeae) assay on the Tigris platform (Hologic, Bedford, MA) that also had a matched culture isolate.

MATERIALS AND METHODS

Bacterial isolates and clinical specimens. Canadian provincial laboratories submit isolates to the National Microbiology Laboratory if they identify resistance to at least one antibiotic or if they do not conduct antimicrobial susceptibility testing (12). From this collection, 241 *N. gonorrhoeae* strains isolated across Canada between 2001 and 2014 were selected for development of the assay along with *N. gonorrhoeae* control strains ATCC 49226, F62, FA19, WHO F, WHO G, WHO K, WHO L, WHO M, WHO N, WHO O, and WHO P (13). Isolates were primarily selected to represent a range of cephalosporin MICs, including DS to ceftriaxone ($n = 55$) and cefixime ($n = 32$). In addition, 193 isolates were included that were susceptible to both ceftriaxone and cefixime and represented a diverse group of *Neisseria gonorrhoeae* multiantigen sequence typing (NG-MAST) types and temporal and geographic distribution. Isolates were cultured (from storage at -80°C in brain heart infusion [BHI] containing 20% glycerol) on GC medium base (Difco Laboratories, Detroit, MI) containing 0.2% BioX and incubated for 18 to 24 h at 35°C in a 5% CO_2 atmosphere.

The SNP genotyping assay was then tested with a total of 58 clinical Hologic Aptima CT/NG NAAT specimens. Ten Aptima specimens, consisting of 5 urethral swabs and 5 urine samples, were obtained from Cadham Provincial Laboratory (Winnipeg, MB, Canada). Forty-eight Aptima specimens were obtained from the British Columbia Centers for Disease Control and consisted of 24 *N. gonorrhoeae*-positive specimens and their corresponding cultured isolates and 24 *N. gonorrhoeae*-negative control specimens selected in consecutive order of receipt.

Fifty different nongonococcal strains were chosen to assay for cross-reactivity based on similarity to *N. gonorrhoeae* sequences or the likelihood of their presence in urine or urogenital specimens (Table 1) (14).

Antimicrobial susceptibility testing. MICs were determined using the agar dilution method as previously described (12). Interpretation of the cephalosporin MICs was based on the criteria of the WHO: cefixime DS MIC of ≥ 0.25 $\mu\text{g/ml}$ and ceftriaxone DS MIC of ≥ 0.125 $\mu\text{g/ml}$ (2). Since there have been recent reports of cephalosporin treatment failures for infections caused by isolates with MICs as low as 0.032 $\mu\text{g/ml}$, we also used MIC cutoffs of 0.032 $\mu\text{g/ml}$ and 0.063 $\mu\text{g/ml}$ for sensitivity and specificity calculations (6).

Real-time PCR assay for SNP genotyping. DNA was extracted from Aptima NAAT specimens using the QIAamp viral RNA minikit according to the manufacturer's instructions (Qiagen, Toronto, ON). Five gene targets were chosen, including 4 associated with cephalosporin resistance, *ponA* (L421P), *mtrR* (-35delA), *porB* (G120/A121), and *penA* (mosaic), along with the *N. gonorrhoeae*-specific *porA* pseudogene as a positive control. SNP targets were selected based on circulating isolates in Canada and previously reported resistance mechanisms. Oligonucleotide primers and probes were chosen for each target region using Primer Express Software version 3.0 (Life Technologies). Gene sequences for *porA* (GenBank accession no. [HE681885.2](#)), *ponA* ([AB727713.1](#)), *mtrR* ([Z25796.1](#)), and *porB* ([M21289.1](#)) were acquired from NCBI. Sequences of *penA* representing a variety of mosaic and nonmosaic *penA* types were aligned using Lasergene

TABLE 1 Cross-reactivity of SNP assays in nongonococcal strains

Species	Strain	Targets showing cross-reactivity ^a			
		<i>ponA</i>	<i>mtrR</i>	<i>porB</i>	<i>penA</i>
<i>Atopobium vaginae</i>	ATCC BAA-55				
<i>Bacteroides ureolyticus</i>	ATCC 33387				
<i>Candida albicans</i>	ATCC 18804				
<i>Corynebacterium glucuronolyticum</i>	ATCC 51860				
<i>Corynebacterium urealyticum</i>	ATCC 43043				
<i>Corynebacterium xerosis</i>	ATCC 373				
<i>Cryptococcus neoformans</i>	ATCC 2517				
<i>Enterobacter aerogenes</i>	ATCC 13048				
<i>Enterococcus faecalis</i>	ATCC 29212				
<i>Enterococcus faecium</i>	ATCC 19434				
<i>Escherichia coli</i>	ATCC 35218				
<i>Gardnerella vaginalis</i>	ATCC 14018				
<i>Klebsiella oxytoca</i>	ATCC 13182				
<i>Lactobacillus crispatus</i>	ATCC 33197				
<i>Lactobacillus gasseri</i>	ATCC 33323				
<i>Lactobacillus iners</i>	ATCC 55195				
<i>Lactobacillus jensenii</i>	ATCC 25258				
<i>Leptotrichia buccalis</i>	ATCC 14201				
<i>Listeria monocytogenes</i>	ATCC 15313				
<i>Mobiluncus curtisii</i>	ATCC 35242				
<i>Moraxella catarrhalis</i>	ATCC 25238		X		
<i>Neisseria animalis</i>	ATCC 49930				
<i>Neisseria animaloris</i>	Clinical				
<i>Neisseria cinerea</i>	ATCC 14685				
<i>Neisseria elongata</i>	ATCC 25295		X		
<i>Neisseria flavescens</i>	ATCC 13120				X
<i>Neisseria lactamica</i>	ATCC 23970				
<i>Neisseria meningitidis</i>	ATCC 13102	X			X
<i>Neisseria mucosa</i>	Clinical				
<i>Neisseria perflava</i>	ATCC 9913				
<i>Neisseria polysaccharea</i>	ATCC 43768	X			
<i>Neisseria sicca</i>	ATCC 29256				
<i>Neisseria subflava</i>	Clinical				X
<i>Neisseria wadsworthii</i>	Clinical				
<i>Neisseria weaveri</i>	Clinical				
<i>Peptococcus niger</i>	ATCC 27731				
<i>Peptostreptococcus anaerobius</i>	ATCC 27337				
<i>Prevotella bivia</i>	ATCC 29303				
<i>Proteus mirabilis</i>	ATCC 7002				
<i>Pseudomonas aeruginosa</i>	ATCC 27853				X
<i>Salmonella typhimurium</i>	ATCC 39183				
<i>Staphylococcus aureus</i>	ATCC 29213				X
<i>Staphylococcus epidermidis</i>	ATCC 14990		X		
<i>Streptococcus agalactiae</i>	ATCC 12386				
<i>Streptococcus gordonii</i>	ATCC 10558				
<i>Streptococcus infantis</i>	ATCC 700779				
<i>Streptococcus oralis</i>	ATCC 35037				
<i>Streptococcus pyogenes</i>	ATCC 19615				
<i>Ureaplasma parvum</i>	ATCC 27815				
<i>Ureaplasma urealyticum</i>	ATCC 27618				

^a Isolates were tested in triplicate.

MegAlign version 11.2.1 (DNASTar, Madison, WI); primers and probes were chosen to detect both the mosaic and nonmosaic sequences. The *ponA* and *mtrR* assays contained probes to detect either the wild-type (WT) or the SNP alleles, while the *porB* assay contained a probe to detect

TABLE 2 SNP assay primers and probes

Target ^a	Primer/ probe ^b	Sequence	Product length (bp)	Probe	Sequence ^c	SNP detected
<i>porA</i>	F R	GTATTTTCAAACGCCACGACG GACCGGCATAATACACATCCG	207	WT	CAGCATTC AATTTGTTC	Indicates isolate is <i>N. gonorrhoeae</i>
<i>ponA</i>	F R	GCGGTCGATAATGAGAAAATGG ATCCAGCGAAACCAAAGCC	131	WT SNP	AGCCGTTGCTGCAGG AGCCGTTGCCGCAGG	WT at L421 L421P
<i>mtrR</i>	F R	TCGAACGGGTTGCAAAGC TCGTTTCGGGTCGGTTTG	141	WT SNP	TGCACGGATAAAAAGT GCACGGATAAAAAGTC	WT at –35 bp –35delA
<i>porB</i>	F R	GCTTGAAGGGCGGCTTC GACAGGTAGCGGTGTTCCC	154	WT Control	TTGGC(G/A)CCGGTGT TGCCGGATTCCCAAGC	WT at G120, A121; if WT probes are absent, implies at least one of G120K, G120R, G120D, A121D, A121G, A121N, A121S Internal positive control detected in all isolates
<i>penA</i>	F1 F2 R	GGCAATCAAACCGTTCGTG TGGATTCGGCAAAGTGG ATAATGCCGCGCACATCC	150 118	WT1 WT2 Mosaic	CCGTGCGCGATAC TGCGCGACGATAC ACCGTACAAGATACCCA	Nonmosaic <i>penA</i> allele 1 Nonmosaic <i>penA</i> allele 2 Mosaic <i>penA</i> allele

^a The *porA* assay contains only one probe. The *ponA* and *mtrR* assays contain one FAM (WT)- and one VIC (SNP)-labeled probe. The *porB* assay contains two FAM (WT)-labeled probes to account for a silent mutation in the WT allele, along with an internal positive control probe. The *penA* assay contains three probes, labeled with FAM, VIC, and NED in a single reaction.

^b F, forward; R, reverse.

^c Bold underlined bases denote SNP locations.

the WT allele, along with an internal positive-control probe that was detected in all isolates (Table 2).

Real-time PCR was performed in a reaction volume of 25 μ l, consisting of 12.5 μ l of 2 $\times$ TaqMan genotyping master mix (Life Technologies), 900 nM each primer (final concentration), 250 nM each probe, 5 μ l of 1 ng/ μ l template DNA, and Ambion nuclease-free H₂O (Life Technologies). PCR amplification and detection of amplification products were performed on a ViiA 7 instrument (Life Technologies). Thermal cycling conditions were as follows: initial preheating at 60°C for 30 s, denaturation at 95°C for 10 min; 45 cycles (40 cycles for *N. gonorrhoeae* culture isolates) of 95°C for 15 s and 60°C for 1 min, and a final elongation step of 60°C for 30 s. Each real-time PCR was performed in triplicate. Results were considered to be positive if they had a quantification cycle (C_q) value of <40 (15) and a relative fluorescence of probe minus the baseline (ΔRn) value of >0.5 for *porA*, *ponA*, *mtrR*, and *penA* and of >0.7 for *porB*.

SNPs detected by the assay were validated by comparison with aligned gene sequences obtained through whole-genome sequencing (WGS) (16).

To determine the limits of detection (LODs) of the real-time PCR assay, DNA from *N. gonorrhoeae* control strains F62, WHO K, and WHO L was extracted using the QIAamp viral RNA minikit and quantified using a Qubit fluorometer (Life Technologies). Ten-fold serial dilutions were performed (0.1 fg/ μ l to 1 ng/ μ l). The LOD was determined for each real-time SNP assay by testing each isolate in duplicate and recording the lowest dilution that produced a positive result as defined above.

Calculation of sensitivity and specificity. Sensitivity measures the percentage of isolates with DS containing the SNP of interest, while specificity represents the percentage of susceptible isolates containing a WT allele. Since treatment failures have been observed with a cefixime MIC of 0.032 μ g/ml (6), sensitivities and specificities were calculated with cutoffs of 0.032 μ g/ml, 0.063 μ g/ml, or 0.125 μ g/ml for each antibiotic. To calculate sensitivity and specificity, isolates were characterized into four categories: true positive (TP), having a high MIC and SNP detected by real-time PCR; false-positive (FP), having a low MIC and SNP detected by real-time PCR; false-negative (FN), having a high MIC and WT result; and

true negative (TN), having a low MIC and WT result. Calculations were performed as follows: sensitivity = TP/(FN + TP) $\cdot$ 100; specificity = TN/(FP + TN) $\cdot$ 100 (17).

NG-MAST. Isolates were characterized by NG-MAST based on the sequence of the *porB* and *tbpB* genes as previously described (18). Aptima specimen NG-MAST types were confirmed using WGS data (16), when available. The sequence type (ST) was determined using the NG-MAST website (www.ng-mast.net).

RESULTS

Bacterial isolates and specimens tested. Overall, 241 clinical *N. gonorrhoeae* isolates from 2001 to 2013, along with 11 *N. gonorrhoeae* reference isolates, representing 77 different STs were tested with our assay. Of the isolates, 103 (40.9%) had ceftriaxone MICs of $\leq$ 0.016 μ g/ml, 94 (37.3%) had MICs of 0.032 to 0.063 μ g/ml, and 55 (21.8%) had DS (MICs of $\geq$ 0.125 μ g/ml). Cefixime susceptibilities were as follows: 108 isolates (42.9%) had MICs of $\leq$ 0.016 μ g/ml, 112 isolates (44.4%) had MICs of 0.032 to 0.125 μ g/ml, and 32 isolates (12.7%) had DS (MICs of $\geq$ 0.25 μ g/ml).

The 10 *N. gonorrhoeae*-positive specimens with no bacterial cultures were obtained from 3 males and 7 females and included 5 urine samples and 5 swabs (3 cervical, 1 vaginal, and 1 penis/urethral). The 24 Aptima specimens with matched bacterial cultures and 24 *N. gonorrhoeae*-negative Aptima controls were obtained from 41 males and 7 females (Table 3). The *N. gonorrhoeae*-negative NAAT specimens were obtained from the penis/urethra (n = 1), urine (n = 13), rectum (n = 4), throat (n = 4), and cervix/vagina (n = 2). Of the 24 matched culture isolates, 16 (66.7%) isolates had ceftriaxone MICs of $\leq$ 0.016 μ g/ml, 7 isolates (29.2%) had MICs of 0.032 to 0.063 μ g/ml, and 1 isolate (4.2%) had DS (MIC of $\geq$ 0.125 μ g/ml), while 15 isolates (62.5%) had cefixime MICs of $\leq$ 0.016 μ g/ml, 8 isolates (33.3%) had MICs of

TABLE 3 SNP assay and NG-MAST results from Aptima NAAT specimens compared with MICs from matched culture isolates^a

Sample no.	Source	Matched isolate MIC ^b		SNP assay results from Aptima NAAT specimens				NG-MAST
		Ceftriaxone	Cefixime	<i>ponA</i>	<i>mtrR</i>	<i>porB</i>	<i>penA</i>	
37200A	Penis/urethra	0.25	0.25	SNP	SNP	SNP	Mosaic	ST-3158
37201A	Rectum	0.004	0.008	WT	WT	SNP	WT	ST-5985
37202A	Rectum	0.032	0.063	UND ^c	UND	SNP	UND	UND
37203A	Rectum	0.032	0.016	SNP	SNP	SNP	WT	ST-9665
37204A	Throat	0.008	0.008	WT	WT	SNP	WT	ST-5985
37205A	Urine	0.008	0.008	WT	WT	SNP	WT	ST-5985
37206A	Urine	0.008	0.016	WT	WT	SNP	WT	ST-5985
37207A	Rectum	0.016	0.016	WT	WT	SNP	WT	ST-5985
37208A	Throat	0.016	0.016	SNP	SNP	SNP	UND	UND
37209A	Rectum	0.032	0.032	SNP	SNP	SNP	WT	ST-8502
37210A	Throat	0.032	0.016	SNP	SNP	SNP	WT	ST-9665
37211A	Urine	0.032	0.016	SNP	SNP	SNP	WT	ST-9665
37212A	Vagina	0.004	0.008	WT	WT	SNP	WT	ST-10169
37213A	Cervix	0.016	0.032	WT	WT	SNP	WT	ST-8890
37214A	Urine	0.008	0.032	SNP	WT	WT	WT	ST-5444
37215A	Urine	0.032	0.063	WT	WT	SNP	WT	ST-6339
37216A	Urine	0.016	0.032	WT	WT	SNP	WT	ST-8890
37217A	Urine	0.008	0.032	WT	WT	WT	WT	ST-2992
37218A	Urine	0.008	0.016	WT	WT	SNP	WT	ST-8890
37219A	Urine	0.002	0.004	WT	WT	WT	WT	ST-10170
37220A	Urine	0.063	0.063	SNP	SNP	SNP	WT	ST-8502
37221A	Urine	0.008	0.008	WT	WT	SNP	WT	ST-8890
37222A	Urine	0.008	0.016	WT	WT	WT	WT	ST-10171
37223A	Urine	0.008	0.016	WT	WT	WT	WT	ST-4643

^a MICs indicating reduced susceptibility are shown in bold.^b MICs were calculated from matched *N. gonorrhoeae* culture isolates.^c UND, undetermined result.

0.032 to 0.125 µg/ml, and 1 isolate (4.2%) had DS (MIC of ≥0.25 µg/ml).

Genetic marker performance. All of the 252 *N. gonorrhoeae* isolates tested positive for the *N. gonorrhoeae*-specific *porA* pseudogene. The assay concordance (percentage of isolates called correctly as WT or SNP) with sequencing for the 252 *N. gonorrhoeae* isolates was 100% for *ponA* (78 WT and 174 SNP) and *penA* (160 WT and 92 mosaic). For the *mtrR* gene, the assay correctly predicted 99.6% of the isolates (90/91 WT and 161/161 SNPs). One isolate gave a false-negative result for *mtrR* instead of a WT result due to the replacement of an A in the 5-A homopolymer at −35 with a C, preventing the probe from binding. For the *porB* gene, 95.2% of the isolates (69/70 WT and 171/182 SNPs) were correctly identified. Twelve isolates gave false-negative results for *porB* due to the presence of the *porB1a* allele rather than the *porB1b* allele.

The SNP assays were tested with 50 non-*N. gonorrhoeae* control species and 24 *N. gonorrhoeae*-negative NAAT specimens. The *N. gonorrhoeae*-specific *porA* pseudogene was negative for all non-*N. gonorrhoeae* species. Cross-reacting species for each SNP assay are listed in Table 1. For the negative Aptima samples, one *porB* and two *penA* assays showed cross-reactivity. These three cross-reacting negative Aptima specimens were all throat swabs.

The LODs were 50 fg/reaction for *porA*, *ponA*, *mtrR*, and *porB*, and 500 fg/reaction for *penA*. Sensitivities and specificities were measured for each SNP assay (Table 4) and for combinations of SNPs (Table 5).

Aptima NAAT specimens. Of the 24 *N. gonorrhoeae*-positive Aptima specimens with matched cultures, assay concordance was 100% for *porB*, 95.8% for *ponA* and *mtrR*, and 91.7% for *penA*

compared to the WGS results (Table 3). One rectal swab specimen gave indeterminate results for *ponA* (neither SNP nor WT was positive), *mtrR* (neither SNP nor WT was positive), and *penA* (both WT2 and mosaic probes produced a positive result). One pharyngeal specimen also had an indeterminate *penA* result due to positive results from both the WT and the mosaic probes. One hundred percent identity was observed between NG-MAST sequences obtained from matched Aptima and culture specimens for specimens that could be typed (Table 3).

DISCUSSION

As NAATs have become the primary method of laboratory diagnosis of gonorrhea, fewer cultures are isolated from patients, providing less information about antimicrobial susceptibilities (19). In this study, we developed real-time PCR assays that permitted us to test *N. gonorrhoeae* cultures and Aptima specimens for SNPs associated with DS to ESCs in gonococcal infections. While this assay cannot replace culture-based MIC determination, it can aid surveillance by providing insight into the prevalence of genes associated with DS to ESCs in *N. gonorrhoeae* NAAT specimens for which no culture is available.

We applied our test to 252 *N. gonorrhoeae* isolates along with 50 non-*N. gonorrhoeae* isolates. The *porA* assay gave positive results for all *N. gonorrhoeae* specimens and isolates and negative results for all non-*N. gonorrhoeae* specimens and isolates, indicating that this is an appropriate *N. gonorrhoeae*-specific genetic marker. The assays were effective in identifying SNPs in both culture and NAAT specimens. The LOD of each real-time PCR assay was approximately 50 fg of DNA, corresponding to approximately

TABLE 4 Sensitivities and specificities of the SNP real-time PCR assays when interpreted individually following testing of *N. gonorrhoeae* culture isolates (*n* = 252)^a

SNP assay	Results for ceftriaxone at an MIC of:						Results for cefixime at an MIC of:					
	≥0.032 µg/ml (<i>n</i> = 149)		≥0.063 µg/ml (<i>n</i> = 116)		≥0.125 µg/ml (<i>n</i> = 54)		≥0.032 µg/ml (<i>n</i> = 143)		≥0.063 µg/ml (<i>n</i> = 112)		≥0.125 µg/ml (<i>n</i> = 90)	
	% sensitivity (TP) ^b	% specificity (TN) ^c	% sensitivity (TP)	% specificity (TN)	% sensitivity (TP)	% specificity (TN)	% sensitivity (TP)	% specificity (TN)	% sensitivity (TP)	% specificity (TN)	% sensitivity (TP)	% specificity (TN)
<i>ponA</i>	98 (146)	72.8 (75)	100 (116)	57.4 (78)	100 (54)	39.4 (78)	95.8 (138)	66.7 (72)	99.1 (111)	55 (77)	98.9 (89)	47.5 (77)
<i>mttR</i>	94.6 (141)	80.4 (82)	98.3 (114)	65.2 (88)	96.3 (52)	44.7 (88)	92.4 (133)	73.8 (79)	98.2 (110)	62.6 (87)	97.8 (88)	54.7 (88)
<i>porB</i>	93.3 (139)	64.8 (59)	94.8 (110)	50.8 (63)	96.3 (52)	36.6 (68)	90.9 (130)	57.7 (56)	92.9 (104)	47.7 (61)	93.3 (84)	42 (63)
<i>penA</i> Mosaic	61.1 (91)	99 (102)	69 (80)	91.2 (124)	75.9 (41)	74.2 (147)	63.2 (91)	99.1 (107)	78.6 (88)	97.1 (136)	91.1 (82)	93.8 (152)

^a Sensitivity = TP/(FN + TP) · 100; specificity = TN/(FP + TN) · 100.
^b TP, true positive (isolates contain the SNP and have high MICs).
^c TN, true negative (isolates do not contain the SNP and have low MICs).

TABLE 5 Sensitivities and specificities of the SNP real-time PCR assays when interpreted in combination following testing of *N. gonorrhoeae* culture isolates (*n* = 252)^a

No. of SNPs ^b	Results for ceftriaxone at an MIC of:						Results for cefixime at an MIC of:					
	≥0.032 µg/ml (<i>n</i> = 149)		≥0.063 µg/ml (<i>n</i> = 116)		≥0.125 µg/ml (<i>n</i> = 54)		≥0.032 µg/ml (<i>n</i> = 143)		≥0.063 µg/ml (<i>n</i> = 112)		≥0.125 µg/ml (<i>n</i> = 90)	
	% sensitivity (TP) ^c	% specificity (TN) ^d	% sensitivity (TP)	% specificity (TN)	% sensitivity (TP)	% specificity (TN)	% sensitivity (TP)	% specificity (TN)	% sensitivity (TP)	% specificity (TN)	% sensitivity (TP)	% specificity (TN)
4 SNPs	56.4 (84)	98.8 (89)	63.8 (74)	91.1 (112)	72.7 (40)	75.5 (139)	58.7 (84)	99 (95)	72.9 (81)	96.9 (123)	84.4 (76)	94 (140)
≥3 SNPs	93.3 (139)	82.2 (74)	98.3 (114)	66.7 (82)	98.2 (54)	45.1 (83)	91.6 (131)	75 (72)	97.3 (109)	63.8 (81)	98.9 (89)	55.7 (83)
≥2 SNPs	98.7 (147)	77.8 (70)	100 (116)	58.5 (72)	100 (55)	39.1 (72)	96.5 (138)	69.8 (67)	99.1 (111)	55.9 (71)	98.9 (89)	47.7 (71)
≥1 SNP	98.7 (147)	54.4 (49)	100 (116)	41.5 (51)	100 (55)	27.7 (51)	97.2 (139)	49 (47)	99.1 (111)	39.4 (50)	98.9 (89)	33.6 (50)

^a Sensitivity = TP/(FN + TP) · 100; specificity = TN/(FP + TN) · 100.
^b Minimum number of SNPs present among *ponA*, *mttR*, *porB*, and *penA*.
^c TP, true positive (isolates contain the SNP and have high MICs).
^d TN, true negative (isolates do not contain the SNP and have low MICs).

25 *N. gonorrhoeae* genomes (20). The low LOD and high sequence identity found between Aptima specimens and their matched culture isolates provide proof of principle that direct molecular characterization can be performed on Aptima specimens. In this assay, two of the *N. gonorrhoeae*-negative pharyngeal specimens and one *N. gonorrhoeae*-positive pharyngeal specimen showed cross-reaction with the *penA* mosaic probe, and one sample showed cross-reaction with the *porB* probe. Although this is a possible limitation in the use of DNA from a NAAT specimen, it should be noted that the non-*N. gonorrhoeae* organisms that gave positive results are not normally found in urine but can be commonly found in the respiratory tract. Balashov et al. (14) also observed that molecular assays may not be applicable for extragenital specimens due to the prevalence of non-*N. gonorrhoeae* species at extragenital sites.

In a study by Allen et al. (6), isolates with cefixime MICs of ≥ 0.125 $\mu\text{g/ml}$ had 25% treatment failures, while failures were 1.9% for MICs of ≤ 0.125 $\mu\text{g/ml}$. One treatment failure isolate had an MIC of 0.032 $\mu\text{g/ml}$. For this reason, we determined the sensitivity and specificity values using MIC cutoffs of 0.032 $\mu\text{g/ml}$, 0.063 $\mu\text{g/ml}$, and 0.125 $\mu\text{g/ml}$. The *ponA*, *mtrR*, and *porB* markers all had high sensitivities for both cefixime and ceftriaxone at the three MICs selected, indicating that organisms with elevated MICs were more likely to contain these SNPs. Specificities for *ponA* and *porB* were lower than those for *mtrR* for both antibiotics. A low specificity for *ponA* is not unexpected, as, although *ponA* L421P is often found in organisms with elevated ESC MICs, particularly those that also contain the *penA* mosaic allele, the L421P variant itself did not produce elevated ESC MICs in transformation experiments (11, 21). This assay tested two adjacent SNPs (PorB 120/121), giving a WT result if there was a WT sequence in both positions or a SNP result if there was variation in at least one position. It is possible that only a specific amino acid change contributes to increased MICs to ESCs at this locus (22–24). In addition, previous studies have shown that *porB* mutations do not affect ESC susceptibility in the absence of the *mtrR* –35del SNP (11, 25). When *porB* mutations are calculated only in the presence of the *mtrR* –35del SNP, sensitivity decreases by $\sim 2\%$ and specificity increases by ~ 4 to 6%. As expected, *mtrR* had the highest specificity of the *ponA*, *mtrR*, and *porB* markers, as a mutation in the *mtrR* promoter sequence of the regulator of the MtrCDE efflux pump has been shown to contribute to an increase in ESC MICs in transformation experiments (11).

The *penA* assay had a high specificity for ceftriaxone at a MIC of ≤ 0.063 $\mu\text{g/ml}$, and at all three cefixime MIC breakpoints tested, implying that the mosaic allele is absent in the majority of low MIC isolates. The presence of the mosaic allele indicates the DS of an isolate. In fact, 98.9% of isolates with the mosaic *penA* allele exhibited ceftriaxone and cefixime MICs of ≥ 0.032 $\mu\text{g/ml}$, with only one isolate exhibiting an MIC of ≤ 0.032 $\mu\text{g/ml}$ for both antibiotics.

There are many limitations to using molecular detection techniques to predict antimicrobial resistance, especially in an organism such as *N. gonorrhoeae*. The mechanisms causing resistance are complex and multifactorial (25–27). *N. gonorrhoeae* is highly recombinogenic and naturally competent, allowing for transformation of DNA from other commensal species (28). Target sequences may cross-react with other similar species, thus causing decreased assay specificities. False-negative results could occur if Aptima specimens do not contain enough *N. gonorrhoeae* DNA template for SNP detection. In addition, a *porA* mutant *N. gonor-*

rhoeae strain that may result in a false-negative result due to sequence variations was recently discovered (29, 30). Care was taken to limit the selection bias of *N. gonorrhoeae* isolates for the validation of the assay by choosing isolates with a range of cephalosporin MICs, NG-MAST types, and temporal and geographic distributions; however, the Aptima specimens are not representative of the population, as they were collected from one region within a limited time frame.

The results of this study highlight the utility of a molecular method of surveillance of antimicrobial susceptibilities in the absence of *N. gonorrhoeae* culture isolates. Upon testing this assay with a wide range of gonococcal isolates, representing a variety of STs and ESC MICs, we found good agreement between the results generated from the SNP assay and the sequence data. In addition, we found *porA* to be a suitable *N. gonorrhoeae*-specific marker. Through detection of resistance determinants on the molecular level, this assay could be used to evaluate trends in the presence of molecular markers associated with ESC DS using the full range of specimens, both NAAT and culture.

ACKNOWLEDGMENTS

This work was supported by internal funds from the Public Health Agency of Canada.

We thank Gary Liu, Pam Sawatzky, and Anton Kowalski from the Streptococcus and STI Unit for their laboratory technical assistance.

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