Genotypic and phenotypic analysis of *Neisseria gonorrhoeae* for the identification of resistance to various antibiotics in pregnant women attending antenatal care at the King Edward VIII Hospital in Durban, KwaZulu-Natal, South Africa.

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy of Medical Science in Medicine

Nelson R. Mandela School of Medicine, College of Health Sciences, University of Kwazulu-Natal, Durban, South Africa

By:

Glynis Oree

Student number: 218086892

2021

Supervisor: Professor Nathlee S. Abbai

Co-Supervisor: Dr Veron Ramsuran

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PREFACE

The experimental work described in this thesis was conducted at the School of Clinical Medicine Laboratory, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, by Glynis Oree from May 2018 to August 2019, under the supervision of Prof Nathlee. S. Abbai.

This work has not been submitted in any form for any degree or diploma to any tertiary institution, where use has been made of the work by others, it is duty acknowledged in the text.

PLAGIARISM DECLARATION

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PERMISSION TO SUBMIT

As the candidate's supervisor, I Professor Nathlee Samantha Abbai have read the thesis and have given our approval for submission for examination

Supervisor: Professor Nathlee S. Abbai

Discipline: Clinical Medicine Laboratory
School of Clinical Medicine
College of Health Sciences
Nelson R. Mandela School of Medicine
Durban, South Africa

PUBLICATION DECLARATION

The following publications (published, in print and/or submitted) that constitute this thesis are presented here.

1. **Oree, G.**, Naicker, M., Maise, H. C., P., Tinarwo, V., Ramsuran and Abbai, N. S. (2020). Comparison of Methods for the Detection of *Neisseria gonorrhoeae* from South African Women Attending Antenatal Care. *International Journal of STD and AIDS*. **Manuscript has been published. Manuscript ID (IJSA-20-329-R1).**

2. **Oree, G**., Naicker, M., Maise, H. C., Tinarwo, P., & Abbai, N. S. (2021). Antimicrobial Susceptibility Patterns in Neisseria gonorrhoeae Isolated from South African Pregnant Women. *Infectious Diseases in Obstetrics and Gynecology*, *2021*.**Manuscript has been published. Manuscript (ID 6684680).**

3. **Oree, G.**, Naicker M., Maise H. C., Tinarwo, P., Ramsuran, V. and Abbai, N. S. (2021). Tracking antimicrobial resistance in *Neisseria gonorrhoeae* from the molecular level using endocervical swabs. *Journal of Laboratory Medicine.* **Manuscript has been accepted and is in printing. Manuscript ID (LABMED-2020-11-U-SCI-0536)**

4. **Oree, G.**, Naicker M., Maise H. C. and Abbai, N. S. (2021). Comparison of endocervical swabs to cultured isolates for the detection of antimicrobial resistance determinants in *Neisseria gonorrhoeae. Journal of Medical Laboratory Science and Technology of South Africa*. **Manuscript has been published. Manuscript ID (JMLSTSA/66).**

Signed: ______________________________ Date: 27 May 2021

Glynis Oree

LIST OF RESEARCH OUTPUTS:

1. **National conference proceedings**

   a. University of KwaZulu-Natal College of Health Sciences Research Symposium 2019. Emerging Patterns of Drug Resistance to *Neisseria gonorrhoeae* in Pregnant Women from Durban, South Africa. Oree, G., Naicker, M., Unemo, M. and Abbai, N. (Second prize winner in PhD. E-Poster Presentation Category).

2. **International conference proceedings**

   ***All conferences to be attended during 2020, cancelled due to Covid-19**

3. **Other Publications emanating from this study**

   a. **Oree, G.**, Naicker, M., Maise, H. C. and Abbai, N. S. (2020). Excellent Correlation between Phenotypic and Genotypic Assays for the Detection of *Neisseria gonorrhoeae* Antimicrobial Resistance Profiles. *Infotex Journal of Infectious Diseases and Therapy*. *2020*(01), 1-5.

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
µM	Micro-molar
AIDS	Acquired Immunodeficiency Syndrome
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
BHI	Brain-Heart-Infused

CDC	Centres for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CRMNG	Chromosomally mediated resistant *Neisseria gonorrhoeae*
CSW	Commercial sex workers
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic Acid
ESCs	Extended Spectrum Cephalosporins
EUCAST	European Committee on Antimicrobial Susceptibility Testing
g	Grams
g	Gravitational force
GASP	Gonococcal Antimicrobial Surveillance Programme
HIV	Human Immunodeficiency Virus
KZN	KwaZulu-Natal
L	Litre
M	Molar
mg	Milligram
MIC	Minimum Inhibitory Concentration
mL	Millilitres
MLST	Multilocus sequence typing
MSM	Men-who-have-sex-with-men
NAATs	Nucleic Acid Amplification Tests
NG-MAST	*Neisseria gonorrhoeae* multi-antigen sequence typing
NYC	New York City
Opa	Opacity
PABA	*p*-aminobenzoic acid
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PID	Pelvic inflammatory disease
PPNG	Penicillinase Producing Neisseria gonorrhoeae
qPCR	Quantitative Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
SNPs	Single Nucleotide Polymorphisms
STD	Sexually Transmitted Disease

STI	Sexually Transmitted Infection
tRNA	Transfer ribonucleic acid
TRNG	Tetracycline Resistant Neisseria gonorrhoeae
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensively- Drug Resistant

ABSTRACT

Introduction: Worldwide antimicrobial resistance (AMR) is making the clinical management of sexually transmitted infections (STIs) increasingly challenging with a particular emphasis on the emergence of antibiotic resistant strains of *Neisseria gonorrhoeae* (*N. gonorrhoeae*). In the current study, the detection and emerging patterns of drug resistant clinical isolates of *N. gonorrhoeae* to previous and current antibiotics to treat cervicitis pathogens as per syndromic management guidelines was investigated.

Methodology: This cross-sectional study was conducted at King Edward VIII Hospital and included 307 antenatal attendees. Two endocervical swabs were collected from each enrolled woman. Each enrolled women also provided data on socio-demographic, behavioural and clinical factors. The first swab was placed in Amies Charcoal media immediately after collection. This swab was used to confirm the identification of *N. gonorrhoeae* clinical isolates using culture based assays. Culture confirmed isolates were grown in Mueller Hinton Broth and were each adjusted to have a bacterial suspension turbidity of a 0.5 McFarland Standard. These isolates were then subjected to antibiotic susceptibility testing to ceftriaxone, tetracycline, spectinomycin, azithromycin, ciprofloxacin, penicillin G and cefixime using the Etest™ method. The second swab was processed for molecular based assays. Extracted DNA from the second swab was subjected to the TaqMan quantitative Polymerase Chain Reaction (qPCR) assay, an in-house *16S ribosomal RNA (rRNA)* PCR and PCR detection of the *opacity (opa)* gene. DNA extracted from the endocervical swabs and cultured isolates were used for the detection of specific targets (genes/plasmids/mutations) associated with resistance to penicillin, tetracycline, ciprofloxacin, spectinomycin, cefixime, azithromycin and ceftriaxone. All statistical analysis performed in this study was conducted in RS Studio.

Results: The prevalence of *N. gonorrhoeae* was 7.8% (24/307) when detected by the TaqMan qPCR assay and 1.9% (6/307) by culture. When compared to culture, PCR for the *opa* gene and PCR for the 16S *rRNA*, the TaqMan qPCR assay was a more superior assay demonstrating a diagnostic accuracy of 94.5%. Susceptibility testing of the six isolates obtained after culturing showed resistant phenotypes for penicillin G (12 - 64 mg/L), tetracycline (1.9 - 32 mg/L) and ciprofloxacin (1.16 - 3 mg/L). Isolates displayed either dual or triple resistance to these 3 antibiotics. However, all isolates showed susceptibility to spectinomycin (>64mg/L), azithromycin (1mg/L), ceftriaxone (>0.125 mg/L) and cefixime (>0.125 mg/L). This study also detected the resistance determinants associated with

penicillin, tetracycline, ciprofloxacin, spectinomycin, cefixime, ceftriaxone and azithromycin from the molecular level using the primary endocervical swab sample. Gene mutations and plasmids associated with resistance to tetracycline (*tetM* gene carried on a plasmid), penicillin G (penicillinase producing plasmid) and ciprofloxacin (Ser-91 mutation) were detected confirming the results obtained with the susceptibility assays. Resistance mutations associated with the remaining antibiotics were not detected. There was a 100% correlation of cultured isolates and endocervical swabs for detecting the specific AMR determinants conferring resistance to tetracycline, penicillin G, and ciprofloxacin.

Conclusion: The TaqMan qPCR assay has the ability to serve as a future diagnostic assay for the detection of *N. gonorrhoeae*. Despite the lack of resistance to spectinomycin, cephalosporins and azithromycin in our study population, continuous surveillance for emerging patterns of resistance to these antibiotics is still required since they form part of the current South African treatment guidelines. The detection of resistance determinants from the molecular level without the need for culture may prove to be more feasible for future epidemiological investigations focused on tracking antimicrobial susceptibility or resistance patterns in *N. gonorrhoeae*.

1.1. THESIS OVERVIEW

1.1.1. Study design and Methodology

Ethics approval for this study was granted by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN), (ethics number: BE355/18). This cross-sectional study involved the recruitment of pregnant women from the King Edward VIII hospital (KEH) in Durban, South Africa. This study site was chosen since previous studies focusing on pregnant women by the same research group were conducted at this site. Staff and patients were familiar with the studies conducted at this facility which ensured a smooth flow of the current study procedures. KwaZulu-Natal is homage to the largest rural population in South Africa and this study site is a primary health care facility which attracts a large number of people seeking medical care, including pregnant women from numerous regions within the province. The first n=307 women who met the eligibility criteria for the study were enrolled. Pregnant women, 18 years and older, willing to provide written informed consent, willing to provide information on socio-demographic, sexual behaviour and clinical history and willing to undergo a pelvic examination to provide two endocervical swabs were eligible for participation. The pelvic examination and sample collection was performed by a specialist gynaecologist. Women who presented with clinical signs of infection were treated using syndromic management. The two endocervical swabs obtained from each participant were processed and tested at the Clinical Medicine Research Laboratory at the Nelson Mandela School of Medicine.

A simple illustration and description of the methodology that was employed throughout this study is displayed in Figure 1.

Informed consent and questionnaire presented to each patient. Followed by gynaecological examination for collection of endocervical swab 1 and 2

Swab 1 placed in Amies charcoal media (LASEC, South Africa) and transported to laboratory for streaking onto New York City (NYC) agar plates (48 hrs at 37°C with 5% CO_2).

Positive colonies sub-cultured onto NYC plates (24 hrs, at 37°C with 5% CO_2). Suspected positive isolates subjected to Gram's staining, microscopy, catalase test, superoxol test and oxidase test.

Confirmed isolates grown on Chocolate Agar plates (24 hrs, at 37°C with 5% CO_2).

Bacterial lawn was scraped into 1 mL Brain-Heart-Infused Broth (LASEC, South Africa) (30mins, at 37°C with 5% CO_2).

Isolation of genomic DNA using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, United States)

Perform PCR with specific primers to track antimicrobial resistance for presence of molecular determinants

Sequencing of PCR amplicons to confirm identity and detect mutations

Colonies suspended in Mueller Hinton Broth (LASEC, South Africa) (McFarland 0.5)

Antimicrobial susceptibility testing using the Etest™ method BioMérieux, France) on Chocolate agar.

Swab 2 placed in 2 mL phosphate-buffered saline and transported to laboratory.

DNA extraction using the PureLink Microbiome DNA Purification kit (ThermoFisher Scientific, United States).

Validation of TaqMan qPCR method for cross-reactivity using different *Neisseria* strains

Detection of pathogen using TaqMan qPCR, conventional *16S rRNA* PCR, *opa* PCR and culture

Limit of detection tests conducted on TaqMan qPCR, conventional *16S rRNA* PCR and *opa* PCR for the detection of *N. gonorrhoeae.*

Sequencing of PCR amplicons to confirm identity

Detection of pathogen using TaqMan quantitative polymerase chain reaction assay, specifically for *N. gonorrhoeae* (Assay ID: Ba04646252_s, ThermoFisher Scientific, United States)

Perform conventional PCR on TaqmMan positive samples with specific primers to track antimicrobial resistance by presence of molecular determinants

Sequencing of PCR amplicons to confirm identity and alignment

Perform real time PCR on TaqmMan positive samples with specific primers to track antimicrobial resistance by presence of molecular determinants

Figure 1: Diagrammatic representation of overall methodology

1.1.2. Structure of the Thesis

The structure of the thesis is in accordance with guidelines that have been specified by the College of Health Sciences at the University of KwaZulu-Natal for the submission of the thesis by manuscript. Each of the manuscripts are formatted in accordance with the guidelines stipulated by the journals to which they have been submitted. The thesis includes the following chapters:

Chapter 1: This chapter includes a description of the rational of the study together with a presentation of a compact yet detailed review of literature relevant to this study. The prevalence and adverse effects of *N. gonorrhoeae* in pregnant women is briefly described. The epidemiology, physical characteristics, transmission, the clinical manifestation of *N. gonorrhoeae* and diagnostic tests used to detect this pathogen is reported. Previously reported antimicrobial treatments, current treatments and mechanisms of resistance to these antimicrobials are discussed in this chapter.

Chapter 2: The manuscript entitled "**Comparison of methods for the detection of *Neisseria gonorrhoeae* from South African women attending antenatal care**" was accepted by the International Journal of STD and AIDS (IJSA-20-329-R1). This chapter details the detection of *N. gonorrhoeae* using the commercially designed TaqMan quantitative Polymerase Chain Reaction (qPCR) assay, culture, an in-house *16S rRNA* PCR and PCR detection of the *opa* gene. This comparative study evaluated the sensitivities and specificities in association with each assay type.

Chapter 3: Currently, there is limited data on *N. gonorrhoeae* susceptibility patterns in pregnant populations from KwaZulu-Natal, South Africa. This chapter provides data on susceptibility patterns to penicillin G, tetracycline, ciprofloxacin, azithromycin, spectinomycin, cefixime and ceftriaxone in pregnant women from our setting. Despite the many *N. gonorrhoeae* AMR studies conducted in South Africa, there is no published data on *N. gonorrhoeae* AMR in pregnant populations, thereby lending novelty to this study. The entitled manuscript "**Antimicrobial susceptibility patterns in *Neisseria gonorrhoeae* isolated from South African pregnant women**" in this chapter is accepted by the Journal of Infectious Disease and Obstetrics (ID 6684680) and is currently under review.

Chapter 4: The manuscript presented in this chapter, investigated the resistance determinants associated with penicillin G, tetracycline, ciprofloxacin, spectinomycin, cefixime, ceftriaxone

and azithromycin from the molecular level using DNA extracted from the primary endocervical swab sample. To the best of our knowledge, there is no published South African study that has used this approach to determine patterns of resistance, especially in pregnant women. This manuscript has been accepted by the journal, Laboratory Medicine (LABMED-2020-11-U-SCI-0536) and is entitled as "**Tracking antimicrobial resistance in *Neisseria gonorrhoeae* from the molecular level using endocervical swabs**".

Chapter 5: Detection of resistance targets and mutations without the need for culture serves as a much more attractive alternative for AMR studies. This chapter compared the primary swab to the cultured isolates for the detection of *N. gonorrhoeae* AMR determinants. To date, there have been no published South African studies that have investigated the sensitivity of the primary endocervical swab against culture for the detection of AMR determinants in this pathogen. This chapter showed that tracking AMR from the molecular level may be a more feasible method for the future. The manuscript in this chapter is entitled "**Comparison of endocervical swabs to cultured isolates for the detection of antimicrobial resistance determinants in *Neisseria gonorrhoeae***" and has been accepted by the Journal of Medical Laboratory Science and Technology of South Africa (JMLSTSA/66).

Chapter 6: The final chapter is a general discussion of the study which focuses on the key findings in association with previously published studies. This chapter discusses limitations, future recommendations and conclusions derived from this study.

CHAPTER 1

1.2. INTRODUCTION

1.2.1. Background on *Neisseria gonorrhoeae*

Gonorrhoea is a sexually transmitted infection (STI) which is caused by the presence of the bacterial pathogen *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and is the second most common STI globally (1–4). The infection rate of this STI has significantly increased globally and has become a public health concern that requires immediate attention on a worldwide scale (5). A global STI surveillance in 2018 which was conducted by the World Health Organization (WHO) revealed an estimated 87 million new gonorrhoea infections globally during 2016, with an incidence of 20 cases per 1000 population (uncertainty interval 14–28) in women (6). Studies conducted in female populations in Africa reported a prevalence of 2.3% with an incidence of 49.7 cases per 1000 and approximately 17 million new cases of this infection in sub-Saharan Africa (7,8). Previous studies conducted in South Africa reported prevalence rates of 3 to 11%, with a higher incidence of 10-31% in women attending STI clinics and sex workers (9–12). A recent study conducted in the KwaZulu-Natal province of South Africa reported prevalence rates of *N. gonorrhoeae* ranging from 3 to 5.4% in female populations (9,10).

Neisseria gonorrhoeae is known to have a short incubation period and high transmission efficiency subsequently promoting the transmission and acquisition of other STIs, including Human Immunodeficiency Virus (HIV) infections (1,5,13–15). Gonococcal infections have severe implications on male and female reproductive health (16,17). This STI is often associated with adverse pregnancy outcomes and detrimental effects to neonates (3,17–22). Most developing countries rely on the syndromic management of this STI, which creates a limitation with a high number of infections going undiagnosed (3,9). Another contributing factor is that low resource settings often lack diagnostic testing which often leads to asymptomatic infections going undetected and untreated, especially in women (3,23). The inability to treat these asymptomatic infections results in long-term clinical complications and ongoing transmission of this STI (23).

The syndromic management of *N. gonorrhoeae* leads to the inappropriate treatment of un-infected and infected individuals consequently increasing costs through misdiagnosis and forms a basis for antimicrobial resistance (AMR) (9). Infections caused by *Neisseria*

gonorrhoeae can be diagnosed by using inexpensive conventional techniques such as culture (3,24,25). However several limitations are associated with conventional culture techniques, such as the invasive collection of clinical samples (endocervical/ urethral), the loss of viability of the gonococcus during transportation, stringent growth requirements and trained clinicians to make the diagnosis (3,24–26). Other non-culture assays, such as Nucleic Acid Amplification Tests (NAATs) are highly efficient, detect non-viable isolates and have the ability to detect multiple pathogens simultaneously (24,26,27). This study provided data on the ability of the TaqMan qPCR assay to detect a higher proportion of *N. gonorrhoeae* positive samples when compared to culture. This assay is therefore more feasible to have in a clinical setting so that infections are detected in a shorter time so that treatment can commence.

Antimicrobial resistant *N. gonorrhoeae* infections have posed as a huge public health hazard over the past 80 years (2,17,28). Quinolones were previously recommended as a first line of treatment for *N. gonorrhoeae* infections, however, it was reported that in 2003 there was a sudden resistance of *N. gonorrhoeae* towards ciprofloxacin with a 22% prevalence (18,29). *N. gonorrhoeae* isolates have shown decreased susceptibility towards third generation cephalosporins, with resistance towards cefixime and ceftriaxone (4,18). This raises concerns since *N. gonorrhoeae* infections may become untreatable in the coming years (17,28). Worldwide AMR is making the clinical management of STIs increasingly challenging with a particular emphasis on drug resistant *N. gonorrhoeae* (28). Antibiotics were previously used for successful treatment of *N. gonorrhoeae* infections, however AMR has emerged globally over the years with various molecular resistance mechanisms that evade previous and even some of the current antibiotics used for treatment of infections (2,4,5,30). The identification and detection of AMR resistance is crucial for the global surveillance of *N. gonorrhoeae*, but poorly developed health systems in some countries creates limitations (2).

Currently a gap in *N. gonorrhoeae* susceptibility data exists, particularly in areas that have a higher prevalence of diseases (3). Determining patterns of resistance regularly is critical for monitoring any emerging resistances that may occur at an early stage, which creates a time frame in which standardized treatment measures can be created for developed and developing countries (3). Different methods are employed worldwide for susceptibility testing depending on the availability of resources and laboratory settings (31). Agar dilution, disc diffusion and Etest methods are all used for the determination of *N. gonorrhoeae* susceptibility, but

minimum inhibitory concentration (MIC) values obtained may differ across the different methods (3,5,24,31). These methods are subject to the availability and cost of reagents to low and high income laboratories (26,31). Whichever methods that are currently employed, data generated in different laboratories must be comparable in order to assess local, regional and global trends in the emergence and spread of resistance (31). Susceptibility data obtained for *N. gonorrhoeae* using these methods in various laboratories are required to be comparable to determine trends in emergence and patterns of resistance (13). Classification of susceptibility and resistance isolates can be determined by the MIC expressed in mg/L, with necessary controls. Reference culture strains have also been established and are used to categorize samples as susceptible, intermediate or resistant using MIC data obtained from the Clinical Laboratory Standards Institute (CLSI) guidelines or with the European Committee on Antimicrobial Susceptibility Testing breakpoints (EUCAST) (32). Currently, NAATs have gained attention on a global scale and have replaced the traditional use of culture for the continuous surveillance of AMR resistance and susceptibility in numerous countries (5,33,34).

The detection of specific genetic markers are used as predictors of AMR and can be used to enhance surveillance and improve individual patient treatment (5,33,34). During 2016, the WHO phenotypically and genotypically characterized gonococcal reference strains for quality assurance and control (33). However, it has been reported that molecular methods are only able to detect existing AMR determinants and therefore cannot completely replace phenotypic assays due to their inability to detect new AMR determinants that may develop in the future (33). Advances in molecular technology can be used in the future to complement phenotypic testing and monitoring of the spread of AMR more rapidly (34).

Presently, in South Africa, antimicrobial resistant *N. gonorrhoeae* infections are a huge public health issue since there are concerns that gonococcal infections may be become untreatable in the coming years. In the current study, emerging patterns of drug resistant clinical isolates of *N. gonorrhoeae* to various antibiotics currently used to treat cervicitis pathogens as per syndromic management guidelines were investigated. In addition, the study identified AMR using phenotypic and genetic determinants. This study was novel since it provided data on antimicrobial susceptibility patterns to a range of antibiotics used against *N. gonorrhoeae* in a population of pregnant women, a currently under-researched area in South Africa. This study also showed that due to the limitations associated with culture based

techniques (such as loss of viability of the gonococcus) for identifying susceptibility/resistance patterns in *N. gonorrhoeae*, detection of resistance determinants from the molecular level without the need for culture may prove to be more feasible for future epidemiological investigations focused on tracking antimicrobial susceptibility/resistance patterns for this pathogen.

1.3. REVIEW OF LITERATURE

1.3.1. Background

Gonorrhoeae is a STI defined by the presence of the pathogenic bacterium *N. gonorrhoeae* (2,35–37). Albert Neisser first discovered this pathogen in 1879 by Gram-staining followed by microscopy of urethral discharge and only successfully cultured this organism in a laboratory in 1882 (38). This STI has gained the attention of researchers globally due to the increased prevalence of this STI in the recent years as well as the pathogen's remarkable ability to acquire antibiotic resistance to previous and current antibiotics (2,35–37).

1.3.2. Epidemiology

The WHO reported an estimated incidence of 78 million new cases of *N. gonorrhoeae* in 2012 (7). In 2018, the WHO conducted a global surveillance which revealed in 2016, an increase to 87 million new infections had occurred in adolescents and adults between the ages of 15 to 49 years old with the highest incidence obtained for sub-Saharan Africa (5,6). High rates of gonococcal infections have been observed over the past few years and various factors have been identified that contribute to the epidemiological diversity and manifestation of *N. gonorrhoeae* (35). These factors include geographical distribution, prevalence of gonococcal infections, sexual orientation, sexuality, sexual preference (interracial or ethnicity), sexual preference by risk category (sexual activity with individuals from high risk groups), access to sex education, economic status and access to testing and treatment (39–43). Additionally, increases in the incidence of gonococcal infections in high-resource settings are due to reckless sexual behaviour due to the availability of antiretroviral treatment for HIV and the misconception that HIV is not life-threatening, leading to lucid sexual behaviour without the use of condoms, increased number of casual sex encounters, increased travel, larger sexual networks and access to sexual services and social media dating services (35,43). Alternatively, factors such as the use of drugs within high-risk sexual networks such as, men-who-have-sex-with-men (MSM) and sex workers contribute to the spread of infections (35).

In recent years, specific population groups have been identified of being at a higher risk of contracting gonococcal infections including MSM, sex workers, migrants and the youth (35). Developed countries such as the United States of America showed a rapid increase in *N. gonorrhoeae* infections by 67% from 2013 (n =333510) to 2017 (n =556413) (44). The prevalence of gonococcal isolates that have been cultured from MSM during the 1990s was 3.9% and drastically increased to 38.5% in the year 2017, supporting the observation that there were changes in sexual behaviour (43). In Southern and Eastern Africa, the prevalence of gonorrhoeae was observed to be 1.7%, however within the same study, it was reported that high risk groups such as sex workers had a higher prevalence of 8.2% for gonococcal infections (45). Third world countries such as South Africa showed that the prevalence of *N. gonorrhoeae* infections in females aged between 15 to 24 years old in clinical community settings was approximately 4.6% (45). Other studies conducted in South Africa have reported prevalence rates for *N. gonorrhoeae* infections in women ranging from 3% -11% in women (9,10,18).

1.3.3. Characteristics of *Neisseria gonorrhoeae*

Gonococcal disease is defined by the presence of *N. gonorrhoeae*, an obligate bacterial pathogen in human samples (16). *N. gonorrhoeae* is a Gram- negative, diplococcal anaerobe that descends from the Neisseriaceae family, and falls in the genus *Neisseria* (35). Humans are the natural hosts for this pathogen and are infected by the colonization and invasion of mucosal surfaces such as the transitional, columnar and squamous epithelia (35). This pathogen comprises outer membrane proteins, lipooligosaccharides, phospholipids and a range of other proteins which facilitate adherence, invasion and resistance towards host cells (46). The pathogen is surrounded by filamentous pili that contribute to the movement along surfaces and adherence of the pathogen, subsequently increasing bacterial pathogenesis (47).

1.3.3.1. Clinical manifestations of *Neisseria gonorrhoeae* in males and females

Gonococcal disease is associated with urethritis and cervicitis in women which is commonly related with a burning sensation accompanied by abnormal urethral or vaginal discharge, respectively (5). Gonococcal infections are also acquired through the mucosa of the eyes, oropharynx and anorectum (3). Endocervical, anorectal, urethritis and pharyngeal infections often go untreated due to their asymptomatic behaviour consequently leading to potentially harmful complications including: infertility, urethral narrowing and epididymitis in males, pelvic inflammatory disease (PID), cervicitis, salpingitis, endometritis, infertility, preterm

rupture of membranes and perihepatitis in females (13,48). This pathogen has a short incubation period with a high transmission efficiency subsequently increasing HIV transmission and acquisition (1,5,15).

1.3.3.2. Clinical manifestations of *Neisseria gonorrhoeae* in neonates

In South Africa, asymptomatic infections occur in almost 50% of STI-infected women who do not seek care and therefore go untreated, directly affecting reproductive and child health (9). These complications often lead to other adverse pregnancy outcomes identified as corneal perforations or blindness, foetal growth retardation, low birth weight, spontaneous abortion, stillbirth, post-partum endometritis, prematurity and increased risk of HIV transmission from mother to child (1,3,5,13,14,17–22). A recent study reported that the maternal to child transmission of HIV is greater in females that are co-infected with *Chlamydia trachomatis* and *N. gonorrhoeae*, with approximately 4000 global annual births outcomes with infants being blind (19). Antenatal care provides an excellent platform to screen for STIs such as *N. gonorrhoeae*, so preventable measures can be employed to reduce adverse outcomes. Research suggests that antenatal screening for STIs is a critical and feasible aspect to consider for the reduction of adverse pregnancy outcomes and to ensure that pregnant women receive appropriate treatment (49–51).

1.3.4. Diagnosis of *Neisseria gonorrhoeae*

Diagnostic testing for the presence of this pathogen can be conducted using several types of tests such as microscopy, culture, nucleic acid-based tests and antigen detection (11,13,24,26,27,52,53). However, each type of test requires different skill sets and techniques for optimal performance which determines the time between testing and the final result (13). Each approach has advantages and disadvantages. Every diagnostic test is dependent on their performance which is determined by sensitivity and specificity calculations, with positive- and negative-predictive values (13).

1.3.4.1. Culture based tests

A large number of laboratories in resource-limited settings make use of conventional, sensitive and inexpensive culture techniques on selective media (54). Presumptive identification and confirmatory tests are used to determine the gonococcus strain present such as carbohydrate utilization tests, oxidase, catalase and superoxol tests (116). Culture was previously considered as the "gold standard" for the detection of gonorrhoeae infections and

the retention of the pathogen by culture is crucial for AMR testing (3,13,24,54). However, the improper management of samples, poor growth and loss of viability of the pathogen results in sensitivities ranging from 85 to 95% for acute infections and declining further to 50% for chronic infections in females by culture (54).

1.3.4.2. Nucleic acid amplification tests

Nucleic acid amplification tests (NAATs) have replaced culture in many laboratories and is currently considered as the "gold standard" (24). NAATs offer the rapid detection of *N. gonorrhoeae* in a single visit from a patient, decreased sample handling resulting in decreased contamination (24,26,27). NAATs have been shown to have higher sensitivities and specificities for the detection of *N. gonorrhoeae* (54). The drawback of NAATs is the lack of detection of AMR and some polymerase chain reactions (PCRs) systems are costly and subject to availability in resource-limited settings, however NAATs have been previously described as an exceptional platform for the detection of *N. gonorrhoeae* in clinical samples (24,26,27). These types of tests include different types of PCRs such as 16S PCRS, PCR assays based on 11 to 13 Opacity (Opa) functional proteins found in *N. gonorrhoeae* strains and ligase chain reactions that were previously used (11,24,26,27,52,53). The *opa* genes are multi-copy genes that harbour conserved regions and encode proteins with physiological functions and are regarded as suitable target sequences for a real-time PCR amplification assay (27). Many Food and Drug Administration (FDA) approved commercial assays are available, however these multiplex assays are costly and simultaneously detect for other STIs in combination with *N. gonorrhoeae* (26,52,53,54,). Other accurate methods includes *N. gonorrhoeae* multi-antigen sequence typing which is employed for the identification of different strains associated with specific populations and Whole Genome Sequencing (WGS) which provides information on local and global epidemiology of gonorrhoeae infections (25,57).

1.3.5. Antimicrobial susceptibility testing

Regular surveillance of antimicrobial susceptibility is implemented for monitoring the susceptibility and resistance patterns to antibiotics that are used for the treatment of this pathogen (3). This provides information about emerging patterns of resistance which assists in the adjustment of the recommended treatments, before treatment failures become problematic (48). Antimicrobial susceptibility testing is conducted in populations groups of regions that have a representative site to recommend treatment regimes, instead of individual

patient management (13,15,58). The Gonococcal Antimicrobial Surveillance Programme (GASP) in partnership with the WHO has laboratories based globally for AMR surveillance (15).

1.3.5.1. Agar Dilution method

The agar dilution method is recognised as the "gold standard" and semi-quantitatively determines MICs of antimicrobials in µg/ml (13,31). This method is not recommended for regular AMR screening of small number of isolates, as it is a lengthy process (5). The Etest method produces comparable data to the agar dilution technique and the Etest method is frequently used although this method is costly, it is a less laborious technique (5).

1.3.5.2. Etest method

The Etest method is a semi-quantitative technique that determines MICs in mg/L or µg/ml (13). This technique utilizes plastic strips that have a MIC scale imprinted on them and the strip is impregnated with a predefined concentration gradient of the antibiotic (13,59). This Etest strip is placed onto agar medium containing *N. gonorrhoeae* and the antibiotic subsequently diffuses in the medium (13). The MIC is determined by the zone of clearance which is read off the gradient scale from the strip (13,59).

The WHO does not recommend disk diffusion methods for antimicrobial susceptibility testing (AST) as appropriate standardization and quality control has not been defined (5,13,32). However this qualitative method can only be used in resource-limited settings where there is no access to MIC determination techniques (31).

1.3.5.3. Molecular determinants

Currently NAATs have not yet been approved for the detection of AMR and are not commercially available (5,13). However, many in-house molecular assays have been developed for the detection of mutations and resistant genes (5,33,60). The main drawback with this method is that this type of molecular testing detects targets that are known and new mutations in the future will not be detected (33). This will require the development of new assays and targets regularly and is not feasible (33). Many advancements with molecular assays for the detection of *N. gonorrhoeae* AMR have been made, however many challenges have to be overcome before they can be approved (5,33).

1.3.5.4. Sequencing platforms

Currently, data generated from WGS, genotyping methods such as multilocus sequence typing (MLST) and *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) are employed to determine the molecular epidemiology, clonal relationships and genetic lineages to control the spread of AMR genotypes of *N. gonorrhoeae* (61). WGS is an accurate method that is employed and has the ability to establish differences and similarities between patients with related isolates and the potential to shed light on the local and global epidemiology of gonorrhoeae (25). The analysis of several housekeeping genes is used for the MLST method for strain typing of *N. gonorrhoeae* and *N. meningitidis* (61). The most feasible method is NG-MAST which is based on the analysis of two loci (*porB* and *tbpB*) which encode the PIB porin and has the ability to identify *N. gonorrhoeae* current and previous isolates that have been analysed (57,61,62). These methods are used predominantly in high-resource settings as they are costly (61). Other inexpensive methods include portable nanopore sequencing devices such as MinION. This nanopore platform provides high yield, high throughput benchtop sequencing, low cost access to the benefits of long-read and real-time DNA sequencing (162). However a limitation associated with this method is the low accuracy and consistency compared to the Illumina and PacBio sequencing reads (162).

1.3.6. Treatment of *Neisseria gonorrhoeae*

The majority of developing countries worldwide have a high prevalence of HIV infections especially in women (63). Sexually transmitted diseases increase the likelihood of acquiring HIV infections and play a critical role in the spread of HIV infections (9,63). This validates that the detection of *N. gonorrhoeae* is critical in areas with high prevalence of other STIs and HIV infections. STIs are declared to be more prevalent in developing countries and the recommendation for the use of the syndromic management of STIs in resource-limited countries was declared by the WHO in the 1990s (64).

1.3.6.1. Syndromic management

The syndromic management of *N. gonorrhoeae* remains the foundation for STI treatment in numerous countries around the world, however, there are several limitations associated with this approach. The syndromic approach is based on the treatment of a constellation of symptoms and therefore fails to diagnose asymptomatic infections which go untreated (9). Studies that have been previously conducted reported that only 5 to 30% of females with trichomonas, chlamydia or gonococcal infections are symptomatic (65–67). This

demonstrates that this approach has poor sensitivity and specificity and misses asymptomatic infections (18,21,68). The syndromic approach was implemented in South Africa at primary health care centres based on the identification of particular clinical manifestations and the following syndromes (1) vaginal or urethral discharge and (2) genital ulcer syndrome (17,29,69). However syndromic management also leads to the over-treatment of females who suffer from vaginal discharge due to the derangement of the vaginal microbiome (9). This consequently increases costs through misdiagnosis and forms a basis for AMR (9).

1.3.6.2. History of treatment for *Neisseria gonorrhoeae* infections

Treatment of STIs such as gonorrhoeae with antimicrobials or antibiotics was successful in the past, but currently an increase in resistance exists towards a range of antibiotics including penicillins, sulfonamides, tetracyclines, quinolones and macrolides (18,34) as shown in Figure 1.

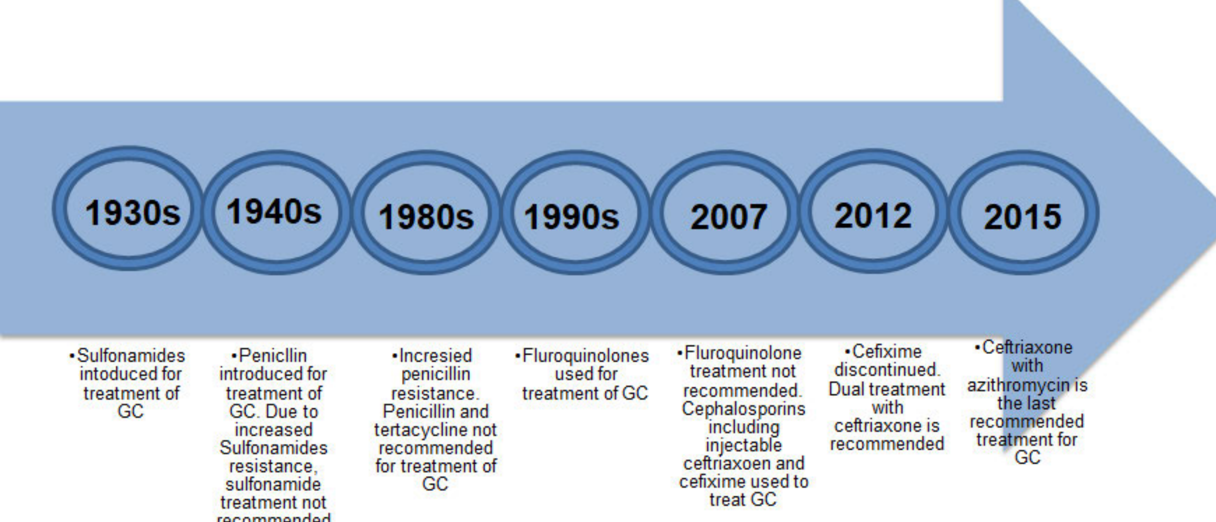

Figure 2: Diagram representing the timeline on antibiotics used for the treatment of Gonorrhoea (Adapted from; Latest data on Antibiotic Resistant Gonorrhoeae, Centres for Disease Control, 2016).

1.3.6.2.1. Treatment with sulfonamides

Sulphonamides were discovered by Gerhard Domagk during 1935 and were the first type of treatment to be used for *N. gonorrhoeae* infections during the 1930s (29,70). These antibiotics compete with *p*-aminobenzoic acid for the enzyme dihydropteroate synthetase (DHPS) during the synthesis of folic acid (71). Sulfanilamide previously treated 80 to 90% of gonorrhoeae infections, followed by sulapyridine from 1940 to 1941 (70). Sulfonamides

rapidly developed a 75% failure in treatment by 1944; however, a combination of sulfonamides with trimethoprim was later used during the 1960s for improved efficacy (72). Trimethoprin was used for inhibition of alternate reactions catalysed by the dihdrofolate reductase enzyme (2).

1.3.6.2.2. Treatment with penicillin

Penicillin was discovered by Alexander Fleming during 1928 and the mechanism of penicillin inhibited cell wall synthesis by binding to penicillin-binding proteins in the periplasm (5,73). However, penicillin was used for the treatment of urethritis and was only introduced as an antibiotic for the treatment of gonorrhoeae infections in 1943 and quickly replaced sulfonamides in cases of treatment failure and was shown to treat over 95% of gonorrhoeae cases (74). During the next two decades decreased susceptibility was observed towards penicillin and doses were gradually increased for treatment (75). This increase in dose resulted in gonococcal strains increasing resistance and increase in treatment failures (75). In 1976, β-lactamase plasmids which originated from Southeast Asia and sub-Saharan West Africa were found in the United States and United Kingdom, these strains exhibited high levels of resistance to penicillin consequently discontinuing the use of penicillin to treat infections (76,77).

1.3.6.2.3. Treatment with tetracycline

Tetracycline, previously known as chlortetracycline (aureomycin) was discovered by Benjamin Minge Dugger and was used as an alternative treatment for patients who were treated with penicillin for gonorrhoeae infections (5,73). Tetracycline was described as the first antimicrobial to inhibit protein synthesis by binding the 30S ribosomal subunit (78). Resistance to tetracycline had evolved over a period due to resistance determinants and acquisition of plasmids with a resistant gene consequently excluding tetracycline from treatment regimens globally (5,79).

1.3.6.2.4. Treatment with spectinomycin

Spectinomycin was first discovered in the 1960's and was used for the treatment of gonorrhoeae subsequent to the emergence of plasmid-mediated resistance towards penicillin (80,81). Spectinomycin is responsible for the inhibition of protein synthesis and elongation by binding to the 30S ribosomal subunit subsequently altering translocation (3,37). The first case of spectinomycin resistance was reported in Netherlands in 1967 followed by the

Philippines in 1981, London and the United Kingdom in 1983 (82,83). This led to the discontinuation of spectinomycin as the first-line monotherapy. A recent study conducted in South Africa showed that spectinomycin effectively treats gonococcal infections; however spectinomycin is unavailable and not used in many countries including South Africa due to high costs and fear of resistance if this antibiotic is introduced (2,5,69).

1.3.6.2.5.. Treatment with quinolones

During the 1960s George Lesher and colleagues discovered synthetic quinolones (5). Quinolones alter the deoxyribonucleic acid (DNA) *gyrase* and *topoisomerase IV* and forms drug-enzyme DNA complexes consequently releasing breaks in double stranded DNA (2,84). Fluoroquinolones are a broader-spectrum of quinolones which include ciprofloxacin and ofloxacin for the treatment of *N. gonorrhoeae* during the 1980s onwards (5). The recommended dose of ciprofloxacin was 250 mg which was further increased to a single dose of 500 mg when decreased susceptibility was observed (48,85). Reduced susceptibility was reported in London during 1989 followed by treatment failures in the 1990s but treatment with this antimicrobial was continued globally for an additional 10-25 years, varying globally (2). The CDC recommendation in 2007 was to discontinue ciprofloxacin for the treatment of gonococcal infections (8).

1.3.6.2.6. Treatment with macrolides

Macrolides were discovered in 1952, with the development of the synthetic derivative of erythromycin referred to as azithromycin in the year 1980 (5). This antimicrobial engages with the P site of the 50S ribosomal subunit resulting in the inhibition of the peptidyl transferase polypeptide chain elongation (2,5,86). Studies which were previously conducted illustrated that erythromycin is not as effective as azithromycin for the treatment of gonorrhoeae infections (87,88). Countries which frequently used azithromycin for gonococcal treatment, reported a decrease in susceptibility and AMR toward this antibiotic during the 1990s (86). However, despite being used in many countries azithromycin monotherapy is not recommended due to adverse effects associated with 2 g of the oral consumption of this antibiotic and rapid resistance that may develop (87,88). A study conducted in 2013 investigated the use of azithromycin as a single dose and illustrated that this antibiotic should be used together with third generation extended spectrum cephalosporins (ESC) for dual-therapy (89–91). Patients who have an allergic reaction to azithromycin can alternatively use doxycycline (5,14).

1.3.6.2.7. Treatment with cephalosporins

Cephalosporins are derived from *Cephalosporium acremonium* and were originally discovered by Giuseppe Brotzu in 1948 (5). The chemical modification of this fungus has generated many useful compounds such as cefixime and ceftriaxone for the treatment of *N. gonorrhoeae* infections (5,14,48). Cefixime is administered orally with a 400 g dose and was introduced prior to ceftriaxone (2). The world's first extensively-drug resistant (XDR) *N. gonorrhoeae* strain was documented to exhibit high levels to both cefixime and ceftriaxone and reported to be from a commercial sex worker (CSW) in Japan (4). The two first cases to exhibit AMR to cefixime in South Africa was during the year 2012 in the MSM population group (2,4). The two patients showed symptoms of persistent urethral discharge and genetic characterisation revealed that one of the two strains (ST1901) was identical to the strain which was associated with XDR in Japan, showing that these resistant clones were spread globally (92). The MSM population group is often associated with the spread of AMR gonorrhoea due to their risky sexual behaviour, participation in global sexual networks and having asymptomatic gonococcal pharyngeal infections (93). The WHO then endorsed a single dose of ceftriaxone (250 mg) as this antibiotic displayed enhanced antimicrobial activity towards this pathogen by having a high binding efficiency, and forms binding complexes to the peptidoglycan transpeptidase 2 (PBP2), preventing crosslinking of PBP2 (2). Additionally, dual therapy of cefixime or ceftriaxone with oral azithromycin/doxycycline (1 g stat) to limit the spread of XDR strains in high risk population groups such as MSM and CSW was advocated (15,69). Recently, AMR towards ceftriaxone-azithromycin dual-therapy has been reported in the United States in a heterosexual man and was genotypically characterized as strain ST1901 and a new ST12133, which was also associated with XDR in Japan (94).

1.3.6.2.8. Future treatment of Neisseria gonorrhoeae infections

Several antimicrobials and compounds have been recently evaluated against ESC-resistant and XDR *N. gonorrhoeae* isolates. These antimicrobials and compounds include fluoroketolide solithromycin, ofertapenem, tigecycline, lipoglycopeptide dalbavacin, gentamicin (single dose of 240 mg, intramuscularly) and gemifloxacin (single dose of 320 mg orally) in combination with 2 g of oral azithromycin (95–99). However, these novel antimicrobials and compounds require further attention including *in vivo* testing, safety,

toxicity, efficacy, optimal dosage, costs and physical and chemical characterisation for treatment of gonococcal infections (35).

1.3.7. Molecular determinants of antimicrobial resistance

N. gonorrhoeae has the remarkable ability to alter its genetic material through the partial or complete transfer of genes via transformation, conjugation and recombination consequently changing its genome by the acquisition of various mutations (5,37). This pathogen has the ability to acquire antigenic variability through obtaining genetic material from other microorganisms thereby contributing to its mechanisms of resistance (37). This pathogen has evolved and acquired resistance mechanisms to nearly all the recommended antimicrobials used for treatment (5). Antimicrobial determinants are predominantly chromosomal, however the bla_{TEM} and *tetM* genes which are responsible for AMR to penicillin and tetracycline are plasmid borne in this pathogen (3,37).

1.3.7.1. Sulfonamide resistance

N. gonorrhoeae resistance towards sulfonamides is associated with the excessive production of *p*-aminobenzoic acid consequently diluting and reducing effects of the antimicrobial (5,35,71). This alters and leads to mutations in the *foIP* gene which specifically encodes the antimicrobial target DHPS (5,35,71). Alterations in DHPS reduces the affinity for sulfonamides ultimately resulting in AMR towards this class of antimicrobials (5,35,71).

1.3.7.2. Penicillin resistance

The overuse of penicillin in the past few decades led to the development of penicillinase-mediated resistance (PPNG) (5). The *penA* gene targets PBP2 and mutations in this gene is identified as a single amino acid insertion D345 in PBP2. In addition, mutations at the carboxyl-terminal of PBP2 consequently reduces PBP2 acetylation (5,87). The *mtrR* gene encodes for an efflux pump and deletion mutations in the promoter region of the *mtrR* gene or a G45D substitution in this gene results in the overexpression and a higher efflux from the MtrCDE efflux pump (2,3,5,37). Mutations in the third loop of porB1b from a single-nucleotide polymorphism (SNP) alter the activity of the *penB* gene that encodes porins and results in the reduced influx of hydrophilic antibiotics (71). The *penB* phenotype is only presented in isolates which exhibit *mtrR* resistance (71). Similarly, mutations in *pilQ* are also associated with the reduced influx of antimicrobials (1,5,100). SNPs in *ponA*, which encodes the penicillin binding protein 1 (PBP1) has been associated with a decrease in PBP1

acetylation (37). The penicillinase producing *N. gonorrhoeae* (PPNG) occurs through conjugation in *N. gonorrhoeae* and contains β-lactamase TEM-1 or TEM-135 which encode for various plasmids including the African, Johannesburg, Asian, Toronto, Nîmes and New Zealand plasmids (76,77,101). The function of these β-lactamase containing plasmids are to hydrolyse the β-lactam ring of penicillin and renders the antimicrobial inactive (5). A recent study conducted in Johannesburg, South Africa revealed that the Toronto plasmid was most present (44.4%) in the investigated population, followed by the African-type (35.2%) and Johannesburg-type (20.3%) (102). The Asian (4.9 kb), African (3.1 kb) and Toronto (2.6 kb) are frequently associated with epidemic outbreaks (102).

1.3.7.3. Tetracycline resistance

Genes which are associated with chromosomally mediated resistance in *N. gonorrhoeae* (CMRNG) to penicillin have the ability to confer tetracycline resistance such as mutations in *mtrR*, *penB*, *pilQ* and substitution mutations in PorB5 (2,5). The 30S ribosomal protein rpsJ modulates tetracycline affinity for its binding site (2). Mutations in the rpsJ protein reduce the binding affinity of tetracycline to the ribosome (2). Tetracycline resistant *N. gonorrhoeae (TRNG)* encoded by the *tetM* gene is harboured by the American and Dutch variants (102–104). *TetM* binds to the 30S ribosomal subunit and inhibits any tetracycline binding to the target. Research studies which have been conducted suggests that the Dutch variant has a higher prevalence in Asian countries and the origin of the American plasmid was from the African continent (105). This is supported by a study which was conducted in a South African male population group which reported a high prevalence of the American type *tetM* plasmid and a lower prevalence of the Dutch type *tetM* plasmid (106). A more recent study conducted in South Africa recently reported the detection of 92% of the isolates with a 90% predominance of the American variant (107,108).

1.3.7.4. Spectinomycin resistance

Spectinomycin resistance is conferred by mutations in the *16S rRNA*, altering the spectinomycin-binding region of helix 34 consequently reducing the affinity of the antibiotic for the ribosome target (2,5). The *rpsE* gene encodes the 30S ribosomal protein S5 and mutations of this gene confers high spectinomycin resistance which is associated with deletions of V25 and K26E, disrupting binding to the ribosome target (5,109,110). A

mutation in the T24P region in the S5 protein inhibits binding of spectinomycin to the *16S rRNA* gene (5,109,110).

1.3.7.5. Quinolone resistance

Quinolone resistance is a result of point mutations in *parC* which reduce the binding of antibiotics such as ciprofloxacin to topoisomerase IV (71,111). The implication of mutations in *gyrA* such as E91K, S88P and D86N reduce the binding affinity of antibiotics to DNA *gyrase* (5,36,112). Additionally, the overexpression of the *NorM* efflux pump produces enhanced MICs for quinolones (5). A study conducted in the year 2003, in males residing in the KwaZulu-Natal province of South Africa used NG-MAST to show novel sequence types (113). The study revealed that the predominant cluster of isolates were from strain ST217, which was previously reported for ciprofloxacin resistance in Europe (113). A global surveillance study revealed that countries within the WHO European region, American and African regions have reported extremely high levels of ciprofloxacin resistance, demonstrating that ciprofloxacin is no longer effective for the treatment of gonococcal infections (1).

1.3.7.6. Macrolide resistance

Macrolide resistance has been linked to point mutations that occur in the *23S rRNA* (C2611T and A2059G) in alleles 1 to 4, reducing the binding affinity of the antibiotic to the 50S ribosomal subunit (89,112). High levels of azithromycin resistance has been observed over the past few years such as the outbreak in England during 2014 and 2015 which affected several heterosexual men and women (114). The isolates were from the same NG-MAST ST9768 and presented a substitution of A2143G in all four *23S rRNA* alleles (114). The *erm* genes encode rRNA methylases responsible for the methylation of nucleotides in the *23S rRNA* which disrupt the binding of macrolides (115). Additionally, mutations in the *mtrR* gene alters the MtrCDE efflux pump and overexpression of the MacAB efflux pump subsequently increases the MICs of antimicrobials. Lastly, mutations in the *mef*-encoded efflux pump increases the MICs for macrolides (5,35).

1.3.7.7. Cephalosporin resistance

Previous studies conducted revealed that cefixime resistance is conferred by mutations in the *penA* gene, with minor contributions by *mtrR* and *porB* (5). Contrastingly, ceftriaxone

resistant *N. gonorrhoeae* strains are equally dependent on all three of these genes (5). Some genes and mutations that may be associated with ESC resistance remain unknown, but most confer penicillin resistance (71). The resistance mechanisms of ESCs are intricate and comprise of various mutation combinations, within single or multiple genes (35). Mosaic *penA* alleles encode PBP2, reducing acetylation and comprise of alterations of approximately 70 amino acids which are acquired by horizontal gene transfer (2,5). Other non-mosaic alleles derived from *penA* mutations (A501V and A501T) were observed to enhance MICs however, this requires extensive research to be proven (2,5).

1.4. Problem Statement

The worldwide clinical management of *N. gonorrhoeae* infections is becoming increasingly challenging due to antimicrobial resistance to various classes of available antibiotic therapy. These include the sulphonamides, penicillins, earlier cephalosporins, tetracyclines, macrolides and fluoroquinolones, which has since been withdrawn (5). In most settings worldwide, ceftriaxone is the last remaining option for empirical first-line antimicrobial monotherapy (5). However, decreasing susceptibility of *N. gonorrhoeae* to ceftriaxone has been reported with the percentage of resistance to ceftriaxone varying extensively, from 1.3% to 55.8% (116). In South Africa, antimicrobial resistant *N. gonorrhoeae* infections has become a major public health issue since there are raising concerns that *N. gonorrhoeae* infections may become untreatable in the near future (28). There is greater concern regarding untreatable infections in the pregnant population within our setting since untreated *N. gonorrhoeae* infections are responsible for complications such as ophthalmic infection in the new-born and severe pregnancy outcomes including, labour and delivery (16). Gonorrhoea has also become a significant cause of first-trimester abortion, thus further contributing to decreasing fertility rates (16). Therefore, it is highly critical that pregnant women undergo antimicrobial susceptibility testing for *N. gonorrhoeae* in order to initiate proper patient management and thus prevent adverse pregnancy outcomes. In the proposed study, emerging patterns of antimicrobial resistance to *N. gonorrhoeae* was investigated using genotypic and phenotypic methods. Additionally, the sensitivity and specificity of NAATs and culture diagnosis of gonorrhoeae infections was evaluated. The resulting information will contribute towards monitoring *N. gonorrhoeae* susceptibility patterns from Durban pregnant women on a national scale.

1.4.1. AIMS AND OBJECTIVES

Aims

To genotypically and phenotypically determine patterns of drug resistance in *N. gonorrhoeae* in women attending antenatal care at the King Edward VIII Hospital in Durban, KwaZulu-Natal, South Africa.

Objectives

(1) To compare the prevalence of *N. gonorrhoeae* infections in pregnant women presenting for antenatal care using different assays of detection.

(2) To determine the susceptibility of pure clinical isolates of *N. gonorrhoeae* to ceftriaxone, tetracycline, spectinomycin, azithromycin, ciprofloxacin, penicillin G and cefixime using the Etest method.

(3) To identify molecular 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.

(4) To compare genotypic to phenotypic methods for determining antimicrobial resistance of *N. gonorrhoeae* clinical isolates to various antimicrobials.

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CHAPTER 2

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Glynis Oree[1], Meleshni Naicker[1], Hopolang C Maise[2], Partson Tinarwo[3], Veron Ramsuran[4] and Nathlee S Abbai[1]

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Comparison of methods for the detection of *Neisseria gonorrhoeae* from South African women attending antenatal care

Glynis Oree [1], Meleshni Naicker. [1], Hopolang C. Maise [2], Partson Tinarwo [3], Veron Ramsuran [4] and Nathlee S. Abbai [1 *]

[1] School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.

[2] Department of Obstetrics and Gynaecology, School of Clinical Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.

[3] *Department of Biostatistics, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.*

[4] School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

Glynis Oree. https://orcid.org/0000-0003-4557-5185

* Corresponding author: Dr Nathlee Abbai, School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Private bag X7, Durban 4001, South Africa. Tel.: +27 31 260 4439. Email address: ABBAIN@ukzn.ac.za

KEYWORDS: *Neisseria gonorrhoeae,* culture, nucleic acid amplification assays, specificity, sensitivity

ABSTRACT

The detection of *Neisseria gonorrhoeae* using culture assays is challenging. This study aimed to compare different assays for the detection of *N. gonorrhoeae*. This cross-sectional study was conducted at King Edward VIII Hospital and included 307 antenatal attendees, each willing to provide two endocervical swabs. The first swab was used for culture identification of *N. gonorrhoeae* and the second swab was processed for the detection of the pathogen by the TaqMan quantitative PCR (qPCR) assay, an in-house *16S rRNA* PCR and PCR detection of the *opa* gene. Culture and the nucleic amplification assays were each used as comparator tests in the analysis. Sensitivity and specificity were calculated using RS Studio. The prevalence of *N. gonorrhoeae* was 7.8%. When compared to the TaqMan assay, the *16S rRNA* PCR exhibited the highest sensitivity of 62%, with a substantial level of agreement (kappa level of agreement: 0.60), followed by the *opa* PCR (38%) with a moderate level of agreement (0.52) and culture exhibiting the lowest sensitivity of 25% with a fair level of agreement (0.38). The diagnostic accuracy of all the assays were >90%. The TaqMan qPCR assay has the ability to serve as a future diagnostic assay for the detection of *N. gonorrhoeae*.

1. INTRODUCTION

Neisseria gonorrhoeae is a pathogen that is associated with cervicitis in women and may lead to endometritis, chronic pelvic pain and pelvic inflammatory disease (PID) resulting in ectopic pregnancy or infertility (1). In 2016, approximately 87 million new cases of gonorrhoeae were reported globally with an incidence of 20 cases per 1000 population in women (2). A study conducted in African female populations reported a 0.7% prevalence for *N. gonorrhoeae* (3). In addition, the incidence rate of *N. gonorrhoeae* is higher in South African women (3.7 per 100 women years) when compared to women from Zimbabwe (1.3 per 100 women years) (3). In KwaZulu-Natal, South Africa, the prevalence of *N. gonorrhoeae* ranges from 3 to 5.4% in female populations (4,5).

Many methods have been used for the detection of *N. gonorrhoeae* infections (6). Culture was previously deemed to be the "gold standard" for the detection of *N. gonorrhoeae* and was described as a cheap, highly sensitive, and specific method in well-developed laboratories (7). The disadvantages associated with this technique is the low successful growth of *N. gonorrhoeae* due to the fastidious nature of the microorganism (7). Due to the limitations associated with culture, nucleic acid amplification tests (NAATs) are currently considered as "gold standard" assays for detection of *N. gonorrhoeae* (8).

Commonly used NAATs include; strand displacement amplification assays, numerous nucleic acid sequence-based amplification tests using various primers and probes, as well as the polymerase chain reaction (PCR) for functional genes such as the *opa* gene present in *N. gonorrhoeae* strains (6–11). Nucleic acid amplification tests offer several advantages over culture such as rapid detection of the pathogen, less sample handling thereby reducing the risk of contamination, flexibility of sampling specimens (urine and genital swabs) and specimen handling (6–8). The disadvantage associated with some NAATs such as the high throughput closed quantitative PCR systems is the high cost and limited availability in resource-poor settings. However NAATs still serve as an excellent platform for the detection of *N. gonorrhoeae* from clinical samples (7,8).

This study compared different methods in terms of sensitivity and specificity for the detection of *N. gonorrhoeae* from endocervical swab samples. The methods included; the microbiological culture assay, gene-specific PCR detection for the *opa* and the 16S *ribosomal RNA* (*rRNA*) genes and the commercially available TaqMan quantitative PCR (qPCR) assay. The TaqMan qPCR assay, a relatively inexpensive, single target assay using a

pre-designed probe and primer mix was compared to culture, *16S rRNA* and *Opa* PCRs for the detection of *N. gonorrhoeae*. This study also provides prevalence estimates for this pathogen in a South African pregnant population.

2. MATERIALS AND METHODS

2.1. Study setting and population

Ethics approval for this study was granted by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN), (BE355/18). The population of this study comprised of pregnant women, who were 18 years and older, willing to provide: a written informed consent, two endocervical swabs, and details on demographics, sexual behaviour and clinical history. All pregnant women attending antenatal care were informed in person about the details of the study by the team and were presented with information brochures about the risks and treatment of gonorrhoeae infections. A high number of women refused to partake in the study as they were uncomfortable with undergoing an endocervical examination (which is not part of the standard of care routine check-up). The gynaecologist informed the women willing to participate, that they would receive treatment if they were symptomatic for this STI and that that the two endocervical samples provided by them would be used for antimicrobial studies.

2.2. Sample collection and processing

Sample collection and processing was conducted from November 2018 to July 2019. All enrolled women underwent a pelvic examination by a specialist gynecologist during sample collection. Women presenting with symptoms of vaginal discharge syndrome were treated using the syndromic management approach. Two endocervical Dacron swabs (LASEC, South Africa) were collected from each woman. The first endocervical swab was placed in Amies Charcoal transport media (LASEC, South Africa) and thereafter inoculated onto New York City agar for culturing of *N. gonorrhoeae*. The plates were inoculated within 4 hours of collection. The second swab was placed in a dry sterile tube containing 2 mL of phosphate buffered saline (pH 7.4); vortexed to dislodge the sample material from the swab and the cervical fluid was stored at -20°C until further molecular analysis. The assays were conducted at the School of Clinical Medicine’s Research Laboratory, UKZN.

2.3. Culture based detection of N. gonorrhoeae

The Amies charcoal swab was inoculated onto New York City agar plates and incubated at 35 °C to 37°C for 24 to 48 hrs in the presence of 5% CO_2. Identification of *N. gonorrhoeae* isolates was determined by microscopic evaluation of Gram stained colonies and biochemical tests such as the catalase, oxidase, carbohydrate and superoxol tests.

2.4. Nucleic acid amplification assays

The stored cervical fluid samples were thawed and subjected to DNA extraction. Prior to the extraction process, the fluid samples were centrifuged for 10 minutes at 14 000 *g* to pellet the sample material and the supernatant was discarded. DNA extraction procedures were performed on the sample pellets using the PureLink Microbiome kit (ThermoFisher Scientific, United States) according to the manufacturer's instructions. The resulting DNA was used for the detection of *N. gonorrhoeae* using the assays described below.

2.5. TaqMan (qPCR) assay

A pre-designed probe and primer mix specific for *N. gonorrhoeae* (Assay ID: Ba04646252_s, ThermoFisher Scientific, United States), was used in this assay. Each reaction comprised 2.5 µL of Fast Start 4x probe master mix, 0.5 µL FAM-labelled probe/primer mix and 2 µL sample DNA to a final volume of 10 µL. Quantitative PCR (qPCR) was performed on the Quant Studio 5 Real-time PCR system (Applied Biosystems, Life Technologies) in a 96 well microtiter reaction plate. Reaction conditions included; 1 cycle at 95°C for 30 seconds followed by 45 cycles of denaturation (95°C for 30 seconds) and annealing (60°C for 30 seconds). Amplified fluorescent products were detected at completion of the annealing period. The raw fluorescent data automatically generated by the PCR Quant Studio 5 PCR system software included cycling threshold (C_T) values.

2.5.1. ***TaqMan (qPCR) assay validation of analytical sensitivity by limit of detection (LoD) studies***

A five-fold dilution series of the WHO-N strain was prepared (undiluted, 1:10, 11:100, 1:1000, 1:10 000 and 1:100 000) and run in replicates of six. The concentration of each dilution was determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, South Africa). The pre-designed commercially available TaqMan qPCR assay, specifically for *N. gonorrhoeae* (Assay ID: Ba04646252_s, ThermoFisher Scientific, United States), was

used (described in section 2.5). Amplification was performed on a Quant Studio 5 Real-time PCR system and was expected to be 100 bp.

2.5.2. Validation of no cross-reactivity for different gonorrhoeae strains in the TaqMan (qPCR) assay

The pre-designed commercially available TaqMan qPCR assay, specifically for *N. gonorrhoeae* (Assay ID: Ba04646252_s, ThermoFisher Scientific, United States) was tested (described in section 2.5) for cross reactivity towards other *Neisseria* strains. The DNA from the following *Neisseria* strains: *N. elongate*, *N. cinera*, *N. weaver* and *N. sicca* were tested, together with the WHO-N *N. gonorrhoeae* strain as a positive control and a no-DNA template sample as the negative control. Amplification was performed on a Quant Studio 5 Real-time PCR system and was expected to be 100 bp.

2.6. Detection of the opa gene from N. gonorrhoeae

Primers which target a conserved region across the 11 opa protein families of *N. gonorrhoeae* were used in the amplification reactions. The primer sequences from Viscidi *et al.,* (2000), were used in this study and were as follows: *Opa* forward primer (OPA-01, 5′-ATGTGCAGGCGGATTTAGCC-3′) and reverse primer (OPA-04, 5′-AATGAGGCTTCGTGGGTTTTG-3′) (12). Each reaction comprised 12.5 µL DreamTaq master mix (ThermoFisher Scientific, United States), 0.5 µL forward primer (10uM), 0.5 µL reverse primer (10uM) and 1 µL of template DNA to a final volume of 25 µL. Amplification included 1 cycle at 95°C for 4 minutes followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds), extension (72°C for 1 minute) and final extension (72°C for 5 minutes). Amplicons were run on a 1% agarose gel at 100 V for an hour and a 100 bp GeneRuler DNA ladder (ThermoFisher Scientific, United States) was used.

2.6.1. Detection of the opa gene from N. gonorrhoeae validation of analytical sensitivity by limit of detection (LoD) studies

The 5 fold serial dilution of G247 was each subjected to the opa PCR in replicates of six, described in section 2.6. This PCR was used to detect the *opa* gene from *N. gonorrhoeae*. The PCR amplicons (650 bp) were run on a on a 1% agarose gel at 100 V for 1 hour and a 100 bp GenerRuler DNA ladder (ThermoFisher Scientific, United States) was used.

2.7. Detection of the 16S rRNA gene from N. gonorrhoeae

An in-house *16S rRNA* assay was used in this study. The primers were designed using the PrimerDesign software using the full-length *16S rRNA* gene of a *N. gonorrhoeae* strain (GenBank: X07714.1). The following primers: forward primer:

(5'-AGGGCTTCACACGTCATACA-3') and the reverse primer (3'-ACTCCTTGCGGTTACCCTAC-5') amplify a 240bp fragment. Each reaction comprised 12.5 µL DreamTaq master mix, 1.25 µL forward primer (10uM), 1.25 µL reverse primer (10uM) and 1 µL template DNA to a final volume of 25 µL. Based on amplicon size and melting temperatures of the primers, the following reaction conditions were used; 1 cycle at 95°C for 2 minutes followed by 30 cycles of denaturation (95°C for 15 seconds), annealing (55°C for 15 seconds), extension (72°C for 1 minute) and final extension (72°C for 5 minutes). Amplicons (600 bp) were run on a 1% agarose gel at 100 V for 1 hour. The DNA ladder was a 100 bp GeneRuler (ThermoFisher Scientific, United States).

2.7.1. Detection of the 16S rRNA gene from N. gonorrhoeae validation of analytical sensitivity by limit of detection (LoD) studies

An in-house *16S rRNA* qPCR assay was used in this study and the 5 fold serial dilution of the WHO-N strain was used as the template DNA and run in replicates of six. The primers were designed using the PrimerDesign software using the full-length *16S rRNA* gene of a *N. gonorrhoeae* strain (GenBank: X07714.1). The following primers: forward primer: (5'-AGGGCTTCACACGTCATACA-3') and the reverse primer (3'-ACTCCTTGCGGTTACCCTAC-5') amplify a 240bp fragment. Each reaction comprised 5 µL SyberGreen, 0.5 µL forward primer (10uM), 0.5 µL reverse primer (10uM) and 2 µL template DNA with the addition of water to a final volume of 10 µL. Reaction conditions included; UDG activation at 50°C for 2 minutes, followed by 1 cycle at 95°C for 2 minutes, followed by 40 cycles of denaturation (95°C for 2 minutes), annealing (55°C for 15 seconds) and final extension at 72°C for 1 minute. Amplified fluorescent products were detected at completion of the annealing period. The raw fluorescent data automatically generated by the PCR Quant Studio 5 PCR system software included cycling threshold (C_T) values.

2.8. Sequencing of 16S amplicons to confirm identity

To confirm the identity of the PCR amplicons obtained with the in-house *16S rRNA* PCR, the amplicons were sequenced at the KwaZulu-Natal Research and Innovation Platform (KRISP) based at UKZN. Sequencing was performed on an ABI3500XL genetic analyser and the raw

sequence data was edited using Chromas software V2.6.5 (Technelysium, Queensland, Australia). The identity of the edited sequences was confirmed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). All sequences showed a >90% identity to *N. gonorrhoeae* strain SH2017009 *16S ribosomal RNA* gene, partial sequence (Genbank accession number; MK620728.1).

2.9. Data analysis

The statistical data analysis was conducted in R statistical computing software version 3.6.2 using the libraries psych and epiR. The categorical characteristics were described using counts and percentage frequencies (Supplementary data, Appendix 1, Table S1). All the tests were conducted at 5% level of significance. Descriptive statistics were presented as merged cross tabulations in the form of counts indicating the comparison of test and comparative assays. A comprehensive understanding of the suitability of the assays was conducted using sensitivity and specificity analysis. In addition, KAPPA test for agreement beyond chance alone. All the tests were conducted at 5% level of significance.

3. RESULTS

3.1. Overview of the study population

The median age of the study population was 29 years with an interquartile range (IQR) of 24-34 (Q1-Q2). A high proportion (80.13%) of the women were asymptomatic (did not present with symptoms of abnormal vaginal discharge). Most women were in the third trimester of pregnancy (64.5%), had attended high school (71.0%) and were unmarried (87.9%). In addition, 61.2% had reported having had 2 to 4 lifetime sex partners and 61.2% reporting not using a condom during their last sex act.

3.2. Prevalence estimates

This study illustrates that out of the 307 samples analysed in this study, 24/307 of the samples, yielded positive results for *N. gonorrhoeae* by the TaqMan qPCR assay resulting in a prevalence of 7.8%. The median age of the women from which the TaqMan qPCR positive isolates (n=24) was 28 years old with an IQR of 25-32. Only 16.67% (4/24) women were symptomatic for *N. gonorrhoeae* with 83.33% (20/24) asymptomatic infections. Most women were in the third trimester of pregnancy with a percentage of 79.16% (19/24) and 20.83% (5/24) in the second trimester of pregnancy (Supplementary data, Appendix 1, Table S1).

This study showed that there was no statistical significance between socio-demographic and behavioural factors with the prevalence of *N. gonorrhoeae* infections.

The prevalence estimates obtained with the other assays were as follows; culture (1.95%), *opa* PCR (2.93%) and *16S rRNA* PCR (7.181%) (Tables 1 to 4). These assays were used for the detection of *N. gonorrhoeae* and were compared against each other.

3.3. Statistical performance of the different assays as comparators against each other

The performance of all four assays for the detection of *N. gonorrhoeae* was evaluated against each other and is described in Tables 1 to 4.

The performance of the TaqMan qPCR assay when compared to the other assays is shown in Table 1.

Table 1: The detection and statistical performance of *N. gonorrhoeae* from endocervical swabs collected from pregnant women using the TaqMan assay as the comparator.

	TaqMan quantitative PCR Assay (Comparator assay)						
Assays	**Correctly classified**		**Kappa agreement**	**Sensitivity (95% Confidence Interval)**	**Specificity (95% Confidence Interval)**	**PPV (95% Confidence Interval)**	**NPV (95% Confidence Interval)**
Culture	Positive	Negative					
Positive	6	0					
Negative	18	283	0.38	0.25 (0.09, 0.47)	1.00 (0.98, 1.00)	1.00 (0.54, 1.00)	0.94 (0.90, 0.96)
Total	**24**	**283**					
opa PCR	Positive	Negative					
Positive	9	0					
Negative	15	283	0.52	0.38 (0.19, 0.59)	1.00 (0.99, 1.00)	1.00 (0.66, 1.00)	0.95 (0.92, 0.97)
Total	**24**	**283**					
16S PCR	Positive	Negative					
Positive	15	8					
Negative	9	275	0.60	0.62 (0.41, 0.81)	0.97 (0.94, 0.99)	0.65 (0.43, 0.84)	0.97 (0.94, 0.99)
Total	**24**	**283**					

Of the 24 samples that tested positive on the TaqMan qPCR assay, only six of the samples yielded a positive result by culture. The remaining 18 positive samples were missed by culture. The *opa* PCR detected nine positive samples, with 15 samples that were undetected

in comparison to the TaqMan qPCR. The *16S rRNA* PCR detected the highest number of 15 *N. gonorrhoeae* positive samples out of the 24 TaqMan qPCR positives. However, eight out of the 15 samples that were detected as positive samples for the *16S rRNA* PCR were shown to be detected as negatives by the TaqMan qPCR as the comparator (Table 1). The highest number of nine positive isolates that corresponded to the TaqMan qPCR was detected by the *opa* PCR. All samples that tested positive for culture and *opa* PCR were positive for the

TaqMan qPCR. The highest number of true positive (9/24) isolates that corresponded to the TaqMan qPCR was detected by the *opa* PCR (Table 1). TaqMan qPCR as the comparator assay (Table 1) showed that a higher number of *N. gonorrhoeae* infections that tested positive from culture, *opa* PCR and *16S rRNA* PCR fell within the 24 TaqMan qPCR positive samples. However, a higher number of the samples that tested positive for TaqMan, *opa* PCR, culture and *16S rRNA*, were shown to be detected as negative by the other comparator assays (Table 2, 3 and 4).

Table 2: The detection and statistical performance of *N. gonorrhoeae* from endocervical swabs collected from pregnant women using the culture as the comparator.

	Culture (Comparator assay)						
Assays	**Correctly classified**		**Kappa agreement**	**Sensitivity (95% Confidence Interval)**	**Specificity (95% Confidence Interval)**	**PPV (95% Confidence Interval)**	**NPV (95% Confidence Interval)**
TaqMan qPCR	Positive	Negative					
Positive	6	18	0.38	1.00 (0.54, 1.00)	0.94 (0.91, 0.96)	0.25 (0.10, 0.47)	1.00 (0.99, 1.00)
Negative	0	283					
Total	**6**	**301**					
***opa* PCR**	Positive	Negative					
Positive	6	3	0.80	1.00 (0.54, 1.00)	0.99 (0.98, 1.00)	0.67 (0.30, 0.93)	1.00 (0.99, 1.00)
Negative	0	298					
Total	**6**	**301**					
***16S* PCR**	Positive	Negative					
Positive	4	19	0.25	0.67 (0.22, 0.96)	0.94 (0.90, 0.96)	0.17 (0.05, 0.39)	0.99 (0.98, 1.00)
Negative	2	282					
Total	**6**	**301**					

Table 3: The detection and statistical performance of *N. gonorrhoeae* from endocervical swabs collected from pregnant women using the *opa* PCR as the comparator.

	***opa* PCR (Comparator assay)**						
Assays	**Correctly classified**		**Kappa agreement**	**Sensitivity (95% Confidence Interval)**	**Specificity (95% Confidence Interval)**	**PPV (95% Confidence Interval)**	**NPV (95% Confidence Interval)**
Culture	Positive	Negative					
Positive	6	0	0.80	0.67 (0.30, 0.94)	1.00 (0.99, 1.00)	1.00 (0.54, 1.00)	0.99 (0.97, 1.00)
Negative	3	298					
Total	**9**	**298**					
TaqMan qPCR	Positive	Negative					
Positive	9	15	0.53	1.00 (0.66, 1.00)	0.95 (0.92, 0.97)	0.38 (0.19, 0.60)	1.00 (0.99, 1.00)
Negative	9	298					
Total	**9**	**298**					
***16S* PCR**	Positive	Negative					
Positive	7	16	0.41	0.78 (0.40, 0.97)	0.95 (0.91, 0.97)	0.30 (0.13, 0.53)	0.99 (0.98, 1.00)
Negative	2	282					
Total	**9**	**298**					

Table 4: The detection and statistical performance of *N. gonorrhoeae* from endocervical swabs collected from pregnant women using the *16S rRNA* PCR as the comparator.

	16S rRNA PCR (Comparator assay)						
Assays	**Correctly classified**		**Kappa agreement**	**Sensitivity (95% Confidence Interval)**	**Specificity (95% Confidence Interval)**	**PPV (95% Confidence Interval)**	**NPV (95% Confidence Interval)**
Culture	Positive	Negative					
Positive	4	2	0.25	0.17 (0.05, 0.39)	0.99 (0.98, 1.00)	0.67 (0.22, 0.96)	0.94 (0.90, 0.96)
Negative	19	282					
Total	**23**	**284**					
TaqMan qPCR	Positive	Negative					
Positive	15	9	0.61	0.65 (0.43, 0.84)	0.97 (0.94, 0.99)	0.63 (0.41, 0.81)	0.97 (0.95, 0.99)
Negative	8	275					
Total	**23**	**284**					
***opa* PCR**	Positive	Negative					
Positive	7	2	0.60	0.30 (0.13, 0.53)	0.99 (0.98, 1.00)	0.78 (0.40, 0.97)	0.95 (0.91, 0.97)
Negative	16	282					
Total	**23**	**284**					

Additional data of sample numbers that were positive for each of the 4 assays is presented (Supplementary data, Appendix 1, Table S2).

3.4. Sensitivity and specificity of the different assays as comparators against each other

Statistical data analysis was performed across the different assays for a comprehensive understanding of the suitability of each assay using sensitivity and specificity analysis shown in Tables 1 to 4.

Culture as the comparator performed poorly with sensitivities from 25 to 67%. TaqMan qPCR displayed the highest sensitivity (100%) when culture (Table 2) and *opa* PCR (Table 3) were comparators (Table 2). The *16s rRNA* PCR obtained a superior sensitivity of 62% than the other assays when TaqMan qPCR was the comparator assay. TaqMan qPCR exhibited the highest sensitivity of 65% when the *16S rRNA* PCR was the comparator (Table 4). Culture showed the highest specificity across all assays as comparators (Tables 1, 3 and 4), with specificities of 100% with TaqMan qPCR and *opa* PCR as the comparators and 99% for the *16S rRNA* PCR as the comparator assay. The *opa* PCR was the second most specific assay displaying a 100% specificity with TaqMan qPCR as the comparator (Table 1) and

99% for both culture and *16S rRNA* PCR (Table 2 and 4) as comparators. The specificity of the TaqMan qPCR and *16S rRNA* PCR was lower and ranged from 94 to 97% across all assays as comparators (Tables 1 to 4). The positive predictive values with TaqMan qPCR as the comparator assay showed that out of all the positive results, those from culture and *opa* PCR were all (100%) genuinely infected but only 65% from the *16S rRNA* PCR were only genuinely infected (Table 1). The culture assay as the comparator showed that the negative predictive value from TaqMan qPCR and *opa* PCR were all (100%) genuinely not infected, but only 99% from the *16S rRNA* PCR. The KAPPA level of agreement for each comparator assay verses other assays ranged from 0.25 to 0.8 (fair to almost perfect level of agreement).

3.5. Diagnostic accuracy of the different assays with the TaqMan qPCR as the comparator method

Diagnostic Accuracy (Area under the curve [AUC]) of culture was used to correctly classify positives as "true positives" and negatives as "true negatives" for all three assays (Supplementary data, Appendix 1, Figures S1 to S4).

3.6. Validation of analytical sensitivity by limit of detection (LoD) studies

The copy number of DNA for each limit of detection assay was calculated using the following formula:

$$number\ of\ copies = (amount\ in\ ng * 6.022x10^{23}\ molecules/mole)/(length\ in\ bp * 1x10^{9}\ ng\ /g * 650\ g/mol\ of\ bp)$$

The table below shows the copy number of DNA that was calculated for the TaqMan and *16S rRNA* qPCRs. The mean CT values for the TaqMan and *16S rRNA* PCR was determined.

Table 5: Limit of detection of the TaqMan and *16S rRNA* qPCRs using the WHO-N *N. gonorrhoeae* strain

Sample	**Concentration (ng/µl)**	**Copy Number of DNA**		**Average CT Values**	
		TaqMan	*16S rRNA* PCR	TaqMan	*16S rRNA* PCR
Undiluted WHO-N	1046.2	$9.69x10^{12}$	$4.04x10^{12}$	12.32	10.86
1:10 WHO-N	83	$7.69x10^{11}$	$3.2x10^{11}$	15.67	9.59
1:100 WHO-N	6.5	$6.02x10^{10}$	$3.51x10^{10}$	19.17	12.830

1:1000 WHO-N	0.5	$4.63x10^9$	$1.93x10^9$	22.41	16.19
1:10000 WHO-N	0.1	$9.26x10^8$	$3.86x10^8$	26.04	19.540
1:100000 WHO-N	0.1	$9.26x10^8$	$3.86x10^8$	29.65	25.40

The data obtained illustrated that the DNA copy number of the samples for the TaqMan assay was higher (ranged from $9.69x10^{12}$ to $9.26x10^8$) in comparison to the *16S rRNA* PCR ($4.04x10^{12}$ to $3.86x10^8$). The TaqMan qPCR was observed to have higher copy number ($9.26x10^8$) for the 1:100000 dilution of the WHO-N strain when the concentration of the template DNA was 0.1 ng/µl, whereas the *16S rRNA* PCR obtained a lower copy number ($3.86x10^8$).

The table below shows the copy number of DNA that was calculated for the conventional opa PCR. The mean CT values were determined.

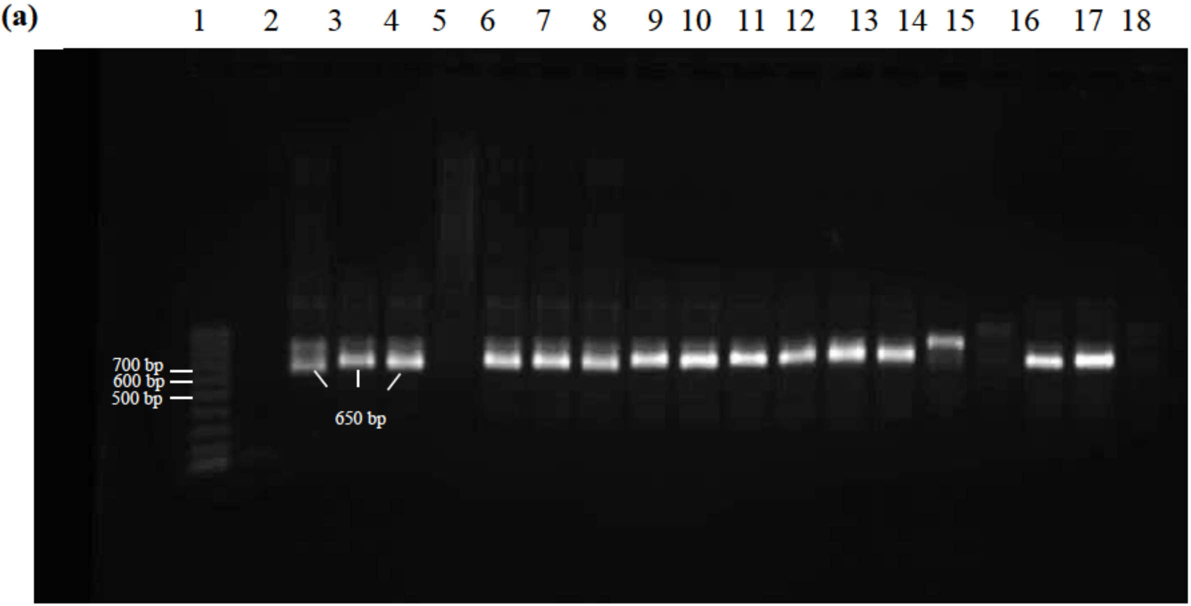
Table 6: Limit of detection of the conventional opa PCR using the G247 *N. gonorrhoeae* strain

Sample	**Concentration (ng/µl)**	**Copy Number of DNA**
		Opa PCR
Undiluted G247	491.4	$7x10^{11}$
1:10 G247	38.9	$5.54x10^{10}$
1:100 G247	4	$5.7x10^9$
1:1000 G247	0.2	$2.85x10^8$
1:10000 G247	0.2	$2.85x10^8$
1:100000 G247	0.1	$1.43x10^8$

The data obtained suggests that the opa PCR produced a copy number of $7x10^{11}$ when the concentration of G247 was 491.4 ng/µl. The data displayed on Table 6 illustrates that the opa PCR produced the least copy number of DNA ($1.43x10^8$) compared to the *16S rRNA* qPCR ($3.86x10^8$) and TaqMan qPCR ($9.26x10^8$) when the concentration of DNA was 0.1 ng/µl.

The amplicons obtained from the Opa PCR for the LOD studies is presented in Figure 1.

Lane:



Lane:

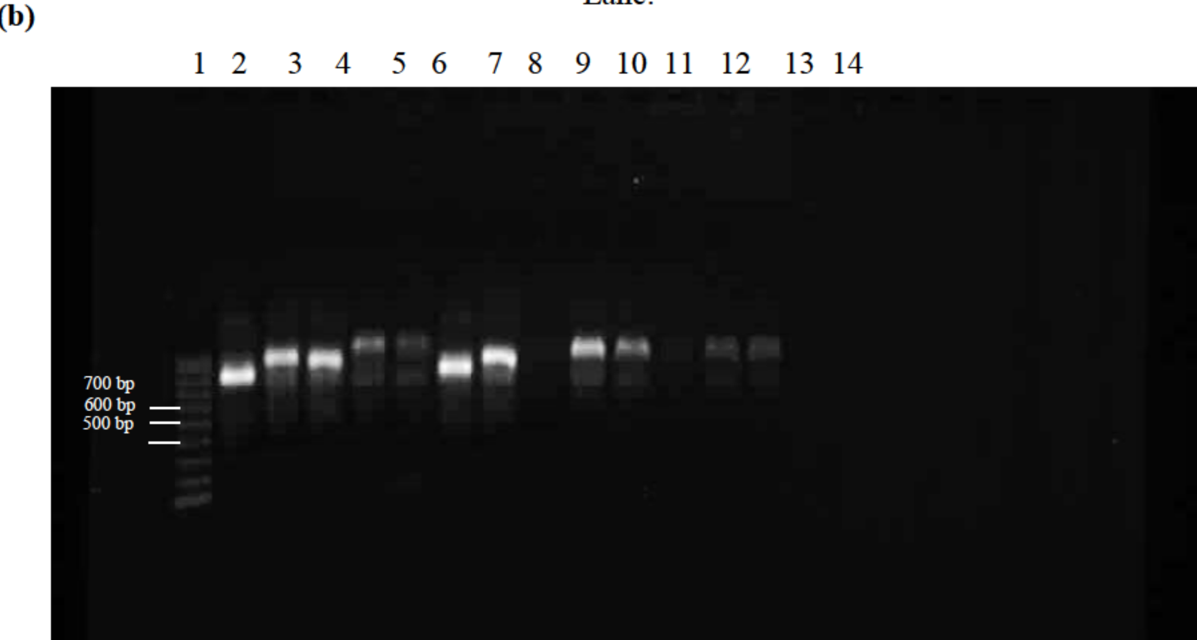


Figure 1: 1% Agarose Gel Electrophoresis of (a) Run 1 of dilution series of *opa* PCR products (b) Run 2 of dilution series of *opa* PCR products. ((a) Lane 1: DNA molecular marker; Lane 2: negative control; Lane 3: undiluted G247; Lane 4: 1:10 dilution G247; Lane 5: 1:10 dilution G247; Lane 6: 1:10 dilution G247; Lane 7: 1:10 dilution G247; Lane 8: 1:10 dilution G247; Lane 9: 1:10 dilution G247; Lane 10: 1:100 dilution G247; Lane 11: 1:100 dilution G247; Lane 12: 1:100 dilution G247; Lane 13: 1:100 dilution G247; Lane 14: 1:100 dilution G247; Lane15: 1:100 dilution G247; Lane 16: 1:1000 dilution G247; Lane 17:

1:1000 dilution G247; Lane 18: 1:1000 dilution G247; Lane 19: 1:1000 dilution G247; Lane 20: 1:1000 dilution G247 (b) Lane 1: DNA molecular marker; Lane 2: 1:1000 dilution G247; Lane 3: 1:10000 dilution G247; Lane 4: 1:10000 dilution G247; Lane 5: 1:10000 dilution G247; Lane 6: 1:10000 dilution G247; Lane 7: 1:10000 dilution G2471; Lane 8: 1:10000 dilution G247; Lane 9: 1:100000 dilution G247; Lane 10: 1:100000 dilution G247; Lane 11: 1:100000 dilution G247; Lane 12: 1:100000 dilution G247; Lane 13: 1:100000 dilution; Lane 14: 1:100000 dilution).

It was observed that nearly all the bands in gel (1a) have bands at approximately 650 bp from the undiluted sample (Lane 3) to the 1:1000 dilution of G247 in lane 20. Gel (1b) showed a band at approximately 650 bp (Lane 2: 1: 1000 dilution of G247). However the 1:10000 (lanes 3 to 8) and the 1: 1000 dilution (lanes 9 to 14) were each observed to have a single bright band accompanied with a additional lighter band.

3.7. Validation of no cross-reactivity for different gonorrhoeae strains in the TaqMan (qPCR) assay

Cross reactivity of the TaqMan qPCR was examined (Table 1) against the following *Neisseria* strains: *N. elongate*, *N. cinera*, *N. weaver* and *N. sicca*, with the *N. gonorrhoeae* WHO-N strain that served as a positive control.

Table 7: TaqMan qPCR CT values of different *Neisseria* strains

Sample	CT value
WHO-N (*N. gonorrhoeae*)	23,110
Negative control	Undetermined
N. elongata	Undetermined
N. cinera	Undetermined
N. weaveri	Undetermined
N. sicca	Undetermined

The CT values obtained showed that the TaqMan assay only detected the WHO-N *N. gonorrhoeae* strain and displayed a CT value of 23.110. The negative control and other *Neisseria* strains were not amplified by the qPCR.

4. DISCUSSION

N. gonorrhoeae is reported as one of the most significant causes of sexually transmitted diseases (STDs) in developing countries (13). Studies conducted in Africa in non-pregnant populations have reported prevalence rates ranging from 3-11% for *N. gonorrhoeae* (4,5,13,14). In Southern Iran, a lower prevalence of 1.3% has been reported for *N. gonorrhoeae* in pregnant women (15). In the present study, the prevalence of *N. gonorrhoeae* in a population of pregnant women was shown to be 7.8% by the TaqMan qPCR assay (Table 1). According to the behavioural and socio-demographics data collected in this study the majority of the women in this study were unmarried, had a secondary level of education, had reported having between 2-4 lifetime sex partners and had only sometimes used condoms during sex (Supplementary data, Appendix A, Table S1). Other studies have shown an association between the prevalence of *N. gonorrhoeae* infections and socio-demographic, behavioural and clinical factors. A study conducted by Karim *et al*., (2018), revealed a high prevalence of *N. gonorrhoeae* in unmarried, educated, urbanised women, suggesting that socio-demographic, behavioural and clinical factors do play a role in the spread of this infection (16). The current study showed that there is no statistical significance between the socio-demographic and behavioural factors in relation to prevalence (Supplementary data, Appendix 1, Table 1).

In this study, a higher proportion of samples tested positive for *N. gonorrhoeae* with the TaqMan qPCR assay. The qPCR assay has been previously described as a specific and sensitive assay for the detection of *N. gonorrhoeae* in clinical samples (6). Other Food and Drug Administration (FDA) approved diagnostic tests such as the BD MAX, GeneXpert, Allplex and Anyplex are multiplex assays that are expensive and are used to simultaneously test for other STIs in combination with *N. gonorrhoeae*, whereas the commercially bought TaqMan probe and primer mix is inexpensive and specifically targets *N. gonorrhoeae* only (17–19). In this study the TaqMan qPCR assay when used as the comparator assay exhibited the highest specificities ranging from 97% for the *16S rRNA* PCR to 100% for both culture and the *opa* PCR. However, sensitivities that were obtained (TaqMan qPCR as the comparator) demonstrated that culture performed the poorest with a sensitivity of 25% compared to the other PCR assays (Table 1). A previous study conducted in men and women from South Africa revealed a lower sensitivity for culture (32%) when compared to NAATs (14). According to Hassanzadeh *et al*., (2013), even under optimal laboratory conditions, the

sensitivity of culture for *N. gonorrhoeae* is low due to bacterial autolysis, poor sampling techniques, and improper specimen storage and transport (20).

This study demonstrated that the TaqMan qPCR assay performed the best with sensitivities ranging from 65 to 100% when performance was evaluated under different assays as comparator; whereas culture performed the best with specificities ranging from 99 to 100% when performance was evaluated under the other comparator assays. Similar to the TaqMan qPCR, lower specificities and higher sensitivities were observed for the in-house *16S rRNA*, followed by the *opa* PCR assay. A previous study which investigated the performance of qPCR, *16S rRNA* PCR and culture for the detection of *N. gonorrhoeae* revealed that culture was the least sensitive method (47 to 69%) in comparison to the *16S rRNA* PCR (77 to 98%) and qPCR assays (90 to 95%) (21). In the current study with the TaqMan qPCR as the comparator assay, the *16S rRNA* PCR exhibited a higher sensitivity than culture (62% versus 25%) which is in keeping with previously published studies.

In this study, the *opa* PCR assay showed a higher sensitivity than culture (38% versus 25%), and a lower sensitivity when compared to the *16S rRNA* PCR (62% versus 38%) (Table 1). However, a previous study found the *opa* qPCR to be more sensitive, specific and reliable than the *16S rRNA* PCR for the detection of *N. gonorrhoeae* (6). A study conducted by Geraats-Peters *et al.,*(2005) showed that the *opa* assay had a fivefold-higher sensitivity in comparison to the *16S rRNA* PCR (6). The conflicting results between the current study data and the findings by Geraats-Peters *et al.*, (2005) could be due to the fact that our study used a conventional PCR approach for detecting the *opa* gene whereas Geraats-Peters *et al.*, (2005) used a qPCR approach and qPCR has been suggested to be more sensitive than conventional PCR. This study revealed that culture ruled out at least 98% of patients that were not infected by *N. gonorrhoeae*, however, culture had the highest false negative rate of 75% followed by *opa* PCR (62.5%). This demonstrates that despite the high specificities for culture and *opa* PCR assays used in this study, for infections such as *N. gonorrhoeae* it is more valuable to have high sensitivity assays such as the *16S rRNA* and qPCR, since missed infections may lead to adverse maternal and infant outcomes in pregnant women (7).

The data obtained for the conventional opa PCR, *16S rRNA* and TaqMan qPCR suggests that the TaqMan qPCR is superior and has the ability to generate a higher copy number of DNA (9.26×10^8) with the availability of 0.1 ng ng/µl. This demonstrates that the TaqMan qPCR has a higher limit of detection (LOD) compared to the other two assays and is most suitable for

the detection of *N. gonorrhoeae* from clinical samples. A study conducted at the University of KwaZulu-Natal by the Department of Medical Microbiology evaluated the TaqMan assay for *Neisseria gonorrhoeae* against the AnyPlex assay (SeeGene) which is an approved diagnostic test for *Neisseria gonorrhoeae* (22). The study found a good comparison between these two assays in terms of the sensitivity, specificity, positive and negative predictive values (22). The TaqMan qPCR exhibited a sensitivity of 91.6%, specificity of 100%, positive and negative predictive values of 100% and 99.58%, respectively (22). The data obtained (Table 7) in this study illustrates that the TaqMan qPCR has no cross-reactivity with other *Neisseria* strains and is specific for the detection of *N. gonorrhoeae*. This assay therefore offers a more cost-effective and accurate alternative for the detection of this pathogen.

The current study showed promise for the use of the TaqMan qPCR assay for the detection of *N. gonorrhoeae* from clinical samples. However, a limitation of this study was that due to financial constraints we did not compare the performance of the TaqMan qPCR to other qPCR platforms approved for the diagnosis of *N. gonorrhoeae.* This can serve as a future research endeavor.

5. CONCLUSION

This study provided data on the ability of the TaqMan qPCR assay to detect a higher proportion of *N. gonorrhoeae* positive samples when compared to culture, *opa* PCR and a *16S rRNA* PCR. This study demonstrates that the TaqMan qPCR assay is sensitive and specific for the detection of the *N. gonorrhoeae* pathogen and is as inexpensive as a conventional PCR but more efficient in producing a result in a shorter time span. This assay is therefore more feasible to have in a clinical setting so that infections are detected in a shorter time so treatment can commence for any pregnant women, therefore minimizing adverse pregnancy outcomes. The majority of developing countries in Africa use the syndromic management of STIs whereas developed countries may use costly, FDA approved tests that can detect multiple STIs. This study is the first reporting on this assay in a South African clinical setting. However, future evaluations on this assay are needed in order to recommend this assay as a future diagnostic tool for *N. gonorrhoeae*.

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7. DECLARATION OF CONFLICTING INTERESTS

The author(s) declare no potential conflicts of interests with respect to the research, authorship, and/or publication of this article.

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10. SUPPLEMENTARY DATA

APPENDIX 1

Table S1 shows socio-demographic and behavioural factors for all 24 of the clinical samples that tested as *Neisseria gonorrhoeae* positive, using the TaqMan assay as the method of detection.

Table S1: Socio-demographic and behavioural factors of the study population stratified by TaqMan qPCR results

	Positive (n=24)	Negative (n=283)	*p*-value	Overall (n=307)
Age				
Mean(CV%)	28±5.5(19.5)	29±6.3(21.3)	0.403	29±6.2(21.2)
Median(Q1;Q2)	28(25;32)	29(24;35)		29(24;34)
Min-Max	20-40	19-45		19-45
Current abnormal discharge				
Yes	4 (16.7%)	57 (20.1%)	0.796	61 (19.9%)
No	20 (83.3%)	226 (79.9%)		246 (80.1%)
Level of education				
Didn't attend school	1 (4.2%)	14 (4.9%)	0.909	15 (4.9%)
Primary school	0 (0%)	6 (2.1%)		6 (2.0%)
High school	19 (79.2%)	199 (70.3%)		218 (71.0%)
College, University	4 (16.7%)	64 (22.6%)		68 (22.1%)
Marital status				
Yes	2 (8.3%)	35 (12.4%)	0.751	37 (12.1%)
No	22 (91.7%)	248 (87.6%)		270 (87.9%)
Has a regular sex partner				
Yes	13 (54.2%)	187 (66.1%)	0.24	200 (65.1%)
No	11 (45.8%)	96 (33.9%)		107 (34.9%)
Cohabitating with partner				
Yes	7 (29.2%)	119 (42.0%)	0.218	126 (41.0%)
No	17 (70.8%)	164 (58.0%)		181 (59.0%)
Age of 1st sex				
<15	0 (0%)	5 (1.8%)	1	5 (1.6%)
15-20	19 (79.2%)	208 (73.5%)		227 (73.9%)
21-25	5 (20.8%)	61 (21.6%)		66 (21.5%)
>25	0 (0%)	9 (3.2%)		9 (2.9%)
Number of lifetime sex partners				
1	8 (33.3%)	76 (26.9%)	0.745	84 (27.4%)
2 - 4	14 (58.3%)	174 (61.5%)		188 (61.2%)
>4	2 (8.3%)	33 (11.7%)		35 (11.4%)
Partner has other partners				
Yes	1 (4.2%)	40 (14.1%)	0.133	41 (13.4%)
Don't know	12 (50.0%)	162 (57.2%)		174 (56.7%)
No	11 (45.8%)	81 (28.6%)		92 (30.0%)
Condom use				
Never	6 (25.0%)	49 (17.3%)	0.737	55 (17.9%)
Rarely	0 (0%)	6 (2.1%)		6 (2.0%)
Sometimes	16 (66.7%)	207 (73.1%)		223 (72.6%)

Always	2 (8.3%)	21 (7.4%)		23 (7.5%)
Condom used during last sex Act				
Yes	10 (41.7%)	100 (35.3%)	0.605	110 (35.8%)
No	14 (58.3%)	175 (61.8%)		189 (61.6%)
Missing	0 (0%)	8 (2.8%)		8 (2.6%)
Smokes				
Yes	0 (0%)	14 (4.9%)	0.613	14 (4.6%)
No	24 (100%)	261 (92.2%)		285 (92.8%)
Missing	0 (0%)	8 (2.8%)		8 (2.6%)
Consumes alcohol				
Yes	1 (4.2%)	25 (8.8%)	0.706	26 (8.5%)
No	23 (95.8%)	258 (91.2%)		281 (91.5%)
Uses other substances to wash genitals				
Yes	1 (4.2%)	11 (3.9%)	1	12 (3.9%)
No	23 (95.8%)	264 (93.3%)		287 (93.5%)
Missing	0 (0%)	8 (2.8%)		8 (2.6%)
Trimester of pregnancy				
1st	0 (0%)	11 (3.9%)	0.246	11 (3.6%)
2nd	5 (20.8%)	93 (32.9%)		98 (31.9%)
3rd	19 (79.2%)	179 (63.3%)		198 (64.5%)
Past preterm delivery				
Yes	5 (20.8%)	56 (19.8%)	1	61 (19.9%)
No	19 (79.2%)	219 (77.4%)		238 (77.5%)
Missing	0 (0%)	8 (2.8%)		8 (2.6%)
Past miscarriage				
Yes	6 (25.0%)	95 (33.6%)	0.343	101 (32.9%)
No	18 (75.0%)	180 (63.6%)		198 (64.5%)
Missing	0 (0%)	8 (2.8%)		8 (2.6%)
Past spontaneous abortion				
Yes	0 (0%)	13 (4.6%)	0.61	13 (4.2%)
No	24 (100%)	262 (92.6%)		286 (93.2%)
Missing	0 (0%)	8 (2.8%)		8 (2.6%)
Experienced past symptoms of STIs				
Yes	10 (41.7%)	112 (39.6%)	0.841	122 (39.7%)
No	14 (58.3%)	171 (60.4%)		185 (60.3%)
Previous treatment for STIs				
Yes	6 (25.0%)	91 (32.2%)	0.469	97 (31.6%)
No	18 (75.0%)	192 (67.8%)		210 (68.4%)

Table S2 shows the raw data of sample numbers that were detected as *Neisseria gonorrhoeae* positive by the culture, *opa* PCR and *16S rRNA* PCR and the TaqMan qPCR assay with CT values. This illustrates that most of the positive samples detected by the other assays, were detected by the TaqMan qPCR assay.

Table S2: Raw data of *Neisseria gonorrhoeae* positive sample numbers

Sample Number	Assay Type				
	TaqMan	**TaqMan CT values**	**Culture**	***Opa* PCR**	***16S rRNA* PCR**
G03	+	33,415	-	-	+
G07	+	38,932	-	-	-
G51	+	26,373	+	+	+
G61	+	29,417	-	+	+
G79	+	29,137	-	+	+
G85	-	UD	-	-	+
G87	-	UD	-	-	+
G88	-	UD	-	-	+
G95	-	UD	-	-	+
G132	+	37,274	-	-	-
G133	+	27,211	-	+	+
G134	+	36,668	-	-	+
G136	+	38,837	+	+	-
G138	+	36,800	-	-	+
G139	-	UD	-	-	+
G141	+	37,792	-	-	+
G142	+	38,029	-	-	+
G143	+	37,812	-	-	-
G144	+	38,695	-	-	+
G145	+	38,097	-	-	+
G148	+	38,919	-	-	-
G151	+	37,878	-	-	-
G153	+	38,846	-	-	-
G154	+	37,457	-	-	-
G156	-	UD	-	-	+
G160	+	39,301	-	-	+
G175	-	UD	-	-	+
G176	+	23,486	+	+	+
G180	+	23,486	+	+	+
G206	+	28,173	+	+	+
G232	-	UD	-	-	+
G247	+	27,406	+	+	-

** UD: undetermined; +: positive; -: negative*

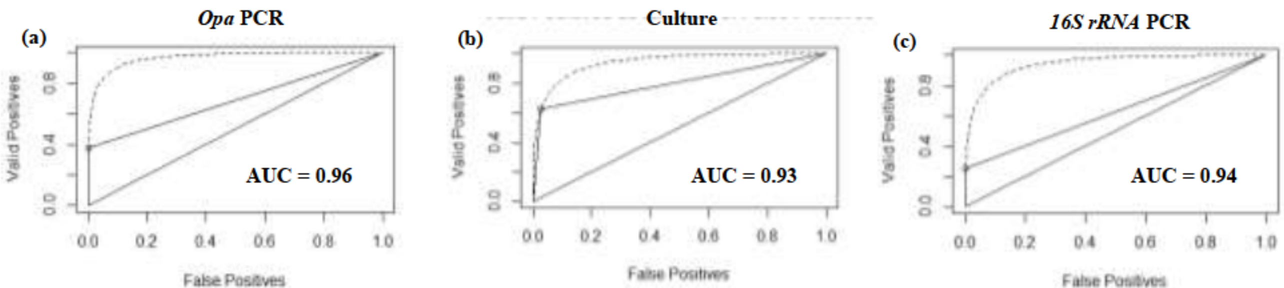


Figure S1: Diagnostic Accuracy of (a) *opa* PCR, (b) culture and (c) *16S rRNA* PCR vs. TaqMan qPCR assay as the comparator. The diagnostic accuracy of (a) *opa* PCR was 95.1%, with 15 samples that were missed and nine positive samples, however there were no false positives. (b) The diagnostic accuracy of culture was 94.1%, six positives and 18 samples that were missed. There were no false positives. (c) The diagnostic accuracy for *16S rRNA* PCR was 94.5% and there were 9 false positives yielded by the *16S rRNA* PCR.

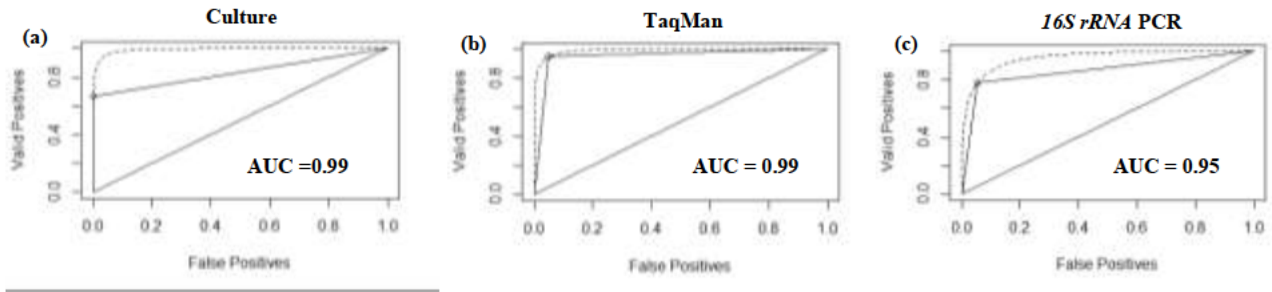


Figure S2: Diagnostic Accuracy of (a) culture, (b) TaqMan qPCR assay and (c) *16S rRNA* PCR vs. *opa* PCR assay as the comparator. The diagnostic accuracy of (a) culture was 99%, with three samples that were missed and no false positives. (b) The diagnostic accuracy of TaqMan qPCR was 95.1%, with nine positives and 15 false positives. (c) The diagnostic accuracy for *16S rRNA* PCR was 94.1% and there were seven positives, two samples were missed and 16 false positives were yielded.

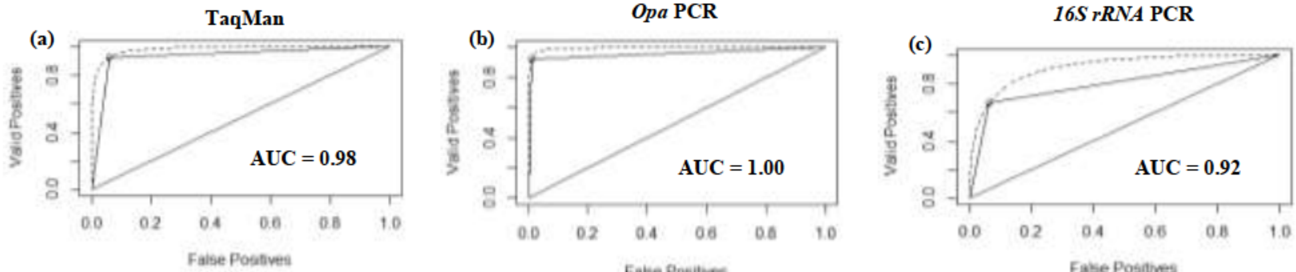


Figure S3: Diagnostic Accuracy of (a) TaqMan qPCR, (b) *opa* PCR and (c) *16S rRNA* PCR vs. culture assay as the comparator. The diagnostic accuracy of (a) TaqMan qPCR was 94.1%, with six positives and 18 false positives. (b) The diagnostic accuracy of *opa* PCR was 99%, with six positives and three false positives. (c) The diagnostic accuracy for *16S rRNA* PCR was 93.2% and there were four positives, two samples were missed and 19 false positives were yielded.

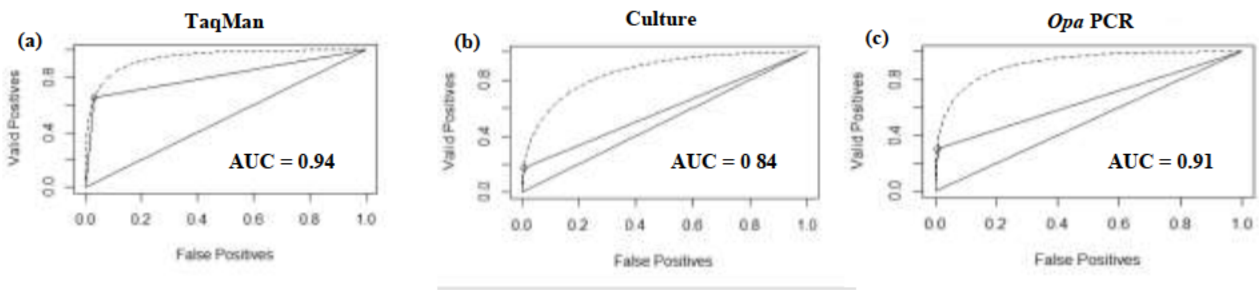
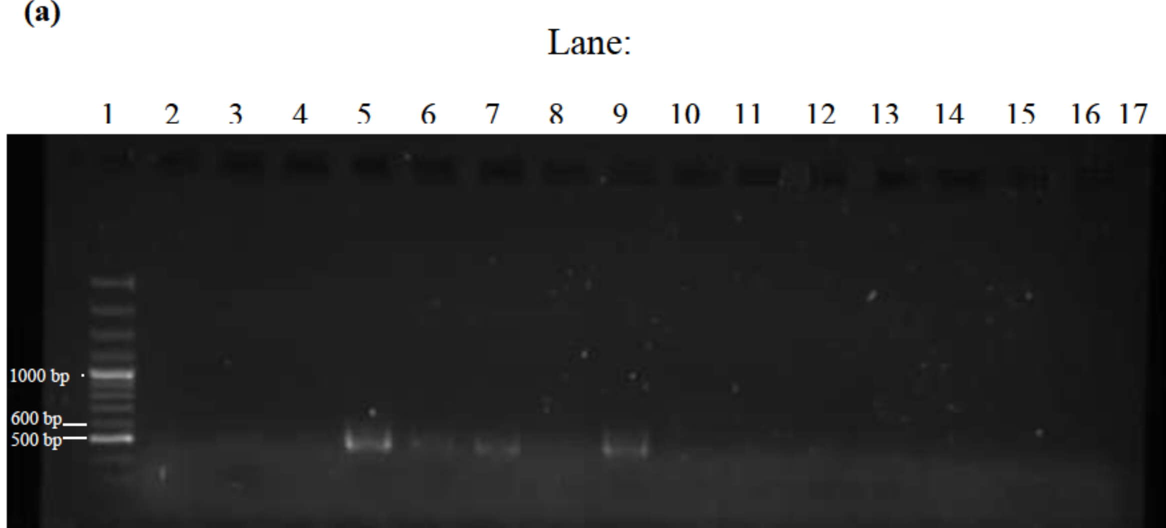


Figure S4: Diagnostic Accuracy of (a) TaqMan qPCR, (a) culture and (c) *opa* PCR vs. *16S rRNA* PCR assay as the comparator. The diagnostic accuracy of (a) TaqMan was 94.5%, with 15 positives, eight samples were missed and nine were false positives. (b) The diagnostic accuracy of culture was 93.2%, with four positives, 19 samples that were missed and two false positives. (c) The diagnostic accuracy for *opa* PCR was 94.1% and there were seven positives, 16 samples were missed and 2 false positives were yielded.

(a)



(b)

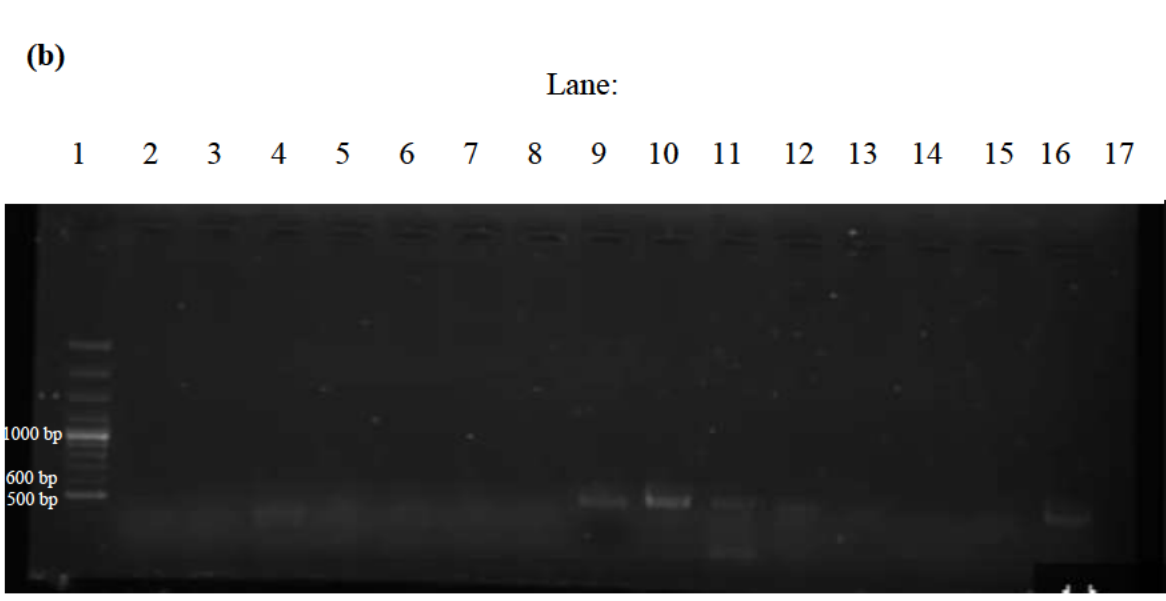


Figure S5: 1% Agarose Gel Electrophoresis of (a) Run 1of *opa* PCR products (b) Run 2 of *opa* PCR products. ((a) Lane 1: DNA molecular marker; Lane 2: negative control; Lane 3: G03; Lane 4: G07; Lane 5: G51; Lane 6: G61; Lane 7: G79; Lane 8: G85; Lane 9: G133; Lane 10: G88; Lane 11: G95; Lane 12: G132; Lane 13: G87; Lane 14: G134; Lane15: G144; Lane 16: G138; Lane 17: G139; (b) Lane 1: DNA molecular marker; Lane 2: G141; Lane 3: G142; Lane 4: G136; Lane 5: G145; Lane 6: G148; Lane 7: G151; Lane 8: G151; Lane 9: G176; Lane 10: G180; Lane 11: G206; Lane 12: G175; Lane 13: G154; Lane 14: G156; Lane 15: G160; Lane 16: G232; Lane 17: G247).

Samples G51, G61, G79, G133, G136, G176, G180, G206 and G247 from figure S5 were each observed to have a band at approximately 600 bp. The negative control in lane 2 on figure S5 (a) showed the absence of any bands. All the remaining sample lanes in figure S5 showed no bands.

CHAPTER 3

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Research Article

Antimicrobial Susceptibility Patterns in *Neisseria gonorrhoeae* Isolated from South African Pregnant Women

Glynis Oree,[1] Meleshni Naicker,[1] Hopolang Clifford Maise,[2] Partson Tinarwo,[3] and Nathlee Samantha Abbai[1]

[1]*School of Clinical Medicine Research Laboratory, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa*
[2]*Department of Obstetrics and Gynaecology, School of Clinical Medicine, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa*
[3]*Department of Biostatistics, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa*

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Antimicrobial susceptibility patterns in *Neisseria gonorrhoeae* isolated from pregnant women from the Durban region of KwaZulu-Natal, South Africa.

Glynis Oree. [1], Meleshni Naicker. [1], Hopolang Clifford Maise. [2], Partson Tinarwo. [3] and Nathlee Samantha Abbai [1*]

[1] *School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa. glynoree@gmail.com; meleshni@yahoo.com; abbain@ukzn.ac.za*

[2] *Department of Obstetrics and Gynaecology, School of Clinical Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa. Maisehc@ukzn.ac.za*

[3] *Department of Biostatistics, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.* TinarwoP1@ukzn.ac.za

*Corresponding author: Professor Nathlee Abbai, School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Private bag X7, Durban 4001, South Africa. Tel.: +27 31 260 4439. Email address: ABBAIN@ukzn.ac.za

ABSTRACT

Background: Gonorrhoeae is a sexually transmitted infection (STIs) caused by an infection with the pathogen *Neisseria gonorrhoeae* and is associated with adverse pregnancy and neonatal outcomes. Emerging resistance towards various antibiotics has been observed globally. However, there is a lack of data on antimicrobial susceptibility patterns in *N. gonorrhoeae* isolated from pregnant women in our setting. This study fills in this gap in the literature.

Methods: The study population included pregnant women, recruited from the antenatal clinic of the King Edward VIII hospital (KEH) in Durban. Endocervical swabs were obtained from 307 women. The swab was placed in Amies Charcoal media for culture assessments. Pure isolates of *N. gonorrhoeae* were subjected to antimicrobial susceptibility testing using the Etest™ method. The Minimum inhibitory concentration (MIC) values were assessed in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019) breakpoints.

Results: The prevalence of *N. gonorrhoeae* by culture was 1.9%. High MIC values to penicillin G (12-64mg/L) indicating a resistant phenotype was observed for all isolates tested, with 50% of the isolates displaying complete resistance. Isolates with intermediate (1mg/L) and resistance (1.9-32mg/L) profiles to tetracycline were observed. Resistance to ciprofloxacin (1.16-3mg/L) was also observed. Isolates displayed either dual or triple resistance to penicillin G, tetracycline or ciprofloxacin. All isolates showed susceptibility to spectinomycin (>64mg/L), azithromycin (1mg/L), ceftriaxone (>0.125 mg/L) and cefixime (>0.125 mg/L).

Conclusion: Despite lack of resistance to ceftriaxone and azithromycin, continuous surveillance for emerging patterns of resistance to these antibiotics is needed since they form part of the treatment guidelines.

KEYWORDS: *Neisseria gonorrhoeae*, penicillin G, ciprofloxacin, tetracycline, spectinomycin, azithromycin, cefixime, ceftriaxone, antimicrobial resistance, pregnant women

1. INTRODUCTION

Neisseria gonorrhoeae is the second most prevalent bacterial sexually transmitted infection (STI) and is a major cause of mortality and morbidity (18). A global STI surveillance in 2018 was conducted by the World Health Organization (WHO) and revealed an estimated 87 million new gonorrhoea infections globally during 2016, with an incidence of 20 cases per 1000 population (uncertainty interval 14–28) in women (6). A study conducted in South Africa and Zimbabwe reported an overall prevalence of 0.7% for *N. gonorrhoeae* infections in women from the general population (118). Other studies conducted in South Africa have reported prevalence rates for *N. gonorrhoeae* from 3%-11% in women (9,10,18). A study conducted exclusively on pregnant women reported a prevalence of 1.3% for *N. gonorrhoeae* (121).

The worldwide clinical management of *N. gonorrhoeae* infections is becoming increasingly challenging due to antimicrobial resistance (AMR) to various classes of available antibiotic therapy (124). Untreated *N. gonorrhoeae* infections are associated with a range of adverse pregnancy outcomes such as neonatal conjunctivitis, foetal growth retardation, spontaneous abortion, stillbirth, prematurity, low birth weight, post-partum endometritis and increased risk of Human Immunodeficiency Virus (HIV) transmission from mother to child during birth (1,3,5,7,13,14,17–22). Therefore it is highly critical that pregnant women undergo antimicrobial sensitivity testing for any *N. gonorrhoeae* AMR in order to initiate proper patient management and thus prevent these adverse pregnancy outcomes.

The withdrawal of sulphonamides, penicillins, earlier cephalosporins, tetracyclines, macrolides and fluoroquinolones led to limited treatment options for this infection (2,5,108). In most settings worldwide, ceftriaxone is the last remaining option for empirical first-line antimicrobial monotherapy (5). However, decreasing susceptibility of *N. gonorrhoeae* to ceftriaxone has been reported with the proportion of resistance to ceftriaxone varying extensively, from 1.3% to 55.8% (116).

Ceftriaxone was the last remaining option for empirical first-line antimicrobial monotherapy (5). South Africa was in accordance with the recommendation made by the WHO which advocated for the replacement of first line treatment with oral cefixime to a single injectable dose (250 mg) of ceftriaxone in 2014 (17). Treatment failures of ceftriaxone monotherapy led to the WHO recommendation of administering dual antimicrobial therapy with the combination of ceftriaxone (250 mg) and azithromycin (1 g stat) (1,14,48,125). However,

decreasing susceptibility of *N. gonorrhoeae* to ceftriaxone has been reported with the proportion of resistance to ceftriaxone varying extensively, from 1.3% to 55.8% (116). In addition, resistance to azithromycin is already prevalent in many settings (5). Therefore dual-antimicrobial therapy cannot ensure long-term effectiveness.

Currently, there is limited data on *N. gonorrhoeae* susceptibility patterns in pregnant populations from KwaZulu-Natal (KZN) in South Africa. This study provides data on susceptibility patterns to penicillin G, tetracycline, ciprofloxacin, azithromycin, spectinomycin, cefixime and ceftriaxone in pregnant women from our setting. In addition, this study provides information on the socio-demographic, behavioural and clinical history in association with AMR.

2. MATERIALS AND METHODS

2.1. Ethical statement

Full ethics approval for this study was granted by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN), (BE355/18).

2.2. Study setting and population

The study population included pregnant women, who were 18 years and older, willing to provide written informed consent, willing to provide biological samples (endocervical swabs), and willing to provide data on their demographics, sexual behaviour and clinical history. The study population was recruited from the antenatal clinic of the King Edward VIII hospital (KEH) in Durban, South Africa from November 2018 to July 2019. Due to the nature of the sample collection, we had a 50% refusal rate during screening. Eventually, the number of women enrolled in this study was 307.

2.3. Sample collection and processing

Each consenting woman was subjected to a clinical examination by a gynaecologist during which endocervical swab samples were collected. The swab was placed in Amies Charcoal transport media (LASEC, South Africa) immediately after collection. The swab was processed within 4 hours after collection at the Clinical Medicine Laboratory at the University of KwaZulu-Natal.

2.4. Culture detection of *N. gonorrhoeae*

Upon arrival at the laboratory, the Amies swabs were streaked onto New York City Agar plates and incubated for 24- 48 hours in the presence of 5% CO_2 at 35-37ºC. After incubation, suspected colonies were sub-cultured onto Chocolate Agar plates and incubated for a further 24 hours in the presence of 5% CO_2 at 35-37ºC. To confirm the identity of the isolates, Gram staining, oxidase, catalase, superoxol and carbohydrate utilization tests were conducted.

2.5. Detection of antimicrobial susceptibility and resistance profiles by the Etest™ method

Culture confirmed isolates were subjected to antimicrobial susceptibility testing. A 0.5 McFarland (ThermoFisher Scientific, United States) inoculum was prepared using each of the *N. gonorrhoeae* culture positive isolates in 1mL Mueller-Hinton Broth (LASEC, South Africa). The Etest method was followed whereby each Etest™ strip (bioMérieux, France) was placed on chocolate agar plates to determine the minimum inhibitory concentrations (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). The plates were then incubated for 24 hours in the presence of 5% CO_2 at 35-37ºC. The WHO kindly provided strains, G, W, X, Y and Z for use as positive controls. The MIC values were assessed in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019) breakpoints.

2.6. Data analysis

The data analysis was conducted using R Statistical computing software (version 3.6.3), a freely available software. Age, the only numerical variable was summarised to show the minimum, maximum and quartiles. The categorical characteristics were described using counts and percentage frequencies. The results were stratified by infection status of *N. gonorrhoeae*, that is, either negative or positive. Due to the skewness of the age distribution, median comparison between the positive and negative groups was conducted using Wilcoxon ranksum test. On the other hand, associations in cross tabulations were tested using either Fisher's exact test for cross tabulations involving counts less than 5 or Chi-Square test, otherwise. All the tests were conducted at 5% level of significance.

3. RESULTS

3.1. Overview and prevalence estimates of the study population

Of the total 307 women who participated in this study, 6/307 isolates were confirmed to be *N. gonorrhoeae* by culture. The prevalence of *N. gonorrhoeae* by culture was 1.9%. There was no significant association between demographic, behavioural and clinical factors and infection status (Table 1). Despite the lack of significance, a large proportion of the study women (80.1%) did not present with symptoms of abnormal vaginal discharge, had reported having between 2-4 lifetime sex partners (61.2%), were unmarried (87.9%), reported "sometimes" using condoms (72.6%), and were not treated for STIs in the past (68.4%) (Table 1).

Table 1: Characteristics of the antenatal women enrolled in this study. The infection status of *N. gonorrhoeae* is based on the data from culture.

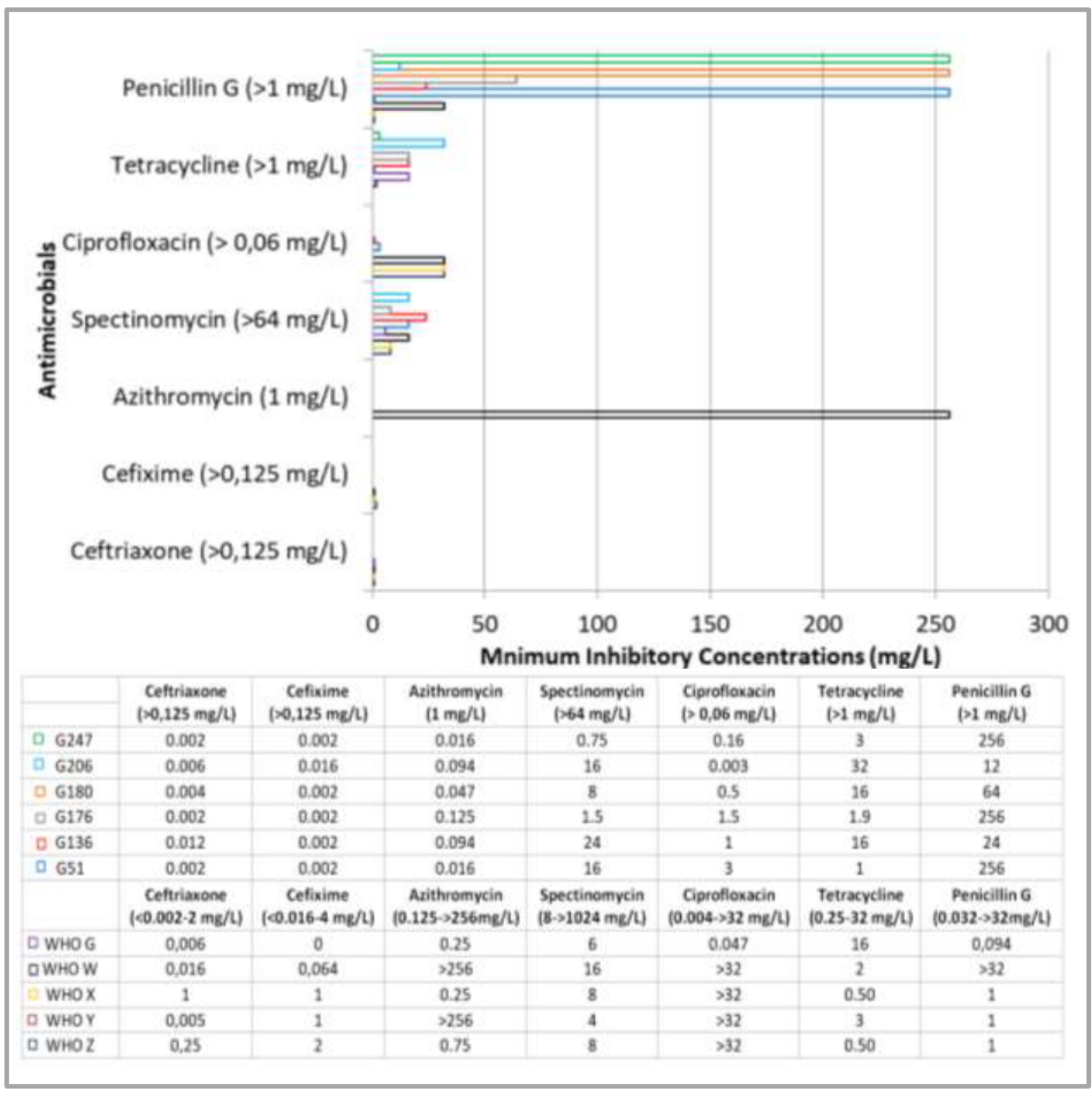
Status	Negative (N=301)	Positive (N=6)	p-value	Overall (N=307)
Age			0.102	
Mean±SD(CV%)	29.4±6.23(21.2)	25.2±3.19(12.7)		29.3±6.21(21.2)
Median(Q1-Q3)	29.0(24.0-34.0)	25.5(22.8-27.5)	Ranksum test	29.0(24.0-34.0)
Min-Max	19.0-45.0	21.0-29.0		19.0-45.0
Current abnormal vaginal discharge			1.000	
No	241 (80.1%)	5 (83.3%)		246 (80.1%)
Yes	60 (19.9%)	1 (16.7%)		61 (19.9%)
Married			1.000	
No	264 (87.7%)	6 (100%)		270 (87.9%)
Yes	37 (12.3%)	0 (0%)		37 (12.1%)
Regular sex partner			0.424	
No	104 (34.6%)	3 (50.0%)		107 (34.9%)
Yes	197 (65.4%)	3 (50.0%)		200 (65.1%)
Co habiting			0.407	
No	176 (58.5%)	5 (83.3%)		181 (59.0%)
Yes	125 (41.5%)	1 (16.7%)		126 (41.0%)
Lifetime sex partners			0.108	
>4	35 (11.6%)	0 (0%)		35 (11.4%)
1	80 (26.6%)	4 (66.7%)		84 (27.4%)
2 to 4	186 (61.8%)	2 (33.3%)		188 (61.2%)
Partner has other partners			0.601	

Status	Negative (N=301)	Positive (N=6)	p-value	Overall (N=307)
Don't know	169 (56.1%)	5 (83.3%)		174 (56.7%)
No	91 (30.2%)	1 (16.7%)		92 (30.0%)
Yes	41 (13.6%)	0 (0%)		41 (13.4%)
Condom use			0.638	
Always	23 (7.6%)	0 (0%)		23 (7.5%)
Never	53 (17.6%)	2 (33.3%)		55 (17.9%)
Rarely	6 (2.0%)	0 (0%)		6 (2.0%)
Sometimes	219 (72.8%)	4 (66.7%)		223 (72.6%)
Trimester			1.000	
1st	11 (3.7%)	0 (0%)		11 (3.6%)
2nd	96 (31.9%)	2 (33.3%)		98 (31.9%)
3rd	194 (64.5%)	4 (66.7%)		198 (64.5%)
Treated for STIs in the past			0.669	
No	205 (68.1%)	5 (83.3%)		210 (68.4%)
Yes	96 (31.9%)	1 (16.7%)		97 (31.6%)

The p-values are based on non-missing cases only (tableStack).

3.4. Antimicrobial susceptibility testing

All 6 isolates produced antimicrobial susceptibility results (Figure 1). WHO reference strains with known MIC values were included as controls. The WHO strains produced the desired results thereby validating the Etest™ MIC results obtained. High MIC values to penicillin G (12-64mg/L) indicating a resistant phenotype was observed for all isolates tested, with 50% of the isolates displaying complete resistance. Of the six isolates, one isolate exhibited an intermediate phenotype for tetracycline (1mg/L) whereas the remaining five isolates showed resistance (1.9-32mg/L). Five of the six isolates showed resistance to ciprofloxacin (1.16-3mg/L) with one isolate still displaying the susceptible phenotype (0.003mg/L). All six isolates displayed either dual or triple resistance to penicillin G, tetracycline or ciprofloxacin. All isolates showed susceptibility to spectinomycin (>64mg/L), azithromycin (1mg/L), ceftriaxone (>0.125 mg/L) and cefixime (>0.125 mg/L). Isolates with complete susceptibility to azithromycin, ceftriaxone and cefixime were observed (Figure 1). The WHO strains (G, W, X, Y and Z) were assessed as positive controls for the validation of the MIC data obtained.



	Ceftriaxone (>0,125 mg/L)	Cefixime (>0,125 mg/L)	Azithromycin (1 mg/L)	Spectinomycin (>64 mg/L)	Ciprofloxacin (> 0,06 mg/L)	Tetracycline (>1 mg/L)	Penicillin G (>1 mg/L)
G247	0.002	0.002	0.016	0.75	0.16	3	256
G206	0.006	0.016	0.094	16	0.003	32	12
G180	0.004	0.002	0.047	8	0.5	16	64
G176	0.002	0.002	0.125	1.5	1.5	1.9	256
G136	0.012	0.002	0.094	24	1	16	24
G51	0.002	0.002	0.016	16	3	1	256
	Ceftriaxone (<0.002-2 mg/L)	Cefixime (<0.016-4 mg/L)	Azithromycin (0.125->256mg/L)	Spectinomycin (8->1024 mg/L)	Ciprofloxacin (0.004->32 mg/L)	Tetracycline (0.25-32 mg/L)	Penicillin G (0.032->32mg/L)
WHO G	0,006	0	0.25	6	0.047	16	0,094
WHO W	0,016	0,064	>256	16	>32	2	>32
WHO X	1	1	0.25	8	>32	0.50	1
WHO Y	0,005	1	>256	4	>32	3	1
WHO Z	0,25	2	0.75	8	>32	0.50	1

Figure 1: Etest™ data of emerging antimicrobial susceptibility/resistance patterns in *N. gonorrhoeae*. Patterns of resistance and susceptibility were determined by the 2019 EUCAST breakpoints.

4. DISCUSSION

This study reported a prevalence estimate of 1.9% for *N. gonorrhoeae* from pregnant women by culture in the Durban region of KwaZulu-Natal, South Africa. Previous studies conducted in countries within Africa have reported rates of 2.3% in non-pregnant women (126). Prevalence rates ranging from 4.9% to 6.1% for *N. gonorrhoeae* have been reported in pregnant women (127,128). In a cohort of pregnant women from Australia, predictors of

being infected with *N. gonorrhoeae* included young age, harmful alcohol use, unwanted pregnancy, low birth weight, perinatal death and co-infection with other STIs during pregnancy (128). Other studies have also shown significant associations with socio-demographic, behavioural and clinical factors and *N. gonorrhoeae* infection (127,129). However, our study showed no statistical significance with socio-demographic, behavioural and clinical factors in relation to *N. gonorrhoeae* infection and may have been due to the small sample size. Although no statistical significance was observed, this data is valuable as it demonstrates that of the five out of the six positive samples (83.3%) were from women that were asymptomatic, all these women were unmarried, 83.3% of these positive women did not know whether their partner had other sexual partners and none of these women used condoms during every sexual intercourse. A study conducted in the Morocco showed that literate, married women from urbanised areas with a high parity were at a higher risk of AMR due to self-medication, easy access to antibiotics and pharmacies (26).

Over the past few years, *N. gonorrhoeae* has acquired AMR to penicillins, tetracyclines and fluoroquinolones (2,5,108). In this study, high MIC values to penicillin G indicating a resistant phenotype was observed for all isolates tested, with half of the isolates displaying complete resistance. Similarly, high MIC values for tetracycline (32mg/L) and ciprofloxacin (3mg/L) was observed in this study. Our findings are similar to other *N. gonorrhoeae* AMR studies conducted in South Africa. A study conducted by Rambaran and colleagues (2019) in men and women presenting with male urethritis syndrome (MUS) and vaginal discharge syndrome (VDS) harboured *N. gonorrhoeae* which were resistant to penicillin, tetracycline, and fluoroquinolones (108). A ten year *N. gonorrhoeae* AMR surveillance study conducted in Johannesburg, South Africa showed high level penicillin and tetracycline resistance in male and female populations (69). A more recent study conducted in Johannesburg, South Africa also revealed the presence of a high number of isolates displaying tetracycline, penicillin and ciprofloxacin resistance (130).

Despite the many *N. gonorrhoeae* AMR studies conducted in South Africa, there is no published data on *N. gonorrhoeae* AMR in pregnant populations, thereby lending novelty to this study. The study by Rambaran *et al.,* (2019) identified isolates with MICs of 32mg/L and 16mg/L to tetracycline in men and women with MUS and VDS, these MICs were considered as high level resistance. In our study, similar MIC values were obtained for the asymptomatic pregnant women. The high level of tetracycline resistance observed could have been the

result of selective pressure by doxycycline which had previously been used in the syndromic management for the treatment of Chlamydia infections (11,106). However, since 2015, doxycycline has been replaced by azithromycin for MUS and VDS in the syndromic management approach. Resistance to azithromycin has been observed in South African men who have sex with men (130). In our study isolates, resistance to azithromycin was not observed. However, there is still a need to monitor susceptibility patterns of this antimicrobial since it is part of syndromic management for the treatment of Chlamydia.

In this study, we identified isolates with a >10-fold increase above the breakpoint for ciprofloxacin. One isolate displayed a MIC of 3mg/L to ciprofloxacin. Previous studies conducted in KwaZulu-Natal, South Africa have reported MIC values of 1mg/L for ciprofloxacin (131). The 10 year *N. gonorrhoeae* AMR surveillance study conducted in Johannesburg, South Africa showed MICs of ≥1mg/L for ciprofloxacin. It was observed that from 2008 to 2016, the prevalence of high-level resistance to ciprofloxacin rose exponentially from 25% to 69% (69). Due to emerging antimicrobial resistance, ciprofloxacin was replaced with cefixime in the syndromic management (132). However, during the year 2012 two cases of decreased susceptibility to cefixime with treatment failure was observed in men who have sex with men (133). In addition, ceftriaxone and spectinomycin were recommended for the treatment of infection with *N. gonorrhoeae* in pregnancy or in those who fail to respond to treatment with ciprofloxacin (113). The current study has not observed any resistance to spectinomycin, cefixime and ceftriaxone. However, more *N. gonorrhoeae* AMR studies need to be conducted on pregnant women since this data is severely lacking both nationally and internationally.

The MIC of the WHO strains (Figure 1) was assessed as positive controls for the validation of the data obtained for the *N. gonorrhoeae* samples. The MIC data obtained for the WHO strains (G, W, X, Y and Z) fell within the MIC range and correlated with the MIC classification described by Unemo and colleagues (36).

5. LIMITATIONS

This study was limited due to a very small number of six *N. gonorrhoeae* positive isolates that were obtained from this study. The reason for this was because only 50% of these women consented for participation as the women had to undergo a pelvic examination in order for the endocervical swab to be conducted. Most women refused this procedure since it was not part of their routine check-up thereby resulting in a 50% refusal rate. It is

recommended that future studies should increase the sample size or conduct studies at more than 1 antenatal clinic to obtain a larger number of positive samples for AMR testing. A risk of "selection" of certain strains is taken into consideration as plasmid mediated resistance was not investigated in this study.

6. CONCLUSION

In this study high MIC values to penicillin G, tetracycline and ciprofloxacin were observed. Currently there are no recent published studies from South Africa that have described *N. gonorrhoeae* AMR profiles in pregnant women. This study thereby provides this missing data. However, this study was limited in terms of the number of culture isolates obtained. Despite this limitation, we were still able to identify resistant phenotypes. This study now provides evidence for the development of larger *N. gonorrhoeae* AMR surveillance studies in pregnant women. Despite the lack of ceftriaxone and azithromycin resistant isolates in the study population, it is still imperative to monitor patterns of emerging resistance since over-treatment in syndromic management can contribute to future resistance.

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8. DECLARATION OF CONFLICTING INTERESTS

The author(s) declare no potential conflicts of interests with respect to the research, authorship, and/or publication of this article.

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CHAPTER 4

Manuscript entitled:

“Tracking antimicrobial resistance in *Neisseria gonorrhoeae* from the molecular level using endocervical swabs“

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Tracking antimicrobial resistance in *Neisseria gonorrhoeae* from the molecular level using endocervical swabs

Oree G. [1], Naicker M. [1], Maise H. C. [2], Tinarwo P. [3], Ramsuran V. [4] and Abbai N. S. [1]

[1] School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.

[2] Department of Obstetrics and Gynaecology, School of Clinical Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.

[3] *Department of Biostatistics, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa*

[4] School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

Corresponding author: Professor Nathlee Abbai, School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Private bag X7, Durban 4001, South Africa. Tel.: +27 31 260 4439. Email address: ABBAIN@ukzn.ac.za

KEYWORDS: *Neisseria gonorrhoeae*, antimicrobial resistance, pregnant women, endocervical swabs

ABSTRACT

Background: The global emergence of drug resistance in *Neisseria gonorrhoeae* has resulted in a range of antibiotics and is now a public health concern since this pathogen may become untreatable in the future. This study aimed to detect antimicrobial resistant determinants in *N. gonorrhoeae* directly from endocervical samples by conventional and real time polymerase chain reactions (PCRs).

Methods: Three hundred and seven pregnant women were enrolled in this study. Endocervical swabs were collected from consenting women and used for the detection of *N. gonorrhoeae*. Molecular indicators associated with penicillin, tetracycline, ciprofloxacin, azithromycin, spectinomycin, cefixime and ceftriaxone resistance were detected by conventional and real time PCR.

Results: Of the 307 women, 24 (7.8%) tested positive for *N. gonorrhoeae.* The *tetM* gene carried on the American type plasmid was shown to be present in all the samples. Approximately 87.5% of the samples carried the penicillinase producing African type plasmid and the *gyrase A* gene carrying the Ser-91 mutation was shown to be present in 37.5% of the samples by conventional PCR. Real time PCR detected 29.1%6 Ser-91 mutations and 8.33% azithromycin mutations. Mutations associated with spectinomycin, cefixime and ceftriaxone resistance were not detected by both PCR methods.

Conclusion: The detection of resistance determinants without the need for culture may prove to be more feasible for future epidemiological investigations focused on tracking antimicrobial susceptibility patterns in *N. gonorrhoeae*.

INTRODUCTION

Neisseria gonorrhoeae, the etiologic agent of gonorrhoeae is reported to be the second most prevalent bacterial sexually transmitted infection (STI) globally [1]. The World Health Organization (WHO) conducted a STI surveillance in 2018 that revealed an estimated 87 million new gonorrhoea infections globally during 2016, with an incidence of 20 cases per 1 000 population (uncertainty interval 14–28 per 1 000 population) in women [2]. A study conducted in South Africa and Zimbabwe reported an overall prevalence of 0.7% for *N. gonorrhoeae* infections in women from the general population [3]. The same study reported an overall incidence rate of 2.4 per 100 woman-years for *N. gonorrhoeae* infections. A higher incidence rate was observed in South African women (3.7 per 100 women years) when compared with the women from Zimbabwe (1.3 per 100 woman-years) [3]. Other studies conducted in South Africa have reported prevalence rates for *N. gonorrhoeae* ranging from 3% -11% in women [4–6].

A population of women attending antenatal care in Shiraz, Southern Iran previously reported a prevalence of *N. gonorrhoeae* to be 1.3% [7]. Untreated *N. gonorrhoeae* infections have been reported to have an association with a range of adverse pregnancy outcomes such as neonatal conjunctivitis, foetal growth retardation, spontaneous abortion, stillbirth, prematurity, low birth weight, post-partum endometritis and increased risk of Human Immunodeficiency Virus (HIV) transmission from mother to child during birth [2–5,8–10]. *Neisseria gonorrhoeae* has been reported to be asymptomatic, thereby allowing the survival of this organism to persist since asymptomatic infections are not treated as per standard of care [8].

The global emergence of drug resistance of *N. gonorrhoeae* to beta-lactams (including cephalosporins), tetracyclines and quinolones is a public health concern since this infection may become untreatable in the future [11,12]. A recent study conducted in KwaZulu-Natal, South Africa demonstrated antimicrobial resistance towards ciprofloxacin (70%), penicillin (60%) and tetracycline (100%) for pure isolates of *N. gonorrhoeae* [10]. The South African STI management guidelines utilises the syndromic approach for the diagnosis and management of STIs [13]. Gonorrhoeae is associated with abnormal vaginal discharge and according to South African guidelines this is treated with an intramuscular single dose of 250 mg ceftriaxone and the oral consumption of 1 g of azithromycin, together with a 12 hour oral doses of 400 mg metronidazole for 7 days[13]. Patients that are allergic to penicillin stop the

use of ceftriaxone and are recommended use a single oral dose (2g) of azithromycin [13]. A gynaecological assessment is suggested if pain persists after 48 to 72 hours of treatment [13].Previous antimicrobial resistance (AMR) studies on *N. gonorrhoeae* have been based on culturing. Culturing for *N. gonorrhoeae* was the gold standard against which other diagnostic tests were compared. However, other non-culture tests were in demand due to the difficulty in maintaining the viability of *N. gonorrhoeae* during transportation and storage [14]. Many countries have now shifted to performing nucleic acid amplification assays for the identification and association of AMR in *N. gonorrhoeae* [10–12,15]. It is highly likely that previous studies that have reported on antimicrobial resistance patterns in *N. gonorrhoeae* may have missed out a significant proportion of positive samples due to the drawbacks of the culturing. Therefore, this study was aimed at detecting the resistance determinants associated with penicillin, tetracycline, ciprofloxacin, spectinomycin, cefixime, ceftriaxone and azithromycin from the molecular level using the primary endocervical swab sample. To the best of our knowledge, there is no published South African study that has used this approach to determine patterns of resistance, especially in pregnant women.

MATERIALS AND METHODS

Ethics approval

Full ethics approval for this study was granted by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN), (BE355/18).

Study setting and population

The study population included n=307 pregnant women recruited from the antenatal clinic of the King Edward VIII hospital (KEH) in Durban, South Africa from November 2018 to July 2019. Each participant was required to provide a signed informed consent together with a questionnaire on demographics and sexual behaviour.

Sample collection and processing

Each enrolled woman underwent a pelvic examination during which time an endocervical swab sample was collected. The swab was collected in a dry sterile tube and was immersed in 2 mL of phosphate buffered saline (pH 7.4), vortexed to dislodge the sample material from swab and the cervical fluid was stored at -20°C until further molecular analysis. The

molecular assays were conducted at the School of Clinical Medicine's Research Laboratory, UKZN.

DNA isolation from the endocervical sample

The PureLink Microbiome DNA Purification kit (ThermoFisher Scientific, United States) was used for the DNA extractions from the swabs. The manufacturer's instructions were followed without any modifications.

Detection of Neisseria gonorrhoeae from the endocervical sample

The pre-designed commercially available TaqMan qPCR assay, specifically for *N. gonorrhoeae* (Assay ID: Ba04646252_s, ThermoFisher Scientific, United States), was used. Amplification was performed on a Quant Studio 5 Real-time PCR system.

Detection of molecular markers/genes associated with antimicrobial resistance by conventional PCR

Detection of beta-lactamase-producing plasmid types

A multiplex PCR was used to detect beta-lactamase-producing plasmid types (Africa, Asia, and Toronto) using specific primers and protocols previously described by Luwang and Tanaka (103,104) (Table 1).

Detection of tetracycline resistance

Tetracycline resistance plasmid types (America and Dutch) were detected by PCR using the primers shown in Table 1. Detection of the specific plasmid type was performed by digestion of the PCR amplicon with *Hinf*I (New England Biolabs, United States).

Detection of ciprofloxacin resistance

The *gyrase A* gene was amplified using primers described in Table 1. Detection of the Ser-91 mutation which confers ciprofloxacin resistance was performed by digestion of the PCR amplicon with *Hinf*I (New England Biolabs, United States).

Detection of azithromycin, spectinomycin, cefixime and ceftriaxone resistance determinants

The 23*S rRNA* gene was amplified in order to identify mutations that are responsible for conferring azithromycin resistance and the *rpsE* gene was amplified to identify spectinomycin resistant determinants (Table 1). The *penA* and *por* genes were amplified to identify resistant determinants for cefixime and ceftriaxone. Primers, PA1 and PA2 primers were used for the amplification of the *penA* gene (Table 1).

Table 1: Primer sequences and targeted genes for the antibiotic resistant determinants investigated in this study

Antibiotic	Primer Name	Primer Sequence (5'-3')	Targeted gene
Azithromycin	gon*rRNA*-F	ACGAATGGCGTAACGATGGCCACA	Point mutations in 23*S rRNA* regions in allele 2 (135)
	23*S rRNAR*-allele 2	GCGACCATACCAAACACCCACAGG	
Cefixime and Ceftriaxone	PA1	CGATATGATCGAACCTGG	*penA* gene (136)
	PA2	ACAATCTCGTTGATACTCG	
Spectinomycin	5S-F	TGGCAAAACATGA AATTGAAG	*rpsE* gene (137)
	5S-R	GCCATGGTTAACTCCCAAAA	
Penicillin G	GC1F	AACTCACGGACAAAATCACGG	β-lactamase producing plasmid (103)
	GC2F	CACCTATAAATCTCGCAAGCC	
	GC3R	AACGCAAGCAGGACGAAATC	
	GC4R	CCTCCACCTTCATCCTCAGC	
Tetracycline	TetMF	ACTGTTGAACCGAGYAAACCT	*TetM* gene (103)
	TetMR	TCTATCCGACTATTTGGACGACG	
Ciprofloxacin	GyrAF	CGGCGCGTACTGTACGCGATGCA	*Gyrase A* gene (104)
	GyrAR	ATGTCTGCCAGCATTTCATGTGAGA	

Real time PCR for the detection of N. gonorrhoeae antimicrobial determinants

The primer sequences described on Table 1 from Donà *et al.,* (2018) were used to detect AMR determinants from the 24 TaqMan qPCR positive samples. Quantitative PCR (qPCR)

was performed on the Quant Studio 5 Real-time PCR system (Applied Biosystems, Life Technologies) in a 96 well microtiter reaction plate. Each reaction comprised 5 μL SyberGreen, 0.5 μL forward primer (10uM), 0.5 μL reverse primer (10uM) and 2 μL template DNA with the addition of water to a final volume of 10 μL. Reaction conditions included; an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation (95°C for 15 seconds), annealing (62°C for 10 seconds) and extension (72°C for 10 seconds).

Table 2: Primers employed for the detection of antimicrobial resistant determinants from *N. gonorrhoeae*

Antibiotic affected	Primer Name:	Primer Sequence (5'-3'):	Associated Target
Decreased susceptibility or resistance to ESCs	545_F	5'-TGGTTAACGGTCGTTACGTCGATT-3'	*penA* Gly545Ser (Mosaic *penA*)
	545_R	5'-GGCCCTGCCACTACACCGTT-3'	
Decreased susceptibility or resistance to ESCs	345_F	5'-GGCAAAGTGGATGCAACCGAT-3'	*penA* Asp345del (Mosaic *penA*)
	345_R	5'-GATAAACGTGGGTATCTTGTACGG-3'	
Moderate azithromycin resistance	C2611_F	5'-AACGTCGTGAGACAGTTTGGTTT-3'	23S rRNA C2611T
	C2611_R	5'-GAACTTAGCTACCCGGCTATGCA-3'	
High azithromycin resistance	A2059_F	5'-TACAGTAAAGGTTCACGGGGTCAC-3'	23S rRNA A2059G
	A2059_R	5'-ATGGCCACACTGTCTCCTCCC-3'	
Ciprofloxacin resistance	gyrA_S91_F	5'-AATACCACCCCCACGGCGATCT-3'	GyrA Ser91Phe
	gyrA_S91_R	5'-TCTATCAGCACATAACGCATAGCG-3'	
Spectinomycin resistance	16S_1192_F	5'-CTTGTCATTAGTTGCCATCATTTG-3'	16S rRNA C1192T
	16S_1192_R	5'-TAAGGGCCATGAGGACTTGATA-3'	

Statistical Analysis

The data analysis was performed in R Statistical Computing software, version 3.6.2. Due to the smaller sample size and consequently a low power, the demographic, behavioural and clinical factors were simply described either as overall or stratified by the resistant profiles. Age was the only numerical measure and skewed which was suggestive of summarisation by minimum, maximum and quartiles only. The other categorical demographic attributes were summarised in the form of counts and percentage frequencies.

RESULTS

Detection of resistance targets by conventional PCR

Of the 307 samples tested, 24 of them produced positive amplification by the TaqMan qPCR assay. The prevalence of *N. gonorrhoeae* among the participants was 7.8% (24/307). Overall, all the samples tested produced a PCR product for at least one of the target genes. Table 3 shows the results of the PCR assays for the targets investigated.

All the 24 positive samples produced a PCR amplicon of 700bp corresponding to the *tetM* gene (Table 3). Sanger sequencing of the PCR amplicons confirmed the identity of the PCR product (99% identity to the American type-*tetM* conjugative plasmid (Accession number: GU479464.1). The restriction digestion pattern (600bp and 93bp) of the *tetM* gene confirmed the presence of the American-type conjugative plasmid in the study samples (Table 3).

An amplicon size of 737bp was obtained with the multiplex PCR for the beta-lactamase-producing plasmid. This amplicon was shown to be present in 87.5% (21/24) of the samples analysed (Table 3). Sanger sequencing confirmed that the samples carried the *N. gonorrhoeae* strain African-TEM 1-MIC16 plasmid PJD5, complete sequence (Accession Number: MK973084.1).

A PCR amplicon of 278bp corresponding to the *gyrase A* gene was amplified in 41.6% (10/24) of the positive samples (Table 3). The amplicons were confirmed to be the *gyrase A* gene from *N. gonorrhoeae*, DNA gyrase subunit A, partial [*N. gonorrhoeae*] (Accession number: APU93729.1). According to the restriction digestion analysis, the 278bp fragment remained undigested in 90.0% (9/10) of the *gyrase A* positive samples indicating the presence of the Ser-91 mutation associated with ciprofloxacin resistance (Table 3).

The *penA* gene which carries mutations associated with cefixime and ceftriaxone resistance was detected in 9/24 (37.5%) of the samples. The *penA* amplicons were confirmed to be the Penicillin-binding protein 2 of *Neisseria gonorrhoeae,* GenBank number: AFJ54623.1. However, none of the mutations associated with resistance to these antibiotics were detected after sequencing of the PCR amplicons indicating a lack of emerging patterns of resistance to ceftriaxone and cefixime. Similarly, mutations associated with resistance to spectinomycin and azithromycin were not detected in the study samples. The PCR amplicons were confirmed to be *Neisseria gonorrhoeae* strain 32380 23S ribosomal RNA gene, complete sequence, Genbank number: KT954110.1 and *Neisseria gonorrhoeae* strain 3mut_SPC 30S subunit ribosomal protein S5 (*rpsE*) gene, Genbank number: GU395615.1.

Table 3: Summary of the antibiotic resistance profiles generated for the endocervical swab samples testing positive for the presence of *N. gonorrhoeae* in the study population by conventional PCR.

Antibiotic	Targeted Gene	Type	n=24
Penicillin G	β-lactamase producing plasmid	African	21/24
Tetracycline	*TetM*	American	24/24
Ciprofloxacin	*GyrA*	Mutation Ser-91	9/24
		Wild type	1/24
Azithromycin	23S allele 2	N/A	0/24
Spectinomycin	*rpsE*	N/A	0/24
Ceftriaxone and cefixime	*penA*	N/A	0/24

N/A – Not applicable

Detection of dual and triple resistant profiles

According to the data, a mono-resistance profile was observed for one sample. Only the *tetM* determinant related to tetracycline resistance was identified in this sample. With respect to samples exhibiting a dual-resistance profile, it was shown that 66.6% (16/24) of the samples carried at least two of the resistant determinants. A lower percentage of the samples, 29.1% (7/24) carried determinants for all resistance markers tested (i.e. *tetM*, *gyrase A* and beta-lactamase producing plasmid) (Table 4).

Table 4: Results of the PCR amplification reactions for the individual samples and resistance profiles generated

	PCR results for resistant determinants									
Participant Identifier	***gyrase A***	**Ser-91 mutation**	***TetM***	**Beta-lactamase producing plasmid**	***23SrRNA***	***23SrRNA* Allele 2 mutation**	***penA gene***	***penA* mutation**	***rpsE* gene**	***rpsE* mutation**
G003	Positive	Yes	Positive	Positive	Negative	Negative	Positive	Negative	Negative	Negative
G007	Negative	-	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
G051	Positive	Yes	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative
G061	Positive	Yes	Positive	Positive	Negative	Negative	Positive	Negative	Positive	Negative
G079	Positive	Yes	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Negative
G132	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G133	Negative	-	Positive	Positive	Negative	Negative	Positive	Negative	Negative	Negative
G134	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G136	Positive	Yes	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G138	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G141	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G142	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G143	Positive	Yes	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
G144	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G145	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G148	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G151	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G153	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G154	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G160	Negative	-	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
G176	Positive	Yes	Positive	Positive	Positive	Negative	Positive	Negative	Negative	Negative
G180	Positive	Yes	Positive	Positive	Positive	Negative	Positive	Negative	Positive	Negative
G206	Positive	No	Positive	Positive	Positive	Negative	Positive	Negative	Positive	Negative
G247	Positive	Yes	Positive	Positive	Positive	Negative	Positive	Negative	Positive	Negative

Samples highlighted in blue indicate dual resistance

Samples highlighted in red indicate triple resistance

The description of behavioural and clinical factors by resistance profile

A description of the 24 positive study participants stratified by overall resistance in relation to demographics, behavioural and clinical factors, shown on Table 5. The results showed that

the majority of them were unmarried 91.7% (22/24), with 79.2% (19/24) of the participants completing high school. A high proportion, 79.2% (19/24) of the women had experienced first sex at an early age of 15 to 20 years of old. On the other hand, condoms were mostly used “sometimes” 66.7% (16/24). Majority, 58.3% (14/24) of the women reported having 2-4 lifetime sex partners. The results also showed that a high proportion of women, 83.3% (20/24) were asymptomatic for infection (i.e. did not present with symptoms of abnormal vaginal discharge). A high proportion of 18/24 (75%) of these women reported no previous treatment of any STIs, however; 41.7% (10/24) of these women, reported past STI symptoms. No woman was reported to have had spontaneous abortion and most of the women 79.2% (19/24) were reported to be in their third trimester of pregnancy.

Table 5: Demographic, behavioural and clinical factors by resistance profiles generated by conventional PCR

Resistance	**Mono (N=1)**	**Dual (N=16)**	**Triple (N=7)**	**Overall (N=24)**
Age				
Median(Q1-Q3)	33.0(33.0-33.0)	28.0(26.0-32.3)	25.0(21.5-27.5)	27.5(24.8-32.3)
Min-Max	33.0-33.0	20.0-40.0	21.0-34.0	20.0-40.0
Current abnormal discharge				
No	1 (100%)	13 (81.2%)	6 (85.7%)	20 (83.3%)
Yes	0 (0%)	3 (18.8%)	1 (14.3%)	4 (16.7%)
Level of education				
Did not attend school	0 (0%)	0 (0%)	1 (14.3%)	1 (4.2%)
High school	1 (100%)	12 (75.0%)	6 (85.7%)	19 (79.2%)
College, University	0 (0%)	4 (25.0%)	0 (0%)	4 (16.7%)
Marital status				
No	1 (100%)	14 (87.5%)	7 (100%)	22 (91.7%)
Yes	0 (0%)	2 (12.5%)	0 (0%)	2 (8.3%)
Has a regular sex partner				
No	0 (0%)	9 (56.2%)	2 (28.6%)	11 (45.8%)
Yes	1 (100%)	7 (43.8%)	5 (71.4%)	13 (54.2%)
Cohabitating with partner				
No	0 (0%)	12 (75.0%)	5 (71.4%)	17 (70.8%)
Yes	1 (100%)	4 (25.0%)	2 (28.6%)	7 (29.2%)
Age of 1st sex				
15-20	1 (100%)	13 (81.2%)	5 (71.4%)	19 (79.2%)
21-25	0 (0%)	3 (18.8%)	2 (28.6%)	5 (20.8%)

Resistance	Mono (N=1)	Dual (N=16)	Triple (N=7)	Overall (N=24)
Number of lifetime sex partners				
1	1 (100%)	4 (25.0%)	3 (42.9%)	8 (33.3%)
2 to 4	0 (0%)	10 (62.5%)	4 (57.1%)	14 (58.3%)
>4	0 (0%)	2 (12.5%)	0 (0%)	2 (8.3%)
Condom use				
Sometimes	1 (100%)	11 (68.8%)	4 (57.1)	16 (66.7%)
Never	0 (0%)	3 (18.8)	3 (42.9%)	6 (25%)
Always	0 (0%)	2 (12.5%)	0 (0%)	2 (8.33%)
Condom used during last sex act				
No	0 (0%)	10 (62.5%)	4 (57.1%)	14 (58.3%)
Yes	1 (100%)	6 (37.5%)	3 (42.9%)	10 (41.7%)
Trimester of pregnancy				
2nd	0 (0%)	3 (18.8%)	2 (28.6%)	5 (20.8%)
3rd	1 (100%)	13 (81.2%)	5 (71.4%)	19 (79.2%)
Past preterm delivery				
No	1 (100%)	11 (68.8%)	7 (100%)	19 (79.2%)
Yes	0 (0%)	5 (31.2%)	0 (0%)	5 (20.8%)
Past miscarriage				
No	1 (100%)	13 (81.2%)	4 (57.1%)	18 (75.0%)
Yes	0 (0%)	3 (18.8%)	3 (42.9%)	6 (25.0%)
Past spontaneous abortion				
No	1 (100%)	16 (100%)	7 (100%)	24 (100%)
Experienced past symptoms of STIs				
No	1 (100%)	11 (68.8%)	2 (28.6%)	14 (58.3%)
Yes	0 (0%)	5 (31.2%)	5 (71.4%)	10 (41.7%)
Previous treatment for STIs				
No	1 (100%)	12 (75.0%)	5 (71.4%)	18 (75.0%)
Yes	0 (0%)	4 (25.0%)	2 (28.6%)	6 (25.0%)

Detection of resistance targets by real time PCR

The AMR determinants of the 24 TaqMan qPCR positive isolates were investigated through real time PCR (Table 6), as described by Donà *et al.,* (2018). Two isolates (8.333%) were observed to have high azithromycin resistance and 7/24 clinical isolates that contained the Ser91 mutation, exhibited ciprofloxacin resistance. All (100%) isolates were susceptible to cefixime, ceftriaxone and spectinomycin (Table 6).

Table 6: Summary of the antibiotic resistance profiles generated for the endocervical swab samples testing positive for the presence of *N. gonorrhoeae* in the study population by real time PCR.

Antibiotic affected	Targeted Gene	n=24
Decreased susceptibility or resistance to ESCs	*penA* Gly545Ser (Mosaic *penA*)	0/24
Decreased susceptibility or resistance to ESCs	*penA* Asp345del (Mosaic *penA*)	0/24
Moderate Azithromycin resistance	*23S rRNA* C2611T	0/24
High Azithromycin resistance	*23S rRNA* A2059G	2/24
Ciprofloxacin	*GyrA* Ser91Phe	7/24
Spectinomycin	*16S rRNA* C1192T	0/24

Detection of dual and triple resistant profiles

Of the 24 samples, two samples (G03 and 176) were observed to harbour the azithromycin resistant determinant, whereas the conventional PCR was unable to detect this. Samples G03 and G176 showed dual resistance towards azithromycin and ciprofloxacin. Samples G03, G61, G79,G176, G180 and G247 corresponded well and picked up the Ser-91 mutation for gyrase in both conventional and real time PCR. However, G133 was observed to have the

gyrase mutation when subjected to real time PCR. Similarly, G51 and G136 were only positive for the gyrase mutation by conventional PCR. The WHO –Y strain served as a positive control for all targets, however no positive control was available for specitnomycin resistance. All samples were negative for the *penA* Gly545Ser, *penA* Asp345del, 23S rRNA A2059G and 16S rRNA C1192T targets which corresponds well to no resistance being observed towards cefixime, ceftriaxone and spectinomycin for the conventional PCR.

Table 7: Antimicrobial resistant determinants from the 24 positive TaqMan qPCR *N. gonorrhoeae* isolates

Sample Name	Target					
	penA Gly545Ser	*penA* Asp345del	23S rRNA C2611T	23S rRNA A2059G	GyrA Ser91Phe	16S rRNA C1192T
G03	Negative	Negative	Negative	Positive	Positive	Negative
G07	Negative	Negative	Negative	Negative	Negative	Negative
G51	Negative	Negative	Negative	Negative	Negative	Negative
G61	Negative	Negative	Negative	Negative	Positive	Negative
G79	Negative	Negative	Negative	Negative	Positive	Negative
G132	Negative	Negative	Negative	Negative	Negative	Negative
G133	Negative	Negative	Negative	Negative	Positive	Negative
G134	Negative	Negative	Negative	Negative	Negative	Negative
G136	Negative	Negative	Negative	Negative	Negative	Negative
G141	Negative	Negative	Negative	Negative	Negative	Negative
G142	Negative	Negative	Negative	Negative	Negative	Negative
G143	Negative	Negative	Negative	Negative	Negative	Negative
G144	Negative	Negative	Negative	Negative	Negative	Negative
G145	Negative	Negative	Negative	Negative	Negative	Negative
G148	Negative	Negative	Negative	Negative	Negative	Negative
G151	Negative	Negative	Negative	Negative	Negative	Negative
G153	Negative	Negative	Negative	Negative	Negative	Negative
G154	Negative	Negative	Negative	Negative	Negative	Negative
G160	Negative	Negative	Negative	Negative	Negative	Negative
G176	Negative	Negative	Negative	Positive	Positive	Negative
G180	Negative	Negative	Negative	Negative	Positive	Negative
G206	Negative	Negative	Negative	Negative	Negative	Negative
G247	Negative	Negative	Negative	Negative	Positive	Negative
G138	Negative	Negative	Negative	Negative	Negative	Negative
WHO-Y	Positive	Positive	Positive	Positive	Positive	-

Samples highlighted in blue indicate dual resistance

DISCUSSION

The worldwide clinical management of *N. gonorrhoeae* infections is becoming increasingly challenging due to resistance to various classes of available antibiotics. These include sulphonamides, beta-lactams, tetracyclines, macrolides, fluoroquinolones and more recently expanded-spectrum cephalosporins [18,19]. 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 [12]. The genotypic variability of *N. gonorrhoeae* has been linked to the acquisition of new genetic material [8].

The emergence of drug resistant *N. gonorrhoeae* is a global concern [20]. For pregnant women, this concern has escalated since untreated *N. gonorrhoeae* infections can have severe consequences on reproductive health, such as an increased risk for acquiring HIV and other STIs as well as obstetric morbidity [21]. Currently, there is limited published data in South Africa on the molecular detection of antimicrobial resistant determinants in *N. gonorrhoeae* from primary genital swab samples. This study provided evidence for the detection of resistant determinants associated with penicillin, tetracycline and ciprofloxacin from the molecular level using the primary endocervical swab sample collected from pregnant women.

In this study, *N. gonorrhoeae* was detected in 24/307 (7.82%) endocervical swab samples. The *tetM* gene was detected in all 24 samples. The prevalence of tetracycline resistance from a genotypic level in this study population was 100%. Similar findings were reported by Rambaran et al. (2019) in which the prevalence of tetracycline resistance was found to be 100% in a population of men and women from South Africa. Further analysis of the *tetM* gene revealed that the *tetM* gene was carried on the American-type plasmid. The findings of our study are similar to other African studies [15,22]. A study conducted in a Moroccan female population, revealed a 100% prevalence of the American type plasmid carrying the *tetM* gene [22]. 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 [15]. Previous studies have suggested that the Dutch type *tetM* plasmid is more prevalent in Asian countries (such as Thailand, Philippines, China and Bangladesh), whereas the American type *tetM* plasmid is more prevalent in European and African countries [23–25]. 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, corresponding with previous

findings that suggest the American variant of the *tetM* originated in Africa [10,26]. 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.

Beta-lactamase-producing plasmids were shown to be present in (87.5%) of the samples analysed in this study (Table 3). The prevalence of penicillin resistance observed in this study is higher than reports from a very recent study conducted in a male population from South Africa in which penicillin resistance was 33% [27].The African type plasmid related to penicillin resistance was observed in the current study. A recent study conducted in South Africa during 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%) [11].

N. gonorrhoeae has the ability to adapt to hostile conditions in which antibiotics may be present and become selective to multiple changes leading to resistance and ultimately to treatment failures [8]. These changes that lead to resistance are either chromosomal or plasmid-mediated [8,18]. Various resistance determinants can co-exist within a single strain of this pathogen consequently increasing the resistance of the organism to numerous antibiotics [8]. Chromosomal mediated resistance has been described to emerge and spread at a slower rate compared to some plasmid-mediated resistance [8]. The first type of chromosomal resistance is drug-specific which involves a single step mutation and the second type involves mutations at multiple loci that determine the level and pattern of resistance [32]. Chromosomal mediated resistance occurs at a slower rate due to the step-wise acquisition of chromosomal changes over years compared to plasmid mediated resistance that occurs in a single step process[8, 32]. Genetic transformation in gonorrhoeae is responsible for the acquisition of drug resistance[32]. Plasmid mediated resistance in tetracyclines and penicillins are acquired through gaining resistant determinants from a plasmid through the process of conjugation [8, 32]. However, not all strains contain conjugative plasmids therefor the different rates of dissemination of plasmid-mediated resistance has been observed [8, 32]. The rapid spread of plasmid-mediated resistance to penicillin and tetracycline has been observed in numerous countries in comparison to the low rate of chromosomal mediated resistance [8, 32]. This study therefore focused on the plasmid-mediated resistance towards tetracycline and penicillin.

Ciprofloxacin resistance has been increasing and has been reported on a worldwide scale, with countries such as Asia (93.8%) and Hefei (100%) exhibiting high levels of resistance

[28,29]. In this study, the *gyrase A* gene was detected in less than half of the study samples (41.6%) by conventional PCR (Table 3). The sensitivity of the PCR for this gene was shown to be low and will require further optimization to obtain the desired results. Mutations within the *gyraseA* gene (Ser-91) has been linked to ciprofloxacin resistance [8,30]. The mutations associated with ciprofloxacin resistance has been previously described [11,12,29,31]. Despite, the limited number of positive amplicons obtained, the Ser-91 mutation which is associated with ciprofloxacin resistance was present in 90% of the samples analysed. 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 [12]. Despite the lack of detection of resistant mutations to ceftriaxone, spectinomycin and azithromycin, PCR detection and DNA sequencing without the need for culture can be used to track emerging resistance to these antibiotics.

According to the behavioural and socio-demographics data collected in this study, it was observed that the majority of women that participated in this study were unmarried, had a secondary level of education, have reported having between 2-4 lifetime sex partners and had sometimes used condoms during sex. In addition, these women that were associated with AMR were predominantly in the third trimester of pregnancy, were asymptomatic and were not treated for STIs in the past. There is a lack of published data that have compared socio-demographic and behavioural risk factors in association with antimicrobial resistance patterns. The majority of the published studies have focused on demographic, behavioural and clinical factors associated with the prevalence of *N. gonorrhoeae* infections [6,30,31]. Our study now provides data on demographic, behavioural and clinical characteristics of pregnant women associated with resistance from Durban, South Africa, thereby adding to the current literature.

The detection of AMR determinants using the SYBR green approach has been described to be inexpensive and requires specific primers for amplification and is thus a more feasible approach in low-resource settings and for point-of-are testing [33]. The results of the real time PCR assay showed a 100% correlation with data obtained from the conventional PCR for ceftriaxone, cefixime and spectinomycin. However, discrepancies were observed for ciprofloxacin and azithromycin resistance between the conventional and real time PCR (Tables 3 and 6). The results obtained from the real time PCR study illustrates that the SYBR green qPCR method rapidly detects AMR determinants, however, further optimization is

required to increase the specificity of the probes that are used. According to Donà and colleagues, this method could potentially be used as a rapid test for point-of-care with further optimization with regards to extra-genital specimens and future studies should include chromosomal resistant determinants for penicillin and tetracycline [33]. This study has illustrated that the detection of AMR determinants by conventional PCR is a time consuming process and the risk of contamination and risk of no PCR product showing on the gel for positive samples.

LIMITATIONS

This study was limited as the researchers focused only on plasmid-mediated resistance for penicillin and tetracycline since plasmid-mediated resistance has previously been described to spread at a more rapid rate in comparison to chromosomal mediated resistance. However, since chromosomal and plasmid mediated resistance can co-exist, both should be investigated in future studies Additionally, study is limited due to genes for azithromycin, ceftriaxone and cefixime which may have not been amplified consequently overlooking a percentage of the study population. The use of molecular techniques for the diagnosis, disease management and surveillance of *N. gonorrhoeae* require further optimization of PCRs for cefixime, ceftriaxone and azithromycin for future use.

CONCLUSION

Due the limitations associated with culture based techniques (such as loss of viability of the gonococcus) for identifying susceptibility/resistance patterns in *N. gonorrhoeae*, detection of resistance determinants from the molecular level without the need for culture may prove to be more feasible for future epidemiological investigations focused on tracking antimicrobial susceptibility/resistance patterns for this pathogen.

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DECLARATION OF CONFLICTING INTERESTS

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ORCID ID: Glynis Oree: https://orcid.org/0000-0003-4557-5185

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CHAPTER 5

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Comparison of endocervical swabs to cultured isolates for the detection of antimicrobial resistance determinants in *Neisseria gonorrhoeae*

G Oree,[1] M Naicker,[1] HC Maise,[2] NS Abbai[1]

[1] *School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, South Africa*
[2] *Department of Obstetrics and Gynaecology, School of Clinical Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, South Africa*

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Comparison of endocervical swabs to cultured isolates for the detection of antimicrobial resistance determinants in *Neisseria gonorrhoeae* using Polymerase Chain Reaction Assays

Glynis Oree. [1], Meleshni Naicker. [1], Hopolang Clifford Maise. [2] and Nathlee Samantha Abbai [1*]

[1] *School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa. glynoree@gmail.com; meleshni@yahoo.com; abbain@ukzn.ac.za*

[2] *Department of Obstetrics and Gynaecology, School of Clinical Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa. Maisehc@ukzn.ac.za*

*Corresponding author: Professor Nathlee Abbai, School of Clinical Medicine Research Laboratory, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Private bag X7, Durban 4001, South Africa. Tel.: +27 31 260 4439. Email address: ABBAIN@ukzn.ac.za

ABSTRACT

Background: The global emergence of antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* to various antibiotics is a public health concern. To date, there have been no published South African studies that have compared the primary swab to the cultured isolates for the detection of *N. gonorrhoeae* AMR determinants. This study provides data on this comparison.

Methods: Paired endocervical swabs were collected from 307 pregnant women. The first swab was stored in Amies charcoal media for culture assessment and the second swab was used for the molecular detection of *N. gonorrhoeae* and resistant determinants. Specific targets (genes/plasmids/mutations) associated with resistance to penicillin, tetracycline, ciprofloxacin, spectinomycin, cefixime, azithromycin and ceftriaxone were detected from cultured isolates and endocervical swabs.

Results: Of the 307 samples tested in this study, only 6 samples were positive by culture. A total of 24 samples tested positive for *N. gonorrhoeae* with the qPCR assay. The 6 samples which were positive for culture fell within the qPCR positives group. Since this study was designed to directly compare the culture swabs to the endocervical swabs for the detection of AMR determinants, the current analysis included only the 6 culture samples and 6 paired endocervical swabs samples (n=6). All 6 isolates were resistant to tetracycline and penicillin G, 5/6 isolates were resistant to ciprofloxacin and all isolates were susceptible to the remaining antimicrobials. There was a 100% correlation between the cultured isolates and endocervical swabs for detecting the specific AMR determinants conferring resistance to tetracycline, penicillin G, and ciprofloxacin.

Conclusion: Based on the findings in this study, tracking emerging patterns of resistance from the molecular level using only the endocervical swabs may serve as an attractive future research direction.

INTRODUCTION

Neisseria gonorrhoeae (*N. gonorrhoeae*), is the causative agent of gonorrhoeae and is reported to be among one of the most prevalent bacterial sexually transmitted infections (STIs) globally (18). *N. gonorrhoeae* has the remarkable ability to acquire antibiotic resistance through chromosomal mediated molecular mutation and by the acquisition of plasmids containing resistant determinants, thereby creating a worldwide public health concern (116,133,143). This STI is included in the World Health Organization (WHO) global priority list of antibiotic-resistant bacteria as it has developed resistance to every antimicrobial drug recommended for treatment since the introduction of the sulphonamides in the 1930s (144).

A 2018 WHO global surveillance study revealed that in 2016, 87 million new infections occurred in adolescents and adults between the ages of 15 to 49 years old, with sub-Saharan Africa displaying the highest incidence for *N. gonorrhoeae* (5,6). South African studies have previously reported prevalence rates for *N. gonorrhoeae* from 3%-11% in women (9,10,18). A study conducted in women attending antenatal care had recently reported a prevalence of 1.3% for *N. gonorrhoeae* (121). Untreated *N. gonorrhoeae* infections are associated with adverse outcomes such as reproductive health issues, obstetric morbidity and acquisition of Human Immunodeficiency Virus (HIV) and other STIs (3,5,14,15,17–22,110).

N. gonorrhoeae has been reported to be asymptomatic thereby allowing the survival of this microorganism to persist (37). The global emergence of drug resistance in *N. gonorrhoeae* to previous and current antibiotic treatment is a public health concern since this infection may become untreatable in the future (102,106,129). This microorganism has phenotypic variability through differential expression of existing parts of the microorganisms genome and genotypic variation through acquiring and incorporating new genetic material by conjugation or transformation (37). Another important characteristic is the antigenic variability that *N. gonorrhoeae* acquires through obtaining genetic material from other organisms which contributes to its various mechanisms of resistance (145). This demonstrates that continuous surveillance of the emerging resistance/susceptibility patterns of this pathogen is critical.

It has been previously reported that plasmid-mediated penicillinase producing *N. gonorrhoeae* (*PPNG*) are β-lactamase producing and associated with three plasmid types that confer penicillin resistance (Asian, African and Toronto types) (102–104). Tetracycline

resistant *N. gonorrhoeae* (*TRNG*) is conferred by the *tetM* gene which is harboured by two conjugative plasmids (American and Dutch) (102–104). Ciprofloxacin resistance has been frequently linked with the *gyrA* and *parC* mutations, with predominance of the Ser-91 *gyrA* status (9,37,141). A transfer of the *rpsE* gene allele encoding the mutant ribosomal protein S5 (RPS5) results in a substitution mutation of Thr-24→Pro, subsequently disrupting the binding of spectinomycin to the ribosomal target thereby promoting resistance (109). High-level azithromycin resistance has been recently observed in association with mutations in the *mef* efflux pump gene, the *mtrR* regulatory and *mtrCDE* structural genes (146,147). Point mutations in the 23*S rRNA* region have been previously described to confer azithromycin resistance (112). Resistance towards third generation cephalosporins such as cefixime and ceftriaxone has been observed in many countries including South Africa (133). A recombination of gonococcal *penA* with *penA* genes of commensal *Neisseria* species produces a mosaic-like *penA* structure, associated with conferring this resistance (4,148). Alternatively, modifications in *mtrR* and *porB* are associated with decreased drug accumulation (149).

Antimicrobial susceptibility studies on *N. gonorrhoeae* have been based on minimum inhibition concentration (MIC) by using culture. However, maintaining the viability of *N. gonorrhoeae* is a challenge due to poor transportation and storage often leading to the unsuccessful growth of the organism (54). Detection of resistance targets and mutations without the need for culture serves as a much more attractive alternative for AMR studies. In this study, pregnant women had provided paired endocervical swabs for culture and molecular assessments. DNA was extracted from both cultured samples and the primary endocervical swabs. The paired extracted DNA was used to detect gene targets and identify determinants associated with resistance to penicillin, tetracycline, ciprofloxacin, spectinomycin, azithromycin, cefixime and ceftriaxone by the polymerase chain reaction (PCR). The aim of this study was to compare the primary swab to the cultured isolates for the detection of *N. gonorrhoeae* AMR determinants. To date, there have been no published South African studies that have investigated the sensitivity of the primary endocervical swab against culture for the detection of AMR determinants in this pathogen.

MATERIALS AND METHODS

Ethics approval

Full ethics approval for this study was granted by the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (UKZN), (BE355/18).

Study setting and population

The study population included n=307 pregnant women from the antenatal clinic of the King Edward VIII hospital (KEH) in Durban, KwaZulu-Natal. The study population was recruited from November 2018 to July 2019. At enrolment, eligibility was based on women who were 18 years and older, willing to provide informed consent, willing to provide data on sexual behaviour, demographics and clinical history by use of a structured questionnaire and willing to undergo a pelvic examination to provide two endocervical swab samples.

Sample collection and processing

Each enrolled women underwent a pelvic examination conducted by a gynaecologist and paired endocervical swab samples were collected. The first swab was placed in Amies Charcoal transport media (LASEC, South Africa) immediately after collection. The second swab was placed in a dry sterile tube containing 2 mL of phosphate buffered saline (pH 7.4), vortexed to dislodge the sample material from swab and the cervical fluid was stored at -20°C until further molecular analysis. The vortexed swab was discarded (59). Both swabs were stored at room temperature before being processed at the School of Clinical Medicine's Research Laboratory, UKZN. The swabs were processed within 4 hours of sample collection. The dry swab was immersed in

Culture detection of N. gonorrhoeae

The Amies swabs were plated onto New York City Agar plates and incubated for 24 to 48 hours, at 35-37°C in the presence of 5% CO_2 (59). Suspected colonies were sub-cultured onto Chocolate Agar plates and incubated for a further 24 hours in the presence of 5% CO_2 at 35-37°C. To confirm the identity of the isolates, Gram staining, oxidase, catalase, superoxol and carbohydrate utilization tests were conducted (59). One bead from each of the lyophilised/freeze-dried vials containing WHO strains was placed in 1 mL of Brain-Heart Infused (BHI) Broth (LASEC, South Africa) and was incubated for 30 minutes at 35-37°C. Sterile technique was applied and a loop full of the suspension was streaked onto New York City Agar plates. The plates were incubated at 35-37°C in the presence of 5% CO2 for 24 to 48 hours.

Preparation of culture DNA for extraction

Culture confirmed clinical isolates were re-plated onto Chocolate Agar plates. A set of WHO control strains were also plated out. The culture plates were incubated for 24 hours at 35-37°C in the presence of 5% CO_2. Following growth on the agar plate, an inoculum with 1 mL Brain-Heart Infused Broth (LASEC, South Africa) was then prepared for the clinical isolates and WHO control strains. The samples were incubated overnight at 35-37°C in 5% CO_2.

DNA extraction from cultured isolates

The inoculum was then used for the isolation of genomic DNA using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, United States) according to the manufacturer's guide. A Nanodrop spectrophotometer (ThermoFisher Scientific, South Africa) was used to assess the concentration and purity of the extracted DNA. The extracted DNA was stored at -20°C until further use.

DNA isolation from the endocervical sample

The stored cervical fluid samples were retrieved from the freezer, allowed to thoroughly thaw and then subjected to DNA extraction. The suspension was centrifuged at 14,000 *g* for 10 min and the supernatant was discarded. Recovered pellets were then subjected to a DNA extraction method using the PureLink Microbiome Kit (ThermoFisher Scientific, Waltham, Massachusetts, US), according to the manufacturer's instructions. A Nanodrop spectrophotometer (ThermoFisher Scientific, South Africa) was used to assess the concentration and purity of the extracted DNA.

Detection of Neisseria gonorrhoeae from the endocervical sample

The pre-designed commercially available TaqMan quantitative PCR (qPCR) assay, specifically for *N. gonorrhoeae* (Assay ID: Ba04646252_s, ThermoFisher Scientific, United States), was used for the detection of this pathogen. Briefly, each reaction comprised 2.5 µL of Fast Start 4x probe master mix, 0.5 µL FAM-labelled probe/primer mix and 2 µL sample DNA in a final volume of 5 µL. Amplification was performed on a Quant Studio 5 Real-time PCR system with 1 cycle at 95°C for 30 seconds followed by 45 cycles of denaturation (95°C for 30 seconds) and annealing (60°C for 30 seconds). Amplified fluorescent products were detected at completion of the annealing period. The raw fluorescent data that was

automatically generated by the PCR Quant Studio 5 PCR system software included cycling threshold (C_T) values.

Detection of molecular markers/genes associated with antimicrobial resistance

The sequences of the primers used for the detection of the individual genes are shown in Table 1. A no template control was used as a negative control and WHO reference strains were used as positive controls to confirm validity of the PCR results. Conventional PCR was performed for all gene targets in a final volume of 25 µl and comprised of: 12.5 µl Dream Taq (2x) Mastermix (ThermoFisher Scientific, United States), 0.5 µl of each (10 µM) primer, 2 µl template DNA and nuclease free water. PCR amplicons and a 100 bp GeneRuler DNA ladder (ThermoFisher Scientific, United States) were separated on a 1% agarose at 100 V for 1 hour by gel by electrophoresis and visualised using a UV transilluminator (Gene Genius, SYNGENE, Maryland, United States).

Table 1: Primer sequences and targeted genes for the antibiotic resistant determinants investigated in this study.

Antibiotic	Primer Name:	Primer Sequence (5'-3'):	Targeted gene	Reference Sequence/positive control
Azithromycin	**gon*rRNA*-F**	ACGAATGGCGTAACGATGGCCACA	Point mutations in 23*S rRNA* regions in allele 2 (135)	WHO-Y
	23*S rRNAR*-allele 2	GCGACCATACCAAACACCCACAGG		
Cefixime and Ceftriaxone	**PA1**	CGATATGATCGAACCTGG	*penA* gene (136)	WHO-Y
	PA2	ACAATCTCGTTGATACTCG		
Cefixime and Ceftriaxone	**PorB1**	AAAGGCCAAGAAGACCTCGGC	*por* gene (136)	WHO-Y
	PorB2	GAGAAGTCGTATTCCGCACCG		
Spectinomycin	**5S-F**	TGGCAAAACATGA AATTGAAG	*rpsE* gene (137)	NG 3.1 30S ribosomal protein S5 (Genbank accession: KF021592)
	5S-R	GCCATGGTTAACTCCCAAAA		
Penicillin G	**GC1F**	AACTCACGGACAAAATCACGG	β-lactamase producing plasmid (103)	-
	GC2F	CACCTATAAATCTCGCAAGCC		
	GC3R	AACGCAAGCAGGACGAAATC		
	GC4R	CCTCCACCTTCATCCTCAGC		
Tetracycline	**TetMF**	ACTGTTGAACCGAGYAAACCT	*TetM* gene (103)	-
	TetMR	TCTATCCGACTATTTGGACGACG		
Ciprofloxacin	**GyrAF**	CGGCGCGTACTGTACGCGATGCA	*Gyrase A* gene (104)	WHO-Y
	GyrAR	ATGTCTGCCAGCATTTCATGTGAGA		

Detection of azithromycin resistance determinants

The 23*S rRNA* gene was amplified in order to identify mutations that are responsible for conferring azithromycin resistance (Table 1). Amplification was performed at an initial denaturation of 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute (denaturation), 60°C for 1 minute (annealing) and 72°C for 1 minutes (extension). A final extension step at 72°C for 5 minutes was included.

Detection of cefixime and ceftriaxone resistance determinants

The *penA* and *por* genes were amplified to identify resistant determinants for cefixime and ceftriaxone. Primers, PA1 and PA2 were used for the amplification of the *penA* gene (Table 1). Amplification was performed with an initial denaturation of 95°C for 3 minutes, followed by 30 cycles of 95°C for 1 minute (denaturation), 48°C for 1 minute (annealing) and 72°C for 1 minute (extension) and final extension step at 72°C for 5 minutes.

Primers, PorB1and PorB2 were used for the amplification of the *por* gene (Table 1). Amplification was performed at an initial denaturation of 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute (denaturation), 56°C for 1 minute (annealing) and 72°C for 1 minutes (extension). A final extension step at 72°C for 5 minutes was included.

Detection of spectinomycin resistance determinants

The *rpsE* gene was amplified to identify spectinomycin resistant determinants (Table 1). Cycling conditions included initial denaturation of 95°C for 1 minutes, followed by 30 cycles of 95°C for 1 minute (denaturation), 48°C for 1 minute (annealing) and 72°C for 1 minutes (extension) and final extension step at 72°C for 5 minutes.

Detection of penicillin resistance determinants

A multiplex PCR was used to detect beta-lactamase-producing plasmid types using 0.25 µL (10 µM) of each primer, with protocols previously described by Luwang and Tanaka (103)'(104). The reaction mixture consisted of 5 µL of template DNA together with all the reagents previously mentioned. The PCR cycling conditions included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 58°C for 1 minute, extension at 72°C for 1 minute and 30 seconds and a final extension at 72°C for 10 minutes.

Detection of tetracycline resistance determinants

Tetracycline resistance plasmid types were detected by PCR using the primers shown in Table 1. The PCR cycling conditions included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minute, extension at 72°C for 1 minute and 30 seconds and a final extension at 72°C for 10 minutes. Detection of the specific plasmid type was performed by digestion of

the PCR amplicon with *Hinf*I (New England Biolabs, United States). The digests were incubated at 37°C for 1 hour followed by heat inactivation at 80°C for 20 minutes and visualized on a 1.5% agarose gel.

Detection of ciprofloxacin resistance determinants

The GyraAF and GyrAR primers were used for the amplification reactions. Cycling conditions included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles each consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, extension at 72°C for 1 minute and 30 seconds and a final extension at 72°C for 10 minutes. Detection of the Ser-91 mutation which confers ciprofloxacin resistance was performed by digestion of the PCR amplicon with *Hinf*I (New England Biolabs, United States). The digests were incubated at 37°C for 1 hour followed by heat inactivation at 80°C for 20 minutes and visualized on a 1.5% agarose gel.

Sequencing of PCR amplicons to confirm identify and alignment

To confirm the identity of the PCR amplicons and identify mutations responsible for conferring resistance, Sanger sequencing was performed. The samples were sequenced at Inqaba Biotechnological Industries in Pretoria, South Africa. Sequencing was performed on an ABI3500XL genetic analyser and the raw sequence data was edited using Chromas software V2.6.5 (Technelysium, Queensland, Australia). The identity of the edited sequences was confirmed using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). Nucleotide sequences were translated using ExPASy translate tool (https://web.expasy.org/translate/). In order to identify resistance conferring mutations, a multiple sequence alignment was performed using ClustalW (https://www.genome.jp/tools-bin/clustalw). The alignment included WHO reference strains which are resistant to the named antibiotics (carrying the mutations) and paired culture and endocervical swab samples.

RESULTS

Detection of N. gonorrhoeae by culture and PCR

Of the 307 samples tested in this study, only six samples were positive by culture. A total of 24 samples tested positive for *N. gonorrhoeae* with the qPCR assay. The six samples which were positive for culture fell within the qPCR positives group. Since this study was designed

to directly compare the culture swabs to the endocervical swabs for the detection of AMR determinants, the current analysis included only the six culture samples and six paired endocervical swabs samples (n=6). The phenotypic resistance profiles for the six culture positive isolates were conducted previously (Supplementary Figure 1). The phenotypic results from the previous study showed that all isolates were penicillin resistant (12-64mg/L), isolates were observed to have intermediate (1mg/L) and resistant (1.9-32mg/L) profiles to tetracycline and ciprofloxacin resistance was observed for five of the six isolates (1.16-3mg/L). All isolates were susceptible to spectinomycin, azithromycin, cefixime and ceftriaxone (0.75-24 mg/L, 0.016-0.125, 0.002-0.016 and 0.002-0.012, respectively).

Identification of mutations associated with azithromycin resistance

The *23S rRNA* was amplified in all cultured samples and 5/6 of the endocervical swab samples. The PCR amplicons were confirmed to be the *Neisseria gonorrhoeae* strain 32380 23S ribosomal RNA gene, complete sequence (Genbank number: KT954110.1). Figure 1 represents the sequence alignment for the WHO-Y strain and the selected paired and endocervical samples. The presence of mutations that are responsible for conferring resistance to azithromycin was absent in the clinical samples analysed, however present in the WHO strain. The WHO strain exhibited a C→T nucleotide substitution which has been associated with azithromycin resistance (Figure 1). This was in line with the observed phenotypic resistance profile previously conducted on these isolates, in which none of the clinical isolates were azithromycin resistant, however, the WHO-Y strain was resistant.

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G180E           ----------------TGTGA-GATGCAATCTACCCGCTGCTAGACGGAAAGACCCCGTG
G176C           -CGAGGTTGAAGTGGTTGTGAAGATGCAATCTACCCGCTGCTAGACGGAAAGACCCCGTG
WHO-Y           ------TTGAAGTGGTTGTGAAGATGCAATCTACCCGCTGCTAGACGGAAAGACCCCGTG
G176E           ---AATTTGAAGTGGTTGTGAAGATGCA-TCTACCCGCTGCTAGACGGAAAGACCCCGCG
                                ***** ****** ***************************** *

G180C           AACCTTTACTGTAGCTTTGCATTGGACTTTGAAGTCACTTGTGTAGGATAGGTGGGAGGC
G180E           AACCTTTACTGTAGCTTTGCATTGGACTTTGAAGTCACTTGTGTAGGATAGGTGGGAGGC
G176C           AACCTTTACTGTAGCTTTGCATTGGACTTTGAAGTCACTTGTGTAGGATAGGTGGGAGGC
WHO-Y           AACTTTTACTGTAGCTTTGCATTGGACTTTGAAGTCACTTGTGTAGGATAGGTGGGAGGC
G176E           AACCTTTACTGTAGCTTTGCATTGGACTTTGAAGTCACTTGTGTAGGATAGGTGGGAGGC
                *** ********************************************************

G180C           TTGGAAGCAGAGACGCCAGTCTCTGTGGAGTCGTCCTTGAAATACCACCCTGGTGTCTTT
G180E           TTGGAAGCAGAGACGCCAGTCTCTGTGGAGTCGTCCTTGAAATACCACCCTGGTGTCTTT
G176C           TTGGAAGCAGAGACGCCAGTCTCTGTGGAGTCGTCCTTGAAATACCACCCTGGTGTCTTT
WHO-Y           TTGGAAGCAGAGACGCCAGTCTCTGTGGAGTCGTCCTTGAAATACCACCCTGGTGTCTTT
G176E           TTGGAAGCAGACACGCCAGTCTCAGTGGAGTCGTCCTTGAAATACCACCCTGGTGTCTTT
                *********** *********** ************************************

G180C           GAGGTTCTAACCCAGACCCGTCATCCGGGTCGGGGACCGTGCATGGTAGGCAGTTTGACT
G180E           GAGGTTCTAACCCAGACCCGTCATCCGGGTCGGGGACCGTGCATGGTAGGCAGTTTGACT
G176C           GAGGTTCTAACCCAGACCCGTCATCCGGGTCGGGGACCGTGCATGGTAGGCAGTTTGACT
WHO-Y           GAGGTTCTAACCCAGACCCGTCATCCGGGTCGGGGACCGTGCATGGTAGGCAGTTTGACT
G176E           GAGGTTCTAACCCAGACCCGTCATCCGGGTCGGGGACCGCGCATGGTAG-----------
                *************************************** *********
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Figure 1: ClustalW multiple sequence alignment for the identification of point mutations in 23*S rRNA* regions in alleles 2 for azithromycin resistance. C: culture DNA, E: endocervical DNA, WHO-Y: positive control. The presence of the mutation (C→T) is shown as highlighted text.

Identification of mutations associated with cefixime/ceftriaxone resistance

The *por* and *penA* genes were amplified in all cultured samples and 5/6 of the endocervical swab samples. For the *penA* amplicons, the sequencing results confirmed its identity to be Penicillin-binding protein 2, partial *[Neisseria gonorrhoeae]* GenBank number: AFJ54623.1. Similarly for *por*, the amplicons were confirmed to be porin [*Neisseria gonorrhoeae*] Genbank number: WP_057321029.1.

According to Figure 2, mutations in the *por* gene responsible for resistance to cefixime and ceftriaxone were absent in the clinical samples. However, the WHO resistant strain was observed to have deletions of tyrosine, lysine and histidine amino acids and substitutions of the following amino acids Gln → Tyr, Val → Ala, Ala → Gly, Ala → Val and Asn → Gln (Figure 2). This also links to the previous phenotypic resistance profile of the isolates in which none of the 6 culture positives showed resistance to either antibiotic.

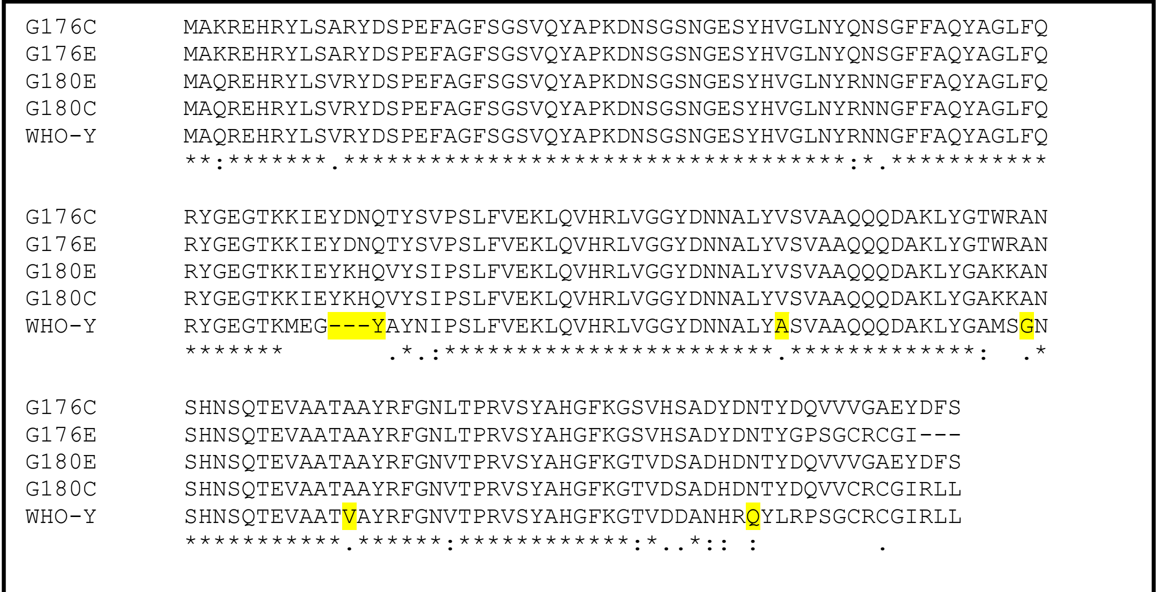

Figure 2: ClustalW multiple sequence alignment for identification of mutations in *por* gene for cefixime/ceftriaxone resistance. C: culture DNA, E: endocervical DNA, WHO-Y: positive control. The presence of the mutations are shown as highlighted text.

Similarly, sequences obtained from the PCR for the *penA* gene showed multiple substitution mutations (Arg→Met, Gly →Thr, Glu→Asp, Ile→Val, Asn→Ser, Arg→Gln, Pro→Lys, Ala→Val, Val→Glu, Leu→Val, Gln→Lys, Glu→Ala, Arg→Lys, Glu→Lys, Pro→Ala, Ala→Pro, Asn→Tyr, Gly→Ala, Gky→Ser, Ile→Asn, Met→Tyr, Ser→Gln, Asn→Glu, Ser→Phe, Thr→Asp, Lys→Gln, Pro→Ala) in the WHO strain, which were not present in the clinical samples (Figure 3).

Figure 3: ClustalW multiple sequence alignment for identification of mutations in *penA* gene for cefixime/ceftriaxone resistance. C: culture DNA, E: endocervical DNA, WHO-Y: positive control. The presence of the mutations are shown as highlighted text.

Identification of mutations associated with spectinomycin resistance

The *rpsE* gene which carries mutations associated with spectinomycin resistance was amplified in the all culture samples and 5/6 endocervical swab samples. The amplicons were confirmed to be *Neisseria gonorrhoeae* strain 3mut_SPC 30S subunit ribosomal protein S5 (*rpsE*) gene, Genbank number: GU395615.1. For this antibiotic, there was no available WHO reference strain which was resistant to spectinomycin, therefore the alignment was conducted using the *rpsE* sequence of the *N. gonorrhoeae* strain NG 3.1 30S ribosomal protein S5 (Genbank accession: KF021592) which carries the mutation for spectinomycin resistance (109). A substitution mutation (Thr-24→Pro) was observed in the KF021592 strain with no mutation observed in both endocervical and culture sequences (Figure 4). This is in keeping

with the AMR data on these isolates, resistance to spectinomycin was not observed in the isolates.

```
G180            ----------------MVAVNRVTKVVKGGRIMAFSALTVVGDGDGRIGMGKGKSKEVPV
G180E           ----------------MVAVNRVTKVVKGGRIMAFSALTVVGDGDGRIGMGKGKSKEVPV
G176E           ----------------MVAVNRVTKVVKGGRIMAFSALTVVGDGDGRIGMGKGKSKEVPV
G176C           ----------------MVAVNRVTKVVKGGRIMAFSALTVVGDGDGRIGMGKGKSKEVPV
KF021592        MAKHEIEERGDGLIEKMVAVNRVPKVVKGGRIMAFSALTVVGDGDGRIGMGKGKSKEVPV
                                *******.************************************

G180            AVQKAMDQARRSMIKVPLKNGTIHHEVIGRHGATKVFMQPAKEGSGVKAGGPMRLVFDAM
G180E           AVQKAMDQARRSMIKVPLKNGTIHHEVIGRHGATKVFMQPAKEGSGVKAGGPMRLVFDAM
G176E           AVQKAMDQARRSMIKVPLKNGTIHHEVIGRHGATKVFMQPAKEGSGVKAGGPMRLVFDAM
G176C           AVQKAMDQARRSMIKVPLKNGTIHHEVIGRHGATKVFMQPAKEGSGVKAGGPMRLVFDAM
KF021592        AVQKAMDQARRSMIKVPLKNGTIHHEVIGRHGATKVFMQPAKEGSGVKAGGPMRLVFDAM
                ************************************************************

G180            GIHNISAKVHGSTNPYNIVRATLDGLSKLHTPADIAAKRGLTVEDILGVNHG
G180E           GIHNISAKVHGSTNPYNIVRATLDGLSKLHTPADIAAKRGLTVEDILGVNHG
G176E           GIHNISAKVHGSTNPYNIVRATLDGLSKLHTPADIAAKRGLTVEDILGVNHG
G176C           GIHNISAKVHGSTNPYNIVRATLDGLSKLHTPADIAAKRGLTVEDILGVNHG
KF021592        GIHNISAKVHGSTNPYNIVRATLDGLSKLHTPADIAAKRGLTVEDILGVNHG
                ****************************************************
```

Figure 4: ClustalW multiple sequence alignment for identification of mutations in *rpsE* gene for spectinomycin resistance. C: culture DNA, E: endocervical DNA, Genbank reference sequence (KF021592). The presence of the mutation is shown as highlighted text.

Identification of penicillin, tetracycline and ciprofloxacin resistance determinants

The beta-lactamase producing plasmid, *tetM* gene and *gyraseA* gene was shown to be present in all the clinical samples tested. The correlation of the PCR assays for the individual genes using the swabs compared to the cultured isolates was 100%. The sensitivity of the PCR assays with both sample types was determined using the following formula: $\frac{number\ of\ true\ positives}{number\ of\ true\ positives + number\ of\ false\ negatives}$ x 100 (150).

The *tetM* gene was shown to be carried on the American type-*tetM* conjugative plasmid (Genbank number: GU479464.1). The multiplex PCR for the beta-lactamase-producing plasmid carried the *N. gonorrhoeae* strain African-TEM 1-MIC16 plasmid PJD5, complete sequence (Genbank Number: MK973084.1). The PCR amplicons corresponding to the *gyraseA* gene was confirmed to be the *gyraseA* gene from *N. gonorrhoeae*, DNA gyrase subunit A, partial [*N. gonorrhoeae*] (Genbank number: APU93729.1). The Ser-91 mutation which was detected by digestion of the *gyraseA* PCR amplicon with *Hinf*I was shown to be present in both culture and endocervical swab samples in 5/6 sample pairs. The presence of

the digestion pattern matched the AMR results since there was one isolate which was susceptible to ciprofloxacin and hence did not carry the mutation.

DISCUSSION

N. gonorrhoeae is one of the most prevalent STIs globally and has acquired AMR to various antibiotics used for treatment over years. Data on plasmid types and antibiotic resistant genes that this pathogen harbours is fundamental for monitoring the emergence and spread of antibiotic resistance (151,152). The majority of studies conducted in previous years have focused on the detection of AMR to antibiotics using culture-based techniques (108,130,132). In this study, a total of 6 isolates of *N. gonorrhoeae* were detected by culture based assays. However, 24 samples tested positive for this pathogen when detected from the endocervical swabs using qPCR. Based on the low recovery rate of isolates with culture, this study investigated the performance of the endocervical swabs against the cultured isolates for the detection of AMR patterns. In this study, specific genes that carry mutations associated with resistance to azithromycin, cefixime, ceftriaxone, spectinomycin and ciprofloxacin were investigated. The presence of plasmids that carry the resistance determinants for penicillin G and tetracycline were also investigated.

Specific mutations associated with the *23S rRNA* is responsible for low- and high-level resistance mutations to azithromycin and erythromycin (110,135,153,154). In this study, a C→T nucleotide substitution was observed only for the WHO azithromycin resistant strain, however, absent in the paired culture and swab samples. This finding correlates well with the AMR profile for these isolates which showed a lack of resistance to azithromycin (Supplementary Figure 1). There was a good correlation (83%) between the cultured isolates and endocervical swabs for detecting the *23S rRNA* for further mutation scanning.

Specific mutations in the *penA* and *por* gene have been associated with resistance to cefixime and ceftriaxone (5). There was a good correlation (83%) between the cultured isolates and endocervical swabs for detecting the *por* gene. However, mutations associated with cefixime and ceftriaxone resistance was absent in the study samples. The mutations (Gln → Tyr, Val → Ala, Ala → Gly, Ala → Val and Asn → Gln) were detected in the WHO resistant control strain indicating the high sensitivity of the PCR and DNA sequencing assays for detecting the desired mutations from the endocervical swab samples. Similarly, another study reported on the following amino acid substitutions Gly-101→Lys, Ala-102→Asp, Gly-101→Lys, Ala-102→Asn,Gly-101→Asn and Ala-102→Asp (136,155). These mutations are often associated

with a decrease in permeability to penicillin, cephalosporins and ciprofloxacin (156,157). The mutation scan was also 100% correlated with the AMR results for the isolates since none of the isolates were resistant to cefixime or ceftriaxone.

A previous study which investigated mutations in the *penA* mosaic allele (PBP 2) which showed reduced susceptibility to cefixime and ceftriaxone in *N. gonorrhoeae* revealed that a substitution of Ala-501→Val was the cause of this reduced susceptibility (155,158). Upon further investigation that study revealed that the PBP 2 pattern XVII contained an Ala-501→Val substitution which was confirmed by a modelling study to cause a conformational alteration of the β-lactam binding pocket leading to a reduction in susceptibility to cephems (158). Our findings are similar to other studies since the Ala→Val substitution was detected in the WHO resistant control strain (158). No mutations were observed with paired culture and endocervical samples despite yielding successful PCR and DNA sequencing results. The lack of resistance conferring mutations correlates well with the AMR patterns for the isolates since susceptibility to cefixime and ceftriaxone was observed in these isolates (Supplementary Figure 1).

Resistance to spectinomycin is associated with mutations in the *rpsE* gene (109). This study used the *N. gonorrhoeae* strain NG 3.1 30S ribosomal protein S5 (Genbank accession: KF021592) as the positive control to determine spectinomycin resistance. A study conducted by Ilina *et al.,* (2013), illustrated that the spectinomycin resistant strain (*N. gonorrhoeae* strain NG 3.1 30S ribosomal protein S5 (Genbank accession: KF021592) carried a substitution mutation of Thr-24→Pro (109,159). The culture and endocervical swab samples investigated in this study lacked these mutations indicating spectinomycin susceptibility. There was a good correlation between the PCR and sequencing assays for both sample types.

Ciprofloxacin resistance has been frequently associated with the *gyraseA* and *parC* mutations, with predominance of the Ser-91 *gyraseA* mutation (9,37,141). The presence of the Ser-91 mutation (identified by restriction digestion analysis) was present in both the endocervical and cultured samples. PCR detection and restriction analysis of the *gyraseA* gene can be used as a future method to track ciprofloxacin resistance from primary endocervical swabs especially in countries where ciprofloxacin is still used for the treatment of gonorrhoea.

A recent study conducted in South Africa by Rambaran *et al*., (2019) showed tetracycline resistance to be associated with the presence of the *tetM* gene carried on a plasmid. The paired cultured and endocervical samples contained the American plasmid variant of the *tetM* gene (identified by restriction digestion analysis). This is in keeping with recent findings from 2019 which showed that 90% of an investigated population contained the American plasmid variant which is in alignment with the premise that the American plasmid variant of the *tetM* gene originated on the African continent (107,108,160,161). Our findings confirm that surveillance for tetracycline resistance can be investigated from primary endocervical swabs since the molecular assays are in concordance with the AMR results for this antibiotic.

N. gonorrhoeae strains with plasmid-mediated resistance to penicillin contain plasmids with a $bla_{\text{TEM-1}}$ gene, encoding a TEM-1-type β-lactamase (5). This enzyme hydrolyses the amide bond of penicillin and renders the penicillin inactive (5). The multiplex PCR employed in this study was successful in detecting the African type beta-lactamase plasmid in both cultured and swab samples. The presence of the African type plasmid is in accordance with previous findings from South Africa (130). The PCR results are also in concordance with the AMR results for this antibiotic.

CONCLUSION

This comparative study between endocervical and cultured samples demonstrated the ability of the endocervical samples as a future sample to track emerging patterns of antimicrobial resistance in *N. gonorrhoeae* since it correlated well with the cultured samples. This approach minimizes the need to culture which requires more stringent measure from sample collection, transportation, sample processing and maintaining viability of the gonococcus. Since the results of the molecular assays correlated well with AMR phenotypic results, tracking emerging patterns of resistance from the molecular level using only the endocervical swabs may serve as an attractive future research direction. However, an important disadvantage associated with molecular methods is their ability to only detect existing AMR determinants and therefore cannot completely replace phenotypic assays due to their inability to detect new AMR determinants that may develop in the future. Another important factor is to carefully consider all AMR determinants for each antimicrobial, as some strains may have more than one resistance determinant. This study was limited by the small sample size (six isolates) therefore it is recommended that future studies should attempt to obtain more isolates. Future studies to be conducted on the molecular determinants for AMR should

consider investigating samples collected from different infection sites and not be subjected to endocervical samples only. Despite this limitation, our study was successful in tracking resistance using molecular determinants from the 6 paired samples.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declare no potential conflicts of interests with respect to the research, authorship, and/or publication of this article.

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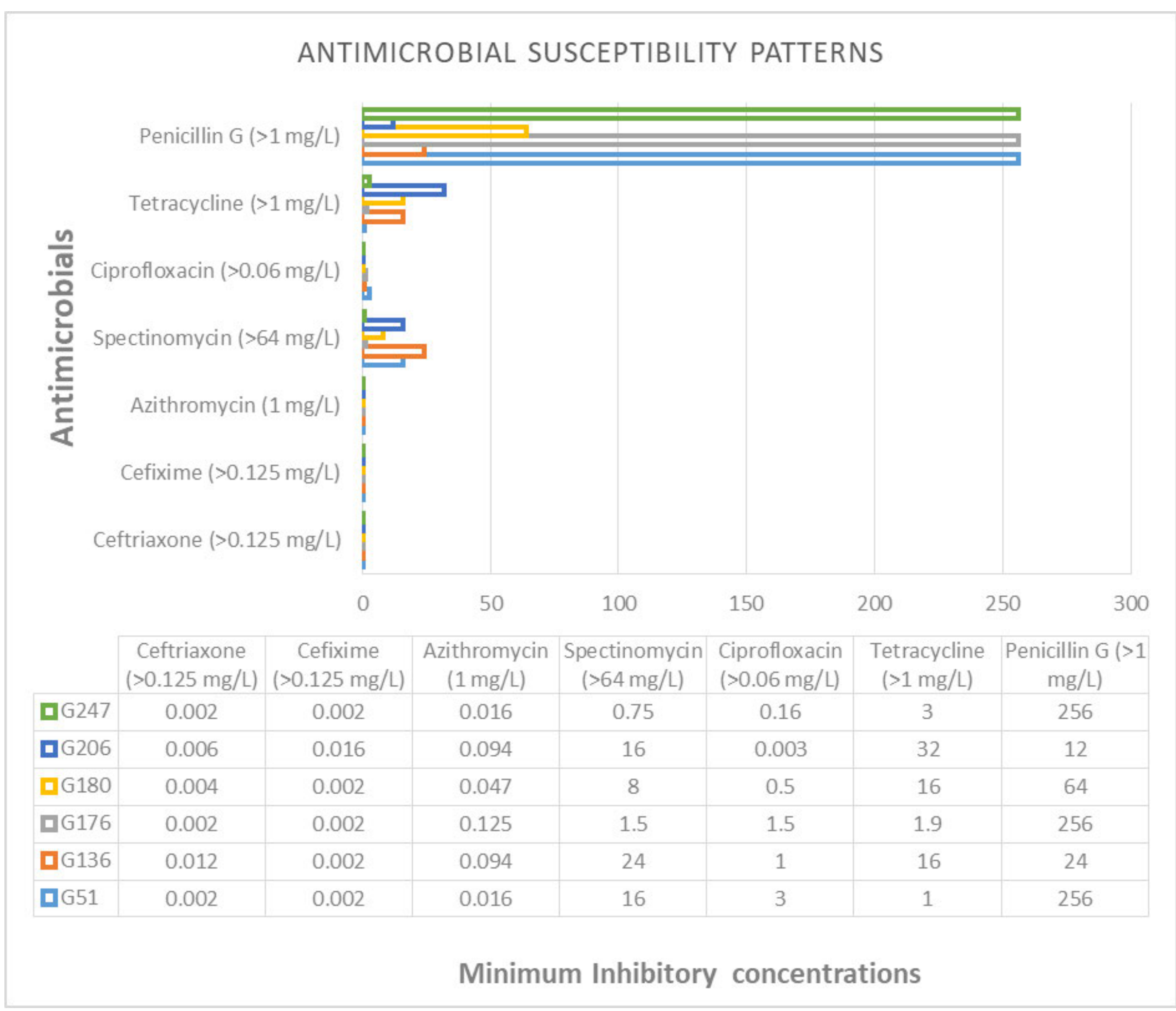
SUPPLEMENTARY DATA



	Ceftriaxone (>0.125 mg/L)	Cefixime (>0.125 mg/L)	Azithromycin (1 mg/L)	Spectinomycin (>64 mg/L)	Ciprofloxacin (>0.06 mg/L)	Tetracycline (>1 mg/L)	Penicillin G (>1 mg/L)
G247	0.002	0.002	0.016	0.75	0.16	3	256
G206	0.006	0.016	0.094	16	0.003	32	12
G180	0.004	0.002	0.047	8	0.5	16	64
G176	0.002	0.002	0.125	1.5	1.5	1.9	256
G136	0.012	0.002	0.094	24	1	16	24
G51	0.002	0.002	0.016	16	3	1	256

Figure S1: Etest™ data of emerging antimicrobial susceptibility/resistance patterns in *N. gonorrhoeae*. Patterns of resistance and susceptibility were determined by the 2019 EUCAST breakpoints.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

N. gonorrhoeae is an STI which poses a threat to males and females globally (15). This bacterial STI has been reported as a health hazard due to its association with high morbidities and rapid increase in resistance to all previous and current antibiotics used for treatment (15,116). The number of *N. gonorrhoeae* cases are much higher worldwide than actual reports and is a result of under-reporting due to the lack of clinical diagnostics and elevated number of asymptomatic cases that remain undetected especially in women (152).

The prevalence of *N. gonorrhoeae* in the pregnant women was 7.8%. This prevalence estimate is relatively high when compared to a study in Africa that had a prevalence of 1.3% (121). The women who tested positive for this pathogen in the current study had a median age of 28 years old. A high proportion of the study women were asymptomatic, reported less condom use, were unmarried, had completed high school and reported having more than one sexual partner. Many studies have reported a significant association between socio-demographic and sexual behaviour with prevalent and incident *N. gonorrhoeae* infections (39–43). However, no statistical significance was observed with these factors in the current study.

This study evaluated methods of detection for *N. gonorrhoeae* using culture which was previously used as the "gold standard" the commercial TaqMan qPCR assay, *16S rRNA* and *opa* PCR. The TaqMan qPCR detected the highest number of infections i.e. 7.8% (24/307) with culture performing the poorest (1.95%, 6/307). The TaqMan qPCR assay was shown to be highly specific and sensitive when compared to the other methods of detection. The TaqMan assay specifically detects *N. gonorrhoeae* only, unlike other FDA approved diagnostic tests which are multiplex assays (55,56,123). This study demonstrated that culture is inferior to the TaqMan qPCR and this finding is in accordance with previous publications (3,13,24,54). This study was the first study to report on the TaqMan assay in a South African population of pregnant women. A limitation of this study was that due to financial constraints we did not compare the performance of the TaqMan qPCR to other qPCR platforms approved for the diagnosis of *N. gonorrhoeae.* This can serve as a future research endeavor.

N. gonorrhoeae is a resilient pathogen and has acquired many mechanisms which increases its ability to evade the host immune system and develop resistance to previous and currently used antibiotics (2,5,71). Many surveillance studies have been conducted on AMR patterns, however there is a lack of data on antimicrobial susceptibility patterns in *N. gonorrhoeae* isolated from pregnant women in our setting (6,36,41,116). For pregnant women, this concern has escalated since untreated *N. gonorrhoeae* infections can have severe implications on reproductive health, acquisition of HIV and other STIs and obstetric morbidity (16). Laboratories on a worldwide scale rely on the use of conventional culture techniques to test for antimicrobial sensitivity on selective media, especially in resource-limited settings (54).

This study identified resistance to penicillin G, tetracycline and ciprofloxacin and susceptibility towards spectinomycin, azithromycin, cefixime and ceftriaxone in the pure isolates obtained through the study. This trend was observed in a previous South African study conducted on men and women presenting with male urethritis syndrome (MUS) and vaginal discharge syndrome (VDS) (130). Our study supports the ineffectiveness and the discontinuation of treatment of *N. gonorrhoeae* infections with tetracycline, penicillin G and ciprofloxacin which is supported by the same observations in a South African surveillance study (69). This study provided AMR profiles for an under researched population group in South Africa, but was limited due to the low number of isolates obtained through culture. Despite this limitation, we were still able to identify resistant phenotypes. This study now provides evidence for the development of larger *N. gonorrhoeae* AMR surveillance studies in pregnant women.

N. gonorrhoeae has been known to acquire mutations and antigenic variability through obtaining genetic material from other microorganisms subsequently increasing its resistance mechanisms (5,37). We investigated the molecular determinants associated with penicillin G, tetracycline, ciprofloxacin, spectinomycin, azithromycin, cefixime and ceftriaxone resistance from non-cultured endocervical swabs. All samples in this study exhibited tetracycline resistance through the acquisition of the *tetM* gene from the American plasmid. This is in keeping with previous findings which showed the presence of the *tetM* gene in 92% of isolates with 90% of these samples containing the American variant, supporting the premise that this variant originated from the African continent (107,108). In addition, 87.5% of the samples contained the African penicillinase-producing plasmid, similar to findings reported by Fayemiwo and partners; who found that plasmid-mediated tetracycline and penicillin

resistance was relatively high in men and women who reside in Johannesburg (102). Lastly, 37.5% of the samples contained the *gyrase A* gene carrying the Ser-91 mutation. Another study conducted in Africa reported a 77.9% prevalence of ciprofloxacin resistance (122). Although no resistance was observed for spectinomycin, azithromycin, cefixime and ceftriaxone it is imperative for ongoing surveillance for AMR determinants. This study demonstrated that the molecular determination of AMR can be potentially used in the future for tracking resistance patterns and may be more feasible as this method is not dependant on the viability of the pathogen. However, a disadvantage associated with using AMR determinants is that MIC has the ability to determine levels of susceptibility and resistance of specific bacterial strains to applied antibiotics of interest and significantly impacts the choice of therapeutic strategy and ultimately affects the efficiency of an infection therapy.

Maintaining the viability of *N. gonorrhoeae* has proved to be challenging due the fastidious nature of the pathogen (24,54). Therefore, the detection of target mutations for AMR is much more attractive for determining the AMR of gonococcal isolates. This study also focused on the comparison of the primary endocervical swab with the paired culture isolates for the detection of AMR from a molecular level.

A 100% correlation was observed between the culture isolates and endocervical swabs for detecting specific AMR determinants. This study was the first to provide a direct comparison between the primary swab and cultured isolates. One of the limitations associated with this study is that NAATs have not yet been approved for AMR testing and many challenges have to be overcome before they can be approved (5,33).

In summary, this study provided prevalence estimates for *N. gonorrhoeae* in a population of pregnant women; investigated the sensitivity and specificity of NAATs versus culture for the detection of this pathogen; determined AMR patterns in cultured isolates using phenotypic methods and detected AMR determinants from non-cultured swabs. The data generated in this study can be used as a foundation for future *N. gonorrhoeae* AMR studies in our setting.

This study had the following limitations;

1. A small number of pure isolates were obtained after culturing for the susceptibility assays, future studies which are based on larger sample sizes may yield a greater number of pure isolates to perform these assessments

2. Only 50% of women participated in this study, due to endocervical examinations not falling under a routine check-up for pregnant women. These women are therefore not obligated to participate. The majority of women at the antenatal clinic were in their third trimester of pregnancy and were uncomfortable to undergo an endocervical examination to be swabbed.
3. The study population was recruited from a single hospital clinic and is not representative of all pregnant in our setting.
4. Chromosomal mediated resistance towards penicillin and tetracycline resistance was not investigated in the current study and only a few resistant determinants were investigated with regards to the other antibiotics.
5. Biases such as the bacterial load of cultures were not assessed. Therefore PCRS were associated with a higher probability of success in samples that have a lower analytical sensitivity.

Future recommendations:

1. Future studies will now take into consideration sampling women from different antenatal clinics in order to achieve higher sample sizes and provide data that is more representative of the pregnant women in KwaZulu-Natal.
2. An incentive should be awarded to those that participate in the study to increase the number of participants.
3. More education should be provided to these women about STIs and why it is beneficial to participate in such studies. The questionnaires and informed consent should be given to all women at the clinic and these women should only disclose whether they want to participate or not once they enter the examination room privately with a doctor. This prevents discrimination by those that are against testing.
4. Future studies should investigate more than 1 resistance mechanism for AMR and these studies should include an array of resistant determinants, both plasmid and chromosomal mediated resistance.
5. Multilocus sequence typing (MLST) should be used in future studies to provide insight on the molecular epidemiology, clonal relationships and genetic lineages to control of *N. gonorrhoeae* isolates collected from this population group.

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7. APPENDICES

1. KwaZulu-Natal Department of Health Ethical Approval

Physical Address: 330 Langalibalele Street, Pietermaritzburg
Postal Address: Private Bag X9051
Tel: 033 395 2805/ 3189/ 3123 Fax: 033 394 3782
Email:
www.kznhealth.gov.za

DIRECTORATE:
Health Research & Knowledge Management

Ref: KZ_201807_036

19 September 2018

Dear Ms G Oree
(UKZN)

<u>Subject: Approval of a Research Proposal:</u>

1. The research proposal titled **'Whole genome sequence analysis of Neisseria gonorrhoeae for the identification of resistance mechanisms of isolates that are resistant to ceftriaxone in pregnant women attending antenatal care at the King Edward VIII Hospital in Durban, Kwa-Zulu Natal, South Africa'** was reviewed by the KwaZulu-Natal Department of Health.

   The proposal is hereby **approved** for research to be undertaken at King Edward VIII Hospital.

2. You are requested to take note of the following:

   a. *Kindly liaise with the facility manager BEFORE your research begins in order to ensure that conditions in the facility are conducive to the conduct of your research. These include, but are not limited to, an assurance that the numbers of patients attending the facility are sufficient to support your sample size requirements, and that the space and physical infrastructure of the facility can accommodate the research team and any additional equipment required for the research.*
   b. *Please ensure that you provide your letter of ethics re-certification to this unit, when the current approval expires.*
   c. *Provide an interim progress report and final report (electronic and hard copies) when your research is complete.*

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Ms G Khumalo on 033-395 3189.

Yours Sincerely

Chairperson, Health Research Committee
Date: 20/09/18

Fighting Disease, Fighting Poverty, Giving Hope

2. King Edward Hospital VII approval

OFFICE OF THE HOSPITAL CEO
KING EDWARD VIII HOSPITAL

Private Bag X02, CONGELLA, 4013
Corner of Rick Turner (Francois Road) & Sydney Road
Tel: 031-3603853, Fax:031-2061457; Email:
www.kznhealth.gov.za

Ref.: KE 2/7/1/(35/2018
Enq.: Mrs. R. Sibiya

28 June 2018

Ms. G. Oree
School of Laboratory Medicine and Medical Sciences
Nelson Mandela - School of Medicine
UNIVERSITY OF KWAZULU-NATAL

Dear Ms. Oree

Protocol: "Whole genome sequence analysis of Neisseria gonorrhea for the identification of resistance mechanisms of isolates that are resistant to ceftriaxone in pregnant women attending antenatal care at the King Edward VIII Hospital in Durban, South Africa".
Degree-PhD; BREC REF. NO. BE355/18

Permission to conduct research at King Edward VIII Hospital is provisionally granted, pending approval by the Provincial Health Research Committee, KZN Department of Health.

Kindly note the following:-

- The research will only commence once confirmation from the Provincial Health Research Committee in the KZN Department of Health has been received.
- Signing of an indemnity form at Room 8, CEO Complex before commencement with your study.
- King Edward VIII Hospital received full acknowledgment in the study on all Publications and reports and also kindly present a copy of the publication or report on completion.

The Management of King Edward VIII Hospital reserves the right to terminate the permission for the study should circumstances so dictate.

Yours faithfully

SUPPORTED/~~NOT SUPPORTED~~ 2018/07/03
DATE

DR. R GREEN-THOMPSON
CLINICAL HEAD – OBSTET&GYNAE.

SUPPORTED/~~NOT SUPPORTED~~ 03/07/2018
DATE

DR. SA MOODLEY
ACTING SENIOR MEDICAL MANAGER

3. University of KwaZulu-Natal Biomedical Research Ethics Committee approval

RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
Durban
4000
KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

21 October 2019

Ms G Oree (218086892)
School of Clinical Medicine
Glyn.oree@gmail.com

Dear Ms Oree

Protocol: Whole genome sequence analysis of Neisseria gonorrhoea for the identification of emerging resistance to various antibiotics in pregnant women attending antenatal care at the King Edward VII Hospital in Durban, Kwa-Zulu Natal, South Africa.
Degree: PhD
BREC Ref No: BE355/18

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 28 September 2019
Expiration of Ethical Approval: 27 September 2020

I wish to advise you that your application for Recertification received on 14 October 2019 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 12 November 2019.

Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc postgraduate administrator: konar@ukzn.ac.za Supervisor: Abbain@ukzn.ac.za

4. Patient informed consent

INFORMED CONSENT DOCUMENT

Whole genome sequence analysis of *Neisseria gonorrhoeae* for the identification of resistance mechanisms of isolates that are resistant to ceftriaxone in pregnant women attending antenatal care at the King Edward VIII Hospital in Durban, KwaZulu-Natal, South Africa.

Version 1.0

PRINCIPAL INVESTIGATOR: Ms Glynis Oree

PHONE: 0832791072

INFORMED CONSENT

You are being invited to take part in a study called: Whole genome sequence analysis of *Neisseria gonorrhoeae* for the identification of resistance mechanisms of isolates that are resistant to ceftriaxone in pregnant women attending antenatal care at the King Edward VIII Hospital in Durban, KwaZulu-Natal, South Africa.

This study is for pregnant women, 18 years and older, below gestational age 28 weeks. Approximately 307 women will be in this study. Before you decide if you want to join this study, we want you to learn about the study. The study staff will talk with you about the study and answer your questions. You may decide not to join or to withdraw from the study at any time.

YOUR PARTICIPATION IS VOLUNTARY

This consent form gives information about the study procedures that will be discussed with you. Your participation is voluntary; you do not have to have the procedures if you do not want to participate in this study. Once you understand the study tests, and if you agree to take part, you will be asked to sign your name on this form.

PURPOSE OF THE STUDY

Neisseria gonorrhoeae is the cause of gonorrhoea infection. Gonorrhoea also promotes the transmission of other STIs, including HIV infection. Currently, in South Africa, antimicrobial resistant *N. gonorrhoeae* infections have become a major public health issue. The emergence of antimicrobial resistance of *N. gonorrhoeae* to various classes of antibiotic therapy has been reported, and this has therefore severely limited treatment options that are currently available in South Africa. For this study, a doctor will collect two samples from your cervix.

WHAT DO I HAVE TO DO IF I DECIDE TO TAKE PART IN THE STUDY?

If you decide to be in this study, we will be able to start the procedures today. Today's study procedures will take approximately **30-60mins**.

You will be asked to:

- Confirm you are able to join the study and that you understand the study requirements.
- You will be asked questions about your yourself and medical history
- You will be asked to provide 2 cervical swabs

RISKS AND/OR DISCOMFORTS

Risks of sample collection: You may feel discomfort during the swab sample collection. We will ensure that your sample is collected by a trained person (doctor).

Other Possible Risks: You may become embarrassed or worried when discussing your sexual behaviour. We will make every effort to make you feel comfortable and protect your privacy and confidentiality whilst you are part of this study. Your visits will take place in private.

CONFIDENTIALITY

We will keep your information confidential. Your personal information may be disclosed if required by law.

Your records may be reviewed by:

Biomedical Research Ethics Committee of the University of KwaZulu-Natal

Study staff

The researchers will do everything they can to protect your privacy.

PROBLEMS OR QUESTIONS

The Biomedical Research Ethics Committee of the University of KwaZulu-Natal has approved this study.

BIOMEDICAL RESEARCH ETHICS ADMINISTRATION

Research Office, Westville Campus

Govan Mbeki Building

University of KwaZulu-Natal

Private Bag X 54001, Durban, 4000

KwaZulu-Natal, SOUTH AFRICA

Tel: 27 31 2602486 - Fax: 27 31 2604609

Email: BREC@ukzn.ac.za

SIGNATURES

If you have read this consent form, or had it read and explained to you, and you understand the information, and you voluntarily agree to participate, please sign your name or make your mark below.

______________________ ___________________________ _______________

Participant Name (print) | Participant Signature | Date

______________________ ___________________________ _______________

Study Staff Conducting Consent Discussion (print) | Study Staff Signature | Date

______________________ __________________________ _______________

*Witness Name *Witness Signature *Date

(print)

* Witness name, signature and date are required on this consent form only when the consenting participant is not able to read (illiterate)

5. Enrolment CRF

ENROLLMENT FORM

Participant Identifier: ☐☐☐☐☐☐

Visit Date: ☐☐/☐☐/☐☐☐☐

1. **How old are you? ________________ years Date of Birth: ________________**

   If younger than 18 years of age, please do not enroll into study…………End of form

2. **Are you currently experiencing any abnormal discharge from your vagina**

No

Yes

3. **What is your highest level of education?**

Did not attend school

Primary school

High school

College, University

4. **Are you married (consensual or legal marriage)?**

No

Yes

5. **Do you have a regular sexual partner?**

No

Yes

6. **Do you currently live with your husband/regular partner?**

No

Yes

7. How old were you when you first had vaginal sex?

<15 years old

15-20 years old

21-25 years old

>25 years old

8. How many male sexual partners have you had in your life?

1 partner

2-4 partners

>4 partners

9. Does your partner have other partners?

No

Yes

Don’t Know

10. How often do you use condoms during sex?

Never

Sometimes

Rarely

11. Did you use a condom during your last sex act?

No

Yes

12. Do you smoke?

No

Yes

13. Do you drink alcohol?

No

Yes

14. Do you wash inside your vagina with substances other than soap and water?

No

Yes

15. Which trimester of pregnancy are you in?

1st trimester

2nd trimester

3rd trimester

16. Have you ever given birth to a preterm baby (<37 weeks)?

No

Yes

17. Have you ever had a miscarriage in the past?

No

Yes

18. Have you had an abortion in the past?

No

Yes

19. Have you ever had abnormal, smelly discharge from your vagina in the past?

No

Yes

20. Have you ever been treated for an infection passed through sex in the past?

No

Yes

END OF FORM