A real-time PCR Assay for Detection of Quinolone-Resistant *Neisseria gonorrhoeae* in Urine Samples

Running title: Real-Time PCR Assay for QRNG

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ABSTRACT

A need exists for development of applicable surveillance tools to detect fluoroquinolone-resistant Neisseria gonorrhoeae (QRNG) from urine samples. We describe a real-time polymerase chain reaction (RT-PCR) assay for detecting mutations in the Ser91 codon of the gyrA gene of N. gonorrhoeae in urine specimens. We tested 96 urine samples collected along with Gonorrhea Isolate Surveillance Project (GISP) urethral swab samples and compared the results with matched minimum inhibitory concentrations (MIC) of ciprofloxacin, as reported by the regional GISP laboratory. We then tested 100 urine specimens, known gonorrhea-positive by nucleic acid amplification testing, provided by females to challenge the RT-PCR assay with urine specimens containing potentially lower target DNA content than specimens from symptomatic males. With an MIC threshold of 0.125µg/mL ciprofloxacin, our assay correctly identified resistance in 41/44 (93.2%, 95% CI 81.3 – 98.6%) corresponding resistant culture specimens, and correctly identified 51/51 (100%, 95% CI 93.0 – 100%) susceptible specimens. One specimen did not amplify. The assay successfully amplified the gyrA amplicon and determined a susceptibility genotype in 72/100 (72%) urine specimens collected from female patients. We developed an assay for detecting QRNG in urine specimens that correlated well with MIC results of cultured specimens and had moderate sensitivity with urine specimens. This methodology might fulfill the need for a QRNG detection system for urine specimens, a useful characteristic in the age of nucleic acid amplification testing for gonococcal infection.

Key Words: Neisseria gonorrhoeae, quinolone, resistance, real-time PCR
Neisseria gonorrhoeae (NG) urogenital infection is an important cause of morbidity, including pelvic inflammatory disease and infertility. Early detection and appropriate treatment of NG infection prevents a substantial proportion of this morbidity. Although fluoroquinolones have been used as an effective treatment of NG infection in the face of widespread penicillin resistance, a growing body of literature has documented the emergence and rapid increase in detecting fluoroquinolone-resistance in N. gonorrhoeae (QRNG) isolates in the United States, Asia, Western Europe, and Israel (2, 3, 5, 6, 12).

In the United States, less than 1% of isolates were QRNG in 1996 compared with more than 7.5% in 2004. However, there are considerable geographic differences in the United States, with cities in the western regions reporting rates of resistance prevalence of over 20% (10). In response to the worldwide dissemination of resistance, CDC has recently begun recommending that all local health clinics with the capacity to do so begin routine surveillance of fluoroquinolone resistance in gonorrhea isolates (1).

Although culture and susceptibility testing of NG isolates continues to be the gold-standard method for detection of antimicrobial resistance, decreases in the use of culture for diagnosis has precipitated the need for alternative methods to detect resistance. In response, methods for detecting QRNG have been developed and reported in the scientific literature including sequencing (16), microarray biochip (9, 17, 19), and other molecular techniques (13, 15). However, each of these techniques has disadvantages in regards to detecting resistance in certain settings. The sequencing and biochip methods might be untenable in locales with limited resources. Real-time polymerase chain reaction (RT-PCR) techniques using Taqman®-style probes (Applied Biosystems, Foster City, California) exclusively detect wild-type strains, with negative results interpreted as presumed resistant strains (13). The lack of internal control might therefore indicate resistance when in actuality, laboratory error or other causes of failed wild-type amplification (e.g. low concentrations of DNA) has caused this result. Alternatively, another RT-PCR assay for QRNG detection using fluorescence resonance energy transfer (FRET) probes and melting curve analysis addresses these problems (15).
However, that technique was not demonstrably effective for use with urine specimens in our laboratory. Hence, such assays might be insensitive to the lower concentrations of DNA in urine specimens. We sought to develop a modified version of the melting curve-based QRNG detection that might be applicable in settings with limited resources and with adequate sensitivity to survey for resistance in urine specimens.

We present a RT-PCR assay for analysis of mutations in the Ser91 region of the gyrA gene by amplification and melting curve analysis with gene-specific primers and probes. Over 99% of QRNG specimens have been shown to have mutations at this site of the gyrA gene (18, 19). This method allows for rapid detection of QRNG without complex laboratory techniques or extensive resources.

MATERIALS AND METHODS

Sample Collection.

We first selected 96 urine samples collected from male patients of the San Francisco City Clinic who had also provided specimens that were analyzed as part of the Gonorrhea Isolate Surveillance Project (GISP) during January 31–November 30, 2005. The goals and methodology of the GISP project have been described previously (2). Briefly, the protocol for the GISP study entails urethral swab specimen collection of the first 25–30 symptomatic patients with NG urethritis to arrive at the San Francisco City Clinic each calendar month. The swab specimens were isolated initially on Thayer-Martin agar plates and then subsequently were sub-cultured onto chocolate agar plates for 24 hours. Colonies were collected from plates and placed into Tryptic Soy Broth with 20% glycerol for freezer storage. Specimens were sent to the regional GISP laboratory in Seattle, Washington for culture and sensitivity testing.

We selected an additional 100 urine specimens from NG-infected female patients who were tested as part of routine clinical care. These samples were included in the study to assess the sensitivity of the assay to detect resistance in urine samples with lower levels of DNA content compared to urine samples from males with urethral discharge. All urine specimens tested positive for NG at the San
Francisco Department of Public Health Laboratory by transcription-mediated amplification (TMA) by using a TIGRIS® System (GenProbe, San Diego, California). Urine specimens were collected and stored in APTIMA Combo 2® (GenProbe, San Diego, California) buffer at -35°C. Selected specimens were collected on the same day as the urethral swab collection for GISP analysis. All specimens in the study were collected and tested as part of routine public health surveillance in accordance with San Francisco Department of Public Health guidelines. Because we studied preexisting diagnostic specimens, this study was not considered human subjects research in accordance with U.S. Department of Health and Human Services guidelines (4).

Real-Time PCR Analysis. For RT-PCR analysis of the gyrA gene, urine specimens (200 µL) were extracted through use of MagNA Pure LC® automated extraction techniques (Roche Diagnostics, Indianapolis, Indiana) by using a MagNAPure LC total nucleic acid isolation kit. Extracted samples (5 µL) were used in RT-PCR. Amplification and melting curve analysis of specimens was performed using a Roche LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN). Amplified regions of the gyrA gene were probed for mutations in the Ser 91 codon through use of melting curve analysis with probes specific to that region of the gyrA gene (Table 1). We originally tested ten urine specimens with the RT-PCR assay developed and published by Li et al. (15), but found crossing points to be suboptimal. The subsequent primers and probes (synthesized by SIGMA-Proligo, The Woodlands, TX) used in this study were selected from prior published reports of similar assays and confirmed by using online BLAST analysis from the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Maryland) (13, 15). The probes for the Ser91 amplicon (gyrA-ser-Flu, gyrA-ser-LC) are a paired set of FRET probes, including a LightCycler Red640 probe and a fluorescein-labeled probe, separated by one base pair. The Red640 oligonucleotide has a maximal absorption and emission of 622 nm and 638 nm respectively.
Reactions were prepared (per reaction) by using 2 µL LightCycler FastStart DNA Master HybProbe mix (Roche Diagnostics, Indianapolis, Indiana), 0.5 µmol (0.5 µL) each of forward and reverse primers, 0.2 µmol (0.2 µL) each of the fluorescein and Red 640 probes, 2.4 µL of 25 mmol MgCl₂, 9.2 µL H₂O, and 5 µL of extracted, purified specimen for a final volume of 20 µL. The LightCycler amplification protocol included the following steps: (1) denaturation at 95°C for 5 minutes; and (2) 45 cycles of (a) denaturation at 95°C for 5 seconds, (b) annealing at 52°C for 5 seconds, and (c) extension at 72°C for 10 seconds with a ramp rate of 20°C/second. Next, we performed melting curve analysis by (3) denaturing at 95°C for 0 seconds; (4) annealing probes at 40°C for 5 seconds; and 5) heating the mixtures to 80°C with a ramp rate of 0.1°C/second.

Melting curve analysis was performed by using LightCycler software, Version 4.0 (Roche Diagnostics, Indianapolis, Indiana). We plotted the negative value of the first derivative of fluorescence per unit time curves to distinguish peak melting temperatures from melting curve plots.

RESULTS
The 96 male urethral swab specimens tested had a range of MIC values from 0.001µg/mL to 32.0µg/mL (Fig. 1). The RT-PCR assay amplified product in 95/96 samples (99.0%). Of those that amplified, two distinct melting curve peaks were identifiable at approximately 56°C and 66°C, corresponding to mutant and wild-type strains (Fig. 2) respectively. Using matched cultured urethral specimens with an MIC threshold of ≥0.125µg/mL ciprofloxacin to differentiate between susceptible and resistant specimens, the assay accurately detected resistance in 41/44 specimens for a sensitivity of 93.2% (95%CI 81.3–98.6%) and it identified 51/51 quinolone-susceptible samples, for a specificity of 100% (95%CI 93.0–100%). The single susceptible specimen that was not correctly analyzed demonstrated no amplification by PCR and therefore was not considered as resistant or susceptible.
The assay successfully amplified the selected portion of the gyrA gene and discerned a quinolone susceptibility genotype in 72/100 (72%) urine specimens from female patients. The remaining 28 specimens did not amplify and therefore no genotype was determined.

We compared the RT-PCR assay described here with that published by Li et al. (15) on ten urine samples. All ten urine samples were amplified using our primers and nine of ten amplified using those described by Li et al. Our assay had an average crossing point differential of 3.44 cycles less than the Li assay (Table 2).

**DISCUSSION**

This novel assay might prove valuable in settings where rapid determination of quinolone resistance to *N. gonorrhoeae* specimens is desired. It is a faster method than that of culture and traditional agar-dilution or disk susceptibility testing and does not require expensive sequencing equipment. Furthermore, in the age of nucleic acid amplification testing for gonococcal and chlamydial infection, using urine as a specimen for drug-resistance genotyping is a valuable capability, precluding laborious culture and isolation methods and supplanting a requirement for urethral swab specimens. In summary, this novel assay provides a rapid and convenient method for detecting fluoroquinolone-resistance in urine samples, enabling laboratories to respond to CDC recommendations for increasing surveillance for QRNG (7).

The two primary regions in the NG genome associated with QRNG are the gyrA and parC genes (8). Others have documented that parC mutations in conjunction with gyrA mutations are associated with higher levels of resistance (MIC≥1.0), while lower levels are almost exclusively associated with gyrA mutations (15, 16). Furthermore, parC mutations in isolation do not appear to be associated with antimicrobial resistance (19). On the basis of these data, and reported estimates of clinically significant resistance for 500mg ciprofloxacin ranging between 0.125–1.0mg (14), we restricted our assay to detection of mutations in Ser91 region the gyrA gene. A recent study corroborating the necessary and sufficient role of Ser91 gyrA mutations in determining QRNG,
documented mutations in that region in 101 of 102 QRNG specimens (MIC ≥0.125mg/mL ciprofloxacin) (18). Though our assay may not differentiate between intermediate and high level resistance, our results indicate that it detects clinically significant resistance.

A previously reported RT-PCR technique implementing melting curve analysis for discrimination of quinolone-resistance genotype also restricted detection to gyrA (13). However, when we attempted to mimic that assay by using DNA-extracted urine samples, we were unsatisfied with the sensitivity of the assay on urine specimens. We hypothesized that the limited sensitivity might have been the result of a large 225bp amplicon size. In response, we restricted our assay to detection of one codon (Ser91), and shortened our amplification length to 133bp in an attempt to increase sensitivity in specimens with lower DNA concentrations. We accomplished this by using a primer already documented as effective in generating amplicons within gyrA (15). Pair-wise comparison of the modified assay described here with that described by Li et. al revealed an average sensitivity increase of 3.44 crossing point values when generating the smaller amplicon (Table 2).

Overall, our assay detected a susceptibility genotype in 72% of female urine specimens and 99% of symptomatic male urine specimens. The most likely source of decreased sensitivity of our RT-PCR assay on female urine samples was the use of the APTIMA®-TMA assay as a gold standard. Transcription mediated amplification assays have been reported to be much more sensitive than standard PCR techniques for detection of genetic content (11). Due to the single copy of gyrA in the NG genome, we are limited to the use of standard PCR for detection of the mutation. Low DNA concentration, DNA degradation, and PCR inhibition are other possible explanations for lack of amplification in the remaining specimens. Overall, the assay exhibited 85.2% (167/196) sensitivity to detect a susceptibility genotype in urine samples, a reasonable proportion for laboratory surveillance of QRNG. Although such a rate is unsuitable for clinical management, it might serve as an appropriate estimate for epidemiologic surveillance purposes when used on known positive specimens.
We document a sensitivity and specificity of 93.2% and 100.0% for detection of QRNG on the basis of correlated, cultured antimicrobial susceptibility testing. Considering the multiple mechanisms by which *Neisseria* attains resistance to quinolones, perfect correlation with culture and sensitivity techniques is not expected by using molecular methods. This assay is also limited by its inability to detect resistance to antibiotics other than fluoroquinolones. However, because of its applicability to urine specimens, this technique has advantages over the established, culture or sequencing-based methods of discrimination and may enable more widespread surveillance of QRNG.
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REFERENCES


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<th>Primers</th>
<th>Oligonucleotide Sequence</th>
<th>Position</th>
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<tr>
<td>NG-\textit{GYRASER91-F}*</td>
<td>5'-cgc-gat-gca-cga-gct-gaa-aaa-3'</td>
<td>174–194</td>
</tr>
<tr>
<td>NG-\textit{GYRASER91-R}$^\dagger$</td>
<td>5'-ttg-cgc-cat-acg-gac-gat-3'</td>
<td>289–306</td>
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<tr>
<td>Probes</td>
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* Primer and probes described previously (11)
† Primer described previously (13)
TABLE 2. Sensitivity analysis comparing crossing points for two real-time polymerase chain reaction assays for the detection of mutations in gyrA from urine specimens.

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Li et al.</th>
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<tr>
<td>1</td>
<td>29.62</td>
<td>27.09</td>
<td>2.53</td>
</tr>
<tr>
<td>2</td>
<td>32.17</td>
<td>28.01</td>
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<tr>
<td>3</td>
<td>30.86</td>
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<td>30.69</td>
<td>27.70</td>
<td>2.99</td>
</tr>
<tr>
<td>5</td>
<td>30.30</td>
<td>27.79</td>
<td>2.51</td>
</tr>
<tr>
<td>6</td>
<td>Undetectable</td>
<td>&gt;40.00*</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>&gt;40.00*</td>
<td>37.13</td>
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</tr>
<tr>
<td>8</td>
<td>&gt;40.00*</td>
<td>34.65</td>
<td>N/A</td>
</tr>
<tr>
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<tr>
<td>10</td>
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*A result of >40.00 corresponds to an apparent log amplification that occurred at more than 40 cycles.*
Fig. 1. Ciprofloxacin minimum inhibitory concentration (MIC) values and melting curve temperature for 96 *Neisseria gonorrhoeae* positive urine isolates.*

Of 96 specimens, 53 had a melting curve temperature of 66 °C, 42 had a melting curve temperature of 56 °C, and one did not amplify†.

DN: did not amplify

GyrA Mut: melting curve temperature of 56°C ±1°C, corresponding to a mutant genotype

GyrA WT: specimens with a melting curve temperature of 66°C ±1°C, corresponding to a wild-type genotype

‡ MIC values (mg/dL Ciprofloxacin) as determined by regional Gonorrhea Isolate Surveillance Project laboratory in Seattle, Washington

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‡ GyrA Mut: melting curve temperature of 56°C ±1°C, corresponding to a mutant genotype
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‡‡ MIC values (mg/dL Ciprofloxacin) as determined by regional Gonorrhea Isolate Surveillance Project laboratory in Seattle, Washington
Fig 2. Melting curve analysis from 14 urine specimens with known minimum inhibitory concentrations (MIC) for ciprofloxacin, including six resistant isolates, seven susceptible isolates, and one negative control.

Resistant isolates were those with an MIC $\geq 0.125$ µg/mL by culture and susceptibility testing.

Sensitive isolates were those with an MIC $< 0.125$ µg/mL by culture and susceptibility testing.