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Research Article

Excellent Correlation between Phenotypic and Genotypic Assays for the Detection of *Neisseria gonorrhoeae* Antimicrobial Resistance Profiles

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Abstract

Background: Antimicrobial testing for *N. gonorrhoeae* is classically performed by first culturing the microorganism. However, nucleic acid amplification assays may be more feasible for determining antimicrobial patterns.

Methods: Paired endocervical swabs were obtained from pregnant women. One swab was cultured to isolate pure colonies of *N. gonorrhoeae* to perform antimicrobial testing to azithromycin, cefixime, ceftriaxone, ciprofloxacin, penicillin G, tetracycline and spectinomycin. The DNA extracted from the second swab was used to determine the presence of genes/mutations associated with resistance. The correlations between phenotypic and genotypic resistance profiles were determined.

Results: Resistance to penicillin, tetracycline and ciprofloxacin was observed across the isolates. A 100% correlation between the phenotypic and genotypic data was observed. For the isolates which displayed resistance to the antimicrobials tested, the respective molecular resistant determinants were shown to be present. The *tetM* gene was also shown to be present in the isolate (G51) which was classified as being intermediate for tetracycline. For the one isolate (G206) which displayed susceptibility to ciprofloxacin, the Ser-91 mutation which confers resistance to this antimicrobial was absent.

Conclusion: Due the extensive time with culture-based techniques for identifying susceptibility/resistance patterns for *N. gonorrhoeae*, detection of resistance determinants from the molecular level which can be accomplished in a shorter time may prove to be more feasible for future antimicrobial studies.

Introduction

Neisseria gonorrhoeae (*N. gonorrhoeae*), the etiologic agent of gonorrhoeae is reported to be the second most prevalent bacterial Sexually Transmitted Infection (STI) globally [1]. The emergence of drug resistance of *N. gonorrhoeae* is a public health concern since this infection may become untreatable in the future [2, 3]. The global clinical management of *N. gonorrhoeae* infections is becoming increasingly challenging due to resistance to various classes of available antibiotics such as sulphonamides, beta-lactams, tetracyclines, macrolides, fluoroquinolones and more recently expanded-spectrum cephalosporins [4, 5]. The ability of *N. gonorrhoeae* to exhibit drug resistance to a wide range of antibiotics is due to this bacterium's remarkable phenotypic and genotypic variability which gives it an added advantage in evading host responses [6]. The genotypic variability of *N. gonorrhoeae* has been

linked to the acquisition of new genetic material through the processes of transformation or conjugation [4, 5].

Antimicrobial testing for *N. gonorrhoeae* is classically performed by first culturing the microorganism. However, a major challenge associated with culture is the loss of viability of the gonococcus during transportation and storage. More recently, Nucleic Acid Amplification Assays (NAATs) for the identification and association of antimicrobial resistance in *N. gonorrhoeae* has been employed [6]. In this study, we performed Minimum Inhibitory Concentration (MIC) testing by the E-test method on cultured clinical isolates. In addition, using the second endocervical swab, we identified determinants associated with antimicrobial resistance patterns in *N. gonorrhoeae* by Polymerase Chain Reaction (PCR) based amplification of specific resistance genes directly from the endocervical swab DNA. This is also the first report on *N. gonorrhoeae* antimicrobial resistance profiles in pregnant women from KwaZulu-Natal, South Africa.

Methods

Samples

A total of 6 pure isolates of *N. gonorrhoeae* were obtained from 307 pregnant women from the antenatal clinic of the King Edward VIII hospital (KEH) in Durban, KwaZulu-Natal from November 2018 to July 2019. Each consenting woman underwent a clinical examination during which two endocervical swab samples were collected. Upon collection, the first swab was placed in 2 mL of phosphate buffered saline (pH 7.4) for molecular testing. The second swab was placed in Amies Charcoal transport media (LASEC, South Africa) for culturing onto New York City agar plates and confirmation of *N. gonorrhoeae* isolates by Gram staining, catalase, oxidase, and glucose utilization tests. Full ethics approval for this study was granted by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal, (BE355/18).

Determination of Antimicrobial Profiles by the E-test Method

A 0.5 McFarland (ThermoFisher Scientific, United States) inoculum of *N. gonorrhoeae* was prepared using Mueller-Hinton Broth (LASEC, South Africa). The E-test method (BioMérieux, France) was used to determine the MICs (mg/L) of azithromycin (0.016 – 256), cefixime, ceftriaxone (0.002 – 32), ciprofloxacin (0.002 – 32), penicillin G (0.016 – 256), tetracycline (0.016 – 256) and spectinomycin (0.064 – 1024). Reference strains, G, W, X, Y and Z were kindly provided by the World Health Organisation (WHO) for use as controls in these experiments. The MIC values were assessed in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019) breakpoints.

Molecular Testing

DNA was extracted from the endocervical swab using the PureLink Microbiome Kit (ThermoFisher Scientific, United States). The extracted DNA was used to detect the presence of the pathogen with the TaqMan quantitative PCR assay along with a predesigned probe and primer mix for *N. gonorrhoeae* (ThermoFisher Scientific, United States). The extracted DNA was also used to determine the presence of genes/mutations associated with resistance.

Results and Discussion

Prevalence Estimates

Of the 307 samples cultured, only 6 samples were shown to be culture positive (2%), however, with the TaqMan quantitative PCR assay, 24 samples (8%) tested positive for the presence of *N. gonorrhoeae*. Other South African studies have reported *N. gonorrhoeae* infection rates that range from 3 to 11% [7, 8].

Antimicrobial Profiles Based on the E-test Method

Table 1 shows the antimicrobial profiles of the *N. gonorrhoeae* tested. All of the isolates displayed susceptibility to azithromycin, cefixime, ceftriaxone, and spectinomycin. Resistance to penicillin G, tetracycline and ciprofloxacin was observed across the isolates. Some isolates showed dual and triple resistant phenotypes (Table 1). For pregnant women, resistant *N. gonorrhoeae* infections can have severe consequences on reproductive health, such as an increased risk for acquiring Human Immunodeficiency Virus (HIV) and other STIs as well as obstetric morbidity [9]. In our population of

pregnant women, we did not observe resistance to any of the antibiotics currently used for the management of Vaginal Discharge Syndrome (VDS) in our setting. Despite, the lack of resistance it is important that ongoing antimicrobial resistance surveillance is carried out since ceftriaxone and azithromycin are used in the management of VDS in South Africa.

Table 1: Antimicrobial susceptibility patterns in *N. gonorrhoeae* based on E-test method. Patterns of resistance and susceptibility were determined by the 2019 EUCAST breakpoints. The MIC denoting resistance is shown in brackets underneath each antibiotic name.

Isolate	Ceftriaxone (>0.125 mg/L)	Cefixime (>0.125 mg/L)	Azithromycin (1 mg/L)	Ciprofloxacin (>0.06 mg/L)	Spectinomycin (>64 mg/L)	Tetracycline (>1 mg/L)	Penicillin G (>1 mg/L)
G51	Complete susceptibility	Complete susceptibility	Complete susceptibility	3 mg/L **	16 mg/L	1 mg/L *	Complete resistance **
G136	0,012 mg/L	Complete susceptibility	0,094 mg/L	1** mg/L	24 mg/L	16 mg/L **	24 mg/L **
G176	Complete susceptibility	Complete susceptibility	0,125 mg/L	1.5 mg/L **	1.5 mg/L	1.9 mg/L **	Complete resistance**
G180	0,004 mg/L	Complete susceptibility	0,047 mg/L	0,50 mg/L **	8 mg/L	16 mg/L **	64 mg/L **
G206	0,006 mg/L	0,016 mg/L	0,094 mg/L	0,003 mg/L	16 mg/L	32 mg/L **	12 mg/L **
G247	Complete susceptibility	Complete susceptibility	Complete susceptibility	0,16 mg/L **	0,75 mg/L	3 mg/L **	Complete resistance**

**Intermediate phenotype*

***Resistant phenotype*

Antimicrobial Profiles Based on PCR Detection

Specific primers targeting resistant determinants for penicillin, tetracycline and ciprofloxacin were investigated since resistant profiles for these antibiotics were observed with the E-test method. The primers and reaction conditions for each of these determinants have been described elsewhere [2, 3]. The *tetM* gene was shown to be present in all samples. Sequencing of the PCR amplicons confirmed the identity of this gene (99% identity to the American type-*tetM* conjugative plasmid- Accession number: GU479464.1). The findings of our study are similar to other African studies [6, 10] A study conducted in a Moroccan female population, revealed a 100% prevalence of the American type plasmid carrying the *tetM* gene [6] A study conducted in South African males also reported a high prevalence of the American type *tetM* plasmid and a lower prevalence of the Dutch type *tetM* plasmid [10]. A recent study conducted in South Africa demonstrated that the *tetM* gene was detected in 92% of their isolates with a 90% predominance of the American variant, correlating with previous findings which suggests that the American variant of the *tetM* originated in Africa [11, 12]. These studies provide evidence on the non-use of tetracycline for future treatment of *N. gonorrhoeae* infections, since there are high levels of resistance to this antibiotic. The *penicillinase producing plasmid* and *gyrase A* gene were successfully amplified from the swab DNA samples. Sequencing of the amplicons revealed a 90% identity to the Asian-TEM1-MIC512 plasmid PJD4 (Accession number: MK973080.1) and the *gyrase A* gene from *N. gonorrhoeae* (99.5% identity: Accession number: MK628693.1). Previous studies conducted in South Africa in 2010 showed the presence of the African-type (35.2%) and the Toronto-type plasmids (44.4%), as well as a new Johannesburg-type (20.3%). The Asian-type was not detected in that study [13]. However, the findings of our study are similar to the Moroccan study which found a 27.8% prevalence of the Asian type plasmid related to penicillin resistance [6].

Of the samples which carried the *gyrase a* gene, 5/6 samples (83%) carried the Ser-91 mutation associated with

ciprofloxacin resistance. A Moroccan study conducted from 2013 to 2015, demonstrated an extremely high level of ciprofloxacin resistance with 77.9% of the samples exhibiting the Ser-91 mutation [6]. South Africa had previously used a 250 mg dose of ciprofloxacin instead of the 500 mg recommended by the WHO and Centers for Disease Control and Prevention. Selection of resistance in bacteria is associated with the duration and total amount of drug utilization in a population and leads to the question whether the rapid decrease in susceptibility may be due to short course or low-dose of ciprofloxacin [14].

Correlation between Antimicrobial Profiles Obtained by Phenotypic and Genotypic Assays

Table 2 shows the correlation between the E-test MIC data and the presence of resistant genes/mutations. From the analysis, a 100% correlation between the E-test MIC data and the PCR data was observed (Table 2). For the isolates which displayed resistance to the antimicrobials tested, the respective molecular resistant determinants were shown to be present. The *tetM* gene was also shown to be present in the isolate (G51) which was classified as being intermediate for tetracycline indicating a potential development of a resistant phenotype. For the one (G206) isolate which displayed susceptibility to ciprofloxacin, the Ser-91 mutation which confers resistance to this antimicrobial was absent, a wild-type *gyrase A* gene was obtained after digestion with *HinfI* (data not shown). The restriction digestion distinguished wild-type from mutant genes as described previously by [2, 3].

Table 2: Correlation between the E-test antimicrobial profiles and presence of genes/mutations associated with penicillin, ciprofloxacin and tetracycline resistance.

Antibiotics tested by E-test method				Molecular determinants associated with resistance		
Isolate	**Ciprofloxacin (>0.06 mg/L)**	**Tetracycline (>1 mg/L)**	**Penicillin G (>1 mg/L)**	***Penicillin producing plasmid***	***tetM gene***	***Ser-91 mutation***
G51	3 mg/L **	1 mg/L *	Complete resistance **	Present	Present	Present
G136	1** mg/L	16 mg/L **	24 mg/L **	Present	Present	Present
G176	1.5 mg/L **	1.9 mg/L **	Complete resistance**	Present	Present	Present
G180	0,50 mg/L **	16 mg/L **	64 mg/L **	Present	Present	Present
G206	0,003 mg/L	32 mg/L **	12 mg/L **	Present	Present	Absent
G247	0,16 mg/L **	3 mg/L **	Complete resistance**	Present	Present	Present

**Intermediate phenotype*

***Resistant phenotype*

Concluding Remarks

Our study was limited in not investigating the genes/mutations associated with susceptibility to cefexime, ciprofloxacin and azithromycin. However, this investigation is planned for the near future. The study was also limited in terms of sample size (6 isolates), future efforts to obtain more pure isolates are planned. However, despite the small sample size, the study was able to provide preliminary data on the correlation between phenotypic and genotypic methods for the detection of antimicrobial resistance in *N. gonorrhoeae*. Due the extensive time (4 days) with culture-based techniques for identifying susceptibility/resistance patterns for *N. gonorrhoeae*, detection of resistance determinants from the molecular level which can be accomplished in 2 days may prove to be more feasible for future antimicrobial studies.

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