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Performance and Verification of a Real-Time PCR Assay Targeting the *gyrA* Gene for Prediction of Ciprofloxacin Resistance in *Neisseria gonorrhoeae*

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In the United States, 19.2% of *Neisseria gonorrhoeae* isolates are resistant to ciprofloxacin. We evaluated a real-time PCR assay to predict ciprofloxacin susceptibility using residual DNA from the Roche Cobas 4800 CT/NG assay. The results of the assay were 100% concordant with agar dilution susceptibility test results for 100 clinical isolates. Among 76 clinical urine and swab specimens positive for *N. gonorrhoeae* by the Cobas assay, 71% could be genotyped. The test took 1.5 h to perform, allowing the physician to receive results in time to make informed clinical decisions.

The sexually transmitted disease gonorrhea is the second most commonly reported notifiable disease in the United States, with 350,062 cases reported in 2014 (1). The etiologic agent of the disease, *Neisseria gonorrhoeae*, has become a major concern worldwide due to the high prevalence of resistance to antimicrobials that were at one time part of the standard treatment for the disease, including the sulfonamides, penicillins, narrow-spectrum cephalosporins, tetracyclines, macrolides, and fluoroquinolones (2). In the United States, multidrug-resistant *N. gonorrhoeae* is categorized as an urgent threat to public health by the Centers for Disease Control and Prevention (CDC) (3). As a result of emerging resistance, the CDC has issued several changes to the treatment guidelines for gonococcal infections, which included the removal of single-dose oral cephalosporins from the 2012 recommendations due to a significant decline in cefixime susceptibility among the gonococci (4). The current CDC treatment guideline now recommends the use of two drugs: a single intramuscular injection of ceftriaxone followed by a single dose of azithromycin administered orally (5).

In the United States, the prevalence of fluoroquinolone-resistant gonococci has declined from its peak in 2007, when the CDC ceased recommending ciprofloxacin as empirical treatment for gonococcal infections (6). For example, the prevalence of ciprofloxacin-resistant *N. gonorrhoeae* isolates in San Francisco decreased dramatically from 44% in 2006 to 9.6% in 2009 (7), which coincided with these changes to national treatment guidelines. Currently, the proportion of U.S. isolates resistant to ciprofloxacin (MIC, ≥1.0 μg/ml) is at 19.2% (CDC, unpublished data), suggesting that ciprofloxacin may still be a viable option in some cases for treatment of infections, the majority of which are caused by ciprofloxacin-susceptible isolates. However, as few laboratories routinely perform culture and susceptibility testing for *N. gonorrhoeae*, a molecular assay is needed to differentiate patients infected with a susceptible strain from those infected with a resistant strain. Resistance to ciprofloxacin is conferred by mutation in the Ser91 codon of the *gyrA* gene in >99% of all resistant isolates investigated to date, making this mutation an attractive target for prediction of ciprofloxacin susceptibility (8). In this study, we evaluated a real-time (RT)-PCR assay of the *gyrA* gene that was coupled with high-resolution melt analysis to differentiate wild-type and mutant *gyrA* sequences at the Ser91 codon (8).

The *gyrA* RT-PCR used in this study applies fluorescence resonance energy transfer (FRET) probes that target the *gyrA* gene in the region coding for amino acid 91 and was performed as previously described (8). Data were analyzed using the Melt Curve Genotyping Module of LightCycler 480 software version 1.5.0 SP3. Melt temperatures were designated either wild type (Ser91) or mutant in the assay's target region.

The accuracy of the *gyrA* RT-PCR in differentiating ciprofloxacin-susceptible isolates from ciprofloxacin-resistant isolates was evaluated against 100 clinical isolates of *N. gonorrhoeae* that were obtained from the *Neisseria* Reference Laboratory (GISP Regional Laboratory, University of Washington Harborview Medical Center, Seattle, WA, USA). These isolates were recovered between 2011 and 2014 from cultures performed using pharyngeal, rectal, urethral, cervical, and urine specimens collected from men with gonococcal infection. Ciprofloxacin MICs were determined for these isolates by agar dilution, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (9). Twenty-three isolates were ciprofloxacin susceptible (MIC, ≤0.012 μg/ml) and 77 ciprofloxacin resistant (MIC range, 1 to >16 μg/ml). A 0.5 McFarland suspension of each isolate was prepared in normal saline solution, and DNA was extracted using a Magna Pure LC 2.0 instrument (Roche Diagnostics, Indianapolis, IN) and a Magna Pure DNA large-volume kit; this DNA was tested by the use of the *gyrA* RT-PCR. The *gyrA* RT-PCR assay classified all 23 ciprofloxacin-susceptible isolates as having a wild-type *gyrA* genotype and all

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TABLE 1 *N. gonorrhoeae*-positive clinical specimens used in this study

N. gonorrhoeae-positive clinical specimen type	No. (%) of specimens with indicated *gyrA* genotype by PCR		
	Wild-type	Mutant	Indeterminate[a]
Urine ($n = 57$)	31 (54.4)	9 (15.8)	17 (29.8)
Swab ($n = 18$)[b]	9 (50.0)	4 (22.2)	5 (27.8)
ThinPrep ($n = 1$)	0 (0.0)	1 (100.0)	0 (0.0)

[a] Indeterminate specimens produced *gyrA* amplicon signal that could not be genotyped as either wild type or mutant.
[b] Data include rectal ($n = 14$), urethral ($n = 2$), cervical ($n = 1$), and vaginal ($n = 1$) swab results.

77 ciprofloxacin-resistant isolates as Ser91 *gyrA* mutants. These results indicated that the assay was 100% accurate for prediction of both ciprofloxacin susceptibility and ciprofloxacin resistance for this collection of contemporary isolates. The specificity of the RT-PCR for the *N. gonorrhoeae gyrA* gene was evaluated *in silico* by BLAST (10) analysis of the primer and probe sequences against the NCBI nucleic acid collection, as well as by testing 6 nongonococcal *Neisseria* species (*Neisseria meningitidis*, *Neisseria sicca*, *Neisseria subflava*, *Neisseria mucosa*, *Neisseria cinerea*, and *Neisseria elongata*) acquired from the UCLA clinical isolate collection. None of the nongonococcal *Neisseria* species demonstrated a fluorescence signal that could be evaluated during the melt phase of the PCR. This parallels the results of the *in silico* analysis, which found homology between the *gyrA* primers and only two *gyrA* genes (those of *N. gonorrhoeae* and *N. meningitidis*), but homology between the resultant amplicon and probe sequences used in the assay was found only for the *N. gonorrhoeae* amplicon.

To evaluate the clinical sensitivity of the *gyrA* RT-PCR, 76 clinical specimens that tested positive for *N. gonorrhoeae* by the Cobas 4800 CT/NG test (Roche Diagnostics) were evaluated. These included 57 urine specimens, 14 rectal swabs, 2 urethral swabs, 1 cervical swab, 1 vaginal swab, and 1 cervical scraping submitted in liquid cytology media (ThinPrep PreservCyt solution). In addition, 12 ciprofloxacin-susceptible and 12 ciprofloxacin-resistant *N. gonorrhoeae* isolates were serially diluted 1:10 in remnant, patient-deidentified urine specimens that had previously tested negative for *N. gonorrhoeae*, to cover the concentration range of 10 to 10^5 CFU/ml. These specimens, seeded ($n = 24$) and clinical ($n = 76$), were processed for the Cobas 4800 CT/NG assay (Roche Diagnostics), and residual DNA from the specimen-processing deep-well plates was collected with care to avoid magnetic particles. This DNA was then used for the *gyrA* RT-PCR assay. Among the clinical specimens ($n = 76$), 54 (71%) were successfully genotyped by the *gyrA* assay (40 as wild type and 14 as mutant), while the rest failed to produce *gyrA* gene amplification signal (Table 1). From these data, 74.1% overall ciprofloxacin susceptibility was found among the gonococci infecting patients at our institution, slightly below the 80.8% reported by the CDC (unpublished CDC data).

In order to evaluate the sensitivity of the *gyrA* RT-PCR assay compared to that of the Cobas 4800 CT/NG assay, we compared amplification by the *gyrA* RT-PCR to the Cobas 4800 CT/NG assay crossing point (Cp) for all 100 specimens described above. Probit analysis was performed using the Probit module on XLSTAT (Addinsoft, New York, NY) on data from all 100 specimens tested (seeded and clinical), which demonstrated that the Cobas 4800 CT/NT assay crossing point of ≤28.15 was associated with a ≥95% detection rate by the *gyrA* RT-PCR. To estimate the *gyrA* RT-PCR positivity rate among clinical specimens that tested positive for *N. gonorrhoeae* with a Cp below 28.15, we collected crossing-point data from all specimens that tested positive for *N. gonorrhoeae* in the Cobas 4800 CT/NG assay during August to October 2015 in our laboratory. Among 83 *N. gonorrhoeae*-positive urine and swab specimens, 61% had crossing points of ≤28, which suggested that the assay should be able to genotype the majority of specimens. For the 39% of specimens that would be predicted to be below the limit of detection for the *gyrA* RT-PCR, physicians can continue to use CDC-recommended empirical treatments. However, for the remaining patients, administration of ciprofloxacin may be considered, provided the isolate has a wild-type *gyrA* genotype.

Clinical specificity of the *gyrA* RT-PCR was evaluated by testing 116 specimens that were negative for *N. gonorrhoeae* in the Cobas 4800 CT/NG assay. These included 98 urine specimens, 15 rectal swab specimens, 2 vaginal swab specimens, and 1 urethral swab specimen. None of these yielded amplification by the *gyrA* RT-PCR, indicating 100% specificity of the *gyrA* assay for *N. gonorrhoeae* in these specimens.

Nucleic acid amplification tests (NAATs) are the standard of care for the diagnosis of *N. gonorrhoeae* infection and are recommended by the CDC as the first-line test to evaluate patients with suspected *N. gonorrhoeae* infection (5), including testing for pharyngeal and rectal infections. However, because clinical laboratories no longer routinely perform culture, there is a lack of available isolates upon which to perform phenotypic antimicrobial susceptibility tests (11). While the CDC continues to recommend performing culture on specimens from patients with suspected treatment failure (5), this recommendation allows only retrospective recognition of antimicrobial resistance, in contrast to prospective testing, which would allow directed therapy at the time of initial diagnosis. Prospective, molecular testing of clinical specimens positive for *N. gonorrhoeae* for ciprofloxacin susceptibility will be useful to inform treatment decisions for asymptomatic patients, as well as for those patients with symptomatic disease who were not treated until test results became available (12, 13). A major limitation of the assay presented here was its sensitivity, which was lower than that of the Cobas *N. gonorrhoeae* screening test, ranging from 61% (predicted from Probit analysis) to 71% (observed value). Asymptomatic patients have a lower bacterial load than symptomatic patients, which may further reduce the sensitivity of the *gyrA* RT-PCR. However, data on file at Roche from the VENUS trial (Vaginal, Endocervical and Urine Screening Trial for CT/NG; Michael Lewinski, personal communication to R. M. Humphries) demonstrated the *N. gonorrhoeae* crossing points observed in the Cobas 4800 CT/NG Roche assay were in the same range for asymptomatic patients ($n = 7$ patients tested, Cp range = 20.9 to 29.7) and symptomatic patients ($n = 60$ patients tested, Cp range = 19.9 to 31.4). While these numbers are low, they suggest that the assay described here should have similar clinical sensitivities for symptomatic and asymptomatic patients. Reflex testing of *N. gonorrhoeae*-positive specimens by the use of the *gyrA* RT-PCR in our hands took an additional 1.5 h to perform following the Cobas assay, a minimal increase in overall turnaround time, considering that most laboratories batch test *N. gonorrhoeae* screening NAATs.

While the antimicrobial resistance mechanisms among *N. gonorrhoeae* are complex and often multifactorial, recent studies have

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delineated key molecular targets that could be used to predict antimicrobial susceptibility of *N. gonorrhoeae* directly from clinical specimens (11, 14). In our current study, we performed a complete performance evaluation of a previously published real-time PCR assay (8), including evaluation of 100 contemporary isolates of *N. gonorrhoeae* and 76 clinical specimens positive for *N. gonorrhoeae*. Our results indicated a 100% correlation between genotypes and *in vitro* susceptibility to ciprofloxacin for all characterized clinical isolates tested—reflecting previously documented data from 2007, when 51/51 (100%) ciprofloxacin-susceptible *N. gonorrhoeae* isolates were appropriately genotyped as wild type by this assay (8). That previous study found that the *gyrA* RT-PCR misclassified 3 of 44 ciprofloxacin-susceptible isolates as resistant, whereas we did not note any such discrepancies in our evaluation (i.e., we found complete concordance between actual and predicted ciprofloxacin resistance and susceptibility data). Importantly, the data presented here demonstrate that, although other mutations have been associated with fluoroquinolone resistance (i.e., Asp95 in *gyrA*, Ser88 or Glu91 in *parC*) (2), evaluation of the Ser91 target in *gyrA* was as sufficient to confirm ciprofloxacin susceptibility among *N. gonorrhoeae* isolates recovered in 2011 to 2014 as it was to confirm ciprofloxacin susceptibility among isolates recovered in 2005. The assessment of the suitability of Ser91 *gyrA* mutation as a marker of ciprofloxacin resistance is also supported by studies performed in Canada (15), Brazil (16), South Africa (17), and Switzerland (18), which demonstrated that the vast majority of ciprofloxacin-resistant isolates contained the Ser91 mutation. In an independent study, Magooa and colleagues evaluated both *gyrA* and *parC* as targets to predict ciprofloxacin susceptibility among a collection of 15 ciprofloxacin-susceptible and 17 ciprofloxacin-resistant isolates of *N. gonorrhoeae* (17). The *gyrA* target was 100% sensitive and specific for ciprofloxacin susceptibility, whereas *parC* was only 95.5% sensitive and 86.1% specific for ciprofloxacin susceptibility. In addition to the prediction of ciprofloxacin susceptibility, our real-time PCR method could potentially be adapted to detect other mutations that may confer antimicrobial resistance. A recent study reported the presence of mosaic *penA* allele XXXIV in all *N. gonorrhoeae* isolates from major population centers in California with elevated MICs to ceftriaxone or cefixime (19), suggesting that this mosaic allele could serve as an appropriate target for prediction of increased extended-spectrum cephalosporin MICs in our local population.

We found that the *gyrA* RT-PCR fit well into our current molecular diagnostic workflow; in particular, the use of remnant DNA extracted from the Cobas 4800 system allows a shorter turnaround time for producing *gyrA* genotype results—an approach demonstrated by others, in a recent report (20). We have adopted this test for routine clinical use in our laboratory and perform the *gyrA* RT-PCR daily, on the evening shift, following completion of the Roche Cobas 4800 CT/NG runs. *N. gonorrhoeae* isolates detected by the Cobas assay are reported as preliminary results, and *gyrA* genotype data are added, 2 to 3 h later, as a final result. For those laboratories that use non-PCR-based *N. gonorrhoeae* screening test platforms, such as the Aptima CT/NG, a DNA extraction step must be added (21) prior to performing RT-PCR on the LightCycler 480 instrument, adding time to the overall process. In conclusion, our study demonstrated successful incorporation of the *gyrA* melt genotyping RT-PCR assay into a molecular diagnostics workflow of our clinical laboratory. Prospective testing to predict antimicrobial susceptibility enables physicians to use antimicrobial agents other than those recommended for empirical treatment by the CDC and could potentially delay further emergence of resistance to ceftriaxone, offer effective options for patients with drug allergies, and improve patient satisfaction (i.e., resulting from treatment with an oral medication rather than by intramuscular injection) (12). We will prospectively evaluate the effect of susceptibility reporting on prescribing behavior of physicians and local antimicrobial resistance trends at our institution. In addition, the value of this test will be assessed in a clinical trial, for asymptomatic patients who test positive for *N. gonorrhoeae* by NAAT.

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