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Real-Time PCR Targeting the *penA* Mosaic XXXIV Type for Prediction of Extended-Spectrum-Cephalosporin Susceptibility in Clinical *Neisseria gonorrhoeae* Isolates

L. K. Wong,[a] P. Hemarajata,[b] O. O. Soge,[c] R. M. Humphries,[b] J. D. Klausner[d]

Emory University School of Medicine, Atlanta, Georgia, USA[a]; Department of Pathology and Laboratory Medicine, David Geffen School of Medicine, UCLA, Los Angeles, California, USA[b]; *Neisseria* Reference Laboratory, University of Washington Harborview Medical Center, Seattle, Washington, USA[c]; Division of Infectious Diseases, Center for World Health and Department of Epidemiology, David Geffen School of Medicine and Fielding School of Public Health, UCLA, Los Angeles, California, USA[d]

ABSTRACT *Neisseria gonorrhoeae* isolates with decreased susceptibility to extended-spectrum cephalosporins (ESCs) are increasing. We developed an assay to predict *N. gonorrhoeae* susceptibility to ESCs by targeting *penA* mosaic XXXIV, an allele prevalent among U.S. isolates with elevated ESC MICs. The assay was 97% sensitive and 100% specific for predicting at least one ESC MIC above the CDC alert value among clinical isolates, and it could be multiplexed with a previously validated *gyrA* PCR to predict ciprofloxacin susceptibility.

KEYWORDS *Neisseria gonorrhoeae*, gonorrhea, extended-spectrum cephalosporins, antimicrobial resistance, ceftriaxone, antibiotic resistance, cephalosporin, molecular methods

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Address correspondence to R. M. Humphries, rhumphries@mednet.ucla.edu.

Gonorrhea is the second most commonly reported infectious disease in the United States, and an estimated 78 million new annual cases of gonorrhea occur globally (1–3). Effective treatment is critical to both infection control efforts and the prevention of long-term consequences of infection (4). *Neisseria gonorrhoeae* demonstrates a remarkable ability to acquire resistance to antibiotics (5). *N. gonorrhoeae* resistance to penicillins, sulfonamides, tetracyclines, and/or fluoroquinolones (6, 7) is widespread, limiting the use of these antibiotics as empirical treatment options. The U.S. Centers for Disease Control and Prevention (CDC) recommends dual therapy with ceftriaxone (single dose of 250 mg) and azithromycin (single dose of 1 g orally) for the treatment of uncomplicated gonococcal infections (1). Although treatment with ceftriaxone is safe and effective at all anatomic sites, with cure rates of >98% in clinical trials (8, 9), *N. gonorrhoeae* isolates with ESC MICs in the alert ranges defined by the CDC (cefpodoxime [CPD] MIC, ≥0.25 μg/ml; cefixime [CFM] MIC, ≥0.25 μg/ml; or ceftriaxone [CRO] MIC, ≥0.125 μg/ml) are being reported with increasing frequency (7, 10–14). These alert values are lower than the nonsusceptible breakpoints set forth by Clinical and Laboratory Standards Institute (CLSI) (CPD, >0.25 μg/ml; CFM, >0.25 μg/ml and CRO, >0.25 μg/ml) and are used by the CDC as criteria for the surveillance of decreased susceptibility to ESCs. Antimicrobial susceptibility testing (AST) is needed in order to evaluate the trend of ESC MICs and track isolates with decreased susceptibility to cephalosporins. Since nucleic acid amplification tests (NAATs) have become the standard of care for the detection of gonococcal infections (15), most clinical laboratories no longer routinely perform culture and susceptibility testing for *N. gonorrhoeae*. As such, there is a need for molecular AST assays that can predict *N. gonorrhoeae*

susceptibility to antimicrobials commonly used to treat gonococcal infections directly from clinical specimens. We recently developed a real-time PCR with fluorescence resonance energy transfer (FRET) hybridization probes to predict susceptibility for ciprofloxacin by targeting the *gyrA* Ser91 mutation, with 100% correlation between genotype and *in vitro* susceptibility to ciprofloxacin for *N. gonorrhoeae* (16, 17). In this study, we sought to develop an assay to predict decreased susceptibility to ESCs in *N. gonorrhoeae*, to be performed as a multiplex test with *gyrA* to allow for simultaneous prediction of susceptibility to both ESCs and ciprofloxacin.

The primary mechanism of *N. gonorrhoeae* resistance to ESCs is alteration of the *penA* gene, which encodes the penicillin-binding protein 2 (PBP2), resulting in reduced affinity for ESCs (18). Those *penA* "mosaic" alleles likely arose from homologous recombination with other saprophytic *Neisseria* species intrinsically resistant to ESCs (19). A study of 1,100 clinical *N. gonorrhoeae* isolates demonstrated that reduced ESC susceptibility is predominantly clonal and associated with the *penA* mosaic XXXIV allele, with a sensitivity of 98% for cefixime and 91% for ceftriaxone, as a result of the international spread of some multidrug-resistant strains (20). Similarly, data from a genomic epidemiology study of *N. gonorrhoeae* in the United States demonstrated that 114 of 118 *N. gonorrhoeae* isolates with reduced susceptibility to cefixime (98%) originated from two distinct lineages that had independently acquired the *penA* mosaic XXXIV allele (21). As a key determinant of susceptibility and resistance to ESCs, the *penA* gene is of particular interest for the development of rapid molecular assays (5, 22).

In order to determine the presence of *penA* mosaic XXXIV and other mosaic types with homologous sequences in the target region, which include X, XXVII, XXXVII, XLII, LI, LII, LIII, LIV, LV, and LVIII (determined *in silico* by performing BLAST [23] against a sequence database of *penA* mosaic patterns, courtesy of Magnus Unemo), some of which have been associated with reduced susceptibility to ESCs (24, 25), we designed a novel real-time PCR based on an existing TaqMan PCR assay (26). Modifications in primer and probe sequences were made to convert the assay to FRET probe format due to the incompatibility of the TaqMan assay with the previously validated FRET probe-based *gyrA* genotyping assay (16) (see Table S1 in the supplemental material). The fluorescent label on the detection probe for the *penA* mosaic XXXIV target (Cy5) was different from that of *gyrA* (LightCycler 640) to allow for simultaneous detection of both genes. The multiplexed PCR was run on the LightCycler 480 (Roche Diagnostics), with the cycle settings described elsewhere (27), with a modification of detection wavelengths to include Cy5. Following amplification, melt curve analysis was performed using the melt curve genotyping module of the LightCycler 480 software (version 1.5.0 SP3). Genotyping analysis of *gyrA* was performed using primers and probes as described elsewhere (27). For the *penA* target, DNA used for the melting standard curve was extracted from a clinical *N. gonorrhoeae* isolate (FQ074) with an alert cefixime MIC value (0.25 μg/ml), in which the presence of the *penA* mosaic XXXIV type had been previously confirmed by Sanger sequencing. Melt curves identical to that of a known sample with the *penA* mosaic XXXIV mutation indicated the presence of the mosaic XXXIV. The *gyrA* served as an internal control for the multiplex PCR. As such, the *penA* mosaic XXXIV PCR result would only be considered if *gyrA* in the isolate could be genotyped as the wild type or mutant.

The accuracy of the multiplex *gyrA-penA* real-time PCR was evaluated against 150 clinical isolates with known MICs to CFM and CRO, 50 of which were obtained from the CDC Antimicrobial Resistance (AR) Collection (Centers for Disease Control and Prevention, Atlanta, GA), and 100 isolates were obtained from the *Neisseria* Reference Laboratory in Seattle (University of Washington Harborview Medical Center, Seattle, WA, USA). CPD MICs were also available for the CDC isolates. The isolates from the *Neisseria* Reference Laboratory were recovered between 2011 and 2014 from cultures obtained from urine and pharyngeal, rectal, urethral, and cervical specimens of gonococcal infections, and they were previously used to evaluate the singleplex *gyrA* assay (16). Thirty-two isolates were ciprofloxacin susceptible (MIC, $\leq$ 0.012 μg/ml), and 118 isolates were ciprofloxacin resistant (MIC range, 1 to $>$16 μg/ml) based on CLSI

breakpoints (28). Sixty-eight isolates (45%) had at least one ESC MIC equal to or above the CDC alert value, 29 of which had CRO MICs equal to or above the alert values. The isolates were grown on chocolate agar plates for 24 h at 35°C in 5% CO_2-enriched atmosphere prior to use in testing. A 0.5 McFarland suspension of each isolate was prepared in normal saline, and DNA was extracted using a MagNA Pure LC 2.0 (Roche Diagnostics, Indianapolis, IN). Extracted DNAs were used as the templates for the multiplex *gyrA-penA* PCR.

The *gyrA* component of the assay classified all 32 ciprofloxacin-susceptible isolates as having a wild-type *gyrA* genotype and all 118 ciprofloxacin-resistant isolates as Ser91Tyr *gyrA* mutants, indicating that the genotyping of *gyrA* in our multiplex PCR remained 100% accurate for the prediction of both ciprofloxacin susceptibility and resistance among these isolates when multiplexed with *penA* detection. The *penA* mosaic XXXIV analysis demonstrated positive results, defined by amplification of the *penA* mosaic XXXIV target, with melt patterns matching the mosaic XXXIV melt standard (FQ074), in 66/68 (97%) isolates with at least one ESC MIC equal to or above the alert value, while all 82 (100%) isolates with ESC MICs under the alert values were negative for mosaic XXXIV (no amplification). One of the two false-negative isolates (CDC AR Bank number 0194) had all ESC MICs equal to or above the alert value (CPD = 2 μg/ml, CFM = 1 μg/ml, CRO = 0.5 μg/ml), all of which fell in the nonsusceptible range according to CLSI breakpoints described above, but it was negative for mosaic XXXIV by our PCR. This isolate harbored mosaic IX according to CDC whole-genome sequencing (WGS) data. The other false-negative isolate (FQ009) was obtained from UW and had CRO MICs that were equal to or above the alert value range (0.25 μg/ml) but was still considered susceptible by the CLSI breakpoints. WGS of this isolate was performed on an Illumina MiSeq with a 2 $\times$ 300-bp version 3 sequencing protocol. Sequence data (submitted under NCBI accession number NGQH00000000) were assembled using SPAdes version 3.5.0 (29) on a local BaseSpace OnSite server (Illumina, Inc., San Diego, CA), annotated using PATRIC (30, 31), and visualized in Geneious version 10.1.3 (Biomatters, Auckland, New Zealand). WGS data demonstrated the presence of mosaic XII in this isolate. Both the IX and XII mosaic types cannot be detected due to 12 mismatches in DNA sequence targeted by the *penA* mosaic XXXIV assay.

To determine the limit of detection of the assay, we utilized contrived samples seeded with various concentrations of an *N. gonorrhoeae* isolate (FQ074) known to harbor mosaic XXXIV. Isolate FQ074 was 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 (n = 23). These specimens were processed for use in the Cobas 4800 CT/NG assay (Roche Diagnostics), and residual DNA was used for the *gyrA-penA* multiplex assay. Probit analysis determined bacterial concentrations associated with a 95% detection rate on *gyrA* and *penA* mosaic XXXIV targets to be 1.1 $\times 10^6$ and 6.44 $\times$ 10^4 CFU/ml, respectively. To determine analytical specificity, we evaluated the assay using deidentified remnant DNA samples positive for *N. gonorrhoeae* on the Cobas NG/CT assay. Among 50 samples for which *gyrA* genotypes were determined on the multiplex assay, only one sample demonstrated amplification on the XXXIV mosaic target. However, the melt curve did not match that of the XXXIV standard (FQ074). Subsequent Sanger sequencing using primers described in Table S1 demonstrated that the target region for *penA* assay in this isolate was homologous to that of wild-type *penA*, suggesting that the signal detected was not due to the amplification of mosaic XXXIV target.

Although NAATs are considered the standard of care for patients with suspected *N. gonorrhoeae* infection (15), these culture-independent methods do not allow for subsequent conventional AST to be performed due to the unavailability of pure bacterial isolates. With the increase in the prevalence of *N. gonorrhoeae* isolates in the United States with non-wild-type azithromycin MICs ($\geq$2 μg/ml, as defined by CLSI epidemiological cutoff value [28]) from 0.6% in 2013 to 2.6% in 2015 (1) and several recent reports of azithromycin treatment failures around the world (15), ESCs are becoming

the only remaining class of antimicrobials still effective for the empirical treatment of *N. gonorrhoeae* infection. As the development of resistance against ESCs becomes an imminent threat, there is a great need for molecular tests that can accurately predict susceptibility to these agents. A rapid molecular screening test would provide information that would allow for real-time prospective monitoring of samples with decreased MICs to ESCs. Our mosaic XXXIV assay demonstrated high sensitivity (97%) and specificity (100%) for predicting whether ESC MICs were equal to or above the CDC alert values, in a panel of clinical isolates, while still maintaining the accuracy of prediction of ciprofloxacin susceptibility among clinical isolates tested when multiplexed with *gyrA* real-time PCR assay. The CDC alert values are lower than the CLSI breakpoints for susceptibility. As such, our assay may be useful for the early identification of decreased susceptibility in a population before isolates become nonsusceptible based on the CLSI breakpoint. The results from seeding recovery experiments suggested that the assay could be used to determine the presence of *N. gonorrhoeae* harboring the mosaic XXXIV allele directly from clinical specimens. Since the mosaic XXXIV allele was suggested to be a product of interspecies recombination between *N. gonorrhoeae* and saprophytic *Neisseria* species (32), the presence of such *Neisseria* species in specimens collected from mucosal surfaces could potentially have a negative effect on the specificity of the assay. However, *in silico* analysis using a BLAST search (23) against the NCBI nonredundant nucleotide database demonstrated the specificity of the mosaic XXXIV primer and probe combination for *N. gonorrhoeae*. The region targeted by FRET probes in non-*gonorrhoeae Neisseria* species differed from that in *N. gonorrhoeae* by at least one nucleotide, a variation detectable by the FRET hybridization probe technology (33), as seen through melt patterns different from a perfect match. Further evaluation using clinical specimens from various anatomical sites may be needed in order to establish the analytical specificity of the assay when performed directly on clinical specimens.

A major limitation of our assay arose from the high specificity for *penA* mosaic XXXIV. First, since the *penA* real-time PCR presented herein was designed to specifically detect the mosaic XXXIV type, the sensitivity of the assay would depend on the prevalence of *penA* mosaic XXXIV among isolates with MIC values in the alert range in the geographical areas in which the assay would be performed. In a recent genotypic surveillance study of clinical *N. gonorrhoeae* isolates in California, alert value extended-spectrum cephalosporin MICs were observed in 29/684 isolates, all of which carried mosaic XXXIV (26). Presuming that the panel of clinical isolates used to validate the mosaic XXXIV PCR served as an appropriate representation of the population of *N. gonorrhoeae* in the United States, the assay should perform well, with high sensitivity and specificity, as presented herein. Second, the results of mosaic XXXIV PCR correlated only with CDC alert values and not CLSI clinical breakpoints, by which all except one isolate (CDC AR Bank number 0194) were still considered susceptible to CFM and CRO. As such, mosaic XXXIV PCR results may not correlate with clinical outcomes, and clinicians should not use these results to guide antimicrobial therapy. Additionally, this assay may have limited utility in regions where mosaic types other than XXXIV contribute to the increased ESC MICs among a population of isolates. An example of such a region is China, in which the prevalence of the *penA* mosaic XXXIV allele was very low (3 isolates in a nationwide collection of 890 isolates) (34). In these regions, the false-negative rate of the assay may increase due to its inability to identify *N. gonorrhoeae* samples with decreased susceptibility to ESCs that harbor *penA* mosaic types other than XXXIV. Nevertheless, the assay may serve as a useful surveillance tool for areas in which *penA* mosaic XXXIV is endemic. The ability to multiplex this assay with the existing *gyrA* PCR would allow for seamless integration into the molecular diagnostic workflow in clinical laboratories and could provide useful susceptibility information to physicians and epidemiologists in real time.

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession no. NGQH00000000. The version described in

this paper is version NGQH01000000. The sequences and annotation of all contigs can be found at the NCBI Nucleotide database under accession numbers NGQH01000001 to NGQH01000122.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01339-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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We declare no conflicts of interest.

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