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Molecular Assay for Detection of Ciprofloxacin Resistance in *Neisseria gonorrhoeae* Isolates from Cultures and Clinical Nucleic Acid Amplification Test Specimens

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We developed a real-time PCR assay to detect single nucleotide polymorphisms associated with ciprofloxacin resistance in specimens submitted for nucleic acid amplification testing (NAAT). All three single nucleotide polymorphism (SNP) targets produced high sensitivity and specificity values. The presence of ≥2 SNPs was sufficient to predict ciprofloxacin resistance in an organism.

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 (1). Strains of *N. gonorrhoeae* have acquired resistance to all of the antibiotics commonly used for treatment (2). In Canada, 27.9% of the *N. gonorrhoeae* isolates obtained from 2008 to 2012 were resistant to ciprofloxacin (3). Fluoroquinolone resistance in *N. gonorrhoeae* is conferred by mutations in subunit A of DNA gyrase (*gyrA*) and the ParC subunit of topoisomerase IV (*parC*) (4).

Beginning in the early 2000s, an increasing number of gonococcal infections have been detected by nucleic acid amplification tests (NAATs), and a decreasing number of laboratories across Canada are culturing *N. gonorrhoeae*. In fact, >70% of the gonococcal infections in Canada are now detected using NAATs, and antimicrobial susceptibility data are not available for these cases (3).

In this study, real-time PCR assays were developed to detect single nucleotide polymorphisms (SNPs) in genes associated with ciprofloxacin resistance. Three SNPs associated with ciprofloxacin resistance (*gyrA* S91, *gyrA* D95, and *parC* D86/S87/S88) were selected and evaluated using *N. gonorrhoeae* cultures. As a proof of principle, all SNPs were also evaluated using clinical specimens tested by the APTIMA Combo 2 assay on the Tigris platform (Hologic, Bedford, MA), which also had a matched culture isolate.

Two hundred fifty-two *N. gonorrhoeae* isolates, 58 clinical Hologic APTIMA Combo 2 CT/NG NAAT specimens (10 *N. gonorrhoeae*-positive APTIMA specimens, 24 *N. gonorrhoeae*-positive APTIMA specimens with corresponding culture isolates [from the same anatomical site, collected on the same day], and 24 *N. gonorrhoeae*-negative APTIMA specimens), and 50 different nongonococcal strains were tested to evaluate efficacy of the assay, as previously described (5). *N. gonorrhoeae* isolates were selected to represent a range of ciprofloxacin MICs and a diverse group of *N. gonorrhoeae* multiantigen sequence types (NG-MAST) and temporal and geographic distribution. The isolates also represent a range of cephalosporin MICs and included isolates with decreased susceptibility to ceftriaxone (MIC, ≥0.125 μg/ml; $n = 55$) and cefixime (MIC, ≥0.25 μg/ml; $n = 32$). The MICs were determined using the agar dilution method, as previously described (6). Sensitivities and specificities were calculated using the ciprofloxacin resistance breakpoint of 1 μg/ml, according to the CLSI guidelines (7).

Three SNP targets associated with ciprofloxacin resistance were chosen: *gyrA* S91, *gyrA* D95, and *parC* D86/S87/S88. Oligonucleotide primers and probes were chosen for each target region using Primer Express software version 3.0 (Life Technologies), with one probe to detect the wild-type (WT) allele and one positive-control probe that should produce a positive signal in all *N. gonorrhoeae* isolates. Both *gyrA* assays were performed using primers *gyrA_F1* (TACGCGATGCACGAGCTG) and *gyrA_R1* (AGTTGCCCTGTCCGTCTATCAG), along with the control probe *gyrA_CTRL* (VIC-CTGGAATGCCGCCTAC) and either the WT probe *gyrA_S91wt* (FAM-ACGGCGATTCCGCA) (FAM, 6-carboxyfluorescein) or *gyrA_D95wt* (FAM-CAGTTTACGACACCATCG). Assays of the *parC* gene were performed using the primers *parC_F1* (GCGCGATATGGGTTTGACG) and *parC_R1* (GGTAAAATCCTGAGCCATGCG) and the probes *parC_wt* (FAM-CGACAGTTCCGCCTAT) and *parC_CTRL* (VIC-CGTGGTCGGCGAGAT). DNA extraction, preparation, real-time PCR, and analysis of the results were performed as previously described (5). The results were considered positive if they had a quantification cycle (*Cq*) value of <40 (8) and a relative fluorescence of the probe

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TABLE 1 Sensitivity and specificity of SNP target assays tested with 252 *N. gonorrhoeae* cultures

SNP assessment[a]	Sensitivity (%) (no. of TP results)[b]	Specificity (%) (no. of TN results)[c]
SNP assay		
gyrA S91	100 (155)	93.8 (91)
gyrA D95	100 (155)	96.9 (94)
parC	96.1 (149)	99 (96)
No. of SNPs		
All 3	96.1 (149)	100 (97)
≥2 SNPs	100 (155)	97.9 (95)
≥1 SNP	100 (155)	91.8 (89)

[a] Susceptible strains, $n = 97$; ciprofloxacin-resistant strains, $n = 155$.
[b] TP, true positive (isolates contain the SNP and have high MIC values). Sensitivity = TP/(FN + TP) × 100.
[c] TN, true negative (isolates do not contain the SNP and have low MIC values). Specificity = TN/(FP + TN) × 100.

TABLE 3 Collection sites of NAAT specimens with no matched culture isolates

Collection site	No. with *N. gonorrhoeae* NAAT result:	
	Positive ($n = 10$)	Negative ($n = 24$)
Urine	5	13
Penis/urethra	1	1
Cervix	3	2
Vagina	1	0
Throat	0	4
Rectum	0	4

minus the baseline (ΔRn) value of >0.7. The limit of detection (LOD) for the PCRs was determined using serial dilutions of DNA from *N. gonorrhoeae* control strains, as previously described (5). SNPs detected by the assay were validated by comparison with aligned gene sequences obtained through whole-genome sequencing (9).

Of the 252 gonococcal isolates tested, 97 isolates (38.4%) were susceptible, with ciprofloxacin MICs of <1 μg/ml, while the other 155 (61.6%) were resistant to ciprofloxacin (MIC, ≥1 μg/ml). The assay concordance for each genetic marker (percentage of isolates called correctly as WT or SNP according to sequencing data) for the 252 *N. gonorrhoeae* isolates was 100% for *gyrA* S91 and *parC* D86/S87/S88 and 99.6% for *gyrA* D95. One isolate was positive for the *gyrA* D95 SNP using the real-time PCR assay, but sequencing identified a different SNP in the probe region, which caused the false-positive SNP assay result. The LODs were 5 pg/reaction for both *gyrA* SNPs and 5 fg/reaction for *parC*. The sensitivities (percentage of resistant isolates containing the SNP of interest) and specificities (percentage of sensitive isolates containing a WT allele) are shown in Table 1.

Of the 24 *N. gonorrhoeae*-positive APTIMA specimens with matched cultures, the assay concordance for each genetic marker was 100% for *parC* and 83.3% for both *gyrA* assays (Table 2). One rectal

TABLE 2 SNP assay results from APTIMA NAAT specimens compared with MICs from *N. gonorrhoeae* matched culture isolates ($n = 24$)

Sample no.	Source	Ciprofloxacin MIC of culture isolate (μg/ml)[a]	SNP assay results from APTIMA NAAT specimens: *gyrA* S91 Result[b]	*gyrA* S91 C_q value (WT/CTRL)[c]	*gyrA* S91 $\Delta Rn > 0.7$ (WT/CTRL)[d]	*gyrA* D95 Result[b]	*gyrA* D95 C_q value (WT/CTRL)[c]	*gyrA* D95 $\Delta Rn > 0.7$ (WT/CTRL)[d]	*parC* Result[b]	*parC* C_q value (WT/CTRL)[c]	*parC* $\Delta Rn > 0.7$ (WT/CTRL)[d]
37200A	Penis/urethra	**16**	**SNP**	40/39	N/Y	**SNP**	>45/39	N/Y	**SNP**	36/31	N/Y
37201A	Rectum	0.004	WT	30/31	Y/Y	WT	31/32	Y/Y	WT	26/26	Y/Y
37202A	Rectum	**8**	UND	>45/>45	N/N	UND	>45/>45	N/N	WT	34/34	Y/Y
37203A	Rectum	**8**	**SNP**	40/39	N/Y	**SNP**	45/38	N/Y	**SNP**	39/34	N/Y
37204A	Throat	0.004	UND	40/40	N/N	UND	41/42	N/N	WT	33/32	Y/Y
37205A	Urine	0.004	WT	31/32	Y/Y	WT	32/34	Y/Y	WT	26/26	Y/Y
37206A	Urine	0.004	WT	30/30	Y/Y	UND	>45/>45	N/N	WT	25/25	Y/Y
37207A	Rectum	0.004	WT	29/30	Y/Y	WT	33/35	Y/Y	WT	25/25	Y/Y
37208A	Throat	**16**	UND	42/40	N/N	**SNP**	38/39	N/Y	**SNP**	34/31	N/Y
37209A	Rectum	0.016	WT	37/37	Y/Y	**SNP**	36/38	N/Y	WT	32/32	Y/Y
37210A	Throat	**16**	**SNP**	40/39	N/Y	**SNP**	37/38	N/Y	**SNP**	39/34	N/Y
37211A	Urine	**16**	**SNP**	40/39	N/Y	**SNP**	37/39	N/Y	**SNP**	36/31	N/Y
37212A	Vagina	0.004	UND	>45/>45	N/N	WT	33/34	Y/Y	WT	30/30	Y/Y
37213A	Cervix	0.008	WT	37/37	Y/Y	WT	34/35	Y/Y	WT	27/27	Y/Y
37214A	Urine	0.004	WT	31/31	Y/Y	WT	33/35	Y/Y	WT	30/29	Y/Y
37215A	Urine	0.004	WT	28/29	Y/Y	WT	30/31	Y/Y	WT	24/23	Y/Y
37216A	Urine	0.008	WT	31/31	Y/Y	WT	33/33	Y/Y	WT	26/26	Y/Y
37217A	Urine	0.004	WT	30/31	Y/Y	WT	36/36	Y/Y	WT	25/25	Y/Y
37218A	Urine	0.016	WT	25/26	Y/Y	WT	28/29	Y/Y	WT	21/21	Y/Y
37219A	Urine	0.004	WT	37/36	Y/Y	**SNP**	>45/37	N/Y	WT	28/28	Y/Y
37220A	Urine	0.016	WT	29/30	Y/Y	WT	33/34	Y/Y	WT	23/23	Y/Y
37221A	Urine	0.008	WT	27/27	Y/Y	WT	28/28	Y/Y	WT	25/25	Y/Y
37222A	Urine	0.004	WT	28/29	Y/Y	WT	30/31	Y/Y	WT	28/28	Y/Y
37223A	Urine	0.004	WT	34/34	Y/Y	WT	35/36	Y/Y	WT	33/33	Y/Y

[a] Bold type indicates MIC of ≥1 μg/ml or the presence of an SNP.
[b] WT, wild type; UND, undetermined (no amplification or no detectable fluorescence after three real-time PCR [RT-PCR] attempts).
[c] WT, wild-type probe; CTRL, control probe.
[d] N = ΔRn value of <0.7. Y = ΔRn value of >0.7.

TABLE 4 Cross-reactivity of SNP assays in nongonococcal strains[a]

gyrA S91	*gyrA* D95	*parC*
N. lactamica(SNP)	*N. lactamica*(SNP)	*N. cinerea*(WT)
N. meningitidis(SNP)	*N. meningitidis*(SNP)	*N. elongata*(WT)
N. subflava(SNP)	*N. subflava*(SNP)	*N. flavescens*(WT)
N. polysaccharea(WT)	*N. polysaccharea*(SNP)	*N. lactamica*(WT)
		N. meningitidis(WT)
		N. mucosa(WT)
		N. perflava(WT)
		N. polysaccharea(WT)
		N. sicca(WT)
		N. perflava(WT)
		Pseudomonas aeruginosa(WT)
		Streptococcus infantis

[a] Results are listed as WT (wild type) or SNP. Isolates were tested in triplicate; only organisms (which belong to the *Neisseria* genus, unless otherwise specified) with positive results shown.

swab specimen gave a false SNP result for *gyrA* D95. The lower genetic marker performances for the *gyrA* APTIMA NAAT specimens were due to false-negative results, likely because of the higher LOD of the *gyrA* assays. Of the 24 *N. gonorrhoeae*-negative APTIMA specimens, two pharyngeal swabs showed cross-reactivity with the *parC* assay (WT result). The isolation sites for the APTIMA specimens are shown in Table 3.

Of the 50 nongonococcal isolates tested, 38 gave negative results for all three assays. Cross-reactive species are shown in Table 4. Cross-reactivity with similar species is expected when using highly conserved genes, such as *gyrA* and *parC*. This is a limitation when using DNA from a NAAT specimen, as false-positive results can occur in specimens of mixed culture, such as pharyngeal swabs, due to the possible presence of nongonococcal *Neisseria* species (10). However, none of the *N. gonorrhoeae*-negative APTIMA specimens gave false-positive results for *gyrA*, and only two showed a cross-reaction for *parC*, both of which were pharyngeal swabs. As only a limited number of *N. gonorrhoeae*-negative genital specimens were tested, additional testing needs to be performed to thoroughly assess the risk of cross-reactivity. Balashov et al. (11) observed that molecular assays may not be applicable to extragenital specimens due to the prevalence of non-*gonorrhoeae Neisseria* species in these specimens.

All ciprofloxacin-resistant isolates contained ≥2 SNPs, while 97.9% of the susceptible isolates had ≤1 SNP present. One hundred percent of the isolates containing all three SNPs were resistant to ciprofloxacin. Based on these values, the presence of any 2 of these SNPs is sufficient to determine if an isolate is resistant to ciprofloxacin. The high specificity and sensitivity of this assay make it useful for predicting ciprofloxacin resistance in a clinical sample. This is proof of principle that the detection of these SNPs could be useful in a test for targeted therapy with ciprofloxacin, as ~70% of Canadian *N. gonorrhoeae* isolates are currently susceptible to this antibiotic (3).

While the elevated prevalence of ciprofloxacin resistance prevents the effective use of fluoroquinolones for empiric gonorrhea treatment, it is important to monitor resistance prevalence, as resistant strains often have elevated MICs to other antibiotics, including third-generation cephalosporins (3). As the use of molecular methods for laboratory diagnosis of gonorrhea becomes increasingly widespread and cultures become less readily available, molecular methods for detecting antimicrobial resistance genes provide an alternative to culture-based antimicrobial susceptibility testing (12). Although the results of this study highlight the utility of this molecular method to determine ciprofloxacin resistance in the absence of *N. gonorrhoeae* culture isolates, caution should be advised, as cross-reactivity has been observed in other *Neisseria* species.

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