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Molecular Screening for *Neisseria gonorrhoeae* Antimicrobial Resistance Makers in Nigerian Men who have Sex with Men and Transgender Women

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Abstract

Antimicrobial resistant *Neisseria gonorrhoeae* (NG) is a global public health issue that threatens the effectiveness of current treatments of gonorrheal infection. Increased use of nucleic acid amplification tests (NAAT) in lieu of cultures for the detection of NG makes obtaining clinical isolates for susceptibility testing difficult, samples collected in commercial transport buffer for NAAT testing typically do not preserve viable organism. Molecular methods of assessing antibiotic susceptibility do not require viable organisms and are more rapid than traditional methods.

We evaluated 243 NG positive samples in Aptima transport samples including oral swabs, rectal swabs, and urine from a cohort of Nigerian men who have sex with men (MSM) for resistance markers to penicillinase producing NG (PPNG), ciprofloxacin (*GyrA* and *ParC* mutations) and extended spectrum cephalosporins (ESCs, *PenA* (mosaic allele X), *PonA*, *mtrR*, *PorB* mutations) by real-time PCR.

NG DNA was recovered in 75% (183/243) of Aptima transport samples. Of these, 93% (171/183) were positive for at least one resistance marker. We observed a prevalence of dual resistance markers to penicillin and ciprofloxacin at 46.2% (79/171), for PPNG only at 27% (47/171) and ciprofloxacin only at 20% (35/171). Six percent of samples (10/171) tested positive for the *PenA*

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mosaic (allele X) ESC marker. Of those, ten percent (1/10) samples were positive for penicillin, ciprofloxacin and ESC resistance markers. All samples testing positive for the *PenA* mosaic underwent additional ESC marker testing for *PonA*, *mtrR* and *PorB* mutations.

These data indicate that antibiotic resistant NG organisms are common in Nigeria. Laboratory and clinical capacity building in Nigeria should include development of methods to culture and identify NG and determine antimicrobial susceptibility.

Introduction

Neisseria gonorrhoeae (NG) is one of the most common sexually transmitted infections (STIs) in the United States and globally.^{1, 2, 3} NG has been highlighted as an important public health concern due to the global spread of antimicrobial resistant (AMR) species. Of particular concern are strains with resistance to extended spectrum cephalosporins (ESCs) such as ceftriaxone, the currently recommended treatment for NG infections.²⁻⁸ These strains represent essentially untreatable NG, as most circulating strains of NG are resistant to penicillin and many are resistant to ciprofloxacin and azithromycin.^{2-6, 9-12} In resource-limited settings, poor retention of patients or lack of appropriate antibiotics can make antibiotic stewardship difficult. Systematic monitoring for resistance is therefore essential.¹³⁻¹⁶

Typically, AMR is determined using agar dilution methods to determine the minimum inhibitory concentration (MIC) of each antibiotic of interest. Although this is the most accurate method of AMR determination, it can be technically challenging due to the expertise required for NG culture and identification, the precision required for antibiotic preparation, the number of antibiotic concentrations required, MIC plate preparation and interpretation. These methods require viable clinical isolates and are costly. While Etests (BioMerieux, Durham, NC) for many antibiotics are available and are less complex than agar dilution, these culture-based methods are still expensive and require viable organism from clinical isolates.¹⁷

With the increased use of nucleic acid amplification testing (NAAT) for STI detection, clinical isolates of pathogenic organisms are infrequently available, since recovering viable organisms from commercial NAAT transport is impossible. Molecular methods that can identify single nucleotide polymorphisms (SNPs) or mutant genotype markers associated with AMR can potentially be used to overcome some of the challenges associated with traditional culture methods and without a need for viable organism from clinical isolates.¹⁸⁻²⁴ While there are limitations to the use of molecular methods for AMR screening, these methods represent a hypothetically valuable tool to gain information about AMR genotypes associated with a pathogen.^{21, 24} These methods have successfully illustrated correlation with MICs, have been useful in AMR surveillance, and have also demonstrated value in terms of their impact on guiding antimicrobial therapy.^{21, 25-27}

Across Sub-Saharan Africa, many countries employ a syndromic approach to STI management as standard of care.¹⁶ Nigeria and other resource-limited settings do not have the infrastructure for advanced STI diagnostics. In Nigeria, men who have sex with men

(MSM) and transgender women (TGW) face a particularly high burden of HIV, NG and other STIs.²⁸ As such, AMR surveillance in these populations is important in order to promote the most efficacious disease treatment. We sought to determine the prevalence of AMR markers for penicillinase producing NG (PPNG)¹⁹, ciprofloxacin resistance (*GyrA* and *ParC* mutations)¹⁸ and ESCs (*PenA*, *PonA*, *mtrR* and *PorB*)^{22, 23} in *PorA* (PAP) pseudogene positive samples²⁹ from a cohort of Nigerian MSM and TGW.

Material and Methods

Study Population

The Lagos site of the TRUST/RV368 cohort study enrolled MSM and TGW using respondent-driven sampling (RDS) as previously described.^{28, 30} Briefly, seven diverse seed participants and each subsequent enrollee were given three coupons to distribute to other MSM. Eligible participants were adults over 18 years old who presented a valid coupon and reported receptive or insertive anal intercourse with a male partner in the preceding 12 months. Participants who enrolled between 28 April 2014 and 19 July 2016 were included in these analyses.

All participants provided written informed consent before enrollment. Institutional review boards at the Nigerian Federal Capital Territory and Nigerian Ministry of Defense, Abuja, Nigeria; the University of Maryland, Baltimore, MD, USA; and the Walter Reed Army Institute of Research, Silver Spring, MD, USA approved the study.

STI Testing and Follow-Up

Participants underwent testing for HIV and other STIs every three months for up to 18 months. Urine, oral swabs, and rectal swabs were tested for NG and *Chlamydia trachomatis* using the Aptima Combo 2® assay (Hologic, Bedford, MA, USA). Fingerstick blood specimens were screened for HIV infection using a parallel algorithm of rapid tests with Determine® (Alere, Waltham, MA, USA) and Uni-gold® (Trinity Biotech, Co Wicklow, Ireland) kits. Testing was conducted according to package inserts.

Molecular Testing for NG DNA and AMR

Samples that tested positive for NG by the Aptima Combo 2® assay underwent nucleic acid extraction utilizing the Roche MagNA Pure LC robot (Roche Diagnostics, Indianapolis, IN) with the DNA I Isolation kit, employing the blood/cells high performance protocol based on manufacturer instructions. A 200 µl sample volume with a 100 µl elution volume was used. All extraction runs contained positive (wild type NG) and negative (molecular grade water) controls. Following nucleic acid extraction, samples were initially screened by PCR for the *porA* pseudogene (PAP) to ensure NG nucleic acid recovery from the Aptima Combo 2® transport medium.²⁹ Samples that were PAP positive were then screened for PPNG, *GyrA* and *ParC* mutations as well as the *PenA* mosaic (allele X) sequence by real-time PCR.^{18, 19, 22} Samples that tested *PenA* positive underwent additional testing for mutations in the *PonA*, *mtrR*, and *PorB* genes.²³

Real-time PCRs used primers and probes as previously described^{18, 19, 22, 23, 29} and were performed on the Applied Biosystems Quant Studio 12K Flex platform (Applied Biosystems, Foster City, CA) as follows, with minor modification from previous conditions: all reactions had a final volume of 50 µl with 10 µl of sample and 40 µl of mastermix. The mastermix included 25 µl of Taqman Fast Advanced Mastermix (Applied Biosystems), 2 µl of each appropriate primer and probe, 2 µl of 25 mM MgCl₂, and nuclease free water to make a final volume of 40 µl. Cycling conditions for the PAP, PPNG and initial *PenA* screening PCRs were as follows: 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 30 seconds. Samples that tested positive for the *PenA* mosaic sequence underwent additional PCRs for *PonA*, *mtrR* and *PorB* as follows: 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 1 minute. PCR conditions for the *GyrA* and *ParC* assays were as follows: 95°C for 20 seconds, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Positive reactions were defined as PCR reactions having a C_t value of <40.

In order to exclude the possibility of false-positive findings due to cross-reactivity with near neighbor bacterial species, whole genome sequencing (WGS) of NG DNA at Rocky Mountain Laboratories (RML, Hamilton, MT) was attempted for samples testing positive for the *PenA* mosaic sequence for confirmation of real-time PCR results. Without viable organism to perform additional testing, WGS would ensure the real-time PCR results indicating *PenA* mosaic sequences, as well as SNP sequences for *PonA*, *mtrR* and *PorB* were associated with the NG present in the samples tested, or would indicate these results were false positives associated with near-neighbor bacterial species present in the samples that can harbor the same mutations.^{21, 23}

Results

Study Population Demographics and STI Testing

A total of 420 participants (128 HIV-uninfected, 292 HIV-infected) underwent STI testing and 37% (157/420) of participants had NG infections as determined by NAAT, which resulted in 243 samples for AMR evaluation with real-time PCR, as some participants were NG positive at more than one anatomic site. Participant demographics are summarized in Table 1. Of the 157 cases of NG infections, 75.7% (119/157) had an NG infection at one anatomic site, while 13.3% (21/157) had both oral and rectal NG, 7% (11/157) had urogenital and rectal NG, 1.3% (2/157) had oral and urogenital NG, and lastly 2.5% (4/157) had simultaneous oral, urogenital and rectal NG.

Real-Time PCR for Molecular AMR Markers

NG DNA was successfully recovered in 75% (183/243) of the samples. Of these, 93% (171/183) were positive for at least one resistance marker. Overall, 73.6% (126) tested positive for PPNG indicating resistance to penicillin, and 67% (114) tested positive for mutations in *GyrA* associated with ciprofloxacin resistance (Chart 1, Figure 1). By sample type, oral swab samples tested positive in 12.8% (22), 3.5% (6) and 2.4% (4) for PPNG, *GyrA* mutations and *GyrA/ParC* double mutations respectively. For urine samples, 7.6% (13), 9.9% (17) and 8.2% (14) tested positive for PPNG, *GyrA* mutations and *GyrA/ParC*

double mutations respectively. Lastly, for rectal swabs, 56.1% (96) and 46.7% (80) tested positive for PPNG, *GyrA* mutations and *GyrA/ParC* double mutations respectively (Chart 1A). For individuals infected with NG at more than one anatomic site, 47.4% (18/38) had the same results for PPNG (N=13), *GyrA* and *ParC* (N=5) across different anatomic sites.

Ten samples tested positive for the *PenA* mosaic (allele X) sequence. Of these ten samples, six were positive for the *PenA* mosaic (allele X) sequence and were also double mutants for *GyrA* and *ParC*, while two samples were positive for the *PenA* mosaic (allele X) sequence and were PPNG positive. Lastly, there were two samples, one positive for the *PenA* mosaic (allele X) sequence, which was PPNG positive, and was a double mutant for *GyrA* and *ParC*, while the other sample was *PenA* mosaic (allele X) positive, as well as PPNG positive and only had a single mutation for *GyrA*.

Additional testing for mutations in *PonA*, *mtrR* and *PorB* was performed on the 10 samples that were positive for the *PenA* mosaic (allele X) sequence (Table 2).²³ Of these, multiple samples tested positive for both wild-type and mutant sequences simultaneously, notably for *PonA* and *mtrR*. Only one of these samples tested positive for wild-type *PorB*. For expanded *PenA* testing, only two samples that initially tested as positive for the *PenA* mosaic (allele X), repeated as positive for the mosaic (allele X) sequence with an alternate assay. Seven tested positive for wild-type *PenA* by expanded assays. WGS for NG was unsuccessful due to insufficient quantity of NG DNA.

Discussion

In this study we assessed the prevalence of AMR markers associated with decreased susceptibility to penicillin, ciprofloxacin and ESCs in 243 samples in Aptima Combo 2 transport media from 157 NG infected MSM using multiple real-time PCR assays.

We found a high prevalence of molecular markers associated with AMR, mostly affecting susceptibility to penicillin and ciprofloxacin, particularly in the rectal swab samples analyzed. Previous studies of AMR in sub-Saharan Africa have illustrated varying rates of AMR, depending on the population. For example, reported rates of ciprofloxacin resistance have included 6.1–18.6% in Zimbabwe^{14,15}, 28.6% in Ethiopia³¹, 83.1% among Ugandan female sex workers³², 100% in a small study in Ghana³³ and 53.2% in Kenya³⁴. Although AMR studies specific to Nigeria are limited, a 2001 study indicated >90% of samples analyzed were susceptible to ciprofloxacin, although that study only examined 31 samples.³⁵ In contrast, we found 67% (114/117) of the samples evaluated in this study tested positive for markers associated with ciprofloxacin resistance, and greater than 50% of the samples positive for mutations associated with ciprofloxacin resistance were from the rectal swabs analyzed in this study. It is possible that AMR prevalence in Nigeria has increased over time.

Neither of the two studies from Zimbabwe included susceptibility testing for penicillin, as they screened for resistance to kanamycin, but our study found a relatively high number of samples that were positive for PPNG and were double mutants for *GyrA* and *ParC*. The additional studies performed in Ethiopia, Uganda, Ghana and Kenya, all analyzed different antibiotics, but each study screened for AMR to antibiotics that would be traditionally used

to treat NG.^{14, 15, 31–34} These prior studies each relied on agar dilution MIC or Etest to assess AMR, in contrast to the molecular techniques employed in this study. As such, it is difficult for direct comparison of our methods to agar dilutions or Etests employed in other studies. It should also be noted that the participants in this study were exclusively MSM and TGW, while the other studies focused on broader populations, and population differences could impact AMR prevalence. Additionally, these prior studies sampled limited anatomic sites, generally not including rectal or oral sampling, whereas our study universally screened participants for infection at the urethral, rectal and oral sites.

The data generated from screening samples in this study for the *PenA* mosaic sequence suggested the possibility of ESC resistance but were ultimately inconclusive. Resistance in NG to ESCs has not yet been reported in many regions in Africa, although multidrug resistant NG has been reported in South Africa.^{14, 15, 16, 31–34, 36} When molecular testing for markers associated with resistance to ESCs was expanded to include additional assays²³, results were still inconclusive as the majority of samples simultaneously tested positive for both wild-type and SNP sequences (Table 2). This is likely because of the presence of mixed bacterial species in swab specimens rather than pure NG cultures, including both near-neighbor *Neisseria* species as well as other commensal bacteria that potentially have acquired these resistance factors. This is supported by the fact that the additional assays employed are capable of cross-reacting with other bacterial species.²³ WGS did not provide additional information regarding the NG in these samples as not enough NG DNA could be recovered. This is possibly because of the use of clinical swab samples as opposed to clinical isolates, as a clinical NG isolate would concentrate the amount of viable pure NG present in the sample and would remove interference from the presence of the human genome or non-pathogenic organisms in sequencing efforts. WGS has shown promise to be a predictive tool for AMR in NG when performed using clinical culture isolates.³⁷

There are several limitations to this study. First, NG DNA was successfully recovered from only 75% of samples. The samples utilized in these analyses were clinical swabs that contained not only the organism of interest (NG), but also human epithelial cells and organisms from other commensal bacterial species. The presence of these additional cellular types can complicate molecular testing, particularly in assays where the target can be found across multiple species. Clinical isolates, in this instance, the isolated NG organism, are typically utilized for AMR determination as this approach ensures that AMR is determined only for the pure organism of interest. Without clinical isolates, we could not perform traditional AMR determination by culture and MIC to verify the accuracy of the molecular methods employed here. Additionally, the molecular methods used in this study were all developed using clinical isolates or NG organism with previous AMR data available, making application of the sensitivity and specificity of these assays to this study swabs problematic. However, previous studies have demonstrated strong agreement between molecular methods and traditional methods for assessing AMR.^{21, 24}

The data generated by assays for wild-type and SNP sequences of *PenA*, *PonA*, *mtrR* and *PorB* as well as the poor quality results from WGS are illustrative of the limitations in applying molecular assays for resistance markers to clinical swab samples as opposed to clinical isolates, since clinical samples contain mixed bacterial species, many of which are

near neighbor species such as *N. meningitidis* that can have the same mutations associated with AMR in NG. WGS could have confirmed the presence or absence of these mutations had viable NG been available for testing. These data also highlight the need for a rapid phenotypic assays for AMR surveillance, not just for NG, but for other pathogenic organisms that exhibit AMR.

Our molecular screening efforts for AMR in these samples was limited to markers for penicillin, ciprofloxacin and ESC, but assessments for resistance to other antibiotics, such as tetracycline (*TetM*) or azithromycin (23S rRNA mutations), could have provided valuable additional information. The molecular methods employed in this study were genotypic as opposed to phenotypic assays, and although the genotypic assay may detect a marker or SNP associated with AMR, the results do not necessarily correlate to phenotype.^{21, 24}

The percentages of resistance to penicillin and ciprofloxacin observed in this study highlight the need for continuous and consistent monitoring for AMR NG. This is particularly critical in populations and regions where transmission and acquisition of AMR NG is a concern, and where antibiotic stewardship is suboptimal. Molecular methods, despite their limitations can be useful tools in properly planned studies with appropriate sample types. Although these are not phenotypic assays, molecular methods represent the best intermediary approach until rapid, practical, affordable and accurate phenotypic assays are developed to monitor for AMR NG.

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Figure 1. Relationship of PPNG, *GyrA* and *ParC* Mutations for 171 NG Positive Samples.

The diagram represents the relationship between observed detections for all PPNG positive detections (126), all *GyrA* mutations (114), all combined *GyrA/ParC* mutations (91), and additional combinations of positives for PPNG, *GyrA* mutations only and combined *GyrA/ParC* mutations (78), PPNG positives and combined *GyrA/ParC* mutations (67) and lastly combined *GyrA* mutations with combined *GyrA/ParC* mutations. The total number of observations for each is listed in parentheses.

Chart 1. Percentage of Molecular Markers for PPNG, *GyrA* and *ParC*.

This chart presents the percentage of samples evaluated where molecular screening for antimicrobial resistance markers for 1) any resistance 2) PPNG detections, 3) *GyrA* mutant detections, 3) *GyrA/ParC* double mutant detections. There were no *ParC* mutant, *GyrA* wild-type detections. Sample testing positive for the *PenA* mosaic sequence (allele X) are not represented in the table and analyses for these samples are found in the Results section and Table 2.

Chart 1A. Percentage of Molecular Markers for PPNG, *GyrA* and *ParC* by Sample Type.

This chart presents the percentage of samples evaluated where molecular screening for antimicrobial resistance markers for 1) PPNG oral swab detections 2) *GyrA* mutant detections oral swab, 3) *GyrA* and *ParC* double mutant detections oral swab, 3) *GyrA/ParC* double mutant detections oral swab 4) PPNG detections urine 5) *GyrA* mutant detections urine 6) *GyrA/ParC* double mutant detections urine 7) PPNG detections rectal swab 8) *GyrA* mutant detections rectal swabs, 9) *GyrA* and *ParC* double mutant detections rectal swab. There were no *ParC* mutant, *GyrA* wild-type detections. Sample testing positive for the *PenA* mosaic sequence (allele X) are not represented in the table and analyses for these samples are found in the Results section and Table 2.

Table 1.Enrollment Characteristics of 157 Participants with Prevalent or Incident *Neisseria gonorrhoeae*.

Characteristic	Level	n=157 (%)
Age, median (IQR^A)		22 (19, 25)
Age	21 years	72 (45.9)
	22–30 years	78 (49.7)
	>30 years	7 (4.5)
Gender Identity	Male	117 (75.5)
	Female	31 (19.7)
	Other/Unknown	9 (5.7)
Sexual Orientation	Gay/Homosexual	78 (49.7)
	Bisexual	78 (49.7)
	Other/Unknown	1 (0.6)
Education	Junior Secondary	4 (2.5)
	Senior Secondary	116 (73.9)
	>Senior Secondary	37 (23.6)
Occupation	Unemployed	37 (23.6)
	Student	42 (26.8)
	Professional/Self-Employed	28 (17.8)
	Entertainment/Hospitality	13 (8.3)
	Driver/Laborer	5 (3.2)
	Other/Unknown	32 (20.4)
Marital Status	Single/Never Married	144 (91.7)
	Married/Living with a Woman	2 (1.3)
	Living with a Man	6 (3.8)
	Divorced/Separated/Widowed/Other	5 (3.2)
HIV Status	Uninfected	40 (25.5)
	Infected	117 (74.5)

^AIQR: interquartile range

Table 2.

Results for Molecular Testing for the *PenA* Mosaic Sequence and Mutations in the *PenA*, *mtrR* and *PorB* Genes. This table presents results from molecular screening performed initially for the *PenA* mosaic (allele X) sequence (1-reference 20), and expanded molecular testing for additional *PenA* (allele X), *PenA*, *mtrR* and *PorB* mutations (3-reference 21). Shaded boxes represent assay results that were positive for the listed wild-type (WT) or single nucleotide polymorphism (SNP).

Sample	<i>PenA</i> ¹ Mosaic Initial Result	<i>PenA</i> WT1 ²	<i>PenA</i> WT2 ²	<i>PenA</i> Mosaic ² Secondary Result	<i>PenA</i> WT ²	<i>PenA</i> SNP ²	<i>mtrR</i> WT ²	<i>mtrR</i> SNP ²	<i>PorB</i> WT1 ²	<i>PorB</i> WT2 ²
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										