NOTES

Use of Applied Biosystems 7900HT Sequence Detection System and Taqman Assay for Detection of Quinolone-Resistant *Neisseria gonorrhoeae*

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Mutations in quinolone resistance-determining regions (QRDRs) have been associated with quinolone-resistant *Neisseria gonorrhoeae* (QRNG). Since diagnostic nucleic acid amplification tests for gonococci are now in frequent use, molecular detection of QRNG could facilitate surveillance in the absence of culture. Here we describe a real-time molecular assay for detecting QRDR mutations in gonococci.

Quinolone-resistant *Neisseria gonorrhoeae* (QRNG) strains are rapidly emerging. Well-characterized quinolone resistance-determining region (QRDR) mutations correlate with decreased gonococcal antimicrobial susceptibility to fluoroquinolones (MIC, ≥ 1 $\mu\text{g/ml}$) (2–6, 8–10, 13, 19, 21, 24, 26–28).

Using well-characterized isolates, we developed a method for detecting QRDR mutations utilizing Taqman chemistry and the ABI 7900HT Prism sequence detector (Applied Biosystems, Foster City, Calif.).

***N. gonorrhoeae* strains.** We evaluated 80 isolates collected in 2000 to 2001 in Israel that were characterized previously (9, 29).

DNA isolation, QRDR amplification, and direct sequencing. Genomic DNA was purified (Promega Wizard, Promega Corp., Madison, Wis.), and QRDRs were amplified (11, 12). The forward primers used were GyrA Forward (NG-GYRA-Z; 5'-CAAATTCGCCCTCGAAACCCT-3'; nucleotides [nt] 30 to 50 of the *gyrA* gene, 368-bp product) and ParC Forward (NG-PARC-Z; 5'-GCCCGTGCAGCGCGCAT-3'; nt 138 to 155 of the *parC* gene, 219-bp product). Reaction mixtures had a 50- μl total volume: 25 μl of PCR Master mix, 22 μl of sterile water, 1 μl each of forward and reverse primers, and 1 μl of DNA template. PCR products were sequenced with forward primers, and data were aligned with QRDR sequences for amino acids (aa) 91 to 95 of *gyrA* (GenBank accession no. U08817) and aa 86 to 92 of *parC* (GenBank accession no. U08907). The three mutation patterns identified are shown in Table 1.

ABI primer and probe design. Primers and probes (Table 2) were developed by using Primer Express 2.0 software (Applied

Biosystems, Foster City, Calif.). Probes encompassed aa 91 and 95 of *gyrA* and aa 86, 87, and 88 of *parC*, the loci most often associated with resistance (2–6, 8–10, 13, 19, 21, 24, 26–28). Using National Center for Biotechnology Information BLAST analysis, primers and probes were designed to match only gonococcal target sequences.

ABI real-time PCR. Diplex PCRs were performed in 96-well plates with the following per well: 25 μl of Promega PCR Master mix, 21 μl of sterile water, 0.2 μl of each set of forward and reverse primers (0.2 μM final concentration of each), 0.5 μl of each probe (0.2 μM final concentration of each), and 1 μl of DNA template, for a total reaction volume of 50 μl . Cycles included one 2-min hold (50°C); a 10-min denaturation (95°C); and 40 cycles of denaturation (95°C for 30s), annealing (60°C for 30s), and extension (72°C for 30s). Control wells included blanks, wild-type (WT) strains, strains with one mutation, and strains with multiple mutations, as determined by sequencing. The lower-level detection limit was established at five genome copies by using published methods (15).

TABLE 1. QRDR mutation patterns for gonococcal samples as determined by direct sequencing and real-time PCR detection with the ABI 7900HT sequence detection system

Sample type	QRDR mutation pattern ^a					
	<i>gyrA</i>			<i>parC</i>		
	aa 91 (Ser) ^b	aa 95 (Asp)	aa 86 (Asp)	aa 87 (Ser)	aa 88 (Ser)	aa 91 (Glu)
A (WT, Cip ^s)	WT	WT	WT	WT	WT	WT
B (mutant, Cip ⁱ)	WT	GAC→AAC (Asp→Asn)	WT	WT	WT	WT
C (mutant, Cip ^f)	TCC→TTC (Ser→Phe)	GAC→GGC (Asp→Gly)	GAC→AAC (Asp→Asn)	WT	WT	WT

^a WT denotes the WT sequence at targeted loci.

^b The WT amino acid is listed in parentheses.

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TABLE 2. Primers and probes for the ABI QRNG QRDR mutation detection system

QRDR primer, probe, or fluorophore	Name	Sequence
<i>gyrA</i>		
Primers		
Forward	GyraABI1	TTG-CGC-CAT-ACG-GAC-GAT
Reverse	GyraABI2	GCG-ACG-TCA-TCG-GTA-AAT-ACCA
Probe	GyrAWT91.95	TGT-CGT-AAA-CTG-CGG-AA
Fluorophore	6-FAM ^a	
<i>parC</i>		
Primers		
Forward	ParCABI1	TGA-GCC-ATG-CGC-ACC-AT
Reverse	ParCABI2	GCG-GAG-ATT-TTG-GGT-AAA-TAC-CA
Probe	ParCWT86.87.88	CGG-AAC-TGT-CGC-CGT
Fluorophore	TET ^b	

^a 6-FAM (6-carboxyfluorescein) emits light at 520 nm.

^b TET (tetrachloro-6-carboxyfluorescein) emits light at 539 nm.

Findings. All 42 ciprofloxacin-resistant (Cip^r) isolates had mutations in both *gyrA* and *parC* QRDRs, identical in 93%. One intermediately resistant (Cipⁱ) isolate had one mutation in *gyrA*. Susceptible strains (Cip^s) were WT (Table 1).

Fluorescence data were analyzed with ABI 7900HT Prism sequence detector software (Applied Biosystems, Foster City, Calif.) and are shown in Fig. 1. Ct is defined as the first PCR cycle in which parametric increases of fluorescence are detected and is an indicator of successful PCR, as well as specific annealing of probe and successful exonucleic cleavage of reporter molecule.

Real-time PCR, sequencing, and MICs correlated 100%.

The amplification plot for a WT strain (Table 1, type A; ciprofloxacin MICs, <0.125 µg/ml) shows exponential signal increase. This indicates WT strains were positively amplified, with mean Cts of 30.6 ± 3.21 cycles ($n = 38$) and 25.2 ± 2.11 cycles ($n = 38$) for the *gyrA* and *parC* loci, respectively.

For mutant strains (Table 1, types B and C), no exponential fluorescence increases were observed ($n = 42$). The Cipⁱ strain (Table 1, type B, ciprofloxacin MIC = 0.25 µg/ml) showed signal amplification for the *parC* locus only (Ct for *parC* = 23.2, Ct for *gyrA* = 40.0). Cip^r strains (Table 1, type C; ciprofloxacin MICs, ≥ 1 µg/ml) showed signal amplification at neither locus (Ct = 40.0). Curves were analogous when either one or two mutations were present.

Multicomponent data analysis was used to set baseline background levels (Fig. 1) and shows acceptable background and significant increases in fluorescence over time for the WT. Combined with relative fluorescence increase, this indicates a successful assay.

Conclusions. With widespread use of nucleic acid amplification tests (NAATs), antimicrobial resistance detection will require molecular methods, as has been described for other organisms (1, 7, 14, 16–18, 25). QRNG detection is a good model for this approach since the resistance mechanisms are based on stepwise accumulation of point mutations correlating with increased MICs (20, 22, 23).

This approach has disadvantages. Because mutations prevent reporter cleavage, negative results would give results similar to those for mutant strains. Thus, this screening tool can only be applied to samples which test gonococcus positive by other methods, such as commercially available NAATs. Our lower detection limit was 5 WT genome copies, similar to that of widely used NAATs. However, further investigations using different NAATs with various gonococcus-DNA concentra-

FIG. 1. QRDR amplification curves and multicomponent data plots from the ABI 7900HT sequence detection system. A, B, and C refer to sample types for which sequence data are given in Table 1. Each plot represents duplex results for one sample. Upper set of curves show relative fluorescence versus PCR cycle number. The lower set of curves depict the relative fluorescence of each fluorophore as compared to that of the background over time.

tions need to be performed. Another limitation is the possibility of detecting synonymous mutations, which translate as “false mutants,” although the frequency of synonymous mutations at these loci appears to be very low. Outlier mutations have been observed at loci not targeted by our probes (6, 19, 27, 28), but these occur infrequently and have only been observed in the presence of at least one mutation detectable with our probes. We intended to develop an assay to screen for clinically important mutations (i.e., those associated with a ciprofloxacin MIC of ≥ 4 $\mu\text{g/ml}$, requiring a change in therapy) and not for definitive genetic analysis. Real-time fluorometric PCR systems can, however, be adapted to screen for resistance-associated gonococcal QRDR mutations and can potentially be applied to NAAT samples.

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