Molecular Characterization of Markers Associated With Antimicrobial Resistance in *Neisseria gonorrhoeae* Identified From Residual Clinical Samples

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Background: The emergence and spread of antimicrobial-resistant (AMR) *Neisseria gonorrhoeae* (NG) is a major public health concern. In the era of nucleic acid amplifications tests, rapid and accurate molecular approaches are needed to help increase surveillance, guide antimicrobial stewardship, and prevent outbreaks.

Methods: Residual urethral swabs, collected prospectively in the Baltimore City Health Department during a 6-month period, were analyzed by real-time polymerase chain reaction assays for NG DNA and AMR determinants to fluoroquinolones, penicillin, and extended-spectrum cephalosporins.

Results: *N. gonorrhoeae* DNA was detected in 34.8% (73/210) of samples, including 67.3% (68/101) of the swabs that had been previously identified as NG positive by culture. Markers associated with decreased susceptibility to fluoroquinolones were detected in 22.4% of the polymerase chain reaction NG-positive samples. The rate of penicillinase-producing NG was very low (1.6%), and no markers associated with decreased susceptibility to extended-spectrum cephalosporins were detected in this cohort of men using the AMR assays herein described.

Conclusions: Detection of molecular markers associated with AMR in NG can be performed directly from residual clinical samples, although the recovery rate of adequate DNA for molecular testing from these samples can be suboptimal. A high number of samples with mutations associated with decreased susceptibility to fluoroquinolones were identified.

Gonorrhea is the second most prevalent bacterial sexually transmitted infection worldwide.[1] In 2015, there were 395,216 cases of *N. gonorrhoeae* (NG) infections reported to the Centers for Disease Control and Prevention, an 11.4% increase from the cases reported in 2014.[2] *N. gonorrhoeae* has progressively developed resistance to a variety of antimicrobials, and the recent emergence of isolates with resistance to extended-spectrum cephalosporins (ESCs), the recommended and last remaining option for the treatment of NG, has prompted worldwide concern regarding the possible spread of antimicrobial-resistant (AMR) NG.[3–5] To combat the spread of resistant strains, there is a need to increase surveillance testing to identify outbreaks and to guide effective therapeutic options.[6]

Since 1986, AMR trends of NG strains in the United States have been monitored through the Gonococcal Isolate Surveillance Project (GISP).[7] However, the data collected by GISP might not provide a complete representation of AMR trends, because GISP only collects the first 25 isolates from more than 24 state and city health departments throughout the country during a given month. In addition, the increased use of nucleic acid amplification tests (NAATs) for NG diagnosis hinders the collection of viable organisms, which are necessary for traditional antibiotic susceptibility testing. To address the need of enhanced susceptibility surveillance in the era of NAATs and resistant strains, molecular approaches to rapidly identify genetic resistance determinants have been previously developed and are widely accepted as suitable alternatives for determination of antimicrobial susceptibility.[8–10] Furthermore, the use of genotypic assays to predict susceptibility is quickly gaining attention as a new tool to monitor AMR NG[11] and help in guiding antimicrobial stewardship.[12]

Ciprofloxacin resistance in NG is mediated by single-nucleotide polymorphisms in the *GyrA* and *ParC* genes.[13] Molecular typing assays have been extensively studied and shown that the wild-type (WT) *GyrA* genotype, especially at codon 91, is a very sensitive and specific predictor of NG susceptibility to ciprofloxacin.[14] For example, a GyrA91-PCR WT assay successfully characterized the GyrA genotype in 100% of NG isolates tested, and when applied to NG NAAT-positive samples and compared with corresponding culture results, the assay had a 99.4% sensitivity for prediction of NG susceptibility to ciprofloxacin.[15] Furthermore, an assay has recently been developed, which can simultaneously detect NG and its ciprofloxacin susceptibility status as a tool to increase surveillance for ciprofloxacin resistance.[16] Implementation of molecular assays for detection of AMR NG could better define the epidemiology of AMR NG, guide antimicrobial treatment options, and provide alternatives to traditional susceptibility methods. In the present study, real-time polymerase chain reaction (PCR) assays were used to detect NG and AMR determinants directly from residual urethral swabs.

MATERIALS AND METHODS

Clinical Samples

A total of 522 urethral swabs were collected at the Baltimore City Health Department (Baltimore, MD) from May to September 2015. The urethral swabs were collected from men seeking testing for sexually transmitted infections, particularly NG. All swabs were initially tested by cultures at the clinic and classified as NG culture–positive or NG culture–negative. After the completion of clinical testing, each swab was stored dry and the swabs' eluate stored frozen at −80°C until they were analyzed by real-time PCR as described hereinafter. The first 150 urethral swabs were tested in a blinded manner for the presence of DNA and all antimicrobial resistance determinants. From the remaining 372 swabs, culture-positive swabs were selected and a matching number of culture negative selected as controls.

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DNA Extraction

Each swab was rehydrated in 500 μL of autoclaved, deionized water, vortexed for 10 seconds, and DNA was extracted from 200 μL of the sample using the automated MagNA Pure LC instrument (Roche Diagnostics, Indianapolis, IN). DNA extraction was carried out with the DNA I Blood Cells High Performance protocol according to the manufacturer's instructions. Positive controls (serial dilutions of NG or the Asian plasmid containing the penicillinase-producing NG [PPNG] target or a plasmid with the *penA* mosaic target) and negative controls (NG-negative urethral swabs) were processed using the same DNA extraction protocol.

Real-Time PCR-Based Detection

Assays Description

Six real-time PCR assays—4 of them carried out as 2 multiplex assays—were used in this study for the detection of NG DNA and markers associated with AMR for fluoroquinolones, penicillin, and ESCs. The primer and probe sequences are shown in Table 1. The multiplex *porA*/*opa* real-time PCR assay was used to detect the presence of NG DNA.[17,18] Previously validated assays targeting the *GyrA* and *ParC* genes were used in a multiplex format to detect mutations in the Quinolone Resistance–Determining Region (QRDR) of NG, which are associated with decreased susceptibility to fluoroquinolones.[13,19] This assay exclusively detects the WT QRDR of the *gyrA* and *parC* genes. When mutation(s) is present in the QRDR, the TaqMan probe does not recognize the target sequence, resulting in negative PCR results.[19] An assay targeting a sequence predicted to be conserved across all NG plasmid types harboring the β-lactamase gene was used to detect PPNG.[20] This assay has been shown to be very sensitive (100%) and specific (98.7%) in comparison to bacterial cultures for detection of PPNG in clinical specimens.[20]

Lastly, the penicillin-binding protein 2 (PBP-2) assay[21] targeted the mosaic structure of the NG *penA* gene, which encodes PBP-2 and has been shown to be associated with decreased susceptibility to cephalosporins.[22]

Real-Time PCR

After the extraction of DNA, samples were tested for the presence of NG DNA using the *porA/opa* assay and, if identified as NG positive by PCR, tested for AMR determinants using the primers and probes described in Table 1. Briefly, PCRs were performed in 96-well plates in a total volume of 50 μL, using 40 μL of PCR master mix and 10 μL of sample. PCR master mix contained 25 μL of 2× TaqMan universal PCR mix (PE Applied Biosystems, Foster City, CA); 1 μL each of 10 μM of the forward primer, the reverse primer, and the TaqMan probe; 2 μL of $MgCl_2$ and water to a final volume of 40 μL. The PCR conditions and cycling parameters for each assay have been previously described.[17–21] Control samples were included in each run. All samples were tested in duplicate. Samples were considered to be positive for NG if either assay (*porA* or *opa*) had an amplification profile with a C_t value of less than <37. *N. gonorrhoeae*–positive samples were further analyzed by real-time PCR for resistance markers associated with fluoroquinolones (*gyrA*/*parC*), penicillin (PPNG), and ESCs (PBP-2). To evaluate the analytical performance of these assays with residual clinical samples, the first 150 swabs were analyzed in a blinded manner. In addition, 47 NG-negative swabs (identified by culture and real-time PCR) were also analyzed using the *gyrA/parC*, PPNG, and PBP-2 assays. The testing of the NG-negative samples was carried out to determine the analytical specificity of the assays.

RESULTS

Of the 522 urethral swabs collected, 210 swabs were analyzed by using the real-time PCR assays. The remainder swabs

TABLE 1. Primer and Probe Sequences for the Real-Time PCR-Based Detection of NG and Antimicrobial Resistance Determinants

Assay/Target	Primers and Probes	Probe Sequence 5′–3′
**PorA*†*	pap-F	CAGCATTCAATTTGTTCCGAGTC
	pap-R	GAACTGGTTTCATCTGATTACTTTCCA
	pap-TM	FAM-CGCCTATACGCCTGCTACTTTCACGC-BHQ1
**Opa*‡*	NGopa-F	TTGAAACACCGCCCGGAA
	NGopa-R	TTTCGGCTCCTTATTCGGTTTAA
	NGopa	TET-CCGATATAATCCGTCCTTCAACATCAG-BHQ1
GyrA§¶	GyrA-F	TTGCGCCATACGGACGAT
	GyrA-R	GCGACGTCATCGGTAAATACCA
	GyrA91-95	FAM-TGTCGTAAACTGCGGAA-BHQ-1
ParC§¶	ParC-F	TGAGCCATGCGCACCAT
	ParC-R	GGCGAGATTTTGGGTAAATACCA
	ParC86-88	TET-CGGAACTGTCGCCGT-BHQ-1
PPNG||	PPNG-F	AGCTGTTCGTTTTTTACTACCAATCA
	PPNG-R	TGATTTAGTCGTTGAGGTTGAACAA
	PPNG-TM	TET-AATTTAAAGAGTGAATAGTACGCCCACGCTTGA-BHQ1
PBP-2**	NG89-F	GTTGGATGCCCGTACTGGG
	NG89-R	ACCGATTTTGTAAGGCAGGG
	NG89-TM	FAM-CGGCAAAGTGGATGCAACCGA-BHQ-1

*The *porA* and *opa* assays were carried out as a multiplex assay.
†Assay adapted from Whiley and Sloots.[17]
‡Assay adapted from Tabrizi et al.[18]
§Assay adapted from Giles et al.[19]
¶The *gyrA* and *parC* assays were carried out as a multiplex assay.
||Assay adapted from Goire et al.[20]
**Assay adapted from Ochiai et al.[21]
FAM indicates carboxyfluorescein; IBHQ-1, Iowa Black Hole Quencher-1; TET, tetrachlorofluorescein; TM, TaqMan.

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(n = 312) were not analyzed by PCR because they were culture NG negative. A total of 101 swabs were positive for NG by culture, but NG DNA was only detected in 68 (67.3%) of the swabs by PCR (Table 2A). The high number of samples (33/101) with false-negative PCR results was likely due to the low concentration of NG cells/DNA in these samples resulting from previous use in routine clinical analysis, or to DNA degradation due to the storage of the swabs under different conditions (dry, frozen) before analysis. Most (99%) culture-positive swabs were collected from symptomatic men. In regard to the NG culture–negative swabs, 104 (95.4%) of 109 were also identified as NG negative by PCR (Table 2A). The 5 NG culture–negative/PCR-positive swabs were found to have NG WT *GyrA* and *ParC* sequences indicative of the presence of NG DNA in these samples. Despite the high number of culture-positive/PCR-negative samples, 91.2% (62/68) of the samples that were identified by PCR as NG positive had usable DNA and were further tested for AMR markers (Table 2B). Six samples were excluded from further analysis because they had *porA/opa* C_t values of greater than 37, which suggested low NG DNA load.

None of the 47 NG culture–negative swabs tested positive using any of the AMR (*gyrA/parC*, PPNG, and PBP-2) assays (Table 2B). Regarding the detection of genetic markers associated with AMR, QRDR mutated sequences were detected in 22.4% (15/67) of the PCR NG–positive swabs—53.3% (8/15) had only mutated *gyrA* sequences, whereas 46.7% (7/15) of the samples had mutated sequences in both genes (Table 2B). Unexpectedly, the prevalence of PPNG was very low because only one sample tested positive (Table 2B). Lastly, the *penA* mosaic associated with decreased susceptibility to cephalosporins was not detected in any of the samples analyzed in this study (Table 2B).

DISCUSSION

In the present study, residual urethral swabs were used to determine the feasibility of detecting genetic markers associated with AMR in NG directly from clinical specimens. Because of the widespread use of NAATs for detection of NG, the use of antimicrobial susceptibility testing for surveillance purposes has significantly decreased in the last decade, thus requiring the development and implementation of molecular approaches for characterization of AMR NG. The data presented here support previous studies reporting that residual or residual clinical samples can be used for performing AMR genotyping using molecular approaches.[8–10,23]

Our study found a high rate (22.4%) of samples with mutations in the *GyrA* and/or *ParC* gene, which are commonly associated with resistance to fluoroquinolones.[3,13,19]

Considering that genotypic prediction of ciprofloxacin susceptibility directly from clinical specimens using PCR is comparable with traditional susceptibility approaches,[24,25] molecular detection of QRDR mutations, such as the approach described here, can be used as an alternative tool for QRNG surveillance.[14] Using a single assay, we found a very low number of PPNG strains in this population. However, mutations in other genes, such *PenA*, *mtr*, and *PenB*, which were not analyzed in the current study, may contribute to chromosomally mediated penicillin resistance.[3] Further analysis is warranted to determine if chromosomally mediated resistance to penicillin is present in this cohort of samples. In regard to resistance to ESCs, we found no strains harboring the *penA* mosaic marker associated with decreased susceptibility to ESCs. This finding is consistent with previous reports that cephalosporins-resistant NG strains in Baltimore City are rare.[26]

Our study has several limitations. First, residual urethral swabs were used in this study, which prevented the recovery of sufficient NG DNA from a large number of samples. Additional studies using prospectively collected samples from both males and females should be carried out to provide a more accurate estimate of AMR NG in Baltimore City. The *penA* mosaic assay may not be able to detect all of the *penA* alleles, which have now been described.[3] In addition, isolates with decreased susceptibility to ceftriaxone due to combined effects of mutations in multiple genes could not be identified using this assay.[27] Susceptibility data for the samples tested were not available; however, as previously noted, genotyping prediction of NG ciprofloxacin susceptibility is highly accurate.[24,25] Genotyping studies with extragenital samples are warranted. Ideally, our study should be replicated with urine NAAT specimens because they are the most common type of NAAT testing for men.

Although fluoroquinolones are no longer recommended by the Centers for Disease Control and Prevention for the treatment of NG,[28] the data reported here and in other studies[14,25,29] suggest that a large percentage of NG infections in the United States could be potentially treated with fluoroquinolones. However, this approach will require the rapid identification of fluoroquinolone-sensitive cases before antimicrobial treatment using a molecular approach. A recent report indicated that the implementation of a genotypic assay could promote targeted ciprofloxacin therapy,[12] but this approach can take days to obtain a result. Faster molecular approaches at the point-of-care are needed, which can simultaneously identify NG infections and predict susceptibility to guide antimicrobial stewardship.[16] The ideal method for AMR testing in NG would be to develop a rapid, phenotypic assay that would directly determine the AMR profile of the NG organism present in a given sample. However, because that technology does not yet exist and given the decreasing number of clinical isolates available for culture due to the increase in NAAT testing for NG infections, molecular methods represent the best intermediary step until a rapid, phenotypic assay is developed. The implementation of a rapid (1–2 hours) detection and genotypic assay should prove useful in enhancing empirical treatment, preventing the overuse of cephalosporins, and helping to prevent the spread of AMR NG.

TABLE 2. Real-Time PCR-Based Detection of Gonorrhea in Comparison to Culture (A) and Detection of Genetic Markers Associated With Antimicrobial Resistance in NG by Real-time PCR (B)

	Culture (+)		Culture (−)	
	PCR (+)	PCR (−)	PCR (+)	PCR (−)
A				
No. swabs tested for gonorrhea (n = 210)	68	33	5	104
B				
No. samples analyzed for resistance determinants	62	0	5	47
GyrA or ParC mutants	15	NT	0	0
GyrA mutants	8			
ParC mutants only	0			
GyrA and *ParC* mutants	7			
PPNG	1			
PBP-2	0			

Urethral swabs were first tested for the presence of gonorrheal DNA with the *porA/opa* multiplex assay. Gonorrhea-positive swabs were further analyzed for genetic markers associated with antimicrobial resistance by PCR.

NT indicates not tested.

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