# MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE GENES IN *NEISSERIA GONORRHOEAE* ISOLATES FROM SELECTED REGIONS IN KENYA THROUGH WHOLE GENOME SEQUENCING

**MARY WANDIA KIVATA**

**DOCTOR OF PHILOSOPHY**

**(Biotechnology)**

**JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY**

**2021**

# Molecular Characterization of Antimicrobial Resistance Genes in *Neisseria gonorrhoeae* Isolates from Selected Regions in Kenya through Whole Genome Sequencing

**Mary Wandia Kivata**

**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology of the Jomo Kenyatta University of Agriculture and Technology**

**2021**

## DECLARATION

This thesis is my original work and has not been presented for degree in any other university

Signature..... Date.....

**Mary Wandia Kivata**

This thesis has been submitted for examination with our approval as the university supervisors

Signature..... Date.....

**Dr. Fredrick Eyase, PhD**

**JKUAT, Kenya**

Signature..... Date.....

**Dr. Margaret Mbuchi, PhD**

**KEMRI, Kenya**

Signature..... Date.....

**Prof. Wallace Bulimo, PhD**

**UoN, Kenya**

Signature..... Date.....

**Dr. Wilton Mbinda, PhD**

**PU, Kenya**

## DEDICATION

I dedicate this thesis to my entire family for their encouragement, emotional and financial support, and above all for their understanding and patience during the times I was absent from home.

## ACKNOWLEDGEMENTS

First and for most I take this opportunity to thank my supervisors Dr. Fredrick Eyase, Dr. Margaret Mbuchi, Prof. Wallace Bulimo, and Dr. Wilton Mbinda for their time, audience, advice, reviews, comments, and for encouraging me to see this PhD through. They have followed up and supported me from the start to the completion of this project. Thank you so much for accepting to supervise my work, availing your time out of your busy schedule to read and discuss my work, to review my manuscripts, and for being my mentors. For all this am eternally grateful.

My special thanks go to Dr. Margaret Mbuchi, the principal investigator of the sexually transmitted infections (STI) surveillance study for allowing me to nest my study on her STI surveillance programme. She availed her time for discussions and reviews of my work until late hours of the night. She oversaw and coordinated my laboratory assays as well as facilitating me to attend trainings and scientific conferences. I could not have made it this far if it weren't for your encouragement and support. Thank you so much and may God bless you as you continue to mentor more people.

I also express my gratitude to Ms. Valerie Oundo, Ms. Esther Waguche, and the late Simon Wachira, STI surveillance laboratory staff who introduced me to various laboratory techniques and helped me in day-to-day activities in the laboratory. I thank the field staff working under the STI surveillance study and who were involved in recruiting and consenting study participants, sample collection and arranging for the transport of specimens to the central laboratory in KEMRI headquarters, Nairobi. Special thanks also go to all study participants who volunteered to provide specimens. Without them this study would not have been feasible.

My sincere gratitude goes to Cecilia Kyany'a, Moses Gathii, Janet Manjanja and Justin Nyasinga who helped me with next generation sequencing. I specifically thank KEMRI Centre for Microbiology Research (CMR) laboratory staff for their invaluable input, advice and assistance throughout the time I was working in the

CMR laboratory. I appreciate my fellow students and interns attached at the CMR laboratory for their moral support and friendship. Thank you or making my time in the laboratory enjoyable and memorable.

Special gratitude goes to the Armed Forces Health Surveillance project under the Global Emerging Infections Surveillance at the US Army Medical Research Directorate-Africa (USAMRD-A) for funding my study. I also acknowledge Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI SERU) and Walter Reed Army Institute of Research (WRAIR) Human Subject Protection Board (HSPB) for approving and allowing me to carry out this study. I too thank KEMRI for allowing me to use their facilities, and for their support during the period I worked in the institution and Henry Jackson and Foundation Medical Research International for program support.

My deepest gratitude goes to my husband, Zadock Wambua and sons Adrian Mutula and David Mumo for their continued love and understanding and for me allowing me to spend more time on my research. I also express my sincere gratitude to my dad, Festus Kivata and Mum, Angelica Kivata who have always inspired and enabled me to pursue my dreams up to this far. I salute my sisters Winfred Nthoki, Catherine Mwongeli, and Elizabeth Nundu, Brothers Benson Mutua, Emmanuel Ndaka and Gabriel Muthiani for their love and support throughout the entire time.

Lastly but not least I thank the almighty God for granting me his mercies, health and strength since the time I started my studies as a young child up to this far. Glory to you almighty God.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF APPENDICES

## ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **AMR** | Antimicrobial Resistance |
| **ATM** | Amplicon Tagment Mix |
| **AZM** | Azithromycin |
| **BEAST** | Bayesian Evolutionary Analysis Sampling Trees |
| **BLAST** | Basic Local Alignment Search Tool |
| **BWA** | Bowtie Wheel Aligner |
| **CAA** | Clean Amplified Plate |
| **CAN** | Clean Amplified NTA Plate |
| **CARD** | Comprehensive Antibiotic Resistance Database |
| **CDC** | Centers for Disease Control and Prevention |
| **CFX** | Cefixime |
| **CGE** | Centre for Genome Epidemiology |
| **CIP** | Ciprofloxacin |
| **CR** | Correia Repeat |
| **CREEs** | Correia Repeat Enclosed Elements |
| **CRO** | Ceftriaxone |
| **DHPS** | Dihydropteroate Synthase |
| **DOX** | Doxycycline |
| **dRS3** | Duplicated repeat sequence 3 |
| **dsDNA** | Double stranded Deoxyribonucleic Acid |
| **DTU** | Technical University of Denmark |
| **DUS** | DNA Uptake Sequence |
| **ESC** | Extended Spectrum Cephalosporins |
| **GCLP** | Good Clinical and Laboratory Practices |
| **gDNA** | Genomic Deoxyribonucleic Acid |
| **GEN** | Gentamycin |
| **GGI** | Gonococcal Genetic Island |
| **GTR** | General-Time Reversible |
| **HGT** | Horizontal gene transfer |
| **HIV** | Human Immunodeficiency Virus |

| | |
|---|---|
| **HPD** | Highest Posterior Density |
| **HS** | High Sensitivity |
| **HSPB** | Human Subjects Protection Branch |
| **Ig** | Immunoglobulins |
| **IRB** | Institutional Review Board |
| **IS** | Insertion Sequences |
| **KEMRI** | Kenya Medical Research Institute |
| **MCC** | Maximum Clade Credibility |
| **MGEs** | Mobile Genetic Elements |
| **MGEs** | Mobile Genetic Elements |
| **MICs** | Minimum Inhibitory Concentrations |
| **MLST** | Multi-locus Sequence Type |
| **MMEs** | Minimal Mobile Elements |
| **MSM** | Man who has Sex with Man |
| **NAATs** | Nucleic Acid Amplification Tests |
| **NCBI** | National Center for Biotechnology Information |
| **NDARO** | National Database of Antibiotic Resistant Organisms |
| **NG-MAST** | *N. gonorrhoeae* Multi-antigen Sequence Typing |
| **NG-STAR** | *N. gonorrhoeae* Sequence Typing for Antimicrobial Resistance |
| **NIMEs** | Neisserial Intergenomic Mosaic Elements |
| **NOR** | Norfloxacin |
| **NPM** | Nextera PCR Master Mix |
| **NTA** | Nextera XT Tagment Amplicon Plate |
| **ORF** | Open reading frame |
| **PAL** | Pooled Amplicon Library |
| **PATRIC** | Pathosystems Resource Integration Center |
| **PBP** | Penicillin Binding Proteins |
| **PBS** | Phosphate Buffered Saline |
| **PCR** | Polymerase Chain Reaction |
| **PEN** | Penicillin |
| **PID** | Pelvic Inflammatory Disease |

| | |
|---|---|
| **PPNG** | Penicillinase Producing *Neisseria gonorrhoeae* |
| **QRDR** | Quinolone Resistance Determining Region |
| **rpm** | Revolutions Per Minute |
| **rRNA** | Ribosomal Ribonucleic Acid |
| **RSB** | Re-suspension Buffer |
| **SERU** | Scientific & Ethics Review Unit |
| **SGP** | StoraGe Plate |
| **SNP** | Single Nucleotide Polymorphism |
| **Spp** | Species |
| **SPT** | Spectinomycin |
| **SSREE** | Spencer-Smith Repeat Enclosed Elements |
| **STI** | Sexually Transmitted Infection |
| **T4SS** | Type IV Secretion System |
| **TET** | Tetracycline |
| **TMCRA** | The Most Recent Common Ancestor |
| **tRNA** | Transfer Ribonucleic Acid |
| **USAMRD-A** | United States Army Medical Research Directorate, Africa |
| **WGS** | Whole Genome Sequencing |
| **WRAIR** | Walter Reed Army Institute of Research |
| **WRP** | Walter Reed Project |
| **XDR** | Extensively Drug Resistant |

## ABSTRACT

Gonorrhea, a sexually transmitted infection is caused by an obligate gram-negative bacterium called *Neisseria gonorrhoeae* (*N. gonorrhoeae*). *N. gonorrhoeae* infect various parts of the body and when left untreated, gonoccocal infections can lead to infertility in women and sterility in men. With the absence of a gonorrheal vaccine, different antibiotics have been used to treat gonorrhea since 1930's. While initially sensitive to newly introduced antibiotics, some strains develop single or multiple mechanisms of resistance over time. In Kenya, a few studies have reported resistance to penicillins, tetracyclines, and recently to fluoroquinolones. Consequently, information on antimicrobial resistance (AMR) in Kenyan gonococci is limited. Drug resistant *N. gonorrhoeae* isolates have been observed in a sexually transmitted infection (STI) surveillance study in Kenya. The underlying molecular mechanisms causing this antimicrobial resistance had not been determined. Therefore, the aim of this study was to determine the molecular mechanisms of antimicrobial resistance as well as the genetic diversity of *N. gonorrhoeae* isolates obtained from Kenya under the STI surveillance program through whole genome sequencing. Forty one archived *N. gonorrhoeae* isolates exhibiting varying antibiotic resistance profiles were obtained and sub-cultured on Modified Thayer-Martin (MTM) agar medium. DNA was extracted and the whole genomes sequenced using the Illumina Miseq platform. Sequence reads were assembled *de novo* using CLC Genomics Workbench. Contigs were mapped to FA1090 reference and searched against the NCBI nucleotide database. Genome annotation was performed using Rapid Annotation Subsystem Technology (RAST). Phylogenetic analyses were performed using both Bayesian Evolutionary Analysis Sampling Trees (BEAST) and core genome multi-locus sequence typing (cgMLST). Sequence typing was done using both *N. gonorrhoeae* multi antigen sequence typing (NG-MAST) and multi-locus sequence typing (MLST). Mutations and amino acid alterations were identified using Bioedit sequence alignment editor. Provided antimicrobial susceptibility results were interpreted with reference to European Committee on Antimicrobial Susceptibility Testing standards (EUCAST) version 8.0, 2018 standards. Core genome phylogeny revealed five distinct clusters among the study isolates all characterized by varied MLST and NG-MAST sequence types (STs). Region based clustering was not observed. Temporal Bayesian phylogenetic tree clustered Kenyan isolates closely together into distinct lineages with USA being the most probable ancestral location. A total of 25 MLSTs STs were identified. Of the 41 sequences, 26 belonged to 14 known MLST STs of which ST-1932 was the most common (12.2%), while 15 belonged to 11 new MLSTs STs. Thirty three NG-MAST STs were identified. Thirty four sequences belonged to 28 novel NG-MAST STs while 6 belonged to 5 previously reported STs. A new NG-MAST, ST-19168 was the most common (10%). Twenty five isolates had both β-lactamase (TEM) and TetM encoding plasmids whereas 3 isolates lacked either of the plasmids. TEM encoding plasmids were identified in 29 isolates (penicillinase producing *N. gonorrhoeae*; PPNG) of which 28 had an African type origin (pDJ5) while 1 had an Asian type origin (pDJ4). Two genotypes of β-lactamase were identified; TEM-1 and a recently described

TEM-239. Mosaic PenA patterns associated with ceftriaxone and cefixime resistance was not observed in the present study. All analyzed isolates had non mosaic *penA* alleles. PonA L421P was identified in 19 isolates. Of the 41 isolates, 34 had TetM encoding plasmids, 33 of which had American TetM determinants, whereas 1 had a Dutch TetM determinant. All isolates had S10 V57M mutation. Isolates expressing TEM and tetM had significantly higher penicillin, tetracycline and doxycycline minimum inhibitory concentrations (MICs) respectively. Double GyrA amino acid substitutions; S91F and D95G/A were identified in 39 of the 41 isolates. All ciprofloxacin resistant isolates expressed these GyrA alterations. Of these 39 isolates 29 had an additional E91G or S87R ParC amino acid substitution and significantly high ciprofloxacin MICs. No genetic determinants specifically associated with azithromycin, spectinomycin and gentamycin resistance were identified. R228S substitution in dihydropteroate synthase associated with sulphonamide resistance in gonococci was identified in 40 of the 41 isolates. G120D/N, A121G/S, N122K, and N deletion at position 122 alterations in PorB were identified in 26 isolates. Two isolates had mutated *mtrR* promoters while G45D, T86A, D79N, H105Y and A39T substitutions were identified in MtrR of 38 isolates. No significant relation was observed between identified PorB, MtrR and PonA alterations and antibiotic MICs. The observed high numbers of different MLST and NG-MAST STs indicate that the sampled gonococci are genetically diverse. The observed non-regional distribution of both MLST and NG-MAST STs indicate a heterogeneous gonococcal population in Kenya. Kenyan gonococci are closely related and have evolved into distinct lineages when compared to other global gonococcal strains. High penicillin and tetracycline resistance in the analyzed gonococcal isolates is mainly mediated by plasmid-borne *blaTEM*, and *tetM* genes. The observed high ciprofloxacin resistance and high norfloxacin MICs is mediated by GyrA S91F, D95G/D95A and ParC E91G or S87R substitutions while the observed low level azithromycin resistance could be caused by reduced drug accumulation. From the findings of this study, the combination of azithromycin and ceftriaxone recommended by both Centre for Disease Control and Kenya Ministry of Health is still useful for treatment of gonoccoacal infections in Kenya, while cefixime, gentamycin and spectinomycin are suitable alternatives. The findings further provide current genotypic information on circulating gonococcal strains in Kenya which is useful in planning of containment and gonorrhea treatment measures. The sequence data generated in this study will allow comparative studies between Kenyan gonococci and other global isolates as well as characterization of other molecular markers in the study isolates.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

*N. gonorrhoeae*, the causative agent of gonorrhea is a piliated, gram-negative betaproteobacterium cocci that typically appear in pairs (Tonjum, 2005). It is an obligate human pathogen that infects and colonizes various human body parts populated by non-ciliated mucosal columnar epithelial cells resulting to cellular invasion (Nassif et al., 1999). These body parts include the genital (urethra in males and the endocervix and urethra in females), ocular, nasopharyngeal, and anal mucosa (Edwards & Apicella, 2004; Edwards & Butler, 2011).

Gonococcal infections are characterized by a purulent discharge. Following gonococcal infection, innate immune responses are activated at the sites of colonization. The damage caused by activation of innate immune responses accounts for the pathology of gonorrheal infections (Edwards & Apicella, 2004). Untreated gonococcal infections can lead to more complicated and serious health conditions such as pelvic inflammatory disease (PID) and infertility in women and epididymitis and sterility in men (Tapsall, 2001). Untreated gonorrhea has also been associated with increased acquisition and transmission of Human Immunodeficiency Virus (HIV) (Cohen et al., 1997; Edwards & Apicella, 2004).

*N. gonorrhoeae* has developed multiple mechanisms to escape the immune responses and resist killing by nearly all classes of antimicrobial agents making gonococcal infections increasingly difficult to treat (Camara et al., 2012; Unemo et al., 2016). Consequently, gonococcal infections are among the most predominant bacterial sexually transmitted infections (STIs) worldwide (Da Ros & Schmitt, 2008). Accordingly, gonorrhea remains a major global public health concern and places a large economic burden on healthcare systems. Lack of a gonococcal vaccine reduces preventive measures for gonorrhea and antibiotics remain the only option for management of gonococcal infections. Unfortunately, the use of antibiotics to

manage gonorrheal infections is challenged by the emergence of multidrug resistant (MDR) *N. gonorrhoeae* isolates which have decreased susceptibility or resistance to one of the currently recommended antibiotics (cephalosporin or azithromycin) plus resistance to at least two other antimicrobials.

Several classes of antibiotics including sulphonamides, penicillins, tetracyclines, macrolides, fluoroquinolones and cephalosporins have been used for the management of gonorrhea since 1930's (Tapsall, 2005; Tapsall et al., 2009). While initially sensitive to these antibiotics, some strains of *N. gonorrhoeae* develop single or multiple resistance mechanisms to the antibiotics. Antibiotic resistance mechanisms employed by *N. gonorrhoeae* include a) modification of the antibiotic either through chemical alteration or destruction of the drug by enzymatic means (Patel et al., 2011), b) modification or protection of the drug sensitive target through mutations, enzymatic alterations (through reactions such as methylation), and replacement or bypassing of the target site all which leads to reduced drug affinity, and (Unemo & Shafer, 2014) c) reduced drug accumulation resulting from active efflux pumps such as MtrCDE and MacAB, or decreased influx due to modification or loss of the porin proteins which transport drugs into the cell (Tenover, 2006; Unemo & Shafer, 2014; Munita & Arias, 2016).

*N. gonorrhoeae* has the ability to alter its DNA through different types of mutations in both plasmid and chromosomal DNA and acquisition of foreign DNA through horizontal DNA transfer. This facilitates the rapid development and spread of resistance to newly introduced antimicrobials (Cehovin & Lewis, 2017). To slow resistance to cephalosporins; the last available monotherapy for treatment of gonorrheal infections and improve treatment efficacy, the Centre for Disease Control (CDC) in 2015 recommended a dual therapy for gonococcal infections with a cephalosporin plus either azithromycin or doxycycline. Nevertheless MDR gonococci and extensively drug resistant (XDR) *N. gonorrhoeae* strains which have decreased susceptibility or resistance to the two currently recommended therapies (cephalosporin and azithromycin) plus resistance to at least two other have been reported in different countries including England (Public Heath England Report,

2018), Japan (Ohnishi *et al.*, 2011), France (Magnus Unemo et al., 2012), Spain (Cámara *et al.*, 2012), and Australia (Whiley et al., 2018).

Since the 1970s, a few studies have reported both chromosomal and plasmid mediated gonococcal penicillin and tetracycline resistance in Kenya (Verhagen et al., 1971; Perine et al., 1980; Brunham et al., 1985; van Hall et al., 1991). Reports from the studies led to the introduction of fluoroquinolones as the first line antibiotics for gonorrhea treatment in 1993 (WHO, 1993). *N. gonorrhoeae* remained susceptible to fluoroquinolones since their introduction in 1993 until 2009 when phenotypic fluoroquinolone resistance was first reported in western Kenya (Mehta et al., 2011). Later in 2011 and 2012 resistance was reported in high risk populations in coastal Kenya including Mombasa and Kilifi, Kisumu, and Nairobi (Duncan et al., 2011; Lagace-Wiens et al., 2012). Following these reports the national treatment guidelines for gonococcal infections were revised in 2013. In these revised guidelines, fluoroquinolones were replaced by ceftriaxone and cefixime as the first line drugs for gonorrhea treatment.

Generally, lack of diagnostic testing at the point of care limits data on antibiotic susceptibility and consequently hinders genotypic characterization of molecular mechanisms of drug resistance. The molecular mechanisms underlying Kenyan gonococcal phenotypic resistance has not been well elucidated. Information on both antibiotic susceptibility profiles and the molecular basis underlying drug resistance is vital for guiding gonorrhea treatment recommendations, and also in the development of molecular based antibiotic resistance tests. Sequence information provides information on circulating *N. gonorrhoeae* genotypes associated with antibiotic resistance including their temporal and spatial spread. Further, it allows isolate sequence comparison through sequence sharing between different laboratories (Mortimer & Grad, 2019).

### 1.2 Problem statement

Due to poor surveillance and syndromic management of STIs in which treatment is based on symptoms and signs rather than the etiological based therapy, data on

antibiotic susceptibility patterns in Kenyan gonococci is limited (Dallabetta et al., 1998). Lack of appropriate diagnostic testing, has hindered molecular characterization of antimicrobial resistance. Consequently, there is limited genotypic data on antimicrobial resistance determinants responsible for causing antibiotic resistance in Kenyan gonococci. Additionally, due to limited genomic data including sequence information of Kenyan gonococci, the origin, genetic relatedness, evolution and spatial spread of drug resistant strains have not been determined.

A few studies in Kenya have reported phenotypic resistance to penicillins, tetracyclines, fluoroquinolones, and increased cephalosporin minimum inhibitory concentrations (MIC's) but the information is limited. *N. gonorrhoeae* isolates resistant to penicillin, tetracycline, ciprofloxacin and azithromycin have been observed in a STI surveillance at the US Army Medical Research Directorate-Africa (USAMRD-A), hosted by Kenya Medical Research Institute (KEMRI). Although *N. gonorrhoeae* isolates showing reduced Minimum Inhibitory Concentrations (MIC's) and resistance to different classes of antibiotics have been reported in Kenya, the molecular basis underlying the resistance have not been elucidated. Information on the circulating gonococcal strains and their associated antibiotic resistance profiles is crucial in the development of gonorrhea treatment guidelines and targeted mitigation strategies.

### 1.3 Justification

In the past five years, extensively drug resistant *N. gonorrhoeae* strains have been reported in Japan, Spain, France and most recently in Australia leading to serious concerns of the emergence of untreatable gonorrhea (Ohnishi, Golparian, et al., 2011; Camara et al., 2012; Magnus Unemo et al., 2012; Allen et al., 2013). The spread of these antibiotic resistance genes pose a challenge in management of gonorrheal infections and highlights the importance of molecular antimicrobial surveillance and the need for genomic characterization (Workowski et al., 2008). Molecular surveillance provides knowledge on the determinants and mechanisms of drug resistance in organisms which is useful in monitoring the emergence and spread of antibiotic resistant strains.

Over the years the epidemiology and spread of multidrug resistant gonococci has been monitored through phenotypic, and molecular based typing techniques in combination with antimicrobial susceptibility (AST) data. Recently a new approach; genomic epidemiology which has a higher resolution was introduced. This approach allows characterization and comparative genome analysis of all antimicrobial resistance (AMR) genes especially in resistant strains (Mortimer & Grad, 2019).

With the current limited genomic and sequence data of Kenyan gonococci, there is need for whole genome characterization of drug resistant *N. gonorrhoeae* isolates which will go a long way to help identify and provide information on circulating *N. gonorrhoeae* genotypes associated with antibiotic resistance, their origin, and evolution, temporal and spatial spread. This study therefore, aimed at; a) determining the whole genomes of antimicrobial resistant *N. gonorrhoeae* isolates from Kenyan as well as their strain sequence types, b) determining the evolution and genetic diversity between Kenyan gonococci and global *N. gonorrhoeae* isolates, and c) characterize the molecular basis underlying phenotypic antibiotic resistance observed in the STI surveillance program through whole genome sequencing. The findings of this study will improve human health, by helping in planning of containment and gonorrhea treatment measures in Kenya. The sequence information generated from this study will further allow isolate sequence comparison through sequence sharing between different laboratories. In addition to providing information on antimicrobial resistance markers, the generated whole genome sequences data will be useful in evaluation of other molecular markers for different purposes.

### 1.4 Research questions

i. What comprises the whole genomes of antimicrobial resistant Kenyan *N. gonorrhoeae* isolates and to which MLST and NG-MAST sequence types do they belong to?
ii. How does Kenyan *N. gonorrhoeae* relate genetically among themselves and with other global gonococcal isolates?
iii. Which antimicrobial resistance determinants mediate antibiotic resistance in Kenyan *N. gonorrhoeae* isolates?

iv. Are the antimicrobial resistance determinants expressed by Kenyan *N. gonorrhoeae* isolates similar to those expressed by other global isolates and what is their association with antibiotic susceptibilities?

## 1.5 Objectives of the study

### 1.5.1 General objective

To characterize AMR genes in *N. gonorrhoeae* isolates from different regions in Kenya through whole genome sequencing.

### 1.5.2 Specific objectives

i. To determine the whole genomes of antimicrobial resistant *N. gonorrhoeae* isolates from Kenyan and characterize their MLST and NG-MAST sequence types
ii. To geo-phylogenetically determine the evolution and genetic diversity between Kenyan gonococci and global *N. gonorrhoeae* isolates
iii. To characterize both chromosomal and plasmid borne antimicrobial resistance determinants expressed by Kenyan *N. gonorrhoeae* isolates.
iv. To identify the differences between AMR determinants expressed by the study isolates and those expressed by other global isolates and evaluate their association with antibiotic susceptibilities

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 *Neisseria gonorrhoeae*

*N. gonorrhoeae,* is a gram negative, non-flagellated, non-sporulating, oxidase and catalase producing betaproteobacterium cocci that typically appear in pairs (Tonjum, 2005; Magnus Unemo et al., 2013). It causes the second most common human sexually transmitted infection after chlamydia; gonorrhea, which places a large economic burden on healthcare systems. It is an obligate human pathogen which infects both men and women (Nassif et al., 1999). The bacterium colonizes and infects mucosal membranes of various parts of the human body including the genital (urethra in males and the endocervix and urethra in females), ocular, nasopharyngeal, and anal mucosa (Edwards & Apicella, 2004; Edwards & Butler, 2011).

The pathology of gonorrheal infections mainly results from damage that is caused by the activation of innate immune responses at the colonization sites (Edwards & Apicella, 2004). The pathogen has over the years developed multiple mechanisms to enable it escape the immune system responses and resist killing by nearly all classes of antimicrobial agents making infection with *N. gonorrhoeae* increasingly difficult to treat (Camara et al., 2012; Unemo et al., 2016).

## 2.2 *N. gonorrhoeae* transmission

Although gonorrheal infections can be transmitted to a neonate via an infected mother's birth canal or via auto-inoculation from the hands of an infected person to their eye, *N. gonorrhoeae* is transmitted almost exclusively through sexual contact. Risk factors which increase gonococcal infection include; sexual contact with an infected person or someone from an endemic area, previous gonorrheal infection, STIs or human immunodeficiency virus (HIV) infection, having multiple sex partners/encounters, being a sex worker, street youth and men who have sex with men (Edwards & Apicella, 2004).

## 2.3 Clinical manifestations

Gonorrheal infection is generally limited to superficial mucosal surfaces lined with columnar epithelium. Gonococcal infections are manifested by different syndromes including, pharyngeal, urogenital, and rectal infections in females and males, and conjunctivitis in both neonates and adults (Brunham et al., 2015; Allan-Blitz et al., 2017). Purulent discharge is observed in mucosal gonococcal infections. When left untreated genital gonococcal infections can cause serious and permanent health problems such as pelvic inflammatory disease (PID) and its associated complications in women and epididymitis and sterility in men (Westrom et al., 1992; Brunham et al., 2015). Untreated gonococcal infections can also disseminate to various tissues causing disseminated gonococcal infection (DGI), which potentially give rise to infectious dermatitis-arthritis syndrome, endocarditis and meningitis (Holmes et al., 1971). Importantly, untreated gonococcal infections are associated with increased acquisition and transmission of HIV (Cohen et al., 1997; Mlisana et al., 2012).

### 2.3.1 Genital gonococcal infections

#### 2.3.1.1 Gonococcal urethritis

In males, the infection of the mucous membranes of anterior urethra, leads to an inflammatory and pyogenic infection called urethritis. The symptoms of urethritis include: An itchy urethra; copious and purulent clear or cloudy urethral discharge; testicular or rectal pain; and dysuria (difficulty in urination). Inflammation of the urethral tissues accounts for the redness, swelling, heat, pain, and intense burning experienced during urination (Edwards & Apicella, 2004).

While most men develop symptomatic gonococcal urethritis, some can have asymptomatic urethritis (carriers). The carriers are important reservoirs for transmission and are at increased risk for developing complications (Handsfield et al., 1974). The incubation period of symptomatic urethritis ranges from 1 to 14 days (Harrison et al., 1979). In males, *N. gonorrhoeae* may invade the prostate resulting in prostatitis, or extend to the testicles resulting in orchitis.

#### 2.3.1.2 Gonococcal cervicitis

In women, endocervical gonococcal infection is the most common form of uncomplicated gonorrhea. It is rarely symptomatic with 50% of the infected women being asymptomatic. When symptoms are present they include, vaginal discharge, dysuria, dyspareunia, abnormal pain, uterine bleeding, and lower abdominal and or rectal pain (Edwards & Apicella, 2004; Edwards & Butler, 2011).The incubation period of symptomatic endocervitisis variable, with symptoms developing within 10 days post exposure (McCormack et al., 1977).

In females, cervical involvement may extend through the uterus to the fallopian tubes resulting in salpingitis or to the ovaries resulting in ovaritis (Eschenbach et al., 1975). When the infection ascends to the endometrium and fallopian tubes, it causes pelvic inflammatory disease which is characterized by vaginal discharge, inter-menstrual bleeding, lower abdominal pain, dyspareunia, and fever (Brunham et al., 2015). Untreated PID increases the risk of ectopic pregnancy, cause chronic pelvic pain and tubal infertility.

### 2.3.2 Pharyngeal and anorectal gonococcal infections

Gonococcal pharyngeal infections result from orogenital intercourse. Mostly the infections are asymptomatic and exudative pharyngitis is rare. When symptoms are present they include: tonsillitis, fever, pharyngitis and cervical adenitis.

Symptomatic rectal infections in males result from anal intercourse and are usually more often in men that have sex with men. In women, rectal infections (proctitis) result from autoinoculation with cervical discharge resulting from gonococcal infection as well as from anal intercourse. Symptoms of proctitis include anal irritation, painful defecation, constipation, scant rectal bleeding, painless mucopurulent discharge, anal pruritus, and tenesmus (Klausner et al., 2004).

### 2.3.3 Ocular and disseminated gonococcal infections

Gonococcal ocular infections present as conjunctivitis which in adults results from autoinoculation in persons who have genital gonococcal infection. The transmission

of gonococcal conjunctivitis occurs in newborns that are exposed to infected secretions in the birth canal during birth. To prevent transmission, silver nitrate or antibiotic ointment or drops are administered into the eyes of newborns immediately after birth. Gonococcal conjunctivitis is characterized by a mild non-purulent infection, which if not treated progress to conjunctival edema, copious purulent discharge and marked conjunctival redness (McAnena et al., 2015). Untreated gonococcal conjunctivitis can cause serious consequences including corneal scarring or perforation, endophthalmitis, and blindness (Palafox et al., 2011).

Disseminated gonococcal infection is a systemic infection which is more common in women than in men. It is caused by gonococci strains that produce bacteremia without associated urogenital symptoms. Patients with complement deficiency have greater risk of developing DGI. Clinical manifestations include skin lesions, arthralgia, tenosynovitis, arthritis, hepatitis, myocarditis, endocarditis, and meningitis (Bleich et al., 2012).

### 2.4 Laboratory diagnosis of gonorrhea

Gonorrheal infections are frequently asymptomatic and when the symptoms are present they are commonly nonspecific. Proper gonorrhea diagnosis is established by detection of *N. gonorrhoeae* or its genetic material in genital or extra genital specimens by microscopy of stained smears, culture, or nucleic acid amplification tests (NAATs).

#### 2.4.1 Gram staining

Gram stain is a presumptive non-culture test for diagnosis of gonorrhea. It is a rapid, cheap, sensitive and specific detection method which identifies gonococci as intracellular Gram-negative diplococci in polymorphonuclear leukocytes by microscopy of Gram or methylene blue stained smears of purulent urethral discharge (Magnus Unemo et al., 2013; Workowski & Bolan, 2015). However, in asymptomatic patients, microscopy is not recommended as the only method for diagnosis because negative results do not exclude infection. Direct microscopic examination is not recommended for the diagnosis of rectal and pharyngeal

infections because of the large number of other organisms present (Workowski & Bolan, 2015). Additionally microscopy does not provide any AMR data (Tapsall, 2001).

### 2.4.2 Gonococcal culture

Culture is the historical gold standard for detection of *N. gonorrhoeae*. It enables complete antimicrobial resistance testing, surveillance, detecting treatment failure and characterizing outbreaks. It is highly specific and sensitive when appropriate species verifying assays are applied. The sample is inoculated on an agar that is selective for gonococci and one which contains antimicrobial agents that inhibit the growth of commensal bacteria and fungi. Such media include; Modified Thayer-Martin; New York City; and Martin Lewis mediums which contain antibacterial agents. The antimicrobials include: vancomycin; colistin; trimethoprim lactate; and antifungal agents such as nystatin and anisomycin or amphotericin B.

Typical *N. gonorrhoeae* growth features include pigmented/opaque, smooth, round, moist, convex uniform grayish white to tan colonies. Due to the sensitivity of gonococci to external environmental factors, conditions for sample collection, transport, storage and the culture process are strictly optimized (Magnus Unemo et al., 2013; Workowski & Bolan, 2015).

### 2.4.3 Biochemical tests for *N. gonorrhoeae* identification

#### 2.4.3.1 Oxidase test

The oxidase test uses Kovac's reagent (a 1% (wt/vol) solution of N, N, N', N' – tetramethyl-ρ-phenylenediamine dihydrochloride) to detect the presence of cytochrome c oxidase in a bacterial organism's respiratory chain; if the oxidase reagent is catalyzed, it turns purple. Due to the presence of cytochrome oxidase, Neisseria species give a positive oxidase reaction. Consequently, gram-negative oxidase-positive diplococci which have been isolated on a gonococcal selective media may be identified presumptively as *N. gonorrhoeae* (Janda et al., 1989).

#### 2.4.3.2 Superoxol /catalase test

Superoxol test uses 30% hydrogen peroxide as a reagent. Reactions of superoxol with *N gonorrhoeae* produce a strong explosive positive reaction with bubbling within 1 to 2 seconds compared with weaker reactions with most non-gonococcal Neisseria species (Janda et al., 1989).

### 2.4.4Nucleic acid detection tests

There are two types of nucleic acid detection tests: non-amplified tests and amplified tests. The nucleic acid amplification tests include polymerase chain reaction (PCR), transcription-mediated amplification, and strand displacement amplification (SDA).

NAATs are used on endocervical specimens from women, urethral specimens from men, and urine specimens from men and women (Bachmann et al., 2010). Amplification of gonococci's nucleic acid using NAATs enables rapid simultaneous detection of several pathogens and offers great sensitivity than microscopy and culture. Additionally it can detect nonviable gonococci and is less demanding regarding specimen collection transportation and storage (Whiley et al., 2006; Bachmann et al., 2010). Unfortunately, these techniques do not allow AMR testing and commercially available NAATs have varied sensitivities. Commensal Neisseria species frequently present in the pharynx, rectum, and urogenital tract have genetic homology with *N. gonorrhoeae* and might cross-react in gonococcal NAATs, and result in false positive reports (Katz et al., 2004; Goire et al., 2014).

Non-amplified tests used for *N. gonorrhoeae* include the DNA probe. In this test, a specific probe binds to a complementary nucleic acid present in the sample and the signal is amplified by means of a chemiluminescent label on the probe (Tapsall, 2001). NAATs are less likely to be affected by transport conditions than culture is, and have the potential for more timely results. These tests are used on endocervical and urethral specimens but are not applicable for rectal, urine and pharyngeal specimens, or, and do not allow AMR testing.

## 2.5 Treatment of gonorrheal infections

In women, the pharynx, and the rectum, gonorrhea is frequently asymptomatic and when symptomatic, the symptoms can be non-specific (Magnus Unemo et al., 2013). Treatment of gonorrhea in many developing countries relies mainly on syndromic management, which is based on classification by groups of symptoms and signs to determine the nature of infection rather than the etiological based therapy (Dallabetta et al., 1998).

World Health Organization (WHO) recommends that any antimicrobial treatment for gonorrhea should be such that the cure rate is about 95% (Dallabetta et al., 1998; Tapsall, 2001). Control of gonococcal infections has relied on effective single dose antibiotic therapy given at the initial presentation in order to obtain better compliance. Treatment of gonorrheal infections is complicated by the ability of *N. gonorrhoeae* to develop resistance to antimicrobials and therefore many international jurisdictions change their treatment guidelines regularly in response to the threat of potentially untreatable gonorrhea emergence (Workowski & Bolan, 2015).

Following *N. gonorrhoeae* resistance development to sulphonamides, penicillins, tetracyclines, macrolides, and fluoroquinolones, cephalosporins are left as the only remaining class of monotherapy antimicrobials available for treatment of gonorrhea in many regions (Campos-Outcalt, 2007). Unfortunately resistance to cephalosporins has been demonstrated in some countries (Forsyth et al., 2011; Ison et al., 2011; Unemo & Shafer, 2011; Camara et al., 2012; Allen et al., 2013). To slowdown the emergence and spread of resistance to cephalosporins as well as improve treatment efficacy, a dual therapy for gonorrhea with a cephalosporin plus either azithromycin or doxycycline was recommended by CDC's in 2015 STD treatment guidelines. Azithromycin is preferred to doxycycline as the second antimicrobial because of the compliance and convenience advantages of single-dose therapy and the substantially higher prevalence of gonococcal resistance to tetracycline than to azithromycin (Committee on Gynecologic, 2015; Workowski & Bolan, 2015).

*N. gonorrhoeae* does not elicit a protective response and does not impart immune memory and individuals can become infected repeatedly. Additionally it suppresses and manipulates the host immune responses (Mandrell et al., 1993; Quillin & Seifert, 2018).Currently there is no gonococcal vaccine and therefore there is no mode for immunoprotection against gonococci. Gonococci extensively vary its surface components through antigenic and phase variations which pose a substantial problem in identification of drug targets and development of effective vaccines against *N. gonorrhoeae* strains (Segal et al., 1986; Snyder et al., 2001). Without a protective vaccine, gonococcal infection rates can be reduced by avoiding multiple sexual partners, early diagnosis and treatment of infected individuals, and screening populations at high risk.

### 2.6 Gonococcal pathogenesis

After *N. gonorrhoeae* transmission the bacteria adhere to columnar epithelial cells, penetrate them, and multiply on the basement membrane (Cornelissen, 2011).

#### 2.6.1 Adherence, colonization and invasion

Piliated, Opa-expressing *N gonorrhoeae* cells establishes infection in the urogenital tracts by interacting with receptors on the surface of non-ciliated mucosal columnar epithelial cells which results in cellular invasion. The initial attachment to host epithelial cells is facilitated by Type IV pili (thin, hair-like pilus appendages found on the outer membrane), while a closer attachment is quickly mediated by the Opa outer membrane protein to form microcolony on the epithelial cell surface (Wang et al., 1998; Griffiss et al., 1999).

After adherence to the epithelial surface the pilus retracts allowing more intimate, Opa mediated attachment of the bacteria with the CD66 antigens located on the mucosal cells (Wolfgang et al., 1998; Maier et al., 2004). Following Opa-mediated attachment to the non-ciliated epithelial cells, the bacteria are surrounded by microvilli which draw them to the surface of the mucosal cell where they are engulfed and internalized into the mucosal cells by a process called parasite-directed endocytosis. During endocytosis the membrane of the mucosal cell retracts and

pinches off a membrane-bound vacuole (phagosome) that contains the bacteria (Billker et al., 2002). The vacuole is transported to the base of the cell, where the bacteria are released by exocytosis into the sub-epithelial tissue (Figure 2.1). Once in the sub-epithelial space, they induce the inflammatory process responsible for the disease symptoms. In males and females, different molecular mechanisms are involved in the establishment of gonococci on the mucosal surfaces of (Edwards & Apicella, 2004).

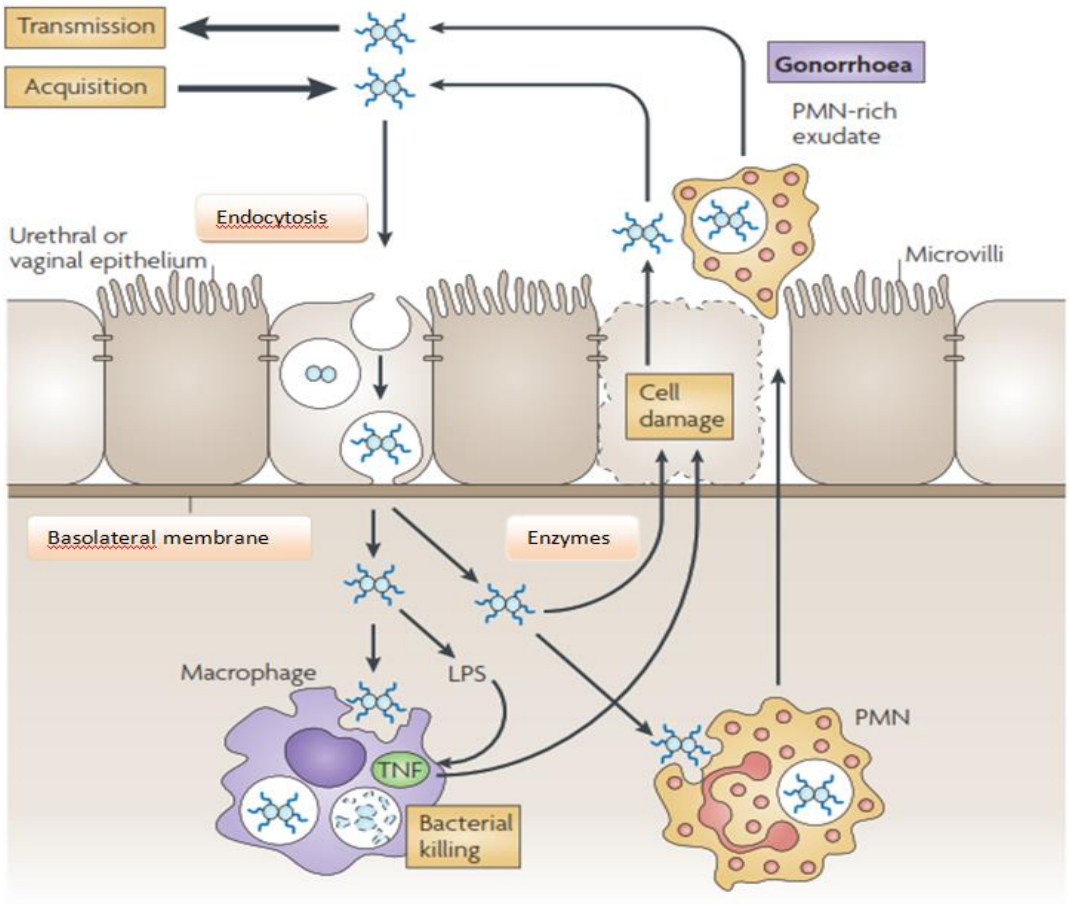



**Figure 2.1: Pathogenesis of gonorrhea.**

Adapted from (Virji, 2009).

Figure 2.1 summarizes the pathogenesis of gonorrhea. After acquisition, piliated Opa expressing gonococcal cells adhere and enter into the columnar epithelial cells through endocytosis. Through exocytosis from the epithelial cells, some gonococcal cells transcytose into the sub-epithelial tissue where they reside and survive inside

PMN cells. After the PMN die the ingested bacterial cells are released in the infectious PMN-rich exudate. In response to infection, the infected PMN cells produce tumor necrosis factor which together with bacterial peptidoglycan and LOS damage both ciliated and non-ciliated epithelial cells.

### 2.6.2 Inflammation

Depending on which Opa protein is being expressed, some gonococci can transcytose to the basolateral side of the mucosal epithelium after internalization, and reside and survive inside the polymorphonuclear (PMN) leukocytes such as neutrophils at least until the neutrophils themselves die and release the ingested bacteria (Densen & Mandell, 1978; Jerse et al., 1994; Edwards & Butler, 2011; Hill et al., 2016). The Neisseriae are not destroyed within the endocytic vacuole, but it is not clear whether they actually replicate in the vacuoles as intracellular parasites (Hill et al., 2016). Inflammation results from the infection since PMN cells are immediately attracted to the site of infection to clear the gonococci. The *N. gonorrhoeae* engulfed by PMN are secreted in the infectious PMN-rich exudate.

The host alternative complement pathway is activated by both gonococcal lipopolysaccharide (LOS) and peptidoglycan while LOS stimulates the production of tumor necrosis factor (TNF) from infected polymorphonuclear cells. The tumor necrosis factor from phagocytes and gonococcal products, such as peptidoglycan and LOS released from gonococcal cell autolysis cause toxic cell damage to both ciliated and non-ciliated epithelial cells of mucosal surfaces. Gonococcal LOS produces mucosal damage by bringing about the release of enzymes, such as proteases and phospholipases that may be important in pathogenesis (Figure 2.1). Together with TNF and other cell wall components, LOS elicit a strong inflammatory response that together with the shedding of damaged urethral epithelial cells accounts for the purulent urethral discharge and give rise the pain associated with genital and rectal infections (Edwards & Butler, 2011).

### 2.6.3 Serum resistance

Several factors including reduction modifiable protein (Rmp), LOS, and immunoglobulin A (IgA) contribute to serum resistance following gonococcal infection. Rmp is an outer membrane protein found in a complex with porin and LOS in all strains of *N. gonorrhoeae*. Antibodies produced against Rmp following Neisserial infection react with their antigenic site on the gonococcal surface blocking bactericidal antibodies against LOS and porin. This protects the bacterium from complement-mediated lysis and increase susceptibility to *N. gonorrhoeae* infection. Following transcytosis, gonococci can enter the bloodstream and utilize host-derived N-acetylneuraminic acid (sialic acid) to sialylate the oligosaccharide component of its LOS, converting a serum-sensitive organism to a serum-resistant one (Mandrell et al., 1993). Although gonococci with nonsialylated LOS are more invasive than those with sialylated LOS, the gonococci with sialylated LOS are more resistant to bactericidal effects of serum (Parsons et al., 1989; van Putten, 1993).

Neisserial LOS shares an antigenic similarity with antigens present on human erythrocytes. This may preclude an effective immune response to these LOS antigens by maintaining the immune-tolerance of the host (Quillin & Seifert, 2018). Gonococci produce two different extracellular IgA1 proteases. These proteases cleave the heavy chain of the human immunoglobulin (Ig) at different points within the hinge region. It is also thought that the Fab fragments of IgA1 may bind to the bacterial cell surface and block the Fc-mediated functions of other immunoglobulins (Halter et al., 1984).

## 2.7 Gonococcal virulence factors

Although *N. gonorrhoeae* does not produce any exotoxins it has a wide range of virulence determinants including both cell surface components (Pili, Porin, Opa proteins, Rmp, Tbp, Lbp and LOS) (Figure 2.2) (Quillin & Seifert, 2018) and extracellular products (IgA serine protease). Figure 2.2 below shows gonococal virulence factors. Gonococcal cell envelope consist of cytoplasmic membrane (inner

membrane), a periplasm (mainly composed of peptidoglycan), and an outer membrane containing LOS, Rmp, PorB, Opa, Tbpand Lpb virulence factors.

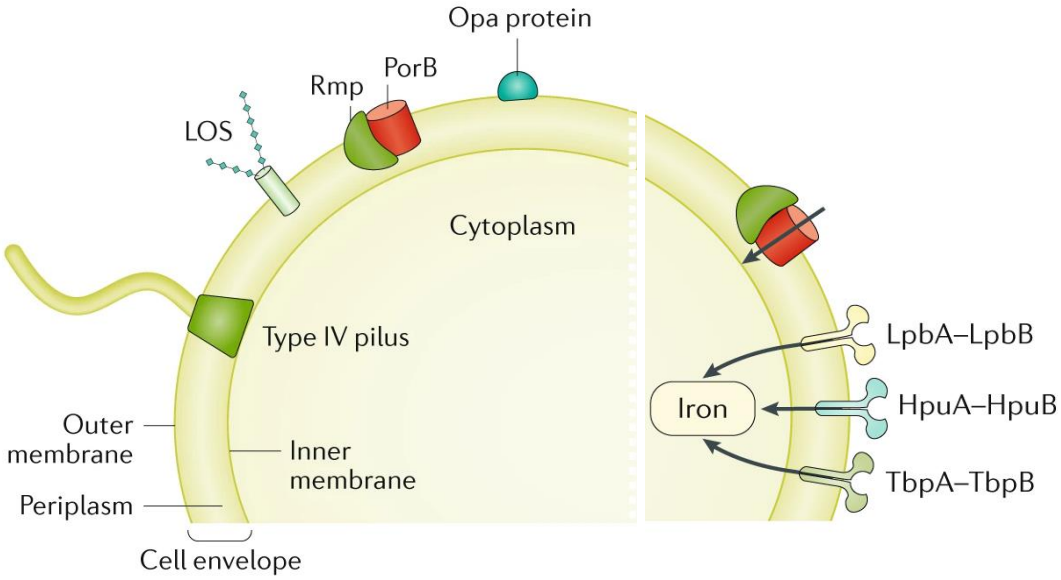



**Figure 2.2: Surface structures involved in gonococcal virulence.**

Adapted from (Quillin & Seifert, 2018)

### 2.7.1 Pili

They are filamentous structures emanating from the bacterial surface and are composed of protein called PilE. The pili in gonococci (fimbriae) are categorized as Type IV pili (Freitag et al., 1995). In gonococci Type IV pili mediate the first stages of infection; adhesion and invasion to host tissues, which is further aided by the outer membrane adhesins, Opa. Additionally, type IV pili are involved in bacterial biofilm formation, twitching motility, and DNA transformation (Taktikos et al., 2015). PilE polypeptide consists of three functional domains based on sequence characteristics (Hill et al., 2016; Shaughnessy et al., 2019); a highly conserved and hydrophobic N-terminal domain comprising the core of the pilus structure; a central partially conserved and structurally aligned β-pleated sheet; and a hydrophilic C-terminal domain which is exposed to the external environment and undergoes antigenic variation allowing the bacteria to avoid recognition by the human host's immune cells (Forest & Tainer, 1997; Hill & Davies, 2009).

### 2.7.2 Opacity-associated outer membrane protein

Opacity-associated outer membrane protein (Opa) proteins are integral outer membrane proteins that cause colonies to appear opaque due to inter-gonococcal aggregation when viewed by phase-contrast microscopy (Fox et al., 2014). These proteins are subject to phase variation (Hill et al., 2016). At any particular time, *N. gonorrhoeae* express zero, one, or several different Opa proteins, and each strain has 10 or more genes for different Opas (Swanson et al., 1992). After initial attachment, the bacteria enter a second stage of tight binding and invasion of epithelial cells which is mediated by Opa proteins (Shaughnessy et al., 2019).

### 2.7.3 Porin proteins

Porins facilitate the movement of nutrients and ions across the outer membrane and can contribute to the survival of the bacteria in host cells (Shaughnessy et al., 2019). The outer membrane porin protein (Por) is the most abundant in the gonococcus accounting for approximately 60% of the total protein content (Hung & Christodoulides, 2013). Although there are variations in PorB size between gonococcal strains, it exists as a single protein species within individual strains and is used for serological classification of gonococci. Two distinct structural classes of Por exist in gonococci (PorA and PorB). PorA is associated with complicated aspects of the gonorrheal infections, whereas PorB is associated with uncomplicated mucosal infections (Hill et al., 2016; Shaughnessy et al., 2019). Gonococcal Por translocate from the outer membrane into epithelial cell membranes after attachment of the cells and can transfer into mitochondria of infected cells (Weel et al., 1991; Muller et al., 2002). This forms porin channels in the mitochondrial inner membrane, and increases the permeability and consequently apoptosis of infected cells (Muller et al., 1999).

### 2.7.4 Lipooligosaccharide

Lipooligosaccharide (LOS) is a major glycolipid of the gonococcus outer membrane, which lacks the repeating O-antigen of lipopolysaccharide (Shaughnessy et al., 2019). Gonococcal LOS is involved in the virulence and pathogenesis of *N.*

*gonorrhoeae* by mediating tissue damage, facilitating bacterial translocation across the mucosal barrier as well as by providing resistance against normal human serum (Shaughnessy et al., 2019). Several antigenic types of LOS are expressed in gonococci (Hung & Christodoulides, 2013).

### 2.7.5 Reduction modifiable protein

Reduction modifiable protein (Rmp) associates with Por in the formation of pores in the cell surface. It protects Por and Los from bactericidal antibodies (Rice et al., 1994; Shaughnessy et al., 2019). The presence of Rmp antibody in women is associated with increased risk for gonococcal infection (Plummer et al., 1993).

### 2.7.6 Transferrin binding protein Tbp1 and Tbp2

*N. gonorrhoeae* acquires iron through well-developed iron acquisition systems which permits it to obtain iron from its host during growth. The iron is necessary to support bacterial invasion. Under low iron conditions gonococci expresses two transferrin binding protein (Tbp1 and Tbp2) and one lactoferrin binding protein (Lbp) (Shaughnessy et al., 2019). These proteins which are found in its outer membrane, are able to directly extract iron from transferrin and lactoferrin, respectively as well as from haemoglobin, and ferritin (Jordan & Saunders, 2009; Noinaj et al., 2012). The expression of transferrin binding protein is required for gonococcal infectivity (Cornelissen et al., 1998).

### 2.7.7 IgA protease

*N. gonorrhoeae* produces Immunoglobin A (IgA) serine protease which is only expressed by pathogenic Neisseria species. Upon release from the cell, the protein undergoes several endo-proteolytic cleavages, leading to maturation of the IgA protease (Hill et al., 2016). During an infection, the mature protease specifically targets and cleaves IgA1 within the proline-rich hinge region of the IgA1 heavy chain (Edwards & Butler, 2011). Neisseria IgA protease also cleaves LAMP1 (a major lysosome associated membrane protein), which leads to lysosome modification and subsequent bacterial survival (Ayala et al., 2002).

## 2.8 Ways used by *N. gonorrhoeae* to change its virulence factors

The state at which gonococci enters host cells depends largely on which surface components are expressed and whether these components are chemically modified or not. *N. gonorrhoeae* modulate the expression or, the chemical character of its surface components via two mechanisms; antigenic variation or phase variation (Meyer & Hill, 2003). Both variation forms result to new variants which the immune system is never able to make an effective antibody response (Meyer & Hill, 2003).

### 2.8.1 Antigenic variations

Antigenic variations are modifications that change the amino acid composition of surface proteins. It allows new variants to arise during the course of an infection, so protective immunity is never generated (Simms & Jerse, 2005). Antigenic variations seen with *N. gonorrhoeae* occur at high frequency in the population and are not necessarily in response to the production of specific antibodies. *N. gonorrhoeae* uses antigenic variation to vary its: pili (PilE) and Opa proteins (Hung & Christodoulides, 2013).

Gonococci change both the amount of pilin subunit produced and the amino acid composition of the subunits through antigenic variation (Hill & Davies, 2009). PilA, (a transcriptional sensor protein) and PilB, (an activator protein) control the amount of PilE produced in gonococcal cells. Gonococci vary the amino acid composition of PilE through homologous recombination and multiple copies of the pilin genes are scattered around the chromosome. Only one of these copies that has a promoter is expressed as a complete gene (*pil*E). The others are non-expressed copies (*pil*S) and serve as storage loci for variable pil sequence. By homologous recombination with *pil*E genes on the same chromosome (or from DNA taken up by transformation), all or part of a silent, *pil*S gene is transferred to the *pil*E site and a new form of gonococci that can express an infinite variety of pili using a limited number of genes is formed (Haas et al., 1992).

The amino acid composition of Opa proteins is also varied by a process of homologous recombination. Most *N. gonorrhoeae* strains have multiple copies of the

*opa* gene; as many as 12, which differ primarily in two hypervariable regions. Homologous recombination can occur between the different copies of the gene to cause antigenic variants (Simms & Jerse, 2005).

### 2.8.2 Phase variation

Phase variation result from frame-shifting within a gene. The frame shift leads to random switching between on/off states of protein expression. Gonococci use slipped strand mispairing to turn on and off the expression of pili, Opa proteins, and LOS. Slipped strand mispairing happens during DNA replication of highly repetitive DNA sequences where when one of the repeats is gained or lost, and a frame shift occurs. Pilus expression can undergo on/off switching ($Pil^+$/$Pil^-$) due to frame shifting either within the *pilE* gene or, within the *pilC* gene (Hill & Davies, 2009; Hung & Christodoulides, 2013). Frame shifting within various glycosyl transferase genes leads to formation of LOS with varied length and carbohydrate chains or other changes in the carbohydrate portion and can render the gonococci serum resistant (Yang & Gotschlich, 1996). Multiple copies of complete *opa* genes are scattered throughout the chromosome. Each of these copies contains repeats of the sequence "CTCTT" at the 5' end of the gene. Addition or subtraction of *opa* repeats resulting from frame shifting bring each individual *opa* gene either in or out of frame ($Opa^+$/$Opa^-$) (Hung & Christodoulides, 2013).

## 2.9 *N. gonorrhoeae* genetics

### 2.9.1 Genome structure

The total size of the gonococcal genome is estimated to be around 2.1-2.3 mega base pairs (Mbp). It is circular in shape and has an average GC content of 52%. Gonococcus is a polyploidy organism and has been shown to possess an average of three chromosome copies per cell (Tobiason & Seifert, 2006; Tobiason & Seifert, 2010).

*N. gonorrhoeae* genomes contain variety of repetitive DNA sequences which serve as phage integration sites or alternative transcription terminators (P. R. Marri et al., 2010). The most common repeat elements in gonococci include; a) DNA uptake

sequence (DUS), a non-palindromic 10-bp sequence which mediate species specific DNA transformation (Duffin Paul & Seifert, 2010), b) Duplicated repeat sequence 3 (dRS3), a 20-bp sequence which flank an intra-chromosomal mobile genetic element called Neisserial inter-genomic mosaic elements (NIMEs) and act as phage integration sites (Parkhill et al., 2000; P. R. Marri et al., 2010), and c) Correia repeat (CR) sequence, a 26-bp sequence that like dRS3 also flanks an intra-chromosomal mobile genetic element called correia repeat enclosed elements (CREEs) (Buisine et al., 2002; Liu et al., 2002).

*N. gonorrhoeae* is naturally competent for transformation and can acquire and integrate naked DNA from other related Neisseria species as well as non-related bacterial species (Spratt et al., 1992). Through horizontal gene transfer (HGT) gonococci are also able to interchange inter-chromosomal DNA elements including gonococcal genetic elements (GGI), bacteriophages as well as plasmids. Consequently, in addition to the chromosomal DNA, some gonococci isolates contain extra chromosomal plasmids DNA (Cehovin & Lewis, 2017).

### 2.9.2 Gonococcal mobile genetic elements

Mobile genetic elements are DNA segments which encode proteins that facilitate movement of DNA within and between bacterial genomes through HGT. In bacteria HGT mainly occur through transformation, transduction and conjugation. Through HGT species evolve and become genetically diverse, acquire virulence and antimicrobial resistance genes as well as other non-survival genes which allow adaptation and survival in specific niches (Frost et al., 2005). Gonococcal mobile genetic elements (MGEs) can be grouped into two; a) MGEs mediating intra-chromosomal rearrangements and b) MGEs mediating inter-chromosomal rearrangements. Intra-chromosomal MGEs include the mobile insertion sequences (IS), and transposons, and immobile Correia repeat enclosed elements (CREEs), Minimal mobile elements (MMEs), Neisserial inter-genomic mosaic elements (NIMEs), and Spencer-Smith repeat enclosed elements (SSREE) (Figure 2.3). The immobile elements are transferred between genomes through homologous recombination. These elements act as recombination hotspots and also carry AMR

cassettes. Figure 2.3 below summarizes the mobile genetic elements found in *N. gonorrhoeae* and the mechanisms through which genetic material is transferred in gonococci.

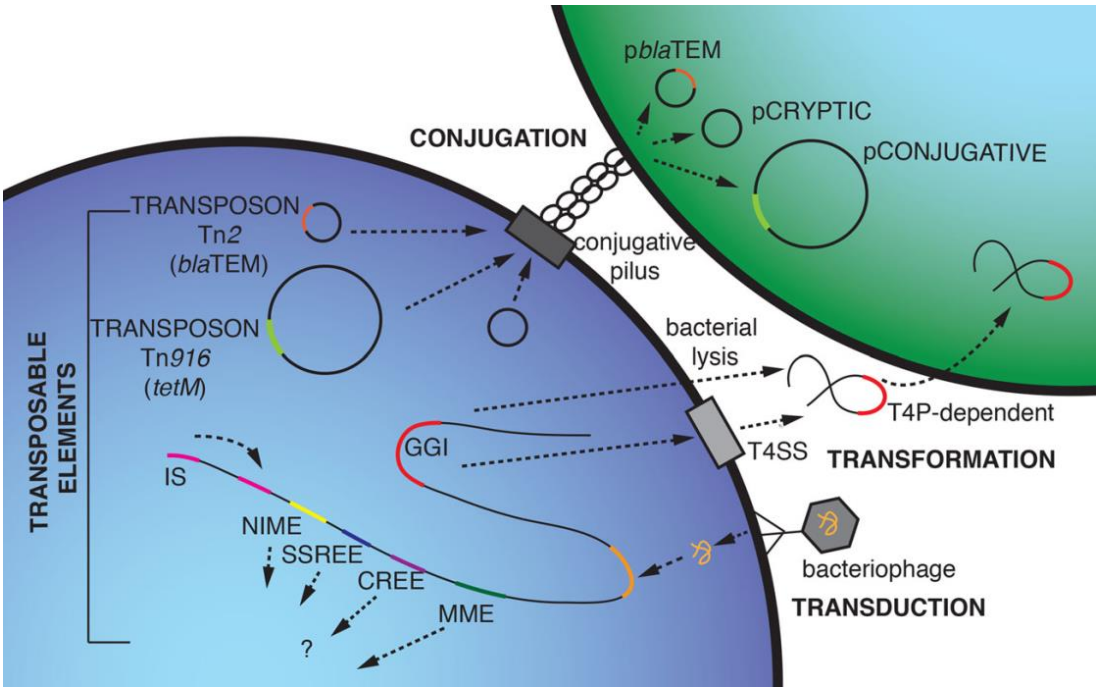



**Figure 2.3: Gonococcal Mobile Genetic Elements and HGT mechanisms.**

Adapted from (Cehovin & Lewis, 2017)

Inter-chromosomal MGEs include bacteriophages, plasmids and gonococcal genetic islands (GGI). They play a role in spreading AMR genes and in bacterial pathogenesis (Figure 2.3) (Cehovin & Lewis, 2017). In addition to intra-genomic transposition, both insertion sequences and transposons can be transferred inter-chromosomally through integration into plasmids or bacteriophages and also through transformation (Siguier et al., 2014).

#### 2.9.2.1 Insertion sequences

Flanked by inverted repeats, insertion sequences (IS) are small transposable elements which can move within the chromosome. As they move, they are capable of mobilizing adjacent genetic elements along with them (Skaar *et al.*, 2005). There are at least 16 different insertion sequences in gonococci which only carry genes which

code for transposition enzymes (Pradeep Reddy Marri et al., 2010). They are used for differentiation between *N. gonorrhoeae*, *N. meningitides*, and *N. lactamica* species (Skaar et al., 2005; Schoen et al., 2008).

#### 2.9.2.2 Transposons

Like insertion sequences, transposons move from one part of a chromosome to another, are flanked by inverted repeats, and carry genes coding for transposases and resolvases which facilitate transposition. Additionally, these elements carry extra genes which confer an added advantage to the carrier bacterium (Cehovin & Lewis, 2017). Two antibiotic resistance genes, *blaTEM* and *tetM* responsible for plasmid mediated penicillin and tetracycline resistance in gonococci are carried by transposons integrated in conjugative gonococcal plasmids (Swartley et al., 1993; Bailey et al., 2011) (Figure 2.3).

#### 2.9.2.3 Correia repeat enclosed elements

Correia repeat enclosed elements (CREEs) also known as *Neisseria* miniature insertion sequence comprise of a 50 to 150 bp non coding variable internal fragment flanked by 26-bp inverted repeat sequence known as correia repeat (CR). Correia repeat enclosed elements lack transposition ability (Buisine et al., 2002). The inverted correia repeats in CREEs have transcription termination effect, while CREEs contain promoter sequences. Consequently CREEs could be involved in regulation of gene expression (Francis et al., 2000; Snyder et al., 2009).

#### 2.9.2.4 Minimal mobile elements

Minimal mobile elements (MMEs) are exchangeable cassettes whose contents differ between strains and species (Snyder et al., 2004). Unlike transposable insertion sequences and transposons, MMEs lack transposition enzymes or terminal inverted repeats. Instead, they are flanked on both ends by conserved protein encoding genes and therefore like the CREEs, they are immobile. Together with the encoded proteins, they are transferred intra-chromosomally through homologous recombination (Snyder et al., 2007).

#### 2.9.2.5 Neisserial intergenomic mosaic elements

Neisserial intergenic mosaic elements (NIMEs) comprise of a 19-214bp repeat sequences flanked by an inverted duplicated repeat sequence 3 (dRS3). Several NIMEs cluster together to form intergenic NIME repeat arrays whose size range from 200 to 2700bp. Like the CREES and MMEs, NIMEs lack transposases and are therefore immobile elements which due to the repetitive nature and presence of recombinase target sequences; dRS3, are hotspots for recombination (Parkhill et al., 2000).

#### 2.9.2.6 Spencer Smith repeat enclosed elements

Spencer Smith repeat enclosed elements (SSREE) are newly described approximately 650bp elements which were found flanking a chromosome fragment that had undergone inversion. They are comprised of an approximately 610bp non-coding internal segment that contain predicted open reading frames (ORFs) flanked by 19bp inverted repeats. Spencer Smith Repeat enclosed elements lack transposase ability and their location in genomes does not differ between genomes. These are thought to serve as homologous recombination templates or recognition sites for insertion sequences (Spencer-Smith et al., 2012). Better understanding of the role of repeat sequences in organisms is being sought through advanced whole genome sequencing techniques.

#### 2.9.2.7 Gonococcal genetic island

Gonococcal Genetic Island (GGI) expressed by 80% of gonococcal isolates, is an approximately 57Kbp genetic element mostly found in pathogenic Neisseria species. It contains DUS sequences and its GC content is less (43%) than that of gonococcal genome (52%). Normally GGI is always integrated at the dif site, a 28bp sequence located near the *ung* gene at the replication terminus. GGI integration duplicates the *dif* gene into a functional *difA* gene and a degenerate *difB gene* (Snyder et al., 2005). Gonococcal genetic island encodes a type IV secretion system (T4SS), which is involved in the secretion of single stranded DNA into the extracellular environment using a self-encoded DNA secretion system and a host site specific recombination

system. T4SS is therefore an important source of transformation substrates. It leads to spread of antibiotic resistance and virulence genes, and enhances antigenic diversity in gonococci. It additionally can be involved in modulation of the host's immune responses (Hamilton et al., 2005).

#### 2.9.2.8 Bacteriophages

Non-lytic and non-transducing double-stranded (dsDNA) lysogenic phages and single stranded (ssDNA) M13 like filamentous phages and prophages have been identified in Neisseria species. For instance the genome of *N. gonorrhoeae* strain FA1090 was found to contain five dsDNA tailed bacteriophages named NgoΦ1–5, and four filamentous phages (Piekarowicz et al., 2007). The bacteriophages are normally flanked by pilin inversion gene (Piv) or invertase-related gene (Irg) sequences while others are integrated in dRS3 repeat sequences. Prophage sequences are thought to play an active or passive role in recombination (Kawai et al., 2005). Since gonococcal phages are non-transducable, the role of bacteriophages in spread of antimicrobial resistance genes in *N. gonorrhoeae* remains to be determined.

#### 2.9.2.9 Gonococcal plasmids

There are two types of plasmids in bacterial cells; a) cryptic plasmids which are small and non-transmissible, and b) conjugative plasmids which are large and can be transferred from one bacterial cell to another through conjugation (Cehovin & Lewis, 2017). In addition to transferring their own DNA, conjugative plasmids can as well co-transfer chromosomal DNA and DNA of other non-transmissible plasmids (Eisenstein et al., 1977). For instance, gonococcal conjugative plasmids have been shown to transfer small non self-transmissible beta lactamase encoding gonococcal plasmids (Pachulec & van der Does, 2010).

*N. gonorrhoeae* harbors both cryptic and conjugative plasmids. Most gonococci carry an approximately 4.2Kbp cryptic plasmid (pJD1), the smallest of the gonococcal plasmids and whose functions are not well known (Hagblom et al., 1986). There are three types of conjugative plasmids in gonococci; 24.5 (39Kbp) MDa plasmid with no detectable marker (pLE2451), and two 25.2 MDa (42Kbp)

plasmids which contain the tetM determinant (Morse et al., 1986). High gonococcal tetracycline resistance is mediated by a transposon-borne (Tn916) class M tetracycline resistance determinant (TetM) which is carried by either of the two 25.2 MDa conjugative plasmids named “American” and “Dutch” type plasmids (Swartley et al., 1993; Chopra & Roberts, 2001).

Resistance to penicillin in gonococci is mediated by a plasmid or transposon borne β-lactamase encoded by *blaTEM* gene. Seven types of non-self-transmissible gonococcal plasmids harbouring the β-lactamase have been established in gonococcal isolates termed as penicillinase producing *N. gonorrhoeae* (PPNG). The plasmids are named based on the geographical areas where they were first described: Asian/pJD4 (7.4Kbp); African/pJD5 (5.6Kbp); Rio/Toronto (5.1Kbp); Nimes (6.8 Kbp); Johannesburg (4.8 Kbp); New Zealand (9.3Kbp) and Australian (3.2 Kbp) (Ashford et al., 1976; Gouby et al., 1986; Brett, 1989; Alam, 2008; Etienne et al., 2011; Trembizki et al., 2014). The Asian type is the ancestral plasmid from which either deletions or insertions gave rise to the other six plasmid types (Ashford et al., 1976; Dillon & Yeung, 1989).

### *2.10 N. gonorrhoeae* epidemiology and antimicrobial resistance evolution

Without a gonococcal vaccine gonorrhea treatment has relied in the use of antimicrobials over the past 80 years. Unfortunately, a major global public health concern has arisen following the emergence of *N. gonorrhoeae* strains multi resistant to previously and currently recommended antimicrobials including sulphonamides, penicillins, tetracyclines, macrolides, fluoroquinolones, and cephalosporins (Figure 2.4) (Tapsall et al., 2009; Unemo, 2015; Unemo et al., 2016).

Sulphonamides were the first antimicrobials used to treat gonorrhea in 1930’s (Kampmeier, 1983). Sulfanilamide, sulfapyridine, and sulfathiazole were successfully used until 1944 when resistance developed (Van Slyke et al., 1941; Dunlop, 1949).

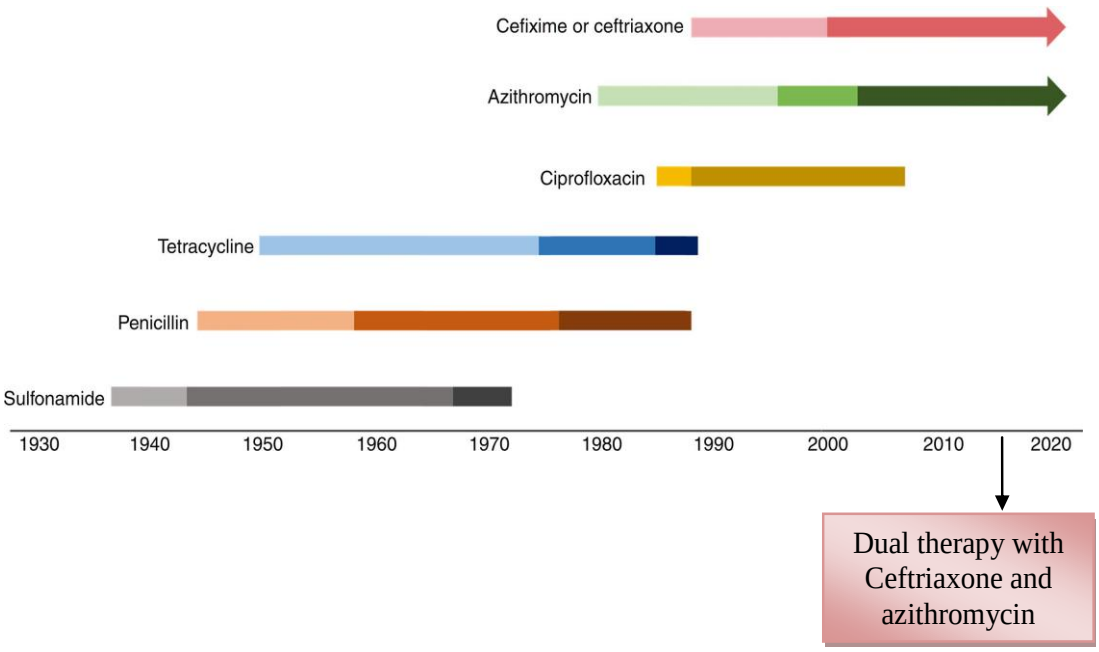



**Figure 2.4: Evolution of antimicrobial resistance in *Neisseria gonorrhoeae*.** Adapted from (Costa-Lourenço et al., 2017)

Following the withdrawal of sulphonamides in treating gonorrhea in the early 1940s, penicillins became the main first line gonorrhea therapy for almost 40 years, initially at very low dosage and subsequently given at higher doses with probenecid (Mahoney et al., 1943; Van Slyke et al., 1943). As penicillin doses increased, plasmid and chromosome mediated resistance developed in the following two decades and tetracycline or erythromycin were used during this period for the management of penicillin allergic patients and treatment of penicillin resistant *N. gonorrhoeae* infections (Amies, 1967; Martin Jr et al., 1970). Chromosome mediated resistance led to reduced tetracycline susceptibility and later in mid-1980's plasmid mediated resistance resulting from *tetM* determinant resulted in the exclusion of tetracycline and penicillin from gonorrhea treatment (Reyn et al., 1958; Roberts et al., 1988).

An aminocyclitol; spectinomycin was then introduced as first line of treatment for penicillinase producing *N. gonorrhoeae* in 1980's but four years later resistance developed and it was abandoned (Stolz et al., 1975; Easmon et al., 1984). This led to introduction of fluoroquinolones; ciprofloxacin and ofloxacin as first line oral

therapy for gonorrhea for the following decade before its resistance developed in 1990's (Gransden et al., 1990; Tanaka et al., 1994; Tanaka et al., 2000). In 2007, fluoroquinolones were abandoned from the Centre for Disease Control (CDC) recommended treatment regimens for gonorrhea (Centers for Disease & Prevention, 2007; Unemo & Shafer, 2011).

The macrolide erythromycin which was introduced in 1950's, was replaced by a more active azithromycin for gonorrhea treatment until later in the 1990's when resistance to both macrolides was reported (Dillon et al., 2006; Dillon et al., 2013; Alirol et al., 2017). Azithromycin though not the recommended monotherapy of gonorrhea following concerns of a rapid resistance selection and possible adverse effects, is one of the two antimicrobials in the introduced dual antimicrobial therapeutic regimens for gonorrhea (Bignell & Fitzgerald, 2011; Bignell & Unemo, 2013).

Following the resistance to fluoroquinolones, cephalosporins were introduced as the last available monotherapy for gonorrhea in the late 1990's (Unemo & Shafer, 2014). Third generation extended spectrum cephalosporins (ESC); ceftriaxone and cefixime are the current international recommended monotherapy for gonorrhea treatment. However, gonococci isolates exhibiting decreased susceptibility and resistance to these cephalosporins have been reported since 2001 (Ohnishi, Saika, et al., 2011).

Cephalosporin resistance is spreading globally and has resulted in the emergence of extensively drug resistant gonococci. Extensively drug resistant (XDR) strains are resistant to two or more of the antibiotic classes that are generally recommended for the treatment of gonorrhea or three or more of the classes that are less frequently used for treatment (Tapsall et al., 2009). *N. gonorrhoeae* H041 and *N. gonorrhoeae* F89 are XDR gonococci reported from Japan, France and Spain and which have high resistance to cefixime and ceftriaxone combined with resistance to nearly all other available therapeutic antimicrobials (Ohnishi, Golparian, et al., 2011; Camara et al., 2012; Magnus Unemo et al., 2012; Allen et al., 2013).

## 2.11 Mechanisms of antimicrobial resistance in *N. gonorrhoeae*

Mechanisms of antimicrobial resistance can generally be grouped into the following categories; a) modification of the antibiotic either through chemical alteration or destruction of the drug by enzymatic means, b) modification or protection of the drug sensitive target through mutations, enzymatic alterations and replacement or bypassing of the target site all which leads to reduced drug affinity, and c) reduced drug accumulation resulting from active efflux pumps or decreased influx due to modification or loss of the proteins which transport drugs into the cell (Tenover, 2006; Munita & Arias, 2016).

Bacterial genomes are dynamic, and are exposed to various genetic events, including, mutations, duplications, inversions, transpositions, recombination, insertion, and deletions. *N. gonorrhoeae* can alter its genome through a) different types of mutations in genes often associated with drug's mechanism of action, and b) acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT) (Spratt et al., 1992).

Being naturally competent it can acquire genetic material from both Neisseria and non Neisserial bacteria species, through transformation and conjugation (O'Rourke & Stevens, 1993; Bilek et al., 2009; Juhas et al., 2009; Corander et al., 2012; Ezewudo et al., 2015). Additionally it has a type IV secretion system that actively transports DNA out of the cell hence contributing to the emergence and spread of virulence and antimicrobial resistance genes (Hamilton & Dillard, 2006). Some gonococcal strains have evolved and acquired or developed resistance to nearly all antimicrobials used for treatment of gonorrhea (Unemo & Shafer, 2014). The mechanisms of resistance to each drug are discussed in the following sections and illustrated in Figure 2.5.

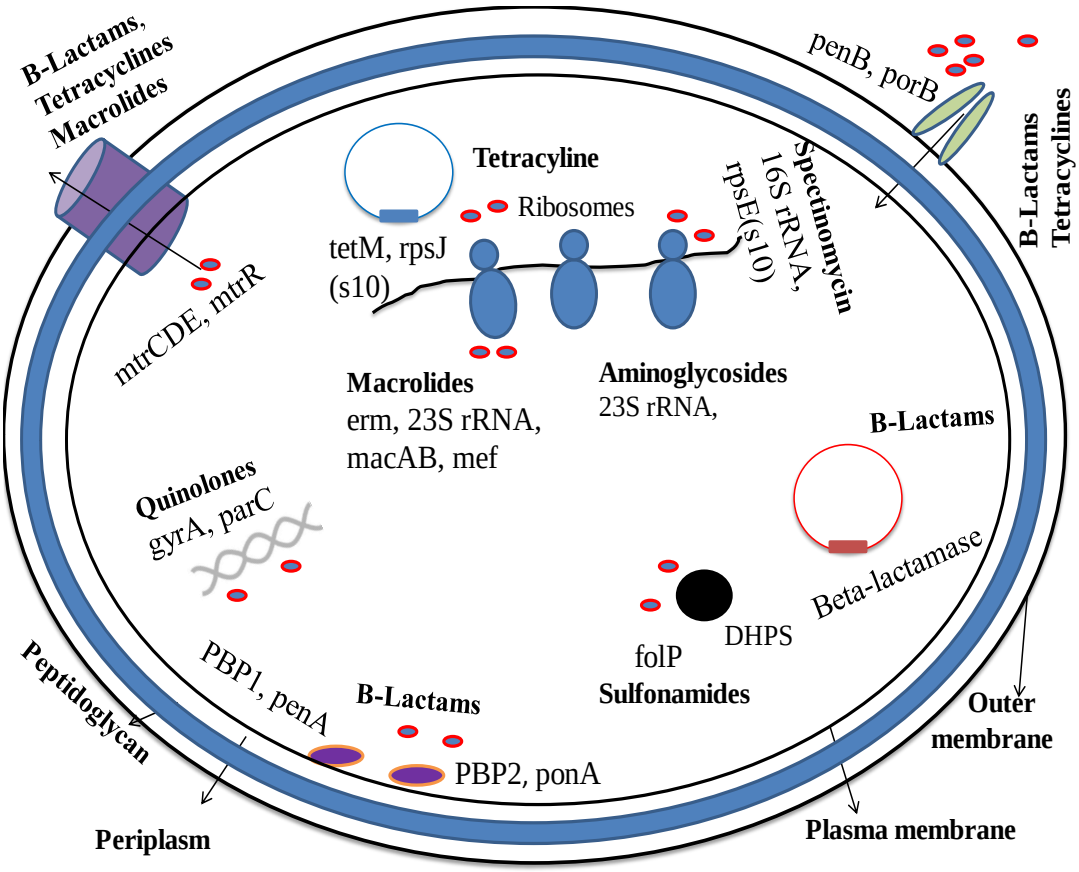



**Figure 2.5: Gonococcal mechanisms of antimicrobial resistance.**

Figure 2.5 is an overview of the main antimicrobial resistance determinants associated with antibiotic resistance in gonococci. The following genes encode: *mtrR*- MtrCDE efflux pump repressor; *porB* - an outer membrane protein (porin); *penA* and *ponA* penicillin binding proteins PBP1 and PBP2 (targets for β-lactams); *gyrA* - gyrase and *parC* - topoisomerase IV (both are quinolone targets); *folP* - dihydropteroate synthase (DHPS, the target for sulphonamides); *tetM* – class M tetracycline resistance determinant; *erm* - rRNA methylase; *macAB*-MacAB efflux pump and *mef* – an efflux pump (both pumps macrolides out of the cell); *rpsJ* – S10 and *rpsE* – S5 ribosomal proteins.

### 2.11.1 Sulphonamides

Sulphonamides the first effective chemotherapeutic agents used for treatment of bacterial infection in humans are compounds which contain $SO_2NH_2$ functional

group and have $RSO_2NH_2$ general formula. Different sulphonamides have been derived from para amino benzene sulphonamide, but their importance has declined in the face of increasing resistance (Lavanya, 2017). Sulphonamides compete with para aminobenzoic acid (PABA) for the enzyme dihydropteroate synthase (DHPS), thereby preventing the formation of tetrahydrofolate which is needed for DNA synthesis (Unemo et al., 2016).

Sulphonamides were introduced as first line drugs for gonorrhea treatment in 1930's but by the 1940's resistance had already emerged mediated by chromosome mutations in *folP* gene which encodes DHPS (Dunlop, 1949; Kampmeier, 1983). Mutated *folP* is translated into a form of DHPS that has a low affinity for sulphonamides (Fiebelkorn et al., 2005). Gonococcal sulphonamide resistance can also arise from over synthesis of paraminobenzoic acid, which dilutes and reduces the concentration of the drug (Johnson & Morse, 1988; Swedberg et al., 1993).

### 2.11.2 Penicillin

Penicillin together with cephalosporins, cephamycins, monobactams, and carbapenems belong to the β-lactam class of antibiotics which contains a four membered β-lactam ring (Kong et al., 2010). Penicillins and cephalosporins are the two major families of β-lactam antibiotics. Alexander Fleming, a bacteriologist who worked at St. Mary's hospital in London discovered the first β-lactam, Penicillin in 1928 (Fleming, 1980). Most penicillins are derivatives of 6-aminopenicillanic acid and differ from one another with respect to the side chain attached to the amino group (Figure 2.6) (Bush & Bradford, 2016). The structure of 6-aminopenicillanic structure is composed of an enclosed dipeptide formed by the condensation of L-cystein and D-valine, resulting in the β-lactam ring and in the thiazolidinic ring. The availability of 6-aminopenicillanic has allowed the creation of hundreds of synthetic and semi synthetic penicillin derivatives and related β-lactam classes of antibiotics.

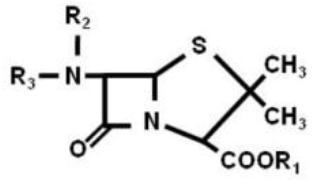



**Figure 2.6: Penicillin core.**

Adapted from (Kong et al., 2010).

Figure 2.6 shows the structure of 6-aminopenicillanic acid to which different side chains are attached to produce variety of penicillins with varied antibacterial activities.

β-lactam antibiotics are bactericidal agents that interrupt bacterial cell-wall formation by interfering with the synthesis of both Gram-negative and Gram-positive bacterial peptidoglycan (Figure 2.5). Peptidoglycan also called murein, is a protective later which preserve bacterial cell integrity and shape as well as preventing the penetration of macromolecules into the cell (Dramsi et al., 2008). Peptidoglycan, located outside bacterial plasma membrane is composed of chains of alternating amino sugars; N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues linked by β-1→4 bonds. In a nascent peptidoglycan, the D-lactoyl group of each NAM residue is substituted by a L-Alanine-D-glutamic acid-meso-diaminopimelic acid/D-lysine-D-Alanine-D-alanine pentapeptide (Figure 2.7). (Vollmer et al., 2008). The amino sugar monomers are covalently cross linked via short peptides by the activity of specific enzymes called transpeptidases or penicillin-binding proteins (PBPs) (Sauvage et al., 2008). The crosslinks provide a net-like conformation which is the basis for peptidoglycan strength (Figure 2.7) (Vollmer et al., 2008).

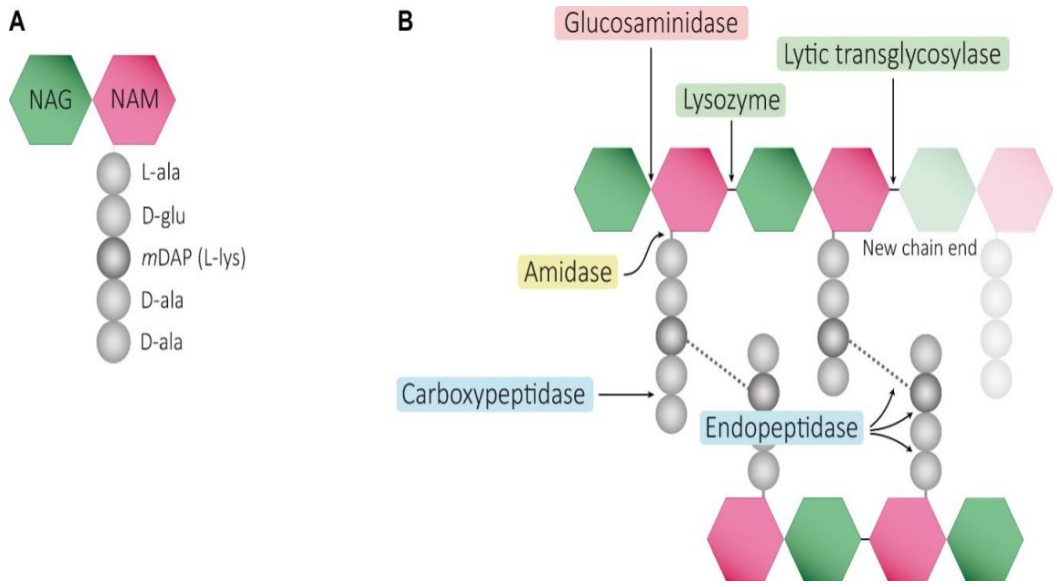



**Figure 2.7: Peptioglycan composition and structure.**

Adapted from (Irazoki et al., 2019)

**A;** peptidoglycan monomer, **B**; peptidoglycan structure and sites of action of different peptidoglycan cleaving enzymes

Penicillin-binding proteins are grouped into classes; A, B, and C. Class A and class B PBPs are transpeptidases which catalyze peptide cross-links formation between adjacent glycan strands of peptidoglycan. While class A PBPs contains additional N-terminal transglycosylase domain activity that polymerizes glycan strands, class B PBPs only possesses the mono-functional transpeptidases activity (Powell et al., 2009). Class C PBPs have an *in vitro* carboxypeptidase activity function to modulate the degree of cross linking in peptidoglycan by removing the terminal D-Alanine of the pentapeptide (Sauvage et al., 2008). The different bacterial species have distinctive set of PBPs that range from 3-8 enzymes per species (Georgopapadakou & Liu, 1980).

In *N. gonorrhoeae* four PBPs have been identified. Two high molecular mass transpeptidases; class A PBP1 and class PBP2, and two low molecular mass class C carboxypeptidases/ endopeptidase PBPs; PBP 3 and PBP 4. (Zapun et al.,

2016).While both PBP3 and PBP4 can be deleted from *N. gonorrhoeae* with only minor effects on cell growth and morphology, PBP1 and PBP2 are essential for cell viability and are therefore the lethal targets of β-lactam antibiotics (Barbour, 1981; Stefanova et al., 2003). The structure of penicillins resembles the terminal D-Alanyl-D-alanine found on the peptide side chain of the peptide monomer. By mimicking these terminal D-alanine residues, penicillin binds irreversibly to the active site of the transpeptidase, thereby preventing the enzyme from cross linking the peptidoglycan strands (Spratt, 1978; Sauvage et al., 2008). Consequently, the formation of peptide bridges which bring about peptidoglycan netlike conformation is inhibited and osmotic stress ruptures the bacterial cell(Spratt, 1978). PBP2 has been shown to be inhibited by low concentrations of penicillin compared to PBP1 and is therefore the primary killing target of penicillin (Barbour, 1981).Gonococcal resistance to penicillin emerged in the 1950's (Franks, 1946; Amies, 1967). Bacterial resistance and reduced susceptibility to β-lactams can be acquired via two routes; chromosomal modifications and acquisition of plasmid borne genes mainly *blaTEM* (Dillon & Yeung, 1989; Spratt et al., 1989; Dillon & Pagotto, 1999).

***Chromosomal modifications;*** chromosomally mediated penicillin resistant strains of *N. gonorrhoeae* arise through the stepwise acquisition of multiple resistance determinants via DNA uptake and homologous recombination. At least five different genes including *pen*A, *pon*A, *mtr*R, *por*B, and *pil*Q are implicated in chromosomally mediated resistance to penicillin in *N. gonorrhoeae* isolates (Unemo & Shafer, 2014).

In *N. gonorrhoeae*, PBP1 and PBP2 (encoded by *ponA* and *penA* genes respectively) are the primary killing target of β-lactams (Barbour, 1981; Stefanova et al., 2003). Chromosomal modifications in these two genes alter the three dimensional structures of PBP1 and PBP2 thereby reducing their affinity for penicillin and consequently lead to reduced susceptibility to β-lactams (Ropp et al., 2002). Acquisition of *penA* sequences with a reduced rate of penicillin acylation from commensal Neisseria spp. through transformation initiate penicillin resistance in gonococci (Brannigan et al., 1990). Common amino acid changes in PenA and which have been associated with

penicillin resistance in gonococci include; an aspartic acid insertion between amino acids 345 and 346 (Asp-345 or D-345) along with four to eight (F504L, A510V, A516G, H541N, P552V, K555Q, I556V, I566V) amino acid substitutions clustered near the carboxyl terminus of the protein (Dowson et al., 1989).

These alterations reduce susceptibility to penicillin by decreasing the acylation rates of PBP2 (transpeptidases) (Ropp et al., 2002; Powell et al., 2009). Recently mosaic *penA* genes containing 60 to 70 amino acid changes compared to a wild-type *penA* gene have been described which result in resistance to both penicillins and cephalosporins (Ameyama et al., 2002; Ohnishi, Golparian, et al., 2011; Camara et al., 2012). Ito et al. (2005) examined and grouped different combinations of this amino acid changes into 10 PBP2 patterns which they numbered as PBP2 patterns I to X. Later in 2007, Whiley et al. (2000) identified 13 more PBP2 patterns which they numbered as PBP2 patterns XI to XXIII (Figure 2.8).

Pattern X which has mainly been associated with cephalosporin resistance particularly ceftriaxone, comprise a mosaic *penA* sequence (Ameyama et al., 2002). Although Pattern XXIII is a mosaic *penA* variation of Pattern X it is not associated with reduced cephalosporin susceptibilities (Whiley et al., 2007b). Although PBP1 has a lower penicillin acylation rate than that of wild-type PBP2, substitution of leucine at position 421 by proline (L421P) in PBP1, encoded by the ponA gene, has been associated with high level of penicillin resistance in gonococci (Ropp et al., 2002).

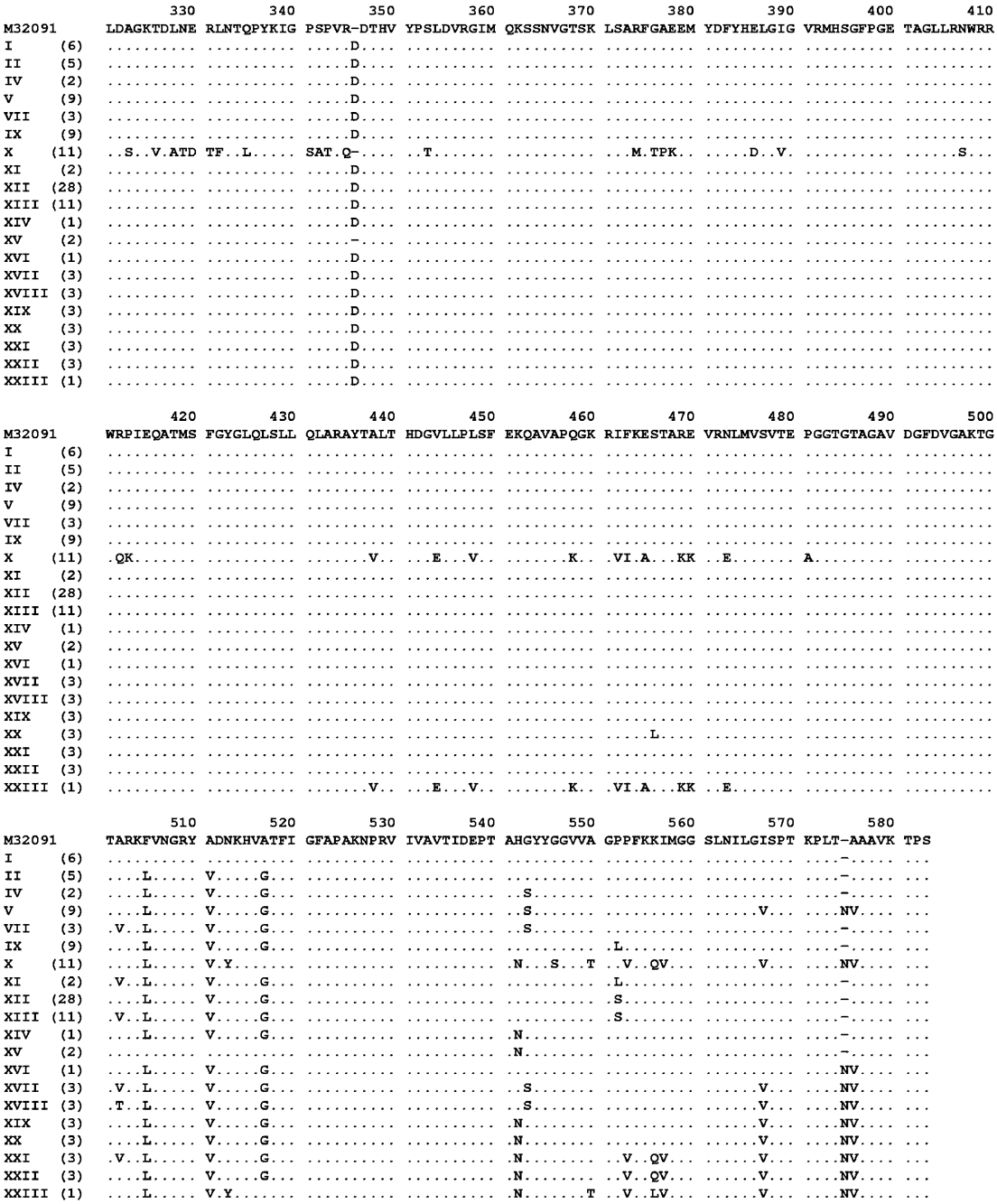



**Figure 2.8: PenA patterns associated with resistance to β-lactam in gonococci.**
Adapted from (Ameyama et al., 2002; Whiley et al., 2007a)

The above Figure shows PenA patterns associated with B-lactan resistance. The alterations include an aspartate insertion at 345 along with 4-8 substitutions clustered near PBP2 carboxyl terminus. Pattern X comprise a mosaic *penA* sequence with 60

to 70 amino acid changes compared to the wild-type and has mainly been associated with cephalosporin resistance particularly ceftriaxone.

Reduced drug accumulation resulting from an active efflux pump or alteration or loss of porins (reduced drug influx) has also been shown to contribute/produce additive effects to drug resistance in *N. gonorrhoeae*. Multiple Transferrable Resistance CDE (MtrC-MtrD-MtrE) efflux pump encoded by *mtrC-mtrD-mtrE* genes has been associated with drug efflux in gonococci. The expression of the pump is repressed by multiple transfer resistance repressor (MtrR) encoded by *mtr*R gene (Figure 2.9) (Unemo & Shafer, 2014).

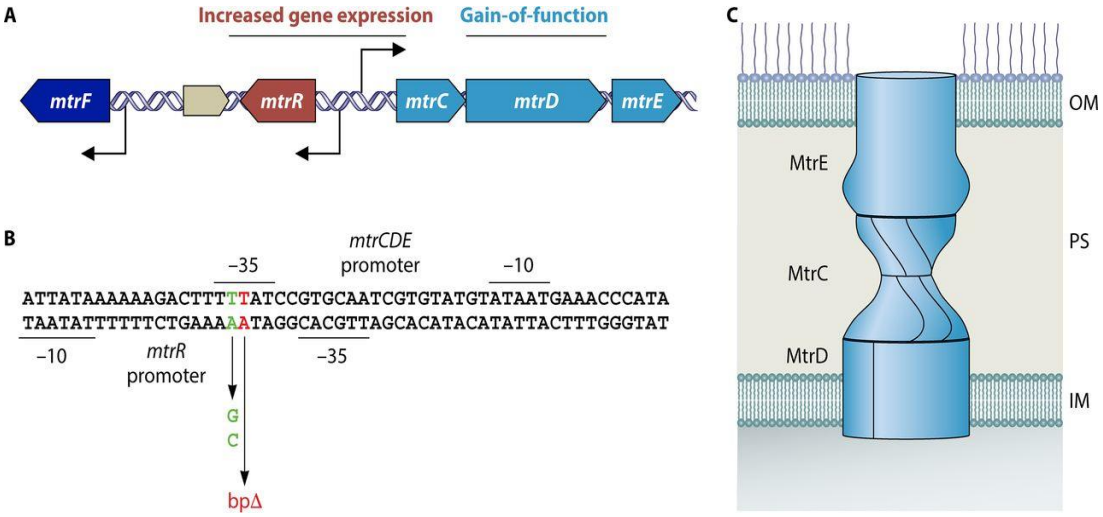



**Figure 2.9: *N. gonorrhoeae mtr* locus.**

Adapted from (Shafer, 2018).

**A**; Structural organization of *mtr* locus and likely impacts of mosaic-like sequences within the *mtrR* and *mtrD* regions, **B;** Overlapping *mtrR* and *mtrCDE* promoters, with highlighted alterations associated with *mtrCDE* over-expression. **C;** Location of MtrC-MtrD-MtrE pump, in the gonococcal membrane

In gonococci, resistance to tetracyclines, penicillins, macrolides, fluoroquinolones and hydrophobic antibacterial agents produced by the host can be mediated by mutations in the promoter or encoding region of the *mtrR* gene. These changes leads

to over expression of the efflux pump and consequently increased drug efflux (Shafer et al., 1995; Lucas et al., 1997; Piddock, 1999; Poole, 2000; Warner et al., 2008). The most common mutations associated with *mtrR* and which are implicated in increased drug efflux in *N. gonorrhoeae* are: a single adenine nucleotide deletion (-A) in the 13bp inverted repeat region between the -10 and -35 hexamers of the *mtrR* promoter, and Ala39Thr, Gly45Asp, and His105Tyr mutations in the *mtrR* structural gene (Corkill et al., 1991; Lucas et al., 1997; Poole, 2000).

Small and charged molecules including antibiotics cross the outer membrane through porinB diffusion channels encoded by *porB* and the cytoplasmic membrane through diffusion (Moniot-Ville et al., 1991; Hooper, 1999). Single nucleotide polymorphisms in the porinB diffusion channels (known as PenA mutations) which ultimately alter its structure can mediate resistance to such molecules which use the channels as a pathway to enter into the cells (Dillon *et al*., 2015). Different studies have suggested though that reduced drug influx resulting from porinB modifications alone cannot account for drug resistance in gonococci, and that for resistance to occur the expression and presence of an endogenous efflux pump such as the MtrCDE is required (Tanaka et al., 1998; Olesky et al., 2006; Shafer & Folster, 2006). Substitutions with charged amino acids at Gly120 and Ala121 and deletions of A121 and N122 in PorB are associated with reduced drug permeation through the porinB protein in *N. gonorrhoeae* (Corkill et al., 1991; Tanaka et al., 1994; Gill et al., 1998; Olesky et al., 2002).

PilQ protein multimers found in gonococcal outer membrane polymerizes and form a pore around the pilus of *N. gonorrhoeae* allowing antibiotics to diffuse into the periplasm. Reduced drug influx result from a missense mutation (E666K) which alters pilQ multimerization, destabilize pore formation and block the entry of antibiotics (Ropp et al., 2002; Helm et al., 2007).

***Plasmid borne enzymes;*** the most widespread mode of clinical resistance development to β-lactam antibiotics is the expression of a group of enzymes specifically designed to degrade and inactivate the antibiotic. These enzymes are

collectively known as penicillinases or β-lactamases. There are many different types of β-lactamases which (Ambler, 1980) are classified into four distinct molecular classes; A, B, C, and D based on the amino acid sequences. The active site of classes A, C and D β-lactamases contain serine, and their structure and mechanism of action is similar to that of the PBPs, suggesting an evolution from PBPs unlike class B whose active site has a binuclear zinc cluster (Fernandes et al., 2013). The most prevalent β-lactamase in gonococci is class A β-lactamase 1 (TEM-1) encoded by *blaTEM-1* gene or its derivatives (Muhammad et al., 2014).These genes are carried by several types of plasmids (discussed in section 0). TEM-1 β-lactamase directly attacks and destroy/inactivate β-lactam drugs including penicillins and early cephalosporins such as cephalothin and cephaloridine by hydrolyzing the amide bond in the β-lactam ring (Coulson, 1985; Bradford, 2001).

In response to the heavy pressure of antibiotic use, β-lactamases continuously mutate leading to the development of more potent derivatives. The extended-spectrum cephalosporins (ESCs) were developed to combat resistance provided by the β-lactamases. While TEM-1 β-lactamase can destroy penicillins it is not active against extended-spectrum cephalosporins (Coulson, 1985), but single nucleotide polymorphisms (SNPs) in *blaTEM-1* resulting in alteration of amino acid configuration around TEM-1 lactamase active site can convert it to an extended broad spectrum lactamase (ESBL)(Knox, 1995).

ESBLs were first reported in the early 1980s in Europe and have since been identified worldwide (Fernandes et al., 2013). They are more stable and potent and can breakdown cephalosporins including ceftriaxone, the last first line monotherapy for treatment of gonorrhea (Wang et al., 2002). *blaTEM-135* encoding a more stable TEM-135 β-lactamase which differs from TEM-1 β-lactamase by one amino acid substitution (M182T) has been described in gonococci from several countries (Wang et al., 2002; Srifeungfung et al., 2009; Ohnishi et al., 2010; Muhammad et al., 2014). It has been described as an intermediate between TEM-1 β-lactamase and extended broad spectrum lactamase (Nakayama et al., 2012). Both *blaTEM-1* and *blaTEM-135*

have mainly been described in the Asian, African and Toronto TEM plasmid types (Yeung et al., 1986; Dillon & Yeung, 1989; Gianecini et al., 2015).

### 2.11.3 Tetracyclines

Tetracyclines, broad-spectrum agents discovered initially from a product of *Streptomyces aureofaciens* in 1945 were used to treat gonorrhea primarily in patients allergic to penicillin (Lewis, 2010). Chlortetracycline and oxytetracycline were the first tetracyclines to be described while other tetracyclines were identified later, either as naturally occurring molecules (tetracycline and demethylchlortetracycline), or as products of semi-synthetic approaches (methacycline, doxycycline, and minocycline) (Chopra & Roberts, 2001). Tetracycline molecules comprise a linear fused tetracyclic nucleus to which a variety of functional groups are attached (2.10). Following the widespread use of tetracycline to treat STIs, resistance arose and spread rapidly from the mid-1980s onwards (Morse et al., 1986; Tapsall et al., 2009; Unemo & Shafer, 2011).

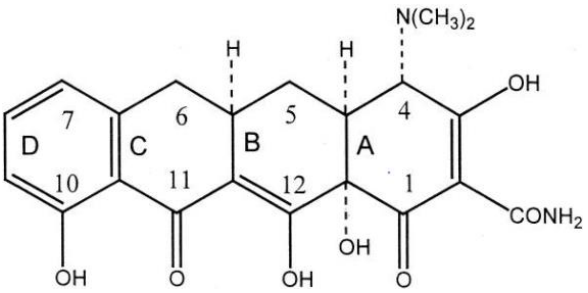



**Figure 2.10: Tetracycline pharmacophore.**

Adapted from (Chopra & Roberts, 2001).

Figure 2.10 shows the core structure of tetracyclines. It comprises of a fused tetracyclic nucleus with four rings numbered A - D. Variety of functional groups can be attached at C5-C9 to produce tetracyclines with varied antibacterial activities.

Tetracyclines inhibit the attachment of aminoacyl-tRNA to the ribosomal acceptor A site in the mRNA-ribosome complex, by mainly binding to the 30S ribosomal

subunit, and accordingly inhibiting the protein synthesis which results in a bacteriostatic effect (Chopra & Roberts, 2001; Unemo & Shafer, 2014).

Tetracycline resistance in gonococci is mediated via two routes; chromosomal modifications and acquisition of transposon-borne *tetM* genes. Chromosomal mediated tetracycline resistance was reported in 1957 (Reyn et al., 1958). The chromosomal modifications include a) V57M amino acid substitution in S10, a 30S ribosomal protein encoded by *rpsJ* gene which results in an altered tetracycline binding site and consequently reduced binding affinity, and b) alterations in genes regulating the expression of efflux pumps and porins and which result into reduced drug accumulation (Hu et al., 2005).

Plasmid mediated tetracycline resistance was reported in 1983 (Morse et al., 1986; Roberts et al., 1988). High level of gonococcal tetracycline resistance is mediated by a transposon-borne (Tn916) class M tetracycline resistance determinant (TetM). It binds to 30S ribosomal subunit thereby blocking tetracycline from binding to its target (Swartley et al., 1993; Chopra & Roberts, 2001). There are two different TetM determinants; American and Dutch which are carried by either of two 25.2 MDa evolutionarily unrelated conjugative plasmids named “American” and “Dutch” type plasmids found in gonococci (Gascoyne et al., 1991; Gascoyne-Binzi et al., 1993).

### 2.11.4 Fluoroquinolones

Fluoroquinolones are more potent and broader spectrum quinolones. Their eighth carbon atom of the backbone is replaced with a nitrogen atom and a fluorine substitution at the sixth position (Ball, 2000). The quinolones are synthetic compounds containing a bicyclic core structure related to the compound 4-quinolone (Figure 2.11). They are developed by modification of 1-alkyl-1,8-naphthyridin-4-one-3-carboxylic acid (Lesher et al., 1962).

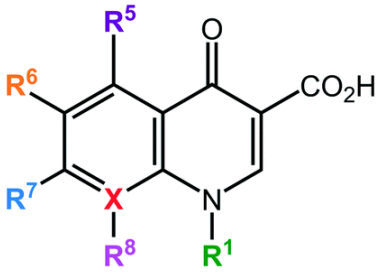



**Figure 2.11: Core structure of quinolone antibiotics.**
Adapted from (Pham et al., 2019)

Figure 2.11 shows the general structure of quinolones. $R_1$, $R_5$, $R_6$, $R_7$, $R_8$ in the structure are important positions for modifications to improve the activity of the resultant antibiotics

The first quinolone, nalidixic acid was introduced in in 1962, and through structural modifications second, third, and fourth generation fluoroquinolones have been developed (Oliphant & Green, 2002). Currently, the commonly prescribed fluoroquinolones include ciprofloxacin, levofloxacin and moxifloxacin (Redgrave et al., 2014). Fluoroquinolones were introduced in the treatment of gonorrhea to replace penicillin in the early 1980s due to their single-dose formulation, minimal side effects, and efficacy at all infection sites, including the pharynx (Lewis, 2010).

Fluoroquinolones block DNA replication by inhibiting the enzymes DNA gyrase (topoisomerase II) and topoisomerase IV (Levine et al., 1998). DNA gyrase catalyzes the untwisting of DNA molecules during DNA replication, and consists of two type A subunits and two type B subunits encoded by *gyrA* and *gyrB* genes (Drlica, 1999). Topoisomerase IV consists of two type C subunits and two type E subunits encoded by *parC* and *parE* genes. This enzyme is involved in the decatenation of covalently closed circular DNA molecules during DNA replication (Levine et al., 1998). The complex formed by DNA, enzyme and fluoroquinolone inhibit the movement of the replication fork; a structure formed by organization of replication proteins thereby disrupting bacterial DNA replication.

Resistance to fluoroquinolones in gonococci was first reported in the late 1980s (Unemo & Shafer, 2014). This resistance is brought about by the gradual accumulation of point mutations in *gyrA* and *parC* quinolone resistance determining region (QRDR). The mutations leads to amino acid substitutions, which alter the three-dimensional structure of the target protein (Piddock, 1999). These mutations reduce the affinity of fluoroquinolone for their targets and consequently to fluoroquinolone resistance in gonococci (Belland et al., 1994; Bodoev & Il'Ina, 2015).

Gonococcal QRDR region lies between amino acids 55-110 and 56-140 in GyrA and ParC respectively (Belland et al., 1994). QRDR amino acid substitutions that have been associated with gonococcal fluoroquinolone resistance include substitutions at S91 by F or Y, and D95 in GyrA protein (Piddock, 1999; Yang et al., 2006). The GyrA alterations initiate gonococcal fluoroquinolone resistance, while additional substitutions in ParC at G85, D86, S87, S88, Q9l and R116 increase the resistance (Bodoev & Il'Ina, 2015).

In addition to altered QRDR, an *in vitro* study by Tanaka et al, (1998) suggested that reduced drug accumulation can also contribute further to the development of fluoroquinolone resistance in gonococci. Fluoroquinolones are small and have charge characteristics that allow them to cross the outer membrane through porin diffusion channels encoded by porB and the cytoplasmic membrane through diffusion (Hirai et al., 1986; Chapman & Georgopapadakou, 1988; Moniot-Ville et al., 1991; Hooper, 1999). Mutations in the PorB can mediate resistance to such molecules which use the channels as a pathway to enter into the cells. Different studies have though suggested that porin alterations alone cannot account for resistance, and that for resistance to occur the expression and presence of an endogenous efflux pump such as the MtrCDE is required (Tanaka et al., 1998).

### 2.11.5 Macrolides

Macrolide is a group of natural and semi-synthetic drugs with a macrocyclic large lactone ring of 12 to 16 atoms which through glycosidic bonds are attached to one or

more sugars (Mazzei et al., 1993; Omura, 2002). The first macrolide, a 14-membered erythromycin A (first generation macrolide), was isolated from cultures of *Streptomyces* in 1952 and introduced into clinical practice (Mazzei et al., 1993). Macrolides with 14, 15 and 16 (second generation macrolides) atoms are the widely used because of their excellent tissue penetration and antimicrobial activity (Bearden & Rodvold, 1999). Azithromycin, a 15-membered second generation macrolide developed in 1980 is one of the drugs in the current WHO recommended dual therapy treatment for gonorrhea (Figure 2.12) (Committee on Gynecologic, 2015; Workowski & Bolan, 2015). The first case of resistance to the dual therapy was reported in the United Kingdom in 2016 (Fifer et al., 2016).

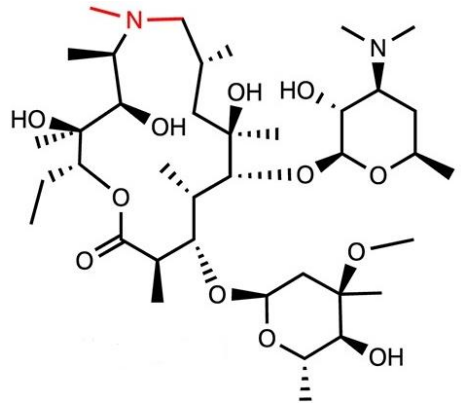



**Figure 2.12: Structure of azithromycin.**
Adapted from (Dinos, 2017)

Figure 2.12 shows the structure of azithromycin, a 15-membered second generation macrolide. Red colour indicates modifications inserted in the erythromycin molecule to generate 14- and 15-membered second generation macrolides.

Macrolides inhibit protein synthesis by interacting with the 4 alleles of 23S rRNA, and causing ribosomes to release incomplete polypeptide. By binding to the 23S rRNA in 50S ribosomal subunit they prevent translocation of peptidyl-tRNA and block peptide exit channel in 50S subunit (Poehlsgaard & Douthwaite, 2005; Kannan et al., 2014).

In gonococci resistance to macrolides result from modification of the ribosomal target, 23S rRNA by either a) methylation of an adenosine residue at position 2058 of 23S rRNA by rRNA methylases encoded by *erm*B, *erm*C and *erm*F genes or (Roberts et al., 1999; Cousin Jr et al., 2003) b) via, A2143G (corresponding to A2059G *Escherichia coli* numbering), and C2599T (corresponding to C2611T*Escherichia coli* numbering) Single Nucleotide Polymorphisms (SNPs) in peptidyltransferase loop in domain V of 23S rRNA (Ng et al., 2002). *N. gonorrhoeae* has four 23S rRNA alleles.

Target modifications block the binding of macrolides to their site on the ribosome. Additionally over expressed efflux pump systems particularly the MtrCDE efflux pump, MacAB and *mefA*-encoded macrolide efflux pump can facilitate macrolide resistance (Zarantonelli et al., 1999; Luna et al., 2000; Rouquette-Loughlin et al., 2005; Unemo & Shafer, 2014). The *mefA* gene which is transferred through conjugation encodes a membrane bound efflux protein and confers resistance to macrolides (Shortridge et al., 1996). MacA, MacB proteins together with TolC outer membrane proteins form an ATP binding cassette (ABC) transporter system that exports macrolides (Rouquette-Loughlin et al., 2005).

MacA is a membrane fusion protein while MacB is an integral membrane protein. Rouquette-Loughlin et al, (2005) reported that, in *N. gonorhoeae* genes encoding MacA (*macA*) and MacB (*macB*) which are separated by 64pb, are transcribed as one unit (*macAB* operon). The operon transcription is controlled by the macA promoter located upstream of macA gene. The -10 hexamer of the macA promoter in *N. gonorrhoeae* and *N. meningitidis* contains a G instead of a normal T (5'TAGAAT3'). This causes a dampening effect (reduced transcription) on *macAB* transcription. This study found that, a G→T mutation in the -10 hexamer (5'TAGAAT3'→5'TATAAT3') of *macAB* operon promoter increased MacAB expression and consequently macrolide resistance. The study also reported that MacAB efflux pump recognize macrolides and together with MtrCDE pump contribute to the decreased gonococcal macrolides susceptibility (Rouquette-Loughlin et al., 2005).

Point mutations in ribosomal proteins L4 and L22 encoded by *rplD* and *rplV* respectively also confer resistance to macrolides (Pardo & Rosset, 1977). L4 and L22 Proteins bind to domain I of 23S rRNA. Mutations in these proteins result into a structure that changes domains II, III, and V conformation thereby affecting the binding of the macrolides to the domain V of 23S rRNA (Gregory & Dahlberg, 1999).

Gonococcal resistance to the macrolide azithromycin was first reported in the 1990s and later from different countries (Wi et al., 2017). This resistance is thought to have developed due to continued use of a lower dosage for treatment of *Chlamydia trachomatis* infections (Handsfield et al., 1994). The efflux pumps, 23S rRNA methylation and C2611T mutation have been associated with low level azithromycin resistance (Zarantonelli et al., 1999; Ng et al., 2002; Cousin Jr et al., 2003). High level azithromycin resistant (MIC $\geq$ 256 mg/L up to 4,096 mg/L) gonococci have been identified in different countries (Unemo & Nicholas, 2012).The high level azithromycin resistance has been associated with an A2059G SNP in three to four alleles of the 23S rRNA gene (Chisholm et al., 2010; Unemo & Nicholas, 2012).

### 2.11.6 Spectinomycin

The aminocyclitol; spectinomycin, is an antibiotic produced by *Streptomyces spectabilis*, is closely related to the aminoglycosides (Easmon et al., 1984). Although it is not a good drug of choice for the treatment of pharyngeal and rectal gonorrheal infection, it is used in human medicine for the treatment of uncomplicated gonorrhea (Tapsall et al., 2009). Spectinomycin binds to the 30S ribosomal subunit and interacts with the 34 helix of 16S rRNA during polypeptide elongation, thereby blocking the EF-G-catalyzed translocation of the peptidyl-tRNA from the A site to the P site (Borovinskaya et al., 2007; Unemo et al., 2016).

Resistance to spectinomycin in gonococci is chromosomally mediated via a) C1192Tmutation in the spectinomycin binding region of helix 34 in16S rRNA genes and the cross linked positions 1063-1066 and 1190-1193 (*E. coli* numbering) (Galimand et al., 2000; Unemo et al., 2009),and b) mutations in the 30S ribosomal

protein S5 encoded by the *rpsE* gene particularly a valine deletion at position 27 and a K28E substitution which have been shown to cause a high level spectinomycin resistance while A T24P substitution has been associated with low level spectinomycin resistance (Ilina et al., 2013; M. Unemo et al., 2013; Unemo & Shafer, 2014). The first case of spectinomycin resistance was reported in 1967 in the Netherlands and later from other countries (Stolz et al., 1975; Eyre et al., 2018).

### 2.11.7 Cephalosporins

Cephalosporins originally isolated in 1948 from the fungus *Cephalosporium* are a family of antibiotics belonging to β-lactam class. They contain a β-lactam structure that is very similar to that of the penicillins (Figure 2.13).

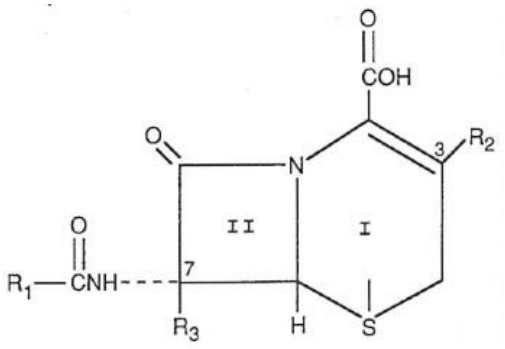



**Figure 2.13: 7-Aminocephalosporanic acid.**

Adapted from (Otto & Noorily, 1994)

Figure 2.13 shows the core structure of cephalosporins. Different side chains are attached at R1, R2 and R3 to produce variety of semi-synthetic broad-spectrum cephalosporins.

Different cephalosporins have been sequentially developed based on the spectrum of activity and broadly categorized into four generations; first generation cephalosporins are more effective against gram-positive pathogens than gram-negatives ones, second generation drugs, developed after the first generation, have improved effects on gram-negative bacteria with some anaerobe coverage, third-generation cephalosporins are particularly effective against gram-negative pathogens,

and some reach the central nervous system, and fourth generation cephalosporins which are broad spectrum with excellent gram-positive and gram-negative coverage and, like their third-generation predecessors, inhibit the growth of the difficult opportunistic pathogen such as *Pseudomonas aeruginosa*. Ceftriaxone, a third generation cephalosporin is the current recommended first line monotherapy for gonorrhea treatment (Bui & Preuss, 2020). Cephalosporins are β-lactams that have a high affinity for gonococcal PBP2, and like penicillins inhibit the cross linking of peptidoglycan within the bacterial cell wall by binding to PBPs (Barry & Klausner, 2009).

Cephalosporin resistance in gonococci arises mainly due to mutations that modify the target PBP2 proteins as well as increased efflux and decreased drug influx. Recombination with *penA* genes of commensal *Neisseria* species has led to development of a mosaic-like *penA* structure which has approximately 60 amino acid alterations from the PBP2 of susceptible strains (Spratt et al., 1992). Studies have associated mosaic *penA* with extended-spectrum cephalosporins (ESCs), cefixime and ceftriaxone resistance in gonococci from different regions (Ameyama et al., 2002; Ito et al., 2005; Ohnishi, Saika, et al., 2011). Substitution of alanine with proline at position 501 of non-mosaic *penA* alleles creates a secondary structure that changes and inhibits the binding of ceftriaxone and cefixime to its site on PBP2 by clashing with their R1 substituent (Magnus Unemo et al., 2012).

Reduced drug accumulation resulting from an active efflux pump and/or altered porins have been shown to contribute to reduced cephalosporin susceptibility (Figure 2.5) (Ohnishi, Saika, et al., 2011; Unemo & Nicholas, 2012). Molecular characterization of *N. gonorrhoeae* H041 isolate by Ohnishi et al. revealed that it harboured previously identified resistance markers including mutations in *porB*, *ponA* and *mtrR* alongside a new *penA* mosaic allele ($penA_{H041}$, encoding mosaic PBP2) (Ohnishi, Golparian, et al., 2011). *N. gonorrhoeae* F89 strain isolated in France and later in Spain was found to harbour similar resistance markers (Camara *et al*., 2012). Due to the high transformation ability of *N. gonorrhoeae* the rise and spread of extensively drug resistant (XDR) strains which appear inevitable will lead

to both intra and extra species spread of the AMR genes which could further lead to the emergency of untreatable gonorrhea (Spratt et al., 1992).

### 2.12 Molecular characterization of gonococcal resistance

The main purpose of typing gonococcal isolates is to understand their epidemiology and investigate the transmission of antibiotic resistant isolates. Molecular methods commonly used to detect and characterize drug resistant *N. gonorrhoeae* isolates can broadly be classified into two; a) gene typing based methods and b) direct detection of genetic markers of AMR.

#### 2.12.1 Gene typing based methods

These methods are based on the analysis DNA sequences. They include; a) *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST), b) Multi-locus sequence typing (MLST), and c) a recently described scheme called *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) (Martin et al., 2004; Ibarz Pavon & Maiden, 2009; Demczuk et al., 2017).

##### 2.12.1.1 *N. gonorrhoeae* multi-antigen sequence typing

NG MAST was designed specifically for typing gonococcal strains (Martin et al., 2004). It involves obtaining partial DNA sequences from hypervariable regions of two outer membrane proteins; a) major outer membrane porin (490pb) encoded by *porB* and, b) outer-membrane lipoprotein (390bp) encoded by *tbpB* and which is involved in iron acquisition. NG-MAST uses an open access database (http://www.ng-mast.net/) for analysis. NG-MAST places organisms into groups with similar AMR profiles and it's therefore not necessarily definitive since susceptibility profiles can potentially vary within a given NG-MAST type (Martin et al., 2004).

##### 2.12.1.2 *N. gonorrhoeae* sequence typing for antimicrobial resistance

NG-STAR is a newly described antimicrobial resistance multi-locus typing scheme based on gonococcal antimicrobial resistance determinants (Demczuk et al., 2017). NG-STAR uses alleles from seven genes associated with resistance to β-lactams, macrolides, and fluoroquinolones antibiotics. The genes include *penA, mtrR, porB,*

*ponA*, *gyrA*, *parC*, and 23S rRNA. This typing scheme allows monitoring of the global dissemination of antimicrobial resistant gonococcal strains (Demczuk et al., 2017)

#### 2.12.1.3 Multi-locus Sequence Typing

MLST is a technique used to type many bacterial species. It uses partial sequence information of seven relatively conserved, slow evolving housekeeping genes which are distributed throughout the genome to assign an isolate to a sequence type (Enright & Spratt, 1999; Ibarz Pavon & Maiden, 2009). The sequences include: a putative ATP transporter (*abcZ*); adenylate kinase (*adk*); shikimate dehydrogenase (*aroE*), fumarate hydratase (*fumC*); glucose-6-phosphate dehydrogenase (*gdh*); pyruvate dehydrogenase subunit (*pdhC*) and phosphoglucomutase (*pgm*) partial genes (Maiden et al., 1998).

### 2.12.2 Direct molecular methods

Direct molecular methods of detecting AMR genetic markers are developed to target DNA sequences that confer resistance to antimicrobials. The methods detect mutations or sequences that are either fundamental to the resistance or highly associated with resistance. Each sequence typically only predicts susceptibility to a single antibiotic. Multiple assays or multiplex polymerase chain reaction (PCR) methods have been developed to generate full resistance profiles (Palmer et al., 2000; Whiley et al., 2006). Both NG-MAST and direct detection of AMR genetic markers may be unsuitable for use on pharyngeal samples because of cross-reaction with commensal Neisseria species (Goire et al., 2014).

### 2.12.3 Next generation whole genome sequencing

A new method; next generation whole genome sequencing, has also been used to characterize AMR genes. This technology allows high resolution characterization and comparative genome analysis of all AMR genes especially in resistant strains (Balloux et al., 2018). In addition it can be used to track the spread of resistance across different geographic locations and populations (Mortimer & Grad, 2019).

Whole genome sequencing enables species identification through the comparison of test genome with a curated multispecies reference database. Additionally a pathogens genome can as well be used to predict the pathogens phenotype (Richter & Rossello-Mora, 2009). Whole genome sequencing also provides a better understanding of immune responses and susceptibility to infections through analyzing patient and infecting pathogen's whole genomes (Davila et al., 2010).

# CHAPTER THREE

# METHODOLOGY

## 3.1 Study design and study isolates

The current study was a retrospective laboratory-based study nested in a STI surveillance program under Armed Forces Health Surveillance at the US Army Medical Research Directorate-Africa. The study isolates were obtained as part of the study entitled "A surveillance study of antimicrobial susceptibility profiles of *N. gonorrhoeae* isolates from patients seeking treatment in selected clinics in Kenya" Walter Reed Army Institute of Research (WRAIR) Human Subject Protection Board (HSPB) Protocol l#1743/Kenya Medical Research Institute Scientific and Ethics Review Unit (SERU) #1908.

Forty one archived and viable *N. gonorrhoeae* isolates exhibiting varying antibiotic resistance profiles were obtained for analysis. The isolates were recovered from both male and female patients seeking treatment in selected clinics between 2013 and 2018. The selected clinics were from four geographic locations in Kenya namely: Nairobi; Coastal Kenya; Nyanza (Kisumu and Kombewa) and Rift Valley. The obtained study isolates were assigned new accession numbers which constituted of two parts; an abbreviation for the sub study title (KNY_NGAMR), and specimen number in the sub-study which were assigned chronologically. Both demographic (host gender, year of isolation and region) and antimicrobial susceptibility data for the obtained isolates were provided by the principal investigator of the parent STI surveillance study (Appendix I). The STI study tested the obtained isolates for susceptibility to a panel of ten drugs namely: ceftriaxone; cefixime; azithromycin; ciprofloxacin; norfloxacin; spectinomycin; tetracycline; doxycycline; penicillin and gentamicin. The minimum inhibitory concentrations (MIC) breakpoints for the provided antimicrobial susceptibility data was interpreted with reference to European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 8.0, 2018 standards (Appendix I I).

### 3.2 Ethical Consideration

Permission to carry out the study was obtained from both Kenya Medical Research Institute Scientific and Ethics Review Unit (SERU) under approval KEMRI/SERU/CCR/0053/3385 (Appendix III) and Walter Reed Army Institute of Research (WRAIR) Human Subject Protection Board (HSPB) (WRAIR#1743A; Appendix IV)

### 3.3 Laboratory procedures

#### 3.3.1 *N. gonorrhoeae* isolation

The obtained frozen isolates were thawed and inoculated on modified Thayer-Martin agar medium supplemented with vancomycin, nystatin, colistin and trimethoprim lactate and incubated at 37°C in 5% $CO_2$ for 18 to 24 hours. The plates with growth were examined for the presence of typical *N. gonorrhoeae* growth features which include; pigmented/opaque, smooth, round, moist, uniform pink/brown colonies. Inoculums were prepared from plates with growth and examined by gram stain to confirm the presence of Gram-negative diplococci organisms. Further presence of *N. gonorrhoeae* in plates with growth was confirmed through oxidase and catalase biochemical tests prior to DNA extraction.

##### 3.3.1.1 Oxidase test

A sterile swab was used to touch and pick a single isolated colony from a fresh culture plate with the swab tip. Pasteur pipette was used to add one drop of oxidase reagent (a 1% (wt/vol) solution of N, N, N', N' –tetramethyl-ρ-phenylenediamine dihydrochloride) to the colony on the swab. Reaction was observed within the first 5-10 seconds. Purple reaction indicated a positive oxidase test.

##### 3.3.1.2 Catalase test

An isolated colony from a fresh culture plate was picked and placed on a clean slide using a sterile inoculating loop. A drop of reagent (30% hydrogen peroxide) was placed onto the colony on the slide using an eye-dropper. A strong "explosive"

reaction with bubbling within 1 to 2 seconds upon contact with the catalase reagent indicated a positive result.

### 3.3.2 DNA Extraction and quantitation

Plasmid and genomic DNA were extracted using and QIAprep Spin Miniprep Kit (Appendix V) and QIAamp DNA Mini Kit (Appendix VI) (QIAGEN, Hilden, Germany) "respectively" according to the manufacturer's instructions. The quality and quantity of extracted DNA was determined by Qubit dsDNA HS Assay using Qubit 3.0 fluorometer, (Thermo Fisher Scientific Inc. Wilmington, Delaware USA) according to the manufacturer's instructions (Appendix VII). The Nextera XT sample preparation kit requires an input of 1ng of DNA. This method allowed the measurement of DNA sample concentrations as low as 10pg/µL. Quantified DNA was stored at -20°C until used for library preparation.

## 3.4 Whole genome sequencing and sequence analysis

### 3.4.1 Library preparation and Illumina MiSeq sequencing

Libraries were prepared from 1ng of quantified DNA of each sample. The DNA was processed by Illumina Nextera XT sample preparation kit (Illumina Inc. San Diego, CA, USA) as described below in sections 3.4.1.1 - 3.4.1.6.

#### 3.4.1.1 DNA tagmentation

A new 96-well Nextera XT Tagment Amplicon Plate (NTA) plate was labeled and 10µl of Tagment DNA (TD) added. 5µl of both diluted input DNA sample (0.2ng/µl) and Amplicon Tagment Mix (ATM) were added sequentially to each sample well labeled. The tubes contents were mixed by gently pipetting up and down 5 times using a multichannel pipette. The NTA plate was tightly sealed using Microseal B and centrifuged at 280 x g at 20°C for 1 minute. The NTA plate was placed in a thermalcycler and incubated at 55°C for 5minutes then held at 10°C. The Microseal 'B' was carefully removed and 5µl of neutralization (NT) buffer added to each well. The tubes contents were mixed by gently pipetting up and down 5 times using a

multichannel pipette. The plate was resealed with Microseal 'B', centrifuged at 280 x g at 20°C for 1minute and left at room temperature for 5 minutes.

#### 3.4.1.2 Library amplification

The NTA plate was placed in a TruSeq Index Plate Fixture and 15µl of Nextera PCR Master Mix (NPM) added to each well of the NTA plate. Using a multichannel pipette, 5µl of both index 2 primers and index 1 primers were added to the NTA plate sequentially. The tubes contents were mixed by gently pipetting up and down 3-5 times using a multichannel pipette, the plate covered with Micoseal 'A' and centrifuged at 280 x g at 20°C for 1minute. PCR was performed using the following conditions: 72°C for 3 minutes; 95°C for 30 seconds; (95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds) x 12 cycles; 72°C for 5 minutes and later held at 10°C.

#### 3.4.1.3 Library clean up

The NTA plate was centrifuged at 280 x g at 20°C for 1minute to collect condensation. A new MIDI plate was labeled CAA (Clean Amplified Plate). The PCR product from the NTA plate was transferred using a multichannel pipette set to 50µl to the CAA plate. AMPure XP beads were vortexed for 30 seconds to ensure even dispersion and 90µl of the beads added to each well of the CAA plate. The tubes contents were mixed by gently pipetting up and down 10 times using a multichannel pipette and incubated at room temperature without shaking for 5 minutes. The plate was then placed on a magnetic stand until the supernatant cleared. Carefully, with the CAA plate on the magnetic stand, a multichannel pipette was used to remove and discard the supernatant and the beads washed with freshly prepared 80% ethanol twice. With the CAA plate still on the magnetic stand, the beads were allowed to air-dry for 15 minutes after which the CAA plate was removed from the magnetic stand and 52.5µl of re-suspension buffer (RSB) added to each well using a multichannel pipette. The tubes contents were mixed by gently pipetting up and down 10 times using a multichannel pipette and incubated at room temperature for 2 minutes. The CAA plate was placed back on the magnetic stand

until the supernatant cleared. A new TCY plate was labeled Clean Amplified NTA Plate (CAN). Using a multichannel pipette 50µl of supernatant from the CAA plate was carefully transferred to the newly labeled CAN.

#### 3.4.1.4 Library normalization

A new MIDI plate was labeled Library Normalization plate (LNP) and 20µl of the supernatant from the CAN plate carefully transferred into the LNP plate using a P20 multichannel pipette and fine tips. 46µl of Library Normalization Additive 1 (LNA1) per sample was added in a clean 15ml conical flask. Library Normalization Beads 1 (LNB1) was re-suspended by thoroughly pipetting up and down using a P1000 pipette set at 1000µl for 15-20 times and 8µl of the LNB1 per sample added to the 15ml conical flask and mixed well by inverting the tube 15–20 times. Using a multichannel pipette 45µl of the LNA1/LNB1 mix was added to each well of the LNP containing the libraries, sealed with Microseal 'B' and shaken on a microplate shaker at 1,800rpm for 30 minutes. The LNP plate was placed on a magnetic stand for 2 minutes. With the LNP plate on the magnetic stand, the supernatant was discarded carefully using a multichannel pipette set at 80µl. The LNP plate was removed from the magnetic stand and the beads washed with Library Normalization Wash 1 (LNW1) twice. The LNP plate was removed from the magnetic stand and 30µl of 0.2N NaOH added to each well to elute the sample. The LNP plate was sealed with Microseal 'B' and shaken on a microplate shaker at 1,800rpm for 5 minutes. The StoraGe Plate (SGP) barcode plate sticker was applied to a new 96-well PCR plate and 30µl of Library Normalization Storage Buffer 1 (LNS1) added to each well to be used in the SGP plate. After 5 minutes of elution and when all the samples are re-suspended, the LNP plate is placed on a magnetic stand for 2 minutes. Using a multichannel pipette set to 30µl, the supernatant from the LNP is transferred into the to the SGP plate, sealed with Microseal 'B' and centrifuged at 1,000 x g for 1 minute.

### 3.4.1 5 Library pooling

A new Eppendorf tube was labeled Pooled Amplicon Library (PAL). The SGP plate was centrifuged at 1000 x g at 20°C for 1 minute and 5µl of each library from the SGP plate transferred to the PAL tube and mixed by inversion.

#### 3.4.1.6 Illumina MiSeq sequencing

The pooled libraries were diluted, denatured and paired end reads generated on Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a paired-end 2×300bp protocol (Sim et al., 2015). Data was uploaded to Illumina's basespace cloud network and the unprocessed sequence reads in the form of FastQ files for each sample downloaded from Illumina's basespace cloud for bioinformatic analysis.

### 3.4.2 Read assembly and genome identification

Sequence reads were checked for quality and assembled *de novo* using both CLC Genomics Workbench version12.0 and assembly pipeline version1.2 available from the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services). Assembled contigs were mapped against FA1090 (GenBank Accession number AE0049969) *N. gonorrhoeae* reference genome and unmapped contigs were assembled *de novo* using CLC Genomics Workbench 12.0. Initial identification of the assembled genomes was performed using Basic Local Alignment Search Tool for nucleotides (BLASTn) in National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov). Identified plasmid sequences were mapped against the ancestral Asian pDJ4 plasmid (GenBank Accession number NC_002098.1) downloaded from NCBI website using Geneious Prime2019 (https://www.geneious.com).

### 3.4.3 Annotation

Assembled genomes were annotated using both Rapid Annotation using Subsystem Technology (https://rast.nmpdr.org) hosted on Pathosystems Resource Integration Center version 3.5.31 (PATRIC) (https://www.patricbrc.org) (Aziz et al., 2008), and Bacterial Isolate Genome Sequence Database (BIGSdb) genomics platform tools

hosted on www.pubmlst.org/neisseria. Plasmid sequences were also annotated using Geneious Prime 2019 (https://www.geneious.com).

### 3.4.4 Multi-Locus Sequence Type analysis

Identification of multi-locus sequence types was performed on assembled genome sequences using MLST version 1.8 (https://cge.cbs.dtu.dk/services/MLST/) available online at Centre for Genome Epidemiology (CGE) (https://cge.cbs.dtu.dk/services) (Ahrenfeldt et al., 2017).

### 3.4.5 *N. gonorrhoeae* Multi-Antigen Sequence Type analysis

A local blast search against NG-MAST *porB* and *tbpB* database was used to identify *porB* and *tbpB* genes from the assembled genomes using Bioedit sequence alignment editor version 7.0.5 (Hall, 2011) . Determination of *porB* and *tbpB* alleles and NG-MAST sequence types (ST) was performed at NG-MAST website (http://www.ng-mast.net/) using correctly trimmed *porB* (490bp) and *tbpB* (390bp) genes. Novel *porB* and *tbpB* alleles and NG-MAST profiles were uploaded to the NG-MAST database.

### 3.4.6 Genome-wide single nucleotide polymorphism phylogeography

Phylogenetic analyses were performed on 74 *N. gonorrhoeae* genomes; 41 from the current study and 33 additional global genomes for comparison. The comparison genomes are from varied geographical regions including; Unites States of America (6), Australia (5), Denmark (6), United Kingdom (3), France (4), Japan (1), Sweden (3), Korea (1), Italy (1), Canada (2), and Malaysia (1) and were retrieved from NCBI. The analyses were restricted to include genomes for which epidemiological information was available.

#### 3.4.6.1 Identification of single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) in the 74 genomes were identified using the CSI phylogeny version 1.4 pipeline available online at Technical University of Denmark (DTU) Centre for Genome Epidemiology (CGE) (https://cge.cbs.dtu.dk/services/CSIPhylogeny). Sequence reads and the additional genome sequences

were mapped to *N. gonorrhoeae* strain NCCP11945 (GenBank accession number CP001050) reference genome, using Bowtie Wheel Aligner (BWA) version v.0.7.17 (Li & Durbin, 2009). SNPs from each genome were called using the mpileup tool from SAMTools version.1.7 (Li et al., 2009). SNPs were filtered out when not matching the following criteria: a minimum distance of 10bp between SNPs, a minimum of 10% of the read depth at SNP positions, mapping quality above 25, and SNP quality above 30. Pruning was disabled and all indels were excluded. The SNPs from each genome were concatenated into a single fasta alignment.

#### 3.4.6.2 Temporal Bayesian phylogenetic tree

The concatenated SNP sequences were used to construct a temporal Bayesian phylogenetic tree using Bayesian Evolutionary Analysis Sampling Trees (BEAST) version1.8.2 (Suchard et al., 2018). The most suitable substitution model (General-Time Reversible nucleotide substitution model with a GAMMA correction; GTR+G) was determined using Jmodel in MEGA version 10.0. To identify the best-fit model, (strict clock, coalescent Bayesian Skyline Plot (BSP), and general-time reversible (GTR) model), independent runs (performed on different selected test models with combinations of different molecular clock) were performed for 100 million generations sub-sampled every 10,000 generations (burn-in). The traces and effective sample size (ESS; all exceeded 250) were checked using Tracer version 1.6.0 to evaluate the Markov chain Monte Carlo convergence (Rambaut et al., 2015), and the log files merged using Log-combiner version 1.8.2. The final Maximum Clade Credibility (MCC) was determined using Tree Annotator version 1.8.2 with 10% of the MCMC steps discarded as burn-in. Statistical confidence was represented by the 95% highest posterior density (HPD) interval. The generated tree was visualized using both Microreact and FigTree version 1.4.3(Rambaut, 2012; Argimon et al., 2016).

### 3.4.7 Core genome-based phylogeny

Genetic diversity among the study isolates was determined by assessing the core genomes. *N. gonorrhoeae* core genome multi-locus sequence typing (cgMLST) v.1.0

hosted on pubmlst.org/Neisseria was used to infer core genome based phylogeny using the "cgMLST scheme". The generated data was visualized using Inkscape.

### 3.4.8 Identification of antimicrobial resistance determinants

To identify AMR genes, sequence reads were uploaded to Pathosystems Resource Integration Center version 3.5.7 (https://www.patricbrc.org) and assembled using both Velvet and Spades assembly algorithms. Annotation of the whole genome was performed with Rapid Annotation using Subsystem Technology in PATRIC. Antibiotic determinants were identified in PATRIC using Comprehensive Antibiotic Resistance Database (CARD), PATRIC and National Database of Antibiotic Resistance Organisms (NDARO) databases and saved into separate feature groups from which they were downloaded in fasta format for further analysis. Acquired resistance genes were identified using Resfinder Version 3.0 (https://cge.cbs.dtu.dk/services).

### 3.4.9 Identification of mutations and amino acid alterations in the identified AMR determinants

For comparison and identification of mutations, reference AMR genes (known AMR determinants) and proteins mediating resistance to the different antibiotics were downloaded from the NCBI database. Together with the identified determinants they were aligned using Bioedit sequence alignment editor version7.0.5 (Hall, 2011; Kumar et al., 2018). In addition Bacterial Isolate Genome Sequence Database (BIGSdb) was also used to assess the loci associated with antimicrobial resistance (Jolley et al., 2018).

## 3.5 Statistical analysis

Statistical tests (Unpaired t tests) were conducted in GraphPad Prism V7.0.4 (www.graphpad.com) to assess the effect of antibiotic resistance determinants on antibiotic MICs. The statistical comparisons were two tailed and were performed with the significance level set at $P<0.05$.

# CHAPTER FOUR

# RESULTS

## 4.1 Genomic features of antimicrobial resistant *N. gonorrhoeae* isolates and characterization of both MLST and NG-MAST sequence types

### 4.1.1 Genomic features of *N. gonorrhoeae* isolates

The complete genomes of 41 *N. gonorrhoeae* isolates were successfully sequenced and assembled. The resultant assemblies were uploaded to the publicly available pubMLST database (http://www.pubmlst.org/neisseria), and linked to the National Centre for Biotechnology Information (NCBI) BioProjects: PRJNA481622 and PRJNA590515 (Appendix VII). The assemblies yielded an average length of 2.15Mbp with an average contig length of 150. KNY_NGAMR42 had the shortest assembly length; 2.03Mbp, whereas KNY_NGAMR2 had the longest assembly length; 2.21Mbp. The average GC content was 52.5% and the average coding regions (CDC) were 2661. All the isolates had 3 rRNAs each and 48-50 tRNAs. Twenty two (53.7%) of the 41 isolates had the gonococcal genetic island (GGI) (Table 4.1).

**Table 4.1: Genomic features of the analyzed isolates.**

| S/no | Isolate | Size | %GC | GGI | SRA BioSample | PubMLST ID |
|---|---|---|---|---|---|---|
| 1 | KNY_NGAMR1 | 2.15 | 52.5 | *Absent* | SAMN09685116 | 60436 |
| 2 | KNY_NGAMR2 | 2.21 | 52.3 | Present | SAMN09685117 | 60501 |
| 3 | KNY_NGAMR3 | 2.19 | 52.4 | Present | SAMN09685118 | 60502 |
| 4 | KNY_NGAMR4 | 2.13 | 52.5 | *Absent* | SAMN09685119 | 60503 |
| 5 | KNY_NGAMR5 | 2.2 | 52.3 | Present | SAMN09685120 | 60504 |
| 6 | KNY_NGAMR6 | 2.1 | 52.7 | *Absent* | SAMN09685121 | 60505 |
| 7 | KNY_NGAMR7 | 2.19 | 52.4 | Present | SAMN09685122 | 60506 |
| 8 | KNY_NGAMR8 | 2.09 | 52.7 | *Absent* | SAMN09685123 | 60507 |
| 9 | KNY_NGAMR9 | 2.14 | 52.6 | Present | SAMN09685124 | 59511 |
| 10 | KNY_NGAMR10 | 2.18 | 52.4 | Present | SAMN09685125 | 60508 |
| 11 | KNY_NGAMR11 | 2.14 | 52.6 | *Absent* | SAMN09685126 | 60509 |
| 12 | KNY_NGAMR13 | 2.14 | 52.5 | *Absent* | SAMN09685127 | 60519 |
| 13 | KNY_NGAMR14 | 2.16 | 52.5 | Present | SAMN09685128 | 60511 |
| 14 | KNY_NGAMR15 | 2.14 | 52.5 | *Absent* | SAMN09685129 | 60516 |
| 15 | KNY_NGAMR16 | 2.2 | 52.4 | Present | SAMN09685130 | 61330 |
| 16 | KNY_NGAMR17 | 2.19 | 52.4 | Present | SAMN09685131 | 60513 |
| 17 | KNY_NGAMR18 | 2.14 | 52.5 | *Absent* | SAMN09685132 | 60514 |
| 18 | KNY_NGAMR19 | 2.18 | 52.4 | Present | SAMN09685133 | 60515 |
| 19 | KNY_NGAMR20 | 2.13 | 52.5 | *Absent* | SAMN09685134 | 60512 |
| 20 | KNY_NGAMR21 | 2.14 | 52.6 | *Absent* | SAMN09685135 | 60517 |
| 21 | KNY_NGAMR22 | 2.09 | 52.9 | *Absent* | SAMN09685136 | 60518 |
| 22 | KNY_NGAMR23 | 2.13 | 52.5 | *Absent* | SAMN09685137 | 60510 |
| 23 | KNY_NGAMR24 | 2.13 | 52.6 | *Absent* | SAMN09685138 | 60520 |
| 24 | KNY_NGAMR25 | 2.19 | 52.4 | Present | SAMN13332056 | 61331 |
| 25 | KNY_NGAMR26 | 2.19 | 52.4 | Present | SAMN09685139 | 60439 |
| 26 | KNY_NGAMR27 | 2.19 | 52.4 | Present | SAMN09685140 | 60440 |
| 27 | KNY_NGAMR28 | 2.2 | 52.2 | Present | SAMN09685141 | 60521 |
| 28 | KNY_NGAMR29 | 2.19 | 52.4 | Present | SAMN09685142 | 60522 |
| 29 | KNY_NGAMR30 | 2.14 | 52.5 | *Absent* | SAMN09685143 | 60441 |
| 30 | KNY_NGAMR31 | 2.18 | 52.4 | Present | SAMN09685144 | 60523 |
| 31 | KNY_NGAMR32 | 2.09 | 52.7 | *Absent* | SAMN09685145 | 60524 |
| 32 | KNY_NGAMR33 | 2.19 | 52.4 | Present | SAMN09685146 | 60442 |
| 33 | KNY_NGAMR35 | 2.19 | 52.4 | Present | SAMN09685147 | 60525 |
| 34 | KNY_NGAMR36 | 2.14 | 52.5 | *Absent* | SAMN09685148 | 60443 |
| 35 | KNY_NGAMR41 | 2.13 | 52.5 | *Absent* | SAMN09685149 | 60526 |
| 36 | KNY_NGAMR42 | 2.03 | 52.7 | Present | SAMN09685150 | 60527 |
| 37 | KNY_NGAMR45 | 2.13 | 52.5 | Present | SAMN09685151 | 60444 |
| 38 | KNY_NGAMR50 | 2.19 | 52.4 | Present | SAMN09685152 | 60528 |
| 39 | KNY_NGAMR52 | 2.1 | 52.7 | *Absent* | SAMN09685153 | 60529 |
| 40 | KNY_NGAMR53 | 2.2 | 52.3 | Present | SAMN09685154 | 60530 |
| 41 | KNY_NGAMR54 | 2.13 | 52.6 | *Absent* | SAMN09685155 | 60531 |
| | Average | 2.15 | 52.5 | | | |

Genome sizes (Mbp), GC contents (GC%), the presence of gonococcal genetic island (GGI) SRA BioSample, and PubMLST IDs of the 41 isolates are indicated

### 4.1.2 Identified plasmids

All the 41 *N. gonorrhoeae* isolates had a plasmid related to previously described cryptic plasmid; either pNGK (4153bp) or pJD1 (4207bp). One isolate, KNY_NGAMR2, had two putative plasmids, the cryptic 4Kbp plasmid and a 39Kbp plasmid related to the putative *N. gonorrhoeae* conjugative plasmid pLE245. Twenty five (61%) isolates had both β-lactamase (TEM) and class M tetracycline resistance determinant (TetM) encoding plasmids whereas 3 isolates lacked either of the TEM or TetM plasmids. Of the 41 isolates, 29 had β-lactamase encoding plasmids (TEM plasmids), whereas 12 lacked this plasmid. Of these 29 TEM plasmids, 28 had a backbone corresponding to that of the African penicillinase producing *N. gonorrhoeae*, plasmid (pDJ5; 5599 bp/ 3.2-3.4 MDa) which carry a β lactamase TEM precursor gene. The remaining 1 TEM plasmid had a backbone corresponding to that of the Asian penicillinase producing *N. gonorrhoeae* plasmid (pDJ4; 7426 bp/ 4.4-4.7 MDa) which carry a broad-spectrum β- lactamase TEM-1 gene.

Of the 41 isolates 34 had TetM encoding plasmids whereas 7 lacked the TetM plasmids. Of these 34, 33 carried the American TetM determinant, whereas 1 had a Dutch TetM determinant. Three isolates, (KNY_NGAMR2, KNY_NGAMR6, and, KNY_NGAMR8) only had the putative plasmids and lacked both TEM and TetM plasmids (Table 4.2).

**Table 4.2: Plasmids identified in the analyzed isolates**

| | Plasmids (bp) | | |
|---|---|---|---|
| Isolate | Putative | TEM | TetM |
| KNY_NGAMR1 | 4143 | *Absent* | *Absent* |
| KNY_NGAMR2 | 4207, 39064 | 7449; Asian | *Absent* |
| KNY_NGAMR3 | 4150 | 5580 | 42998; American |
| KNY_NGAMR4 | 4207 | 5673 | 42992; American |
| KNY_NGAMR5 | 4197 | 5600 | 43020; American |
| KNY_NGAMR6 | 4153 | *Absent* | *Absent* |
| KNY_NGAMR7 | 4140 | 5596 | 43120; American |
| KNY_NGAMR8 | 4209 | *Absent* | *Absent* |
| KNY_NGAMR9 | 4153 | *Absent* | 43120; American |
| KNY_NGAMR10 | 4190 | 5597 | 43120; American |
| KNY_NGAMR11 | 4209 | 5594 | 43131; American |
| KNY_NGAMR13 | 4207 | 5599 | 43048; American |
| KNY_NGAMR14 | 4206 | 5597 | 43120; American |
| KNY_NGAMR15 | 4207 | 5594 | 43048; American |
| KNY_NGAMR16 | 4137 | *Absent* | 42944; American |
| KNY_NGAMR17 | 4142 | *Absent* | 43119; American |
| KNY_NGAMR18 | 4207 | 5605 | 42992; American |
| KNY_NGAMR19 | 4150 | *Absent* | 41456; Dutch |
| KNY_NGAMR20 | 4188 | 5599 | 43127; American |
| KNY_NGAMR21 | 4207 | 5592 | 43118; American |
| KNY_NGAMR22 | 4207 | 5598 | 42900; American |
| KNY_NGAMR23 | 4207 | 5599 | 43127; American |
| KNY_NGAMR24 | 4207 | 5598 | 42993; American |
| KNY_NGAMR25 | 4166 | *Absent* | 42994; American |
| KNY_NGAMR26 | 4190 | 5600 | 42944; American |
| KNY_NGAMR27 | 4143 | 5600 | 42983; American |
| KNY_NGAMR28 | 4161 | 5598 | 43120; American |
| KNY_NGAMR29 | 4207 | *Absent* | 42944; American |
| KNY_NGAMR30 | 4207 | 5592 | 43119; American |
| KNY_NGAMR31 | 4207 | *Absent* | 43119; American |
| KNY_NGAMR32 | 4207 | 5591 | *Absent* |
| KNY_NGAMR33 | 4153 | *Absent* | 42944; American |
| KNY_NGAMR35 | 4207 | 5596 | 43121; American |
| KNY_NGAMR36 | 4207 | 5807 | *Absent* |
| KNY_NGAMR41 | 4207 | 5605 | 43091; American |
| KNY_NGAMR42 | 4154 | 5598 | 43289; American |
| KNY_NGAMR45 | 4203 | 5647 | 42999; American |
| KNY_NGAMR50 | 4207 | 5597 | 43120; American |
| KNY_NGAMR52 | 4207 | 5592 | *Absent* |
| KNY_NGAMR53 | 4207 | 5597 | 42943; American |
| KNY_NGAMR54 | 4207 | *Absent* | 42991; American |

Size in basepairs (bp)

### 4.1.3 Molecular typing

#### 4.1.3.1 N. gonorrhoeae multi-locus sequence genotyping

A total of 25 multi locus sequence types (MLSTs) representing 3 *abcZ*, 2 *adk*, 4 *aroE*, 6 *fumC*, 5 *gdh*, 3 *pdhC*, and 2 *pgm* different alleles were determined. Of the 41

sequences, 26 belonged to 14 known MLST STs; ST-1928, ST-1893, ST-1588, ST-1599, ST-11367, ST-11366, ST-11365, ST-1932, ST-11242, ST-1921, ST-8133, ST-11976, ST-11750, and ST-8111,while the remaining 15 sequences belonged to 11 new STs; ST-13613, ST-13614, ST-13763, ST-13764, ST-13765, ST-13766, ST-13778, ST-13779, ST-13780, ST-13781, and ST-13782 (Table 4.3).

**Table 4.3: Identified MLST sequence types and their allelic combination.**

| Isolate | *abcZ* | *adk* | *aroE* | *fumC* | *gdh* | *pdhC* | *pgm* | ST |
|---|---|---|---|---|---|---|---|---|
| KNY_NGAMR1 | 59 | 39 | 170 | 237 | 148 | 153 | 65 | *13613* |
| KNY_NGAMR2 | 126 | 39 | 67 | 111 | 149 | 153 | 65 | 1928 |
| KNY_NGAMR3 | 59 | 39 | 67 | 157 | 147 | 153 | 133 | 1893 |
| KNY_NGAMR4 | 59 | 39 | 67 | 158 | 148 | 71 | 65 | 1588 |
| KNY_NGAMR5 | 59 | 39 | 67 | 157 | 148 | 153 | 65 | 1599 |
| KNY_NGAMR6 | 59 | 39 | 67 | 111 | 189 | 153 | 65 | 11367 |
| KNY_NGAMR7 | 59 | 39 | 67 | 111 | 147 | 71 | 65 | 11366 |
| KNY_NGAMR8 | 59 | 112 | 67 | 158 | 148 | 71 | 65 | 11365 |
| KNY_NGAMR9 | 59 | 112 | 67 | 111 | 189 | 153 | 65 | *13614* |
| KNY_NGAMR10 | 59 | 39 | 67 | 78 | 189 | 153 | 65 | 1932 |
| KNY_NGAMR11 | 59 | 112 | 67 | 158 | 148 | 71 | 65 | 11365 |
| KNY_NGAMR13 | 59 | 112 | 937 | 158 | 148 | 71 | 65 | *13782* |
| KNY_NGAMR14 | 59 | 39 | 170 | 78 | 189 | 153 | 65 | 11242 |
| KNY_NGAMR15 | 59 | 112 | 937 | 158 | 148 | 71 | 65 | *13782* |
| KNY_NGAMR16 | 109 | 39 | 67 | 157 | 150 | 153 | 133 | *13766* |
| KNY_NGAMR17 | 59 | 39 | 67 | 111 | 148 | 71 | 65 | 1921 |
| KNY_NGAMR18 | 59 | 39 | 170 | 158 | 148 | 71 | 65 | 8133 |
| KNY_NGAMR19 | 59 | 39 | 67 | 157 | 147 | 153 | 133 | 1893 |
| KNY_NGAMR20 | 59 | 112 | 67 | 158 | 147 | 71 | 65 | *13780* |
| KNY_NGAMR21 | 59 | 112 | 67 | 158 | 148 | 153 | 65 | 11976 |
| KNY_NGAMR22 | 59 | 39 | 170 | 158 | 148 | 71 | 65 | 8133 |
| KNY_NGAMR23 | 59 | 112 | 67 | 158 | 147 | 71 | 65 | *13780* |
| KNY_NGAMR24 | 59 | 39 | 170 | 158 | 148 | 71 | 65 | 8133 |
| KNY_NGAMR25 | 59 | 39 | 67 | 157 | 147 | 153 | 65 | *13781* |
| KNY_NGAMR26 | 109 | 39 | 67 | 983 | 150 | 153 | 65 | *13779* |
| KNY_NGAMR27 | 59 | 39 | 806 | 157 | 150 | 153 | 133 | *13778* |
| KNY_NGAMR28 | 59 | 39 | 67 | 78 | 189 | 153 | 65 | 1932 |
| KNY_NGAMR29 | 109 | 39 | 67 | 157 | 150 | 153 | 65 | 11750 |
| KNY_NGAMR30 | 59 | 112 | 67 | 157 | 148 | 71 | 65 | *13763* |
| KNY_NGAMR31 | 59 | 39 | 67 | 78 | 189 | 153 | 65 | 1932 |
| KNY_NGAMR32 | 59 | 112 | 937 | 158 | 148 | 71 | 65 | *13782* |
| KNY_NGAMR33 | 109 | 39 | 67 | 157 | 150 | 901 | 133 | *13764* |
| KNY_NGAMR35 | 59 | 39 | 67 | 78 | 189 | 153 | 65 | 1932 |
| KNY_NGAMR36 | 59 | 112 | 67 | 78 | 148 | 71 | 65 | *13765* |
| KNY_NGAMR41 | 59 | 39 | 170 | 158 | 148 | 71 | 65 | 8133 |
| KNY_NGAMR42 | 59 | 39 | 67 | 157 | 148 | 153 | 65 | 1599 |
| KNY_NGAMR45 | 59 | 39 | 806 | 157 | 150 | 153 | 133 | *13778* |
| KNY_NGAMR50 | 59 | 39 | 67 | 78 | 189 | 153 | 65 | 1932 |
| KNY_NGAMR52 | 59 | 39 | 67 | 158 | 148 | 71 | 65 | 1588 |
| KNY_NGAMR53 | 109 | 39 | 67 | 157 | 150 | 153 | 65 | 11750 |
| KNY_NGAMR54 | 126 | 39 | 170 | 158 | 148 | 71 | 65 | 8111 |

The 11 new (identified for the first time in the present study) STs are in bold and italics**.**

Overall, ST-1932 was the most common (*n* = 5, 12.2%) followed by ST-8133 (*n* = 4, 9.76%) and a novel ST-13782 (*n* = 3, 7.32%, (Figure 4.1). Twenty six isolates formed ten clusters (two or more same ST) while 15 isolates formed singular STs (Figure 4.1). Unlike ST-1932 and ST-13782, ST-8133 comprised of isolates recovered from one region; Nyanza. Other STs which comprised of isolates from a single region include: ST-11365 (Kombewa); ST-13780 and ST-13778 (Kisumu) and ST-1588 (Coastal Kenya) (Figure 4.3).

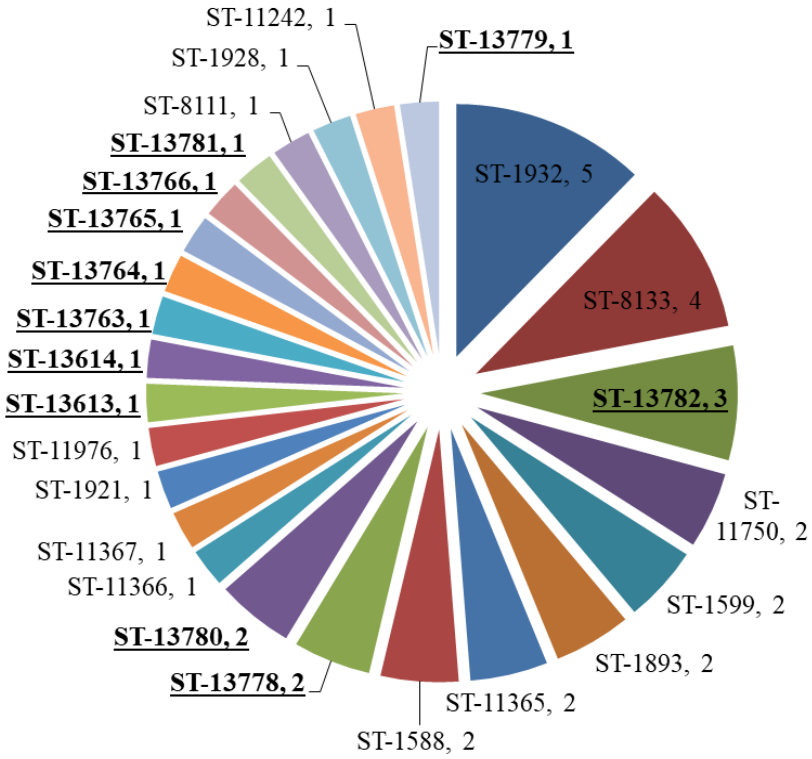



**Figure 4.1: Clusters of identified MLST STs.**

The bold and underlined numbers are the novel STs identified in the present study

### 4.1.3.2 *N. gonorrhoeae* multi-antigen sequence genotyping

As shown in Table 4.4 and Figure 4.2, a total of 33 *N. gonorrhoeae* multi-antigen sequence types (NG-MASTs) representing 25 *porB* and 23 *tbpB* different alleles were identified. Ten of the 25 *porB* and 7 of the 23 *tbpB* alleles were novel (Table 4.4). Thirty four (82.9%) isolates belonged to 28 novel NG-MAST STs; ST-18599, ST-19087, ST-19166-ST-19170, ST-19254-ST-19273 and ST-19727, six (14.6%)

isolates belonged to 5 known STs; ST-10134, ST-355, ST-11752, ST-6202, and ST-15514 while one isolates could not be typed (Table 4.4).

**Table 4.4: Identified NG-MAST sequence types and their allelic combination.**

| Isolate | *porB* | *tbpB* | ST (NG MAST) |
|---|---|---|---|
| KNY_NGAMR1 | 90 | 2905 | *19254* |
| KNY_NGAMR2 | 11156 | 362 | *19265* |
| KNY_NGAMR3 | 11157 | 1807 | *19266* |
| KNY_NGAMR4 | 11158 | 191 | *19267* |
| KNY_NGAMR5 | 2824 | 1806 | *19167* |
| KNY_NGAMR6 | 11159 | 9 | *19268* |
| KNY_NGAMR7 | 121 | 726 | *18599* |
| KNY_NGAMR8 | 970 | 2907 | *19258* |
| KNY_NGAMR9 | 11155 | 9 | *19264* |
| KNY_NGAMR10 | 2298 | 726 | 10134 |
| KNY_NGAMR11 | 11160 | 2908 | *19269* |
| KNY_NGAMR13 | 90 | 133 | 355 |
| KNY_NGAMR14 | 11161 | 145 | *19270* |
| KNY_NGAMR15 | 123 | 133 | *19087* |
| KNY_NGAMR16 | 5286 | 2911 | *19263* |
| KNY_NGAMR17 | 11162 | 5 | *19271* |
| KNY_NGAMR18 | 5746 | 29 | *19168* |
| KNY_NGAMR19 | 3440 | 612 | *19169* |
| KNY_NGAMR20 | 90 | 2909 | *19255* |
| KNY_NGAMR21 | 90 | 190 | 11752 |
| KNY_NGAMR22 | 5746 | 29 | *19168* |
| KNY_NGAMR23 | 90 | 2909 | *19255* |
| KNY_NGAMR24 | 5746 | 29 | *19168* |
| KNY_NGAMR25 | 11164 | 1534 | *19273* |
| KNY_NGAMR26 | 5286 | 2906 | *19261* |
| KNY_NGAMR27 | 3671 | 60 | 6202 |
| KNY_NGAMR28 | 4263 | 726 | *19260* |
| KNY_NGAMR29 | 5286 | 2910 | *19262* |
| KNY_NGAMR30 | 2855 | 133 | *19259* |
| KNY_NGAMR31 | 11163 | 145 | *19272* |
| KNY_NGAMR32 | 9388 | 191 | *19170* |
| KNY_NGAMR33 | 5286 | 60 | *19166* |
| KNY_NGAMR35 | 2298 | 726 | 10134 |
| KNY_NGAMR36 | 90 | 2543 | 15514 |
| KNY_NGAMR41 | 5746 | 29 | *19168* |
| KNY_NGAMR42 | 2037 | 9 | *19727* |
| KNY_NGAMR45 | | 1534 | |
| KNY_NGAMR50 | 90 | 726 | *19256* |
| KNY_NGAMR52 | 2824 | 1806 | *19167* |
| KNY_NGAMR53 | 5286 | 2910 | *19262* |
| KNY_NGAMR54 | 139 | 2051 | *19257* |

The bolded alleles and ST numbers were described for the first time in the present study

A novel ST, ST-19168 comprised of four isolates from Kisumu was the most common (10%). Additionally, these four isolates belonged to an existing MLST ST-8133 which was the second common MLST (Figure 4.1). Five NG MAST ST clusters were formed by 12 isolates, while the rest formed singular STs (Figure 4.2).

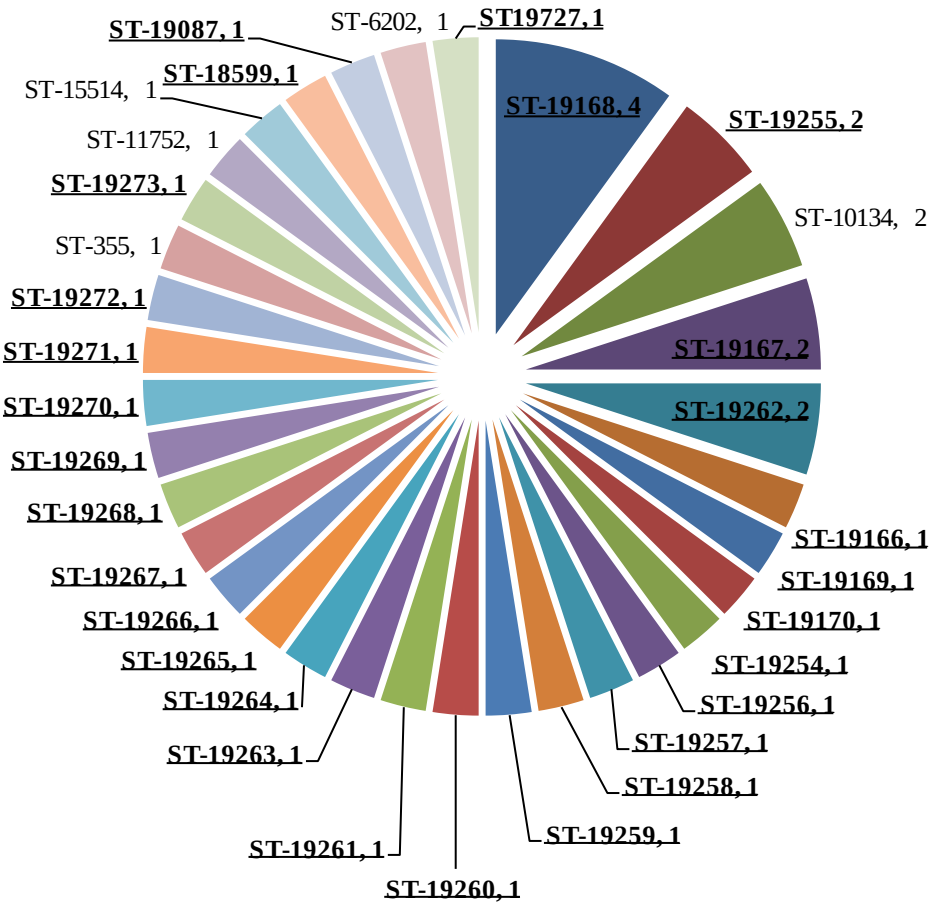



**Figure 4.2: Clusters of identified NG MAST STs.**

The bold and underlined numbers are the novel STs identified in the present study

Neighbor-joining tree generated from concatenated MLST and NG MAST allele nucleotide sequences identified region based clustering in two novel NG-MASTs; ST-19168 and ST-19255 and two MLST STs; 13780 and 8133. These two clusters comprised of isolates recovered from Nyanza (Figure 4.3).

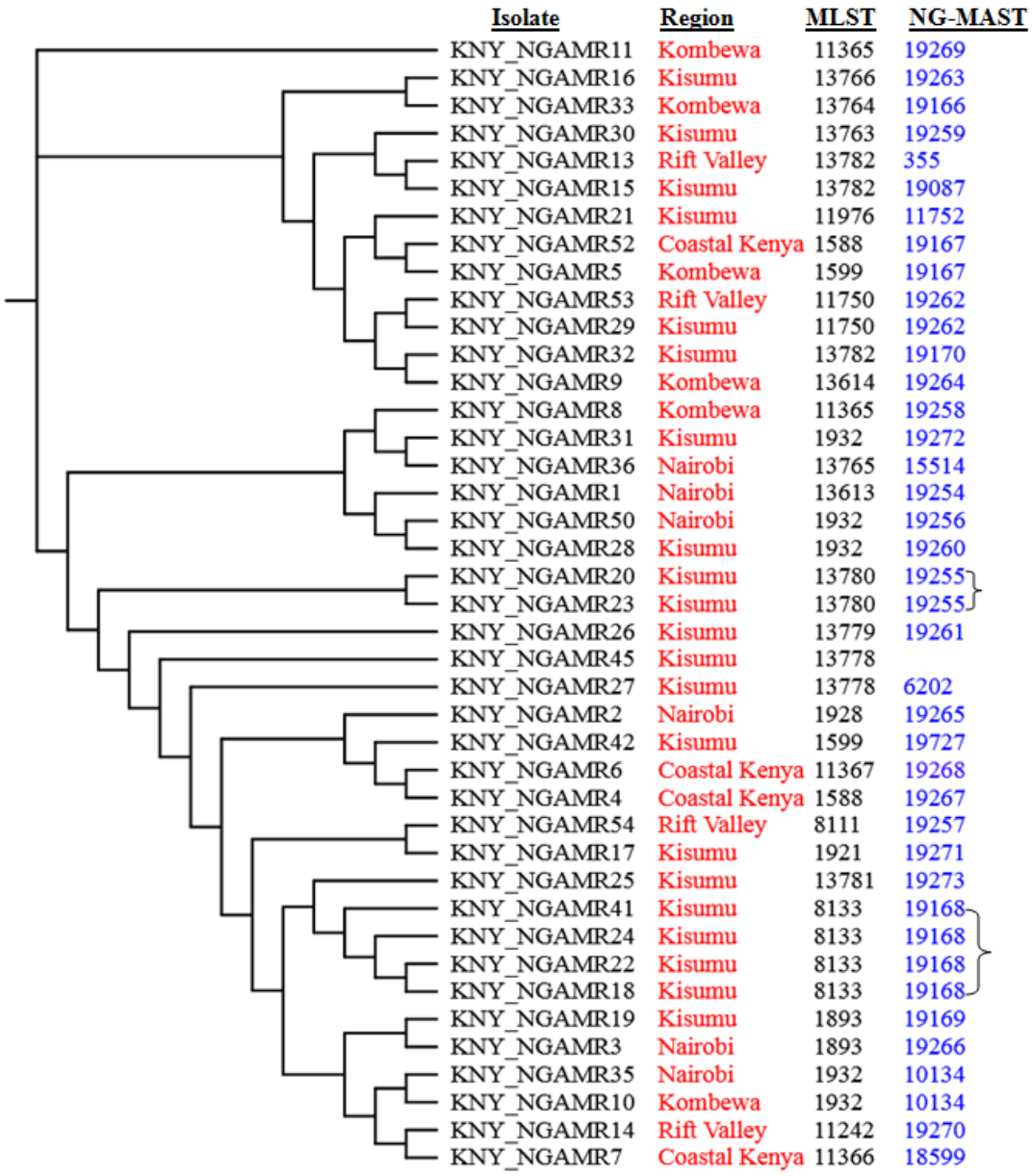



**Figure 4.3: MLST and NG MAST based phylogeny.**

The tree in Figure 4.3 was generated from concatenated MLST and NG MAST allele sequences using the neighbor-joining method. The symbol (}) locates isolates which displayed both NG-MAST and MLST STs. region based clustering. The branch lengths were ignored when generating the tree.

## 4.2 Evolution and genetic diversity between Kenyan gonococci and global *N. gonorrhoeae* isolates

### 4.2.1 Single nucleotide polymorphisms

When, seventy four sequences comprising of 41 study genomes and 33 comparison genomes were mapped to *N. gonorrhoeae* NCCP1194, a total of 23805 SNPs were detected. Among the analyzed Kenya gonococci, Isolates KNY_NGAMR2 and KNY_NGAMR42 were the most genetically diverse with a SNP difference of 5366, while KNY_NGAMR20 and KNY_NGAMR23 were closely related with a SNP difference of 5 (Appendix IX).

### 4.2.2 Root location distribution

For the root location distribution, 95% highest posterior density (HPD) consisted of 2 locations (USA & Kenya), with a strong indication that USA might be the most probable ancestral location (root) with over 84% probability (Table 4.5, underlined).

**Table 4.5: 95% HPD root location distribution**

| Country | 95% HPD root location distribution |
|---|---|
| Canada | 8.999100089991E-4 |
| USA | 0.8480151984801519 |
| Sweden | 5.999400059994001E-4 |
| Japan | 1.9998000199980003E-4 |
| UK | 1.9998000199980003E-4 |
| Malaysia | 2.9997000299970003E-4 |
| Denmark | 4.999500049995E-4 |
| Italy | 6.999300069993001E-4 |
| Kenya | 0.14578542145785423 |
| France | 0.0010998900109989002 |
| Australia | 9.99900009999E-4 |
| Korea | 6.999300069993001E-4 |

### 4.2.3 Temporal Bayesian phylogenetic tree

Thirty nine Kenyan isolates formed five distinct clades, while two isolates, KNY_NGAMR1 & KNY_NGAMR5 were genetically diverse from the rest of the Kenyan isolates and had evolved independently (Figure 4.4).

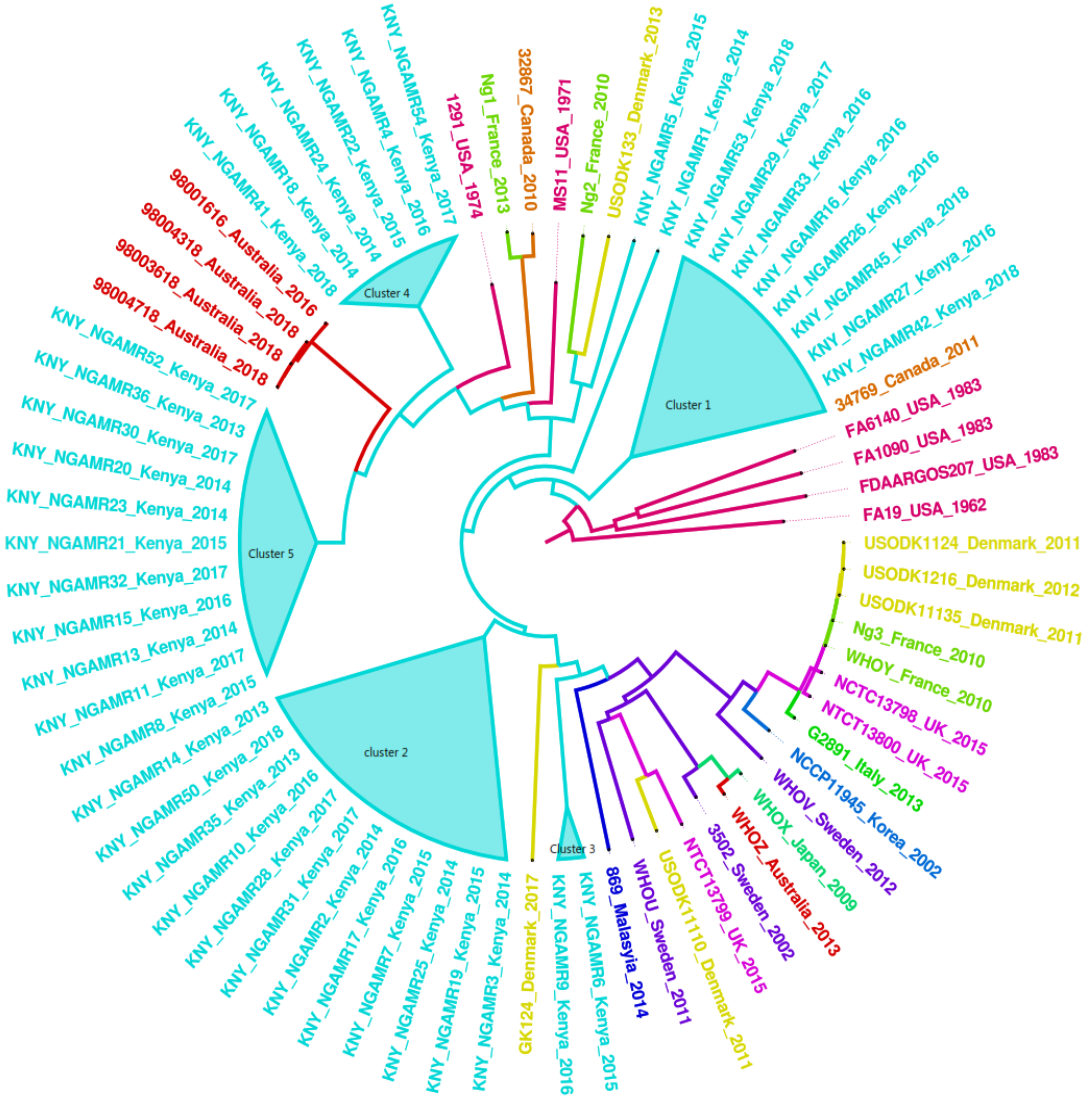



**Figure 4.4: Collapsed temporal Bayesian phylogenetic tree.**

Figure 4.4 shows whole genome SNP based Phylogeny. Isolate names are colored based on the country of isolation. With exception of 2 isolates, the Kenyan isolates formed five distinct clusters which are chronologically numbered based on the year of evolution from cluster 1-5.

According to temporal Bayesian phylogenetic tree, emergence of the most recent common ancestor (TMCRA) for the analyzed isolates was estimated to have taken place in ~1786 (235 years ago). In ~1803, the bayesian phylogenetic tree diverged into two clades, a major clade and a minor basal clade comprised of USA isolates

only. In ~1814, the major clade diverged further into two clades, a minor clade comprised mostly of Kenyan isolates; cluster 1 (n= 8) and a major clade (Figure 4.5).

In ~1838, divergence in the major clade gave rise to the second Kenyan clade; cluster 2 (n= 12) which has since evolved into its own lineage. In ~1873 an ancestor of the third Kenyan clade evolved and later diverged in (~1974) into two Kenyan isolates. An evolution in ~1922 gave rise to the fourth Kenyan clade; cluster 4 (n= 6) and a sister clade which evolved further in ~1930 into a major Kenyan clade; cluster 5 (n= 11) and a minor clade comprised of Australian isolates.

The Most Common Recent Ancestor (TMCRA) of the Kenyan isolates seemed to have been present in (~2013) giving rise to KNY_NGAMR20 and KNY_NGAMR23 which belong to same MLST and NG-MAST STs. The 5 Kenyan clusters further divided to form subsequent lineages (Figure 4.5). TMCRA for KNY_NGAMR1 existed in ~1824 while that of KNY_NGAMR5 and two more isolates each from France and Denmark seemed to have existed in ~1893.

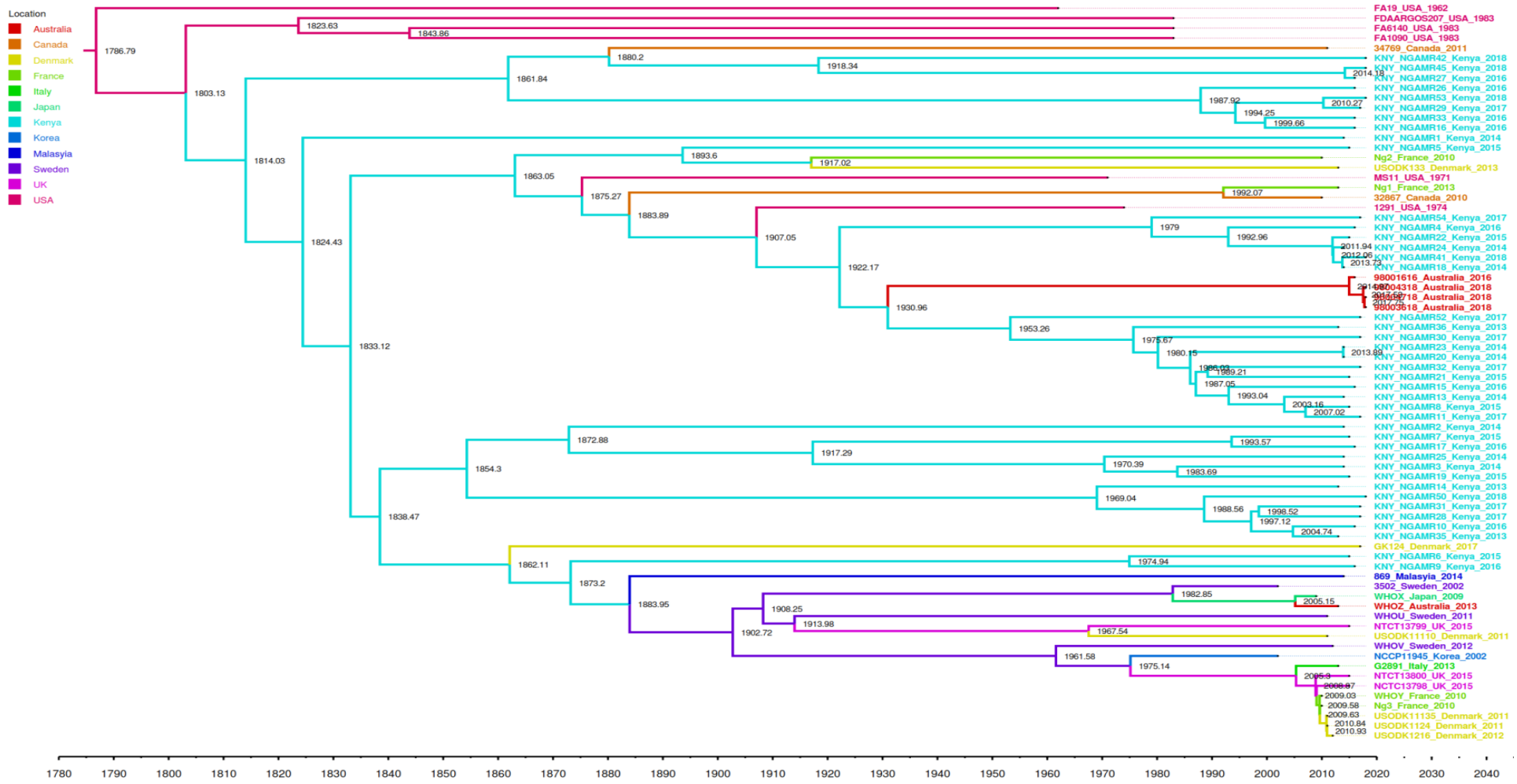



**Figure 4.5: Temporal Bayesian phylogenetic tree**

Whole genome SNP based Phylogeneny. The Most Common Recent Ancestor (TMCRA) for the phylogeny emerged in ~1786. The Kenyan isolates portrayed an evolution pattern leading to their own distinct lineages with the TMCRA having evolved in 2013. Four USA isolates formed a basal clade. The tree was visualized using FigTree version 1.4.3.

Although Kenyan isolates clustered together, there was no location based clustering (Figure 4.6).

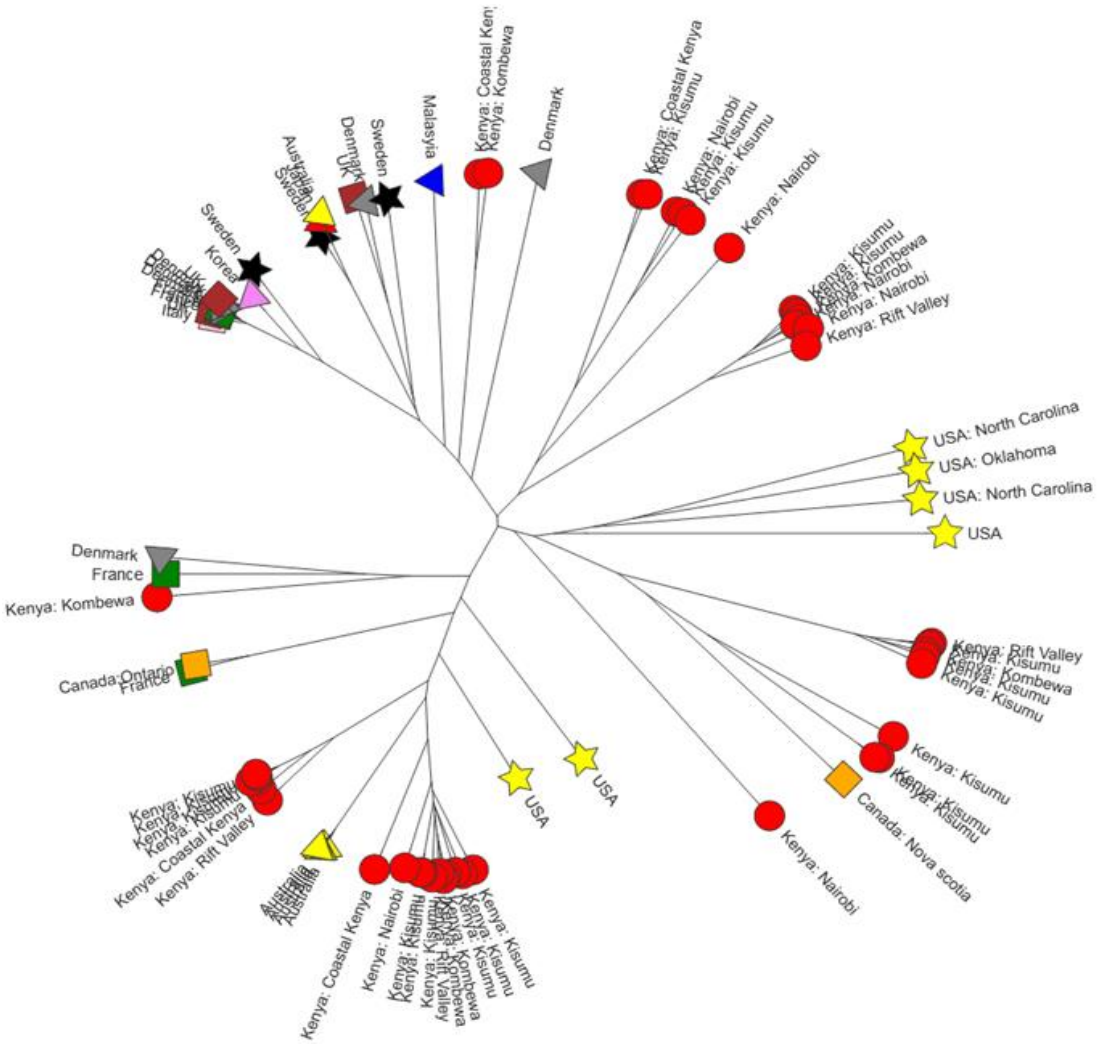



**Figure 4.6: Region based phylogeny.**

In Figure 4.6 above, differently colored shapes show the different countries where the analyzed isolates were isolated. Kenyan regions from where the 41 isolates were recovered are indicated in the leaf terminal narrative. No region based clustering was observed in the present study. The tree was visualized using Microreact.

### 4.2.4 cgMLST based phylogeny

BIGSdb Genome comparator tool used to compare whole genomes of the 41 Kenyan gonococci identified 1470 loci as core. To understand sequence distribution and phylogenetic relationships among the study isolates *N. gonorrhoeae* cgMLST v.1.0

was used to infer phylogeny using "cgMLST scheme". cgMLST phylogeny identified five distinct clusters (group of more than 3 isolates) among the study isolates. Both the SNP and core genome based phylogenies had different topologies. However, the isolates clustered into similar groups in both the core genome and SNP based phylogenies. As observed in SNP based phylogeny, no regional based clustering was observed in the core genome based phylogeny. The five core genome clusters are shown in colored and numbered oval shapes in Figure 4.7. All the five clusters had isolates from Kisumu.

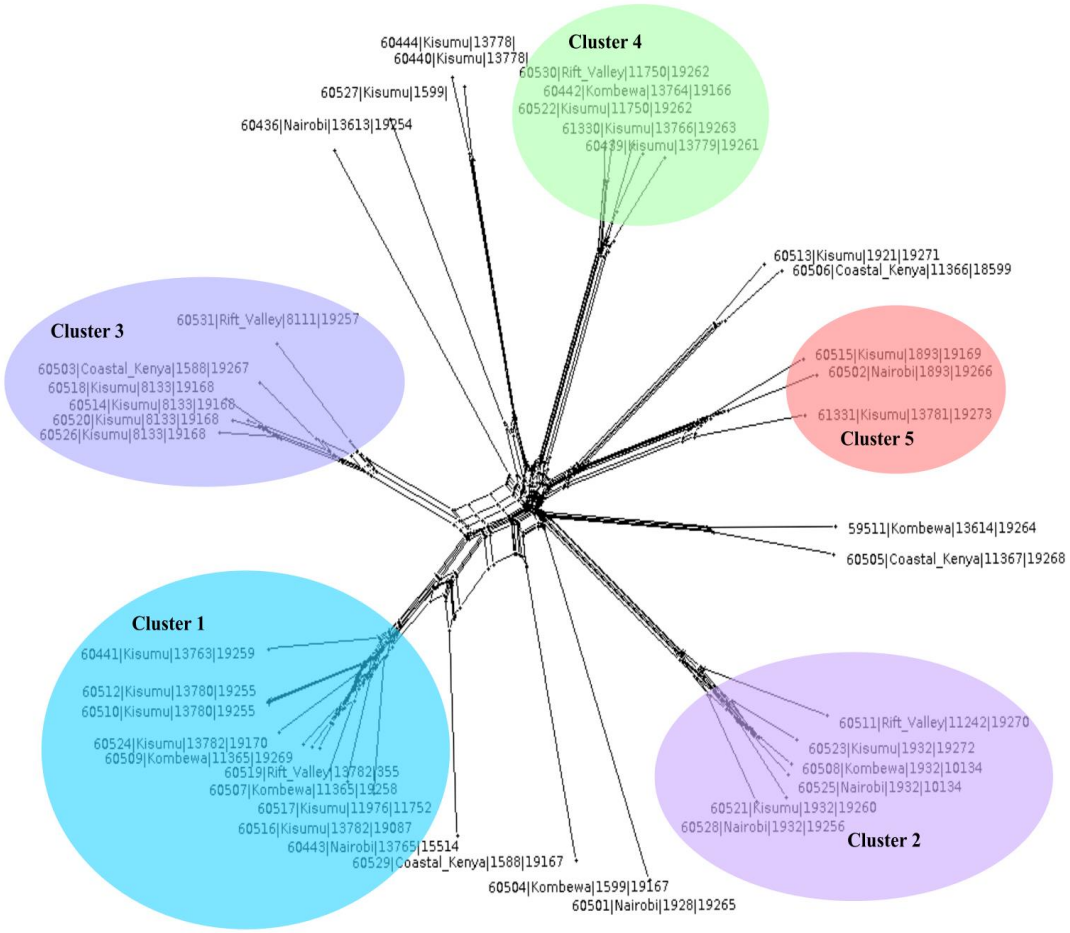



**Figure4.7: Core genome based Phylogeny.**

Figure 4.7 shows phylogeny inferred from 1470 loci identified as core genomes using cgMLST *N. gonorrhoeae* v.1.0. The five identified clusters are indicated by large differently colored oval shapes. The leaf terminals contain a code consisting of

PubMLST ID of the isolate, region of isolation, MLST and NG-MAST STs numbers respectively.

Cluster 1 (n = 10), comprised of 8 isolates from Nyanza (Kisumu 6, and Kombewa 2), 1 from Nairobi, and 1 from Rift valley regions of Kenya (Figure 4.7). This cluster was the most diverse in terms of MLST and NG-MAST types. The 10 isolates belonged to 4 novel MLST STs *viz*: 13765, 13782, 13780, and 13763 and 2 existing MLST STs; 11976 and 11365 (Figure 4.7). Furthermore, the cluster comprised of two existing NG-MAST STs; 11752 and 355 and seven novel NG-MAST STs; 15514, 19259, 19170, 19255, 19087, 19258, and 19265 (Figure 4.7 ).

Clusters 2 and 3 were both formed by six isolates. Cluster 2 was formed by isolates from Nyanza (Kisumu, 2 and Kombewa, 1), Nairobi (2), and Rift valley, (1). The isolates belonged to two existing MLST STs (1932 and 11242), one existing NG-MAST ST (10134) and 4 novel NG-MAST STs (19270, 19256, 19272 and 19260). Cluster 3 comprised of isolates from Nyanza (Kisumu 4), Coast (1), and Rift valley (1). These isolates belonged to 3 existing MLST STs (8133, 1588, and 8111), and 3 novel NG-MAST ST (19168, 19267, and 19257). The four isolates from Kisumu belonged to MLST ST-8133 (Figure 4.7).

Cluster 4 was formed by five isolates from Nyanza (Kisumu 3, and Kombewa 1) and Rift valley (1) belonging to an existing (11750) and 3 novel (13766, 13764, and 13779) MLST STs. They all belonged to 4 novel NG-MAST STs (19262, 19263, 19166, and 19261) (Figure 4.7)

Cluster 5 comprised of 2 isolates from Nyanza and 1 from Nairobi. They belonged to an existing (1893) and a novel (13783) MLST STs, and 3 novel NG-MAST STs (19266, 19169, and 19273) (Figure 4.7).

## 4.3 Characterization of chromosomal and plasmid borne antimicrobial resistance determinants expressed by Kenyan *N. gonorrhoeae* isolates and their association with antibiotic susceptibilities

### 4.3.1 β-Lactams

The tested β-lactam antibiotics included; two 3rd generation cephalosporins namely; cefixime (CFX) and ceftriaxone (CFO), and one penicillin Benzylpenicillin G (PEN). Analysis of the previously determined antimicrobial susceptibility test (AST) data (Appendix I) showed that, two (5%) isolates were penicillin susceptible (MICs ≤ 0.06mg/L), and twenty six (67%) isolates were penicillin resistant (MIC>1 mg/L), while 11 (28%) had an intermediary penicillin resistance (MIC>0.06-1 mg/L). Although reduced ceftriaxone susceptibility (MICs >0.04-0.094 mg/L) was observed in 6 isolates, all tested isolates were ceftriaxone and cefixime susceptible (≤ 0.125 mg/L) (Appendix X)

The following Antimicrobial Resistance (AMR) determinants were identified;

**i. PenA amino acid changes**

The non-mosaic *penA* mutations associated with resistance to penicillin were identified in all isolates (Figure 4.8). The mutations observed belonged to five known patterns, namely; Pattern XXII (D346, F505L, A511V, A517G H542N, P553V, K556Q, I557V, I567V, N575, A576V), Pattern IX (D346, F505L, A511V, A517G, P552L), Pattern XIX (D346, F505L, A511V, A517G, H542N, I567V, N575, A576V), Pattern XIV (D346, F505L, A511V, A517G, H542N), and Pattern II (D346, F505L, A511V, A517G) (Ameyama et al., 2002; Whiley et al., 2007a) (Appendix XI, Table 4.6) . Pattern IX which has a P552L amino acid substitution was only identified in penicillin resistant isolates (Figure 4.17). Pattern XIV was the most common and was identified in 21 (51%) isolates (Table 4.6). Mosaic PenA patterns associated with ceftriaxone and cefixime resistance were not observed in the present study. A501V/T substitution which has been associated with reduced susceptibility to ceftriaxone was also not observed in the present study (Figure 4.8).

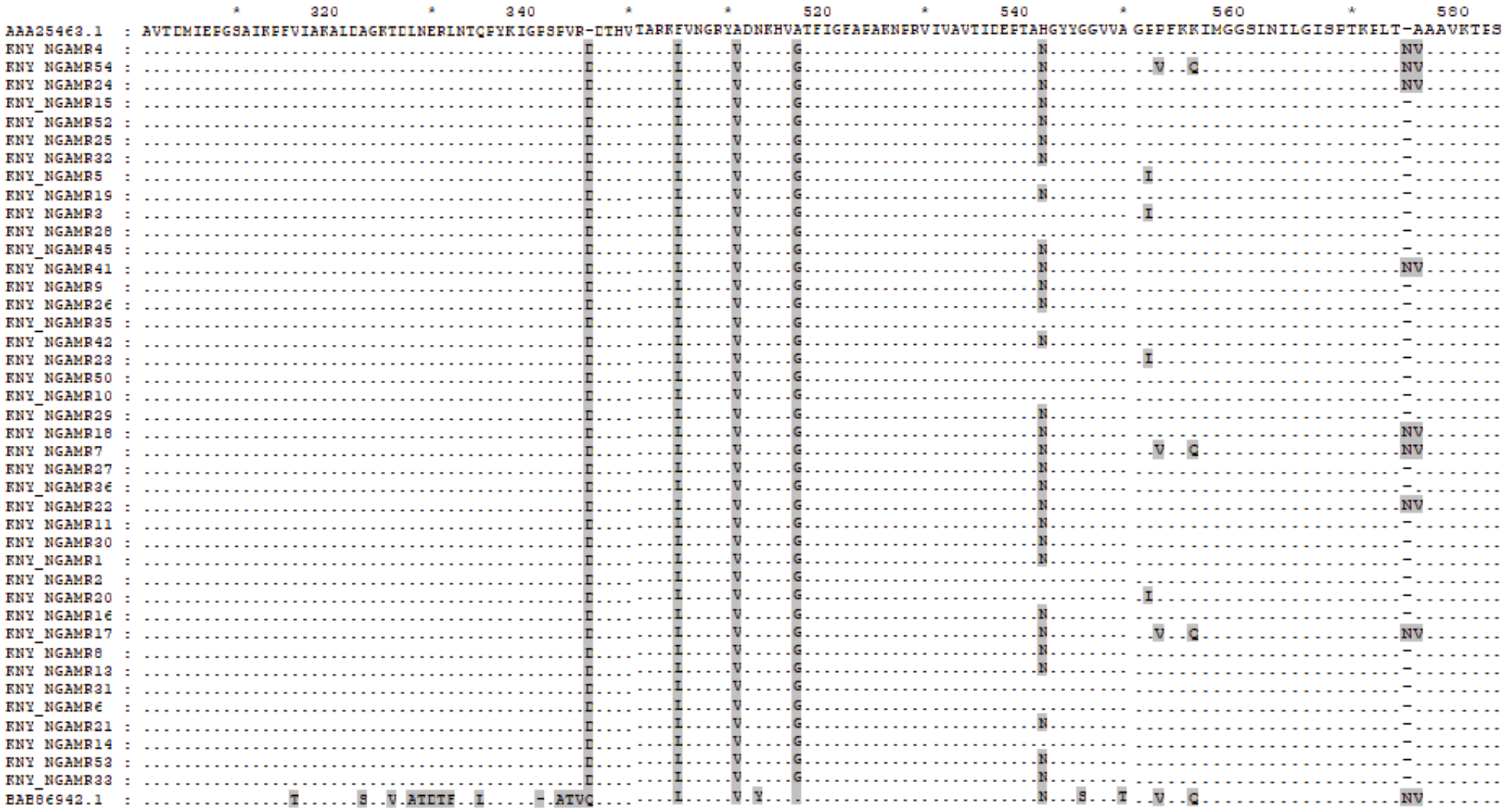


**Figure 4.8: Amino acid changes in PenA.**

In Figure 4.8 above, a wild type PenA from a penicillin susceptible strain (accession no. AAA25463.1) and a mosaic PenA from a strain with reduced susceptibility to cefixime (accession no. BAB86942.1) were included as control sequences. Amino acid changes associated with β-lactam resistance are shaded in gray.

## Table 4.6: AMR determinants mediating resistance to β-Lactams.

| | MICs (mg/L) | | | Genetic determinants of antibiotic resistance | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample ID | CFX | CRO | PEN | *penA* | PonA | PorB | MtrR | TEM |
| KNY_NGAMR1 | <0.016 | <0.002 | 0.064 | XIV | - | A121G, G120D, -N122 | - | NP |
| KNY_NGAMR2 | <0.016 | 0.002 | 64 | II | L421P | G120D | G45D, -A13 (Pro) | TEM-1* |
| KNY_NGAMR3 | <0.016 | NT | 48 | IX | L421P | - | A39T | TEM-239 |
| KNY_NGAMR4 | <0.016 | <0.002 | 8 | XIX | - | A121S, N122K | A39T | TEM-1 |
| KNY_NGAMR5 | <0.016 | *0.006* | 3 | IX | L421P | A121G, G120N, -N122 | H105Y | TEM-1 |
| KNY_NGAMR6 | <0.016 | <0.016 | 0.094 | II | - | - | A39T | NP |
| KNY_NGAMR7 | <0.016 | *0.008* | >256 | XXII | L421P | A121S, N122K | - | TEM-239 |
| KNY_NGAMR8 | <0.016 | 0.002 | 0.064 | XIV | L421P | A121G, G120D, -N122 | A39T | NP |
| KNY_NGAMR9 | <0.016 | *0.004* | 0.094 | XIV | - | A121G, -N122 | A39T | NP |
| KNY_NGAMR10 | <0.016 | <0.016 | >256 | II | - | - | A39T | TEM-1 |
| KNY_NGAMR11 | <0.016 | <0.016 | 12 | XIV | L421P | A121G, G120D, -N122 | A39T | TEM-1 |
| KNY_NGAMR13 | <0.016 | <0.002 | 8 | XIV | L421P | A121G, G120D, -N122 | A39T | TEM-1 |
| KNY_NGAMR14 | <0.016 | <0.002 | 64 | II | - | A121S, N122K | A39T | TEM-1 |
| KNY_NGAMR15 | <0.016 | <0.016 | 48 | XIV | L421P | A121G, -N122 | A39T | TEM-1 |
| KNY_NGAMR16 | <0.016 | <0.016 | 0.19 | XIV | - | A121G, -N122 | A39T | NP |
| KNY_NGAMR17 | <0.016 | <0.016 | 0.38 | XXII | L421P | - | T86A, D79N, H105Y | NP |
| KNY_NGAMR18 | <0.016 | <0.016 | 0.5 | XIX | - | - | A39T | TEM-1 |
| KNY_NGAMR19 | <0.016 | *0.094* | 0.19 | XIV | L421P | - | A39T | NP |
| KNY_NGAMR20 | <0.016 | *0.004* | 12 | IX | L421P | A121G, G120D, -N122 | A39T | TEM-1 |
| KNY_NGAMR21 | <0.016 | <0.016 | 32 | XIV | L421P | A121G, G120D, -N122 | A39T | TEM-1 |
| KNY_NGAMR22 | <0.016 | *0.004* | 2 | XIX | - | - | A39T | TEM-1 |
| KNY_NGAMR23 | <0.016 | <0.002 | 12 | IX | L421P | A121G, G120D, -N122 | A39T | TEM-1 |
| KNY_NGAMR24 | <0.016 | <0.016 | 2 | XIX | - | - | A39T | TEM-1 |
| KNY_NGAMR25 | NT | NT | 0.094 | XIV | L421P | - | A39T, G→A (Pro) | NP |
| KNY_NGAMR26 | <0.016 | <0.016 | 8 | XIV | - | A121G, -N122 | A39T | TEM-239 |
| KNY_NGAMR27 | <0.016 | <0.016 | 1 | XIV | - | - | A39T | TEM-239 |
| KNY_NGAMR28 | <0.016 | <0.002 | 96 | II | - | A121G, -N122 | A39T | TEM-1 |
| KNY_NGAMR29 | <0.016 | <0.002 | 0.094 | XIV | L421P | A121G, -N122 | A39T | NP |
| KNY_NGAMR30 | <0.016 | <0.002 | 12 | XIV | L421P | A121G, -N122 | A39T | TEM-1 |
| KNY_NGAMR31 | <0.016 | <0.002 | 0.38 | II | - | A121G, G120D, -N122 | A39T | NP |
| KNY_NGAMR32 | <0.016 | <0.002 | 8 | XIV | L421P | A121G, G120D, -N122 | A39T | TEM-1 |
| KNY_NGAMR33 | <0.016 | <0.016 | 3 | XIV | - | A121G, -N122 | A39T | NP |
| KNY_NGAMR35 | NT | NT | >256 | II | - | - | A39T | TEM-1 |
| KNY_NGAMR36 | <0.016 | <0.016 | NT | XIV | L421P | A121G, G120D, -N122 | A39T | TEM-1 |
| KNY_NGAMR41 | <0.016 | <0.016 | 32 | XIX | - | - | A39T | TEM-1 |
| KNY_NGAMR42 | <0.016 | <0.016 | 32 | XIV | - | - | T86A, D79N, H105Y | TEM-1 |
| KNY_NGAMR45 | NT | NT | NT | XIV | - | - | - | TEM-239 |
| KNY_NGAMR50 | <0.016 | <0.016 | 16 | II | - | A121G, G120D, -N122 | A39T | TEM-1 |
| KNY_NGAMR52 | <0.016 | <0.016 | 0.38 | XIV | - | A121G, G120N, -N122 | T86A, H105Y | TEM-1 |
| KNY_NGAMR53 | <0.016 | <0.016 | 6 | XIV | L421P | A121G, -N122 | A39T | TEM-239 |
| KNY_NGAMR54 | <0.016 | <0.016 | 6 | XXII | - | - | A39T | NP |

The following indicate: CFX, cefixime; CRO, ceftriaxone; PEN, penicillin; (*), TEM carried by an Asian type TEM plasmid; (-), No changes observed; NT, not tested; -N122, deletion of N at position 122; -A13. Adenine deletion in the *mtrR* promoter; and Pro, promoter mutation

## ii. PonA amino acid changes

In gonococci, the substitution of Leucine by Proline at position 421 of PonA protein reduces the rate of penicillin acylation and is associated with high level of penicillin resistance (Barbour, 1981; Ropp et al., 2002). The L421P amino acid substitution was identified in 19 (46%) of the 41 isolates (Danielsson et al., 1986; Ropp et al., 2002; Shaskolskiy et al., 2019) (Figure 4.9). Four of the 19 isolates had reduced ceftriaxone susceptibility (MICs >0.04-0.094 mg/L) (Table 4.6).

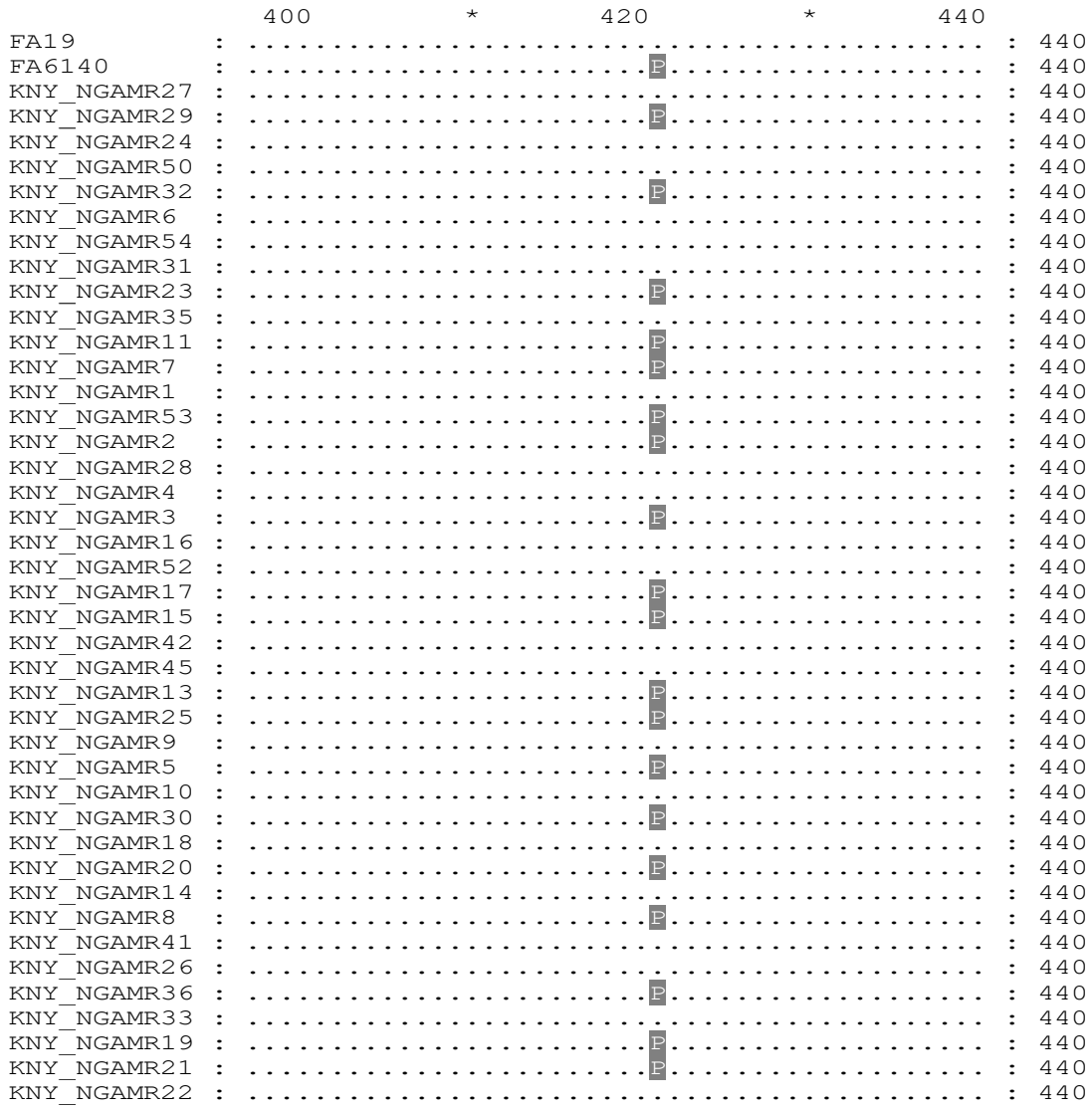



**Figure 4.9: L421P amino acid substitution in PonA.**

In Figure 4.9, altered PonA from a high-level penicillin-resistant strain FA6140 and a wild type PonA from a penicillin-susceptible strain FA19 were included as the control sequences.

No significant increase in penicillin MICs (t (37) = 0.3733, $p$ = 0.7110) was observed in the isolates harbouring PonA L421P amino acid changes (M=28.99, ±SEM = 14.11) when compared to isolates expressing wild type PonA (M=37.32, ±SEM = 16.73) (Figure 4.10).

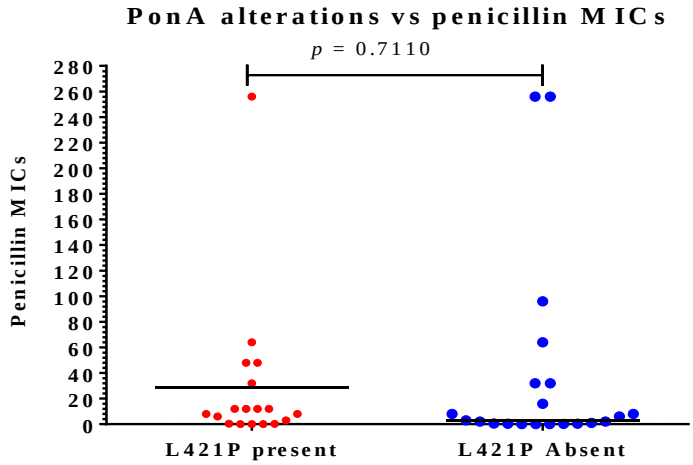



**Figure 4.10: Effects of altered PonA on penicillin susceptibility.**
The central bars across each group of points locate the mean for that group.

### iii. PorB amino acid changes

PorinB (PorB) is a protein which mediates influx of compounds into bacterial cells. Amino acid changes associated with reduced drug permeability in bacterial cells in PorB also termed as PenB changes were analyzed at positions; 120, 121 and 122. While 15 isolates lacked PorB amino acid alterations associated with gonococcal drug resistance (G120, A121, and -N122), five different patterns of PorB amino acid changes were observed in 26 isolates (Table 4.6). These include: Pattern I; *G120D* (1 isolate), pattern II; *A121G, G120D, and -N122* (11 isolates), pattern III; *A121G, G120N, and -N122* (2 isolates), pattern IV; *A121S, and N122K (*3 isolates), and pattern V; *A121G, and -N122* (9 isolates). G120 was substituted with charged amino acids D in 12 isolates, and N in 2 isolates (Appendix XI, Table 4.6).

Although A121 amino acid deletion in PorB associated with resistance to antibiotics was not observed in any of the study isolates, 22 isolates had the A121G amino acid substitution while 3 isolates had the A121S amino acid substitution. N122K amino acid substitution was only identified in the 3 isolates which also expressed A121S amino acid substitution. Meanwhile, N122 deletion was observed only in the 22 isolates expressing A121G amino acid substitution (Figure 4.11). Isolates expressing pattern I and IV PorB amino acid changes were all penicillin resistant (Figure 4.17, Table 4.6).

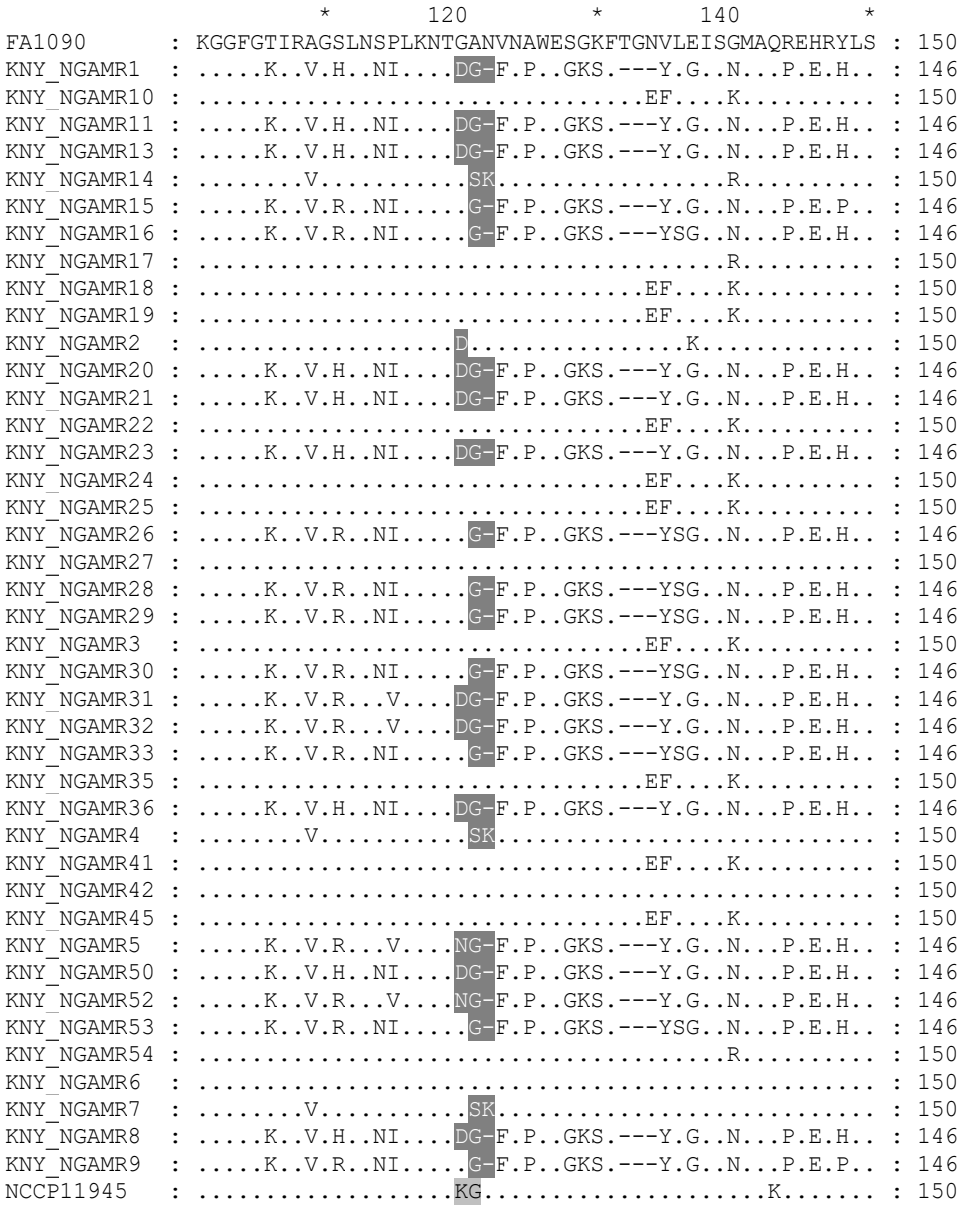



**Figure 4.11: Amino acid changes in the 90-150 hyper variable region of PorB.**

Compared to the *N. gonorrhoeae* FA1090 wild type strain, the study isolates had changes at the G120, A121, and N122 positions. Amino acid changes associated with β-lactam resistance are shaded in gray in Figure 4.11.

No significant increase in penicillin MICs (t (37) = 0.8111, $p$ = 0.4225) was observed in the isolates harbouring the above described PorB amino acid changes (M=26.77, ±SEM = 10.76) when compared to isolates without the described PorB alterations (M=45.45, ±SEM = 24.20) (Figure 4.12).

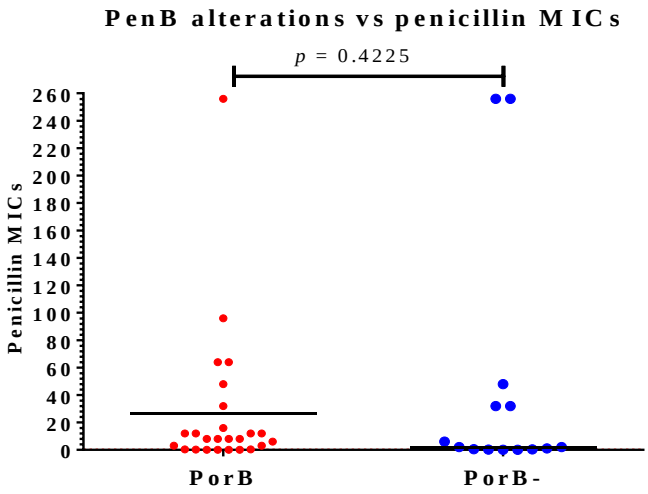



**Figure 4.12: Effects of altered PorB on penicillin susceptibility.**
The central bars across each group of points locate the mean for that group. PorB indicates presence of alterations while PorB- indicates absence of alterations in porinB

### iv. MtrR amino acid changes

The *mtrR* gene encodes a repressor protein; MtrR which represses the expression of the *mtrCDE* operon. This operon encodes a pump which has been associated with drug efflux and therefore resistance to antibacterial agents (Lucas et al., 1997). Polymorphism analysis of the *mtrR* structural gene and its promoter showed that; thirty eight (93%) isolates had amino acid alterations in MtrR repressor protein while 3 isolates had wild type MtrRs (Table 4.6). Adenine deletion (-A13) in the 13bp inverted repeat region between the -10 and -35 hexamers of the *mtrR* promoter, and

MtrR G45D amino acid substitution were identified in only KNY_NGAMR2 of the 41 isolates analyzed. The isolate was penicillin resistant (Figure 4.17, Table 4.6). One isolate with intermediate penicillin susceptibility, KNY_NGAMR25 had a G→A *mtrR* mutation (Figure 4.13).

```
                          *        20         *        40
pro_NEIS1635_6 : TTGCACGGATAAAAAGTCTTTTTTATAATCCGCCCTCGTCAAACCGACC : 49
KNY_NGAMR1     : ..................................................... : 49
KNY_NGAMR9     : ..................................................... : 49
KNY_NGAMR26    : ..................................................... : 49
KNY_NGAMR27    : ..................................................... : 49
KNY_NGAMR30    : ..................................................... : 49
KNY_NGAMR33    : ..................................................... : 49
KNY_NGAMR36    : ..................................................... : 49
KNY_NGAMR45    : ..................................................... : 49
KNY_NGAMR3     : ..................................................... : 49
KNY_NGAMR4     : ..................................................... : 49
KNY_NGAMR5     : ..................................................... : 49
KNY_NGAMR6     : ..................................................... : 49
KNY_NGAMR7     : ..................................................... : 49
KNY_NGAMR8     : ..................................................... : 49
KNY_NGAMR10    : ..................................................... : 49
KNY_NGAMR11    : ..................................................... : 49
KNY_NGAMR23    : ..................................................... : 49
KNY_NGAMR14    : ..................................................... : 49
KNY_NGAMR20    : ..................................................... : 49
KNY_NGAMR17    : ..................................................... : 49
KNY_NGAMR18    : ..................................................... : 49
KNY_NGAMR19    : ..................................................... : 49
KNY_NGAMR15    : ..................................................... : 49
KNY_NGAMR21    : ..................................................... : 49
KNY_NGAMR22    : ..................................................... : 49
KNY_NGAMR13    : ..................................................... : 49
KNY_NGAMR24    : ..................................................... : 49
KNY_NGAMR28    : ..................................................... : 49
KNY_NGAMR29    : ..................................................... : 49
KNY_NGAMR31    : ..................................................... : 49
KNY_NGAMR32    : ..................................................... : 49
KNY_NGAMR35    : ..................................................... : 49
KNY_NGAMR41    : ..................................................... : 49
KNY_NGAMR42    : ..................................................... : 49
KNY_NGAMR50    : ..................................................... : 49
KNY_NGAMR52    : ..................................................... : 49
KNY_NGAMR53    : ..................................................... : 49
KNY_NGAMR54    : ..................................................... : 49
KNY_NGAMR16    : ..................................................... : 49
KNY_NGAMR25    : ..A.................................................. : 49
pro_NEIS1635_8 : ..A.................................................. : 49
KNY_NGAMR2     : ..........-........................................... : 48
pro_NEIS1635_3 : ..........-........................................... : 48
```

**Figure 4.13: G→A and –A13 *mtrR* promoter mutations.**

In Figure 4.13, two mutated *mtrR* promoter alleles; pro_NEIS1635_3 (with –A13) and pro_NEIS1635_8 (with G→A mutation) and a wild type *mtrR* allele pro_NEIS1635_6 were included as control sequences.

A39T amino acid substitution was identified in 33 of the 41 isolates (Figure 4.14). Twenty two isolates harbouring A39T amino acid substitution were penicillin resistant, 9 had intermediary penicillin MICs while one was penicillin susceptible (Table 4.6, Figure 4.14). There was no significant relationship between MtrR A39T substitution and penicillin resistance in the present study ($p$ =0.4677).

H105Y amino acid substitution was identified in 4 isolates. Three of these 4 isolates harboured T86A parallel substitution, one of which was penicillin resistant while the remaining 2 had intermediary penicillin MICs (Figure 4.17). Two of the 3 isolates expressing T86A substitution, had an additional D79N mutation. Isolates expressing T86A, D79N, and H105Y substitutions lacked A39T amino acid substitution. Two penicillin resistant isolates, KNY_NGAMR5 and KNY_NGAMR10 had A29P and C66Y amino acid substitutions which both have not yet been associated with gonococcal drug resistance (Figure 4.14).

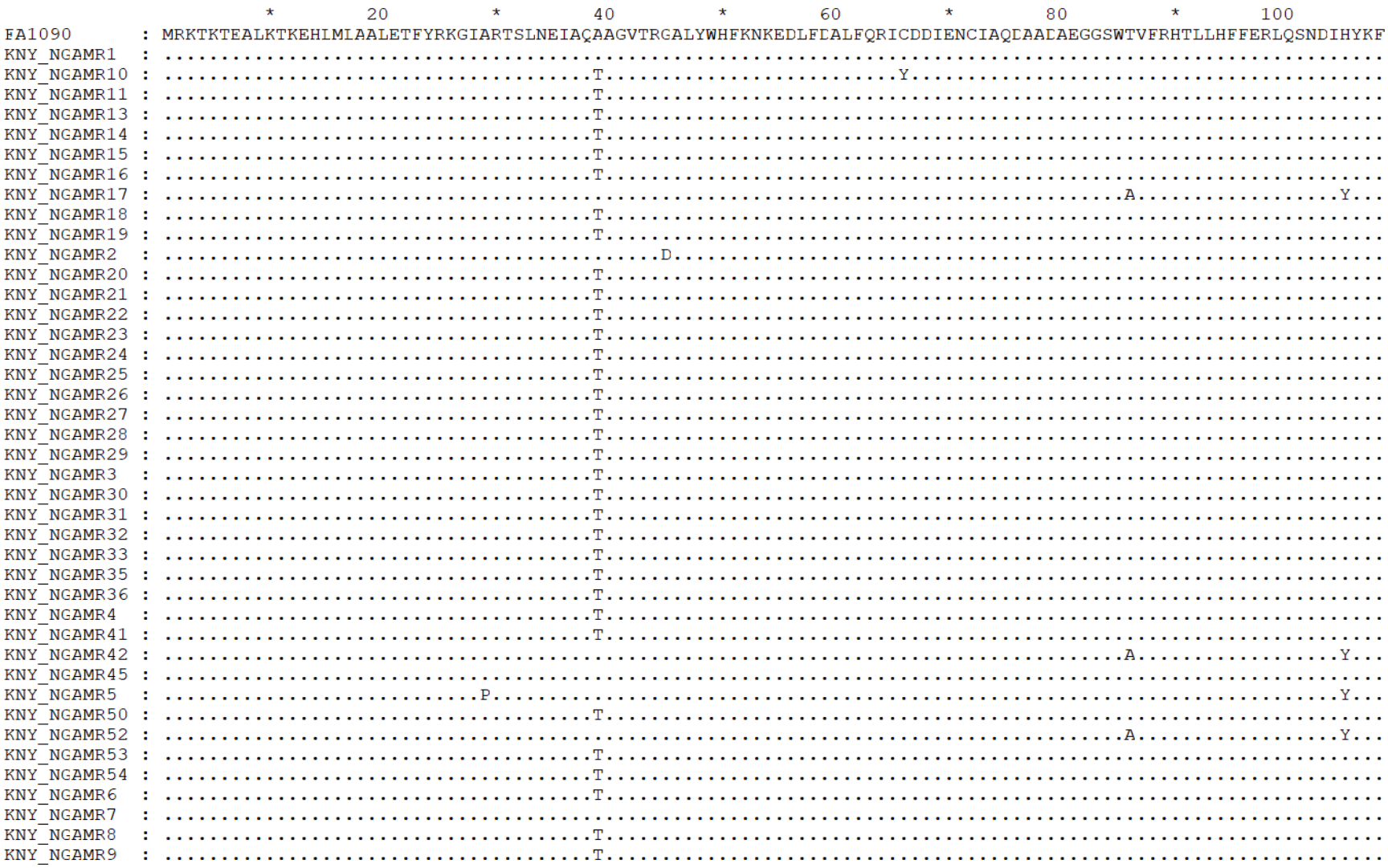



**Figure 4.14: Amino acid changes in MtrR.**

A wild type MtrR from *N. gonorrhoeae* strain FA 1090 is included as the control sequence in Figure 4.14

### v. PenC amino acid changes

E666K amino acid substitution in PilQ, also known as PenC was not observed in any of the study isolates.

### vi. β-Lactamase

Of the 41 isolates, 29 (71%) had β-lactamase encoding plasmids and were therefore penicillinase producing *N. gonorrhoeae* (Table 4.6). Two genotypes of β-lactamases were identified; TEM-1 encoded by *blaTEM-1* gene was identified in 23 of the 29 PPNG, and a new β-lactamase encoded by a recently described *blaTEM-239* allele (NEIS2357 allele 10) (Cehovin et al., 2018) was identified in the remaining 6 PPNG (Table 4.6). This new *blaTEM-239* allele which has only been described in Kenyan *N. gonorrhoeae* isolates and has a Q5 insertion, and a single H6Y amino acid substitution (Figure 4.15).

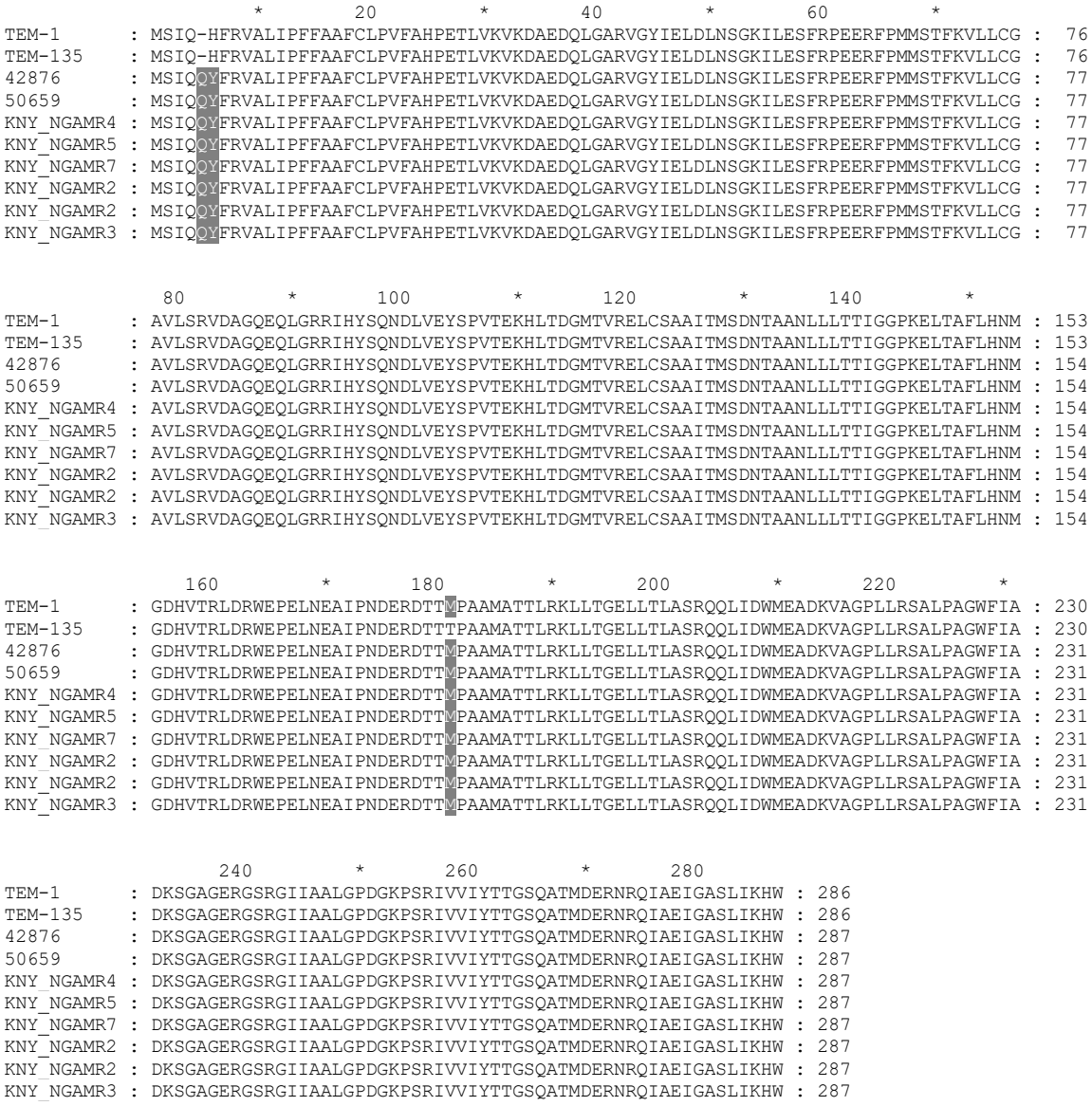



**Figure 4.15: Q5 insertion and H6Y amino acid substitution in TEM-239.**

In Figure 4.15, the alignment is formed by: TEM-1; TEM-135; and eight TEM-239s, six of which are from the current study (KNY_NGAMR) and two from the Coastal Kenya study (50659, and 42876).

All the new *blaTEM* alleles were carried by African type TEM plasmids (pDJ5), while the *blaTEM-1* genes were carried by both African (pDJ5) and Asian (pDJ4) type TEM

plasmids. The sequence of the new *blaTEM-239* allele was deposited under GenBank accession number MK497256l and assigned as class A β-lactamase TEM-239 (*blaTEM*) gene, *blaTEM-239* allele with a protein accession number QBC36181 (Kivata et al., 2020).

Twenty four PPNG were penicillin resistant (MIC>1 mg/L), while 3 had intermediary penicillin resistance (MIC>0.06-1mg/L). The three isolates with intermediary penicillin MICs expressed TEM-1 (n=2) and TEM-239 (n=1) indicating that they were slow penicillinase producing isolates. Two of the 12 non-PPNG were penicillin resistant. One of the two penicillin resistant non-PPNGs, KNY_NGAMR33 expressed altered PenA, MtrR and PorB while KNY_NGAMR54 expressed altered PenA, and MtrR (Table 4.6).

Penicillin MICs of isolates expressing β-lactamase (M=47.96, ±SEM=15.13) were significantly higher (t(37)=2.058, $p$ = 0.0467*) compared to those of the non PPNG (M=0.8870, ±SEM=0.5220) (Figure 4.16).

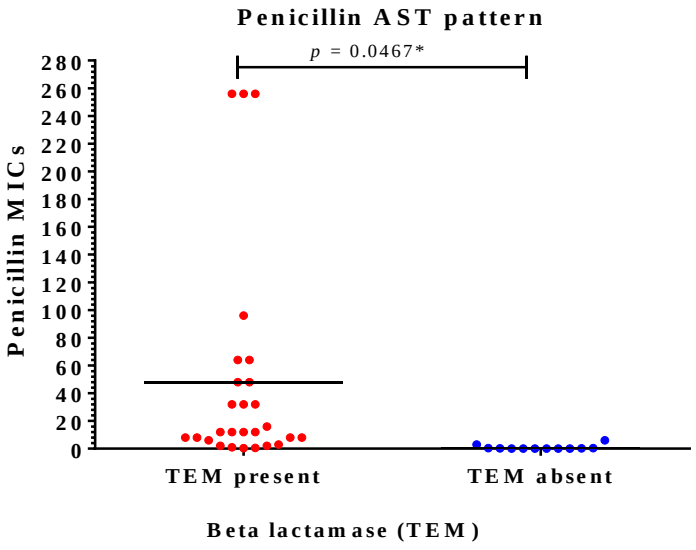
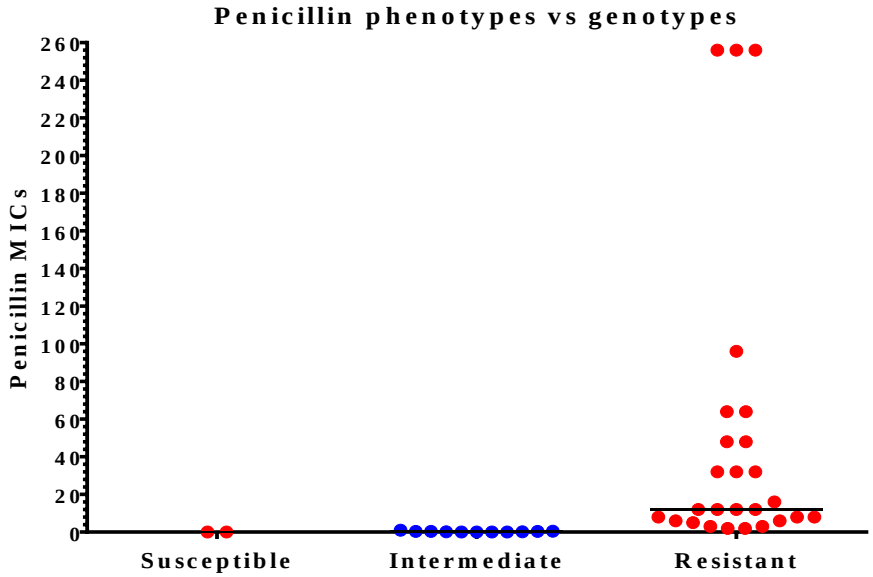



**Figure 4.16: Effects of β-lactamase on penicillin susceptibility.**
The central bars across each group of points locate the mean for that group.




| | | | |
|---|---|---|---|
| PenA | XIV: n=2 | XXII: n=1<br>XIX: n=1<br>XIV: n=7<br>II: n=2 | XXII: n=2<br>XIX: n=4<br>XIV: n=10<br>II: n=6<br>**IX**: n=4 |
| PonA | L421P: n=1 | L421P: n=4 | L421P: n=13 |
| PenB | II: n=2 | II: n=1<br>III: n=1<br>V: n=4 | **I:** n=1<br>II: n=7<br>III: n=1<br>**IV**: n=3<br>V: n=5 |
| MtrR | A39T: n=1 | A39T: n=9<br>T86A: n=2<br>D79N: n=1<br>H105Y: n=2<br>G→A (pro): n=1 | A39T: n=22<br>T86A: n=1<br>D79N: n=1<br>H105Y: n=2<br>G45D,-A13: n=1 |
| TEM | – | TEM1: n=2<br>TEM239: n=1 | TEM1: n=20<br>TEM239: n=4 |

**Figure 4.17: Association of penicillin MICs with identified AMR determinants.**

In Figure 4.17 above, PorB pattern I and IV, and PenA pattern IX amino acid changes were identified only in penicillin resistant isolates (bolded and italicized in the Figure 4.17). Three isolates with intermediary penicillin MICs expressed TEM-1 (n=2) and TEM-239 (n=1)

### 4.3.2 Tetracyclines

The tested tetracycline antibiotics included; tetracycline (TET) and doxycycline (DOX). Analysis of the previously determined Antimicrobial Susceptibility Test (AST) data (Appendix I) showed that, 33 (84.6%) of the tested isolates were tetracycline resistant, 1 (2.6%) had intermediary resistance while 5 (12.8%) were tetracycline susceptible (Appendix XII).

Of the 41 isolates, 34 (82.9%) expressed plasmid borne ribosomal protection TetM protein. They were all tetracycline resistant (MIC >1mg/L) and had increased doxycycline MICs (up to 96mg/L) (Table 4.7, Figure 4.21). Isolates lacking TetM had intermediary to susceptible tetracycline MICs (0.064-0.75mg/L) (Figure 4.21). Dutch type TetM was only identified in one isolate while American type TetM was identified in 33 isolates. Isolates expressing TetM had significantly higher tetracycline (M=22.67, ±SEM = 2.367, t (37) = 3.962, $p$ = 0.0003*) and doxycycline MICs (M=18.24, ±SEM = 2.705, t (37) = 2.722, $p$ = 0.0098*) when compared to the tetracycline (M = 0.4273, ±SEM = 0.0972) and doxycycline MICs (M = 0.7817, ±SEM = 0.1821) of non-TetM expressing isolates (Figure 4.18).

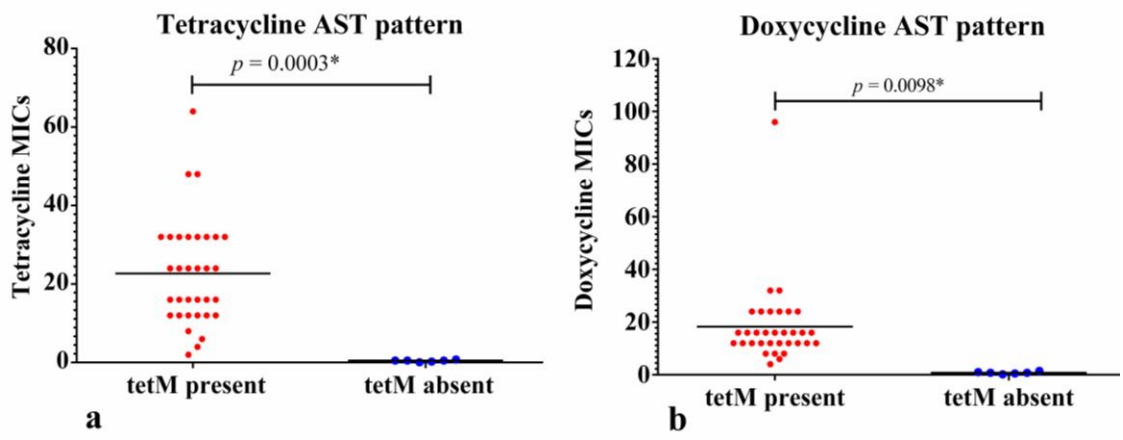



**Figure 4.18: Effects of TetM on; Tetracycline (a) and doxycycline (b) susceptibilities.**

The central bars across each group of points locates the mean for that group

One isolate, KNY_NGAMR19 which had a Dutch TetM determinant had the highest doxycycline and tetracycline MICs (96mg/L and 64mg/L respectively) (Table 4.7). Chromosomal V57M amino acid substitution was identified in S10 ribosomal protein of all isolates both the tetracycline susceptible and tetracycline resistant (Figure 4.19).

```
                    60        *       80        *       100
FA1090      : ILRSPHVNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR18 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR6  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR14 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR11 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR10 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR1  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR5  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR52 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR9  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR16 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR3  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR13 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR4  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR54 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR2  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR21 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR31 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR45 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR19 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR27 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR41 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR17 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR26 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR20 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR35 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR7  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR23 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR24 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR28 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR32 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR30 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR29 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR25 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR53 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR15 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR22 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR42 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR36 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR33 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR50 : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
KNY_NGAMR8  : ILRSPHMNKTSREQLEIRTHLRLMDIVDWTDKTTDALMKLDLPAGVDVEI : 100
```

**Figure 4.19: V57M amino acid substitution in S10 ribosomal protein.**

In Figure 4.19, a wild type S10 ribosomal protein from *N. gonorrhoeae* strain FA 1090 is included as the control sequence

PorB patterns III, IV and V (Appendix XI) were only observed in tetracycline resistant isolates (Figure 4.21). No significant increase in tetracycline (M=16.31, ±SEM = 2.615, t (37) = 1.682, $p$ = 0.1009) and doxycycline MICs (M=12.96, ±SEM = 1.728, t (37) = 1.406, $p$ = 0.1681) was observed in the isolates harbouring PorB amino acid changes when compared to tetracycline (M=24.48, ±SEM = 4.524) and doxycycline MICs (M=20.20, ±SEM = 6.208) of isolates without the described PorB alterations (Figure 4.20).

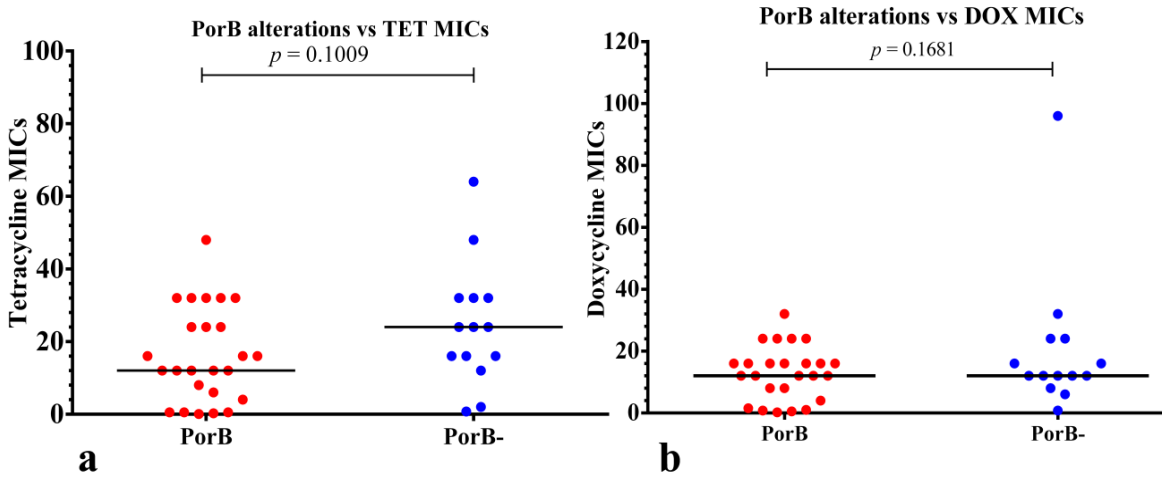


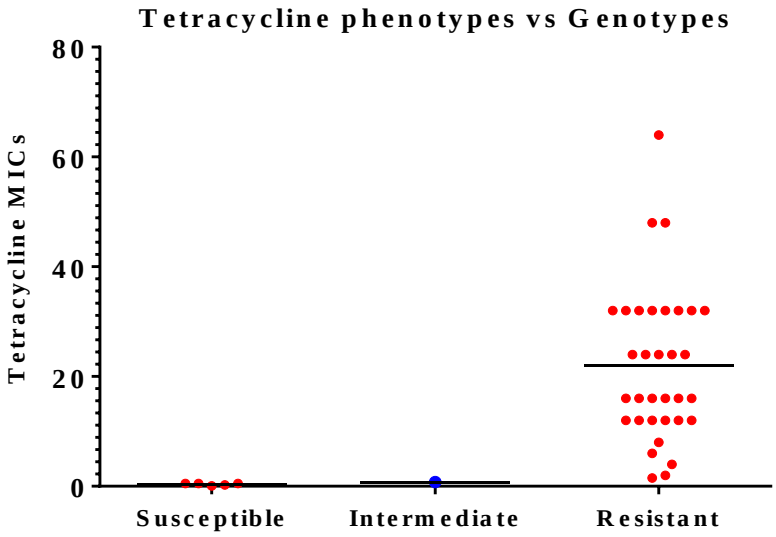

**Figure 4.20: Effects of altered PorB on; tetracycline (a) and doxycycline (b) susceptibilities.**

The central bars across each group of points locate the mean for that group. PorB indicates presence of alterations while PorB- indicates absence of alterations in porinB

**Table 4.7: AMR determinants mediating resistance to tetracyclines.**

| | MICs (mg/L) | | Genetic determinants of antibiotic resistance | | | |
|---|---|---|---|---|---|---|
| Sample ID | DOX | TET | TetM | S10 | PorB | mtrR |
| KNY_NGAMR1 | 0.75 | 0.064 | *Absent* | V57M | A121G, G120D, -N122 | - |
| KNY_NGAMR2 | 1.5 | 0.5 | *Absent* | V57M | G120D | G45D, -A13 ((Pro) |
| KNY_NGAMR3 | 6 | 16 | Present | V57M | - | A39T |
| KNY_NGAMR4 | 16 | 12 | Present | V57M | A121S, N122K | A39T |
| KNY_NGAMR5 | 12 | 16 | Present | V57M | A121G, G120N, -N122 | H105Y |
| KNY_NGAMR6 | 0.75 | 0.75 | *Absent* | V57M | - | A39T |
| KNY_NGAMR7 | 12 | 16 | Present | V57M | A121S, N122K | - |
| KNY_NGAMR8 | 0.5 | 0.5 | *Absent* | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR9 | 16 | 32 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR10 | 24 | 32 | Present | V57M | - | A39T |
| KNY_NGAMR11 | 12 | 12 | Present | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR13 | 12 | 12 | Present | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR14 | 8 | 24 | Present | V57M | A121S, N122K | A39T |
| KNY_NGAMR15 | 16 | 32 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR16 | 8 | 4 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR17 | 12 | 16 | Present | V57M | - | T86A, D79N, H105Y |
| KNY_NGAMR18 | 32 | 24 | Present | V57M | - | A39T |
| KNY_NGAMR19* | 96 | 64 | Present | V57M | - | A39T |
| KNY_NGAMR20 | 16 | 24 | Present | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR21 | 16 | 24 | Present | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR22 | 12 | 32 | Present | V57M | - | A39T |
| KNY_NGAMR23 | 16 | 8 | Present | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR24 | 16 | 16 | Present | V57M | - | A39T |
| KNY_NGAMR25 | 12 | 24 | Present | V57M | - | A39T, G→A (Pro) |
| KNY_NGAMR26 | 24 | 32 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR27 | 16 | 32 | Present | V57M | - | A39T |
| KNY_NGAMR28 | 24 | 32 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR29 | 4 | 12 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR30 | 12 | 12 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR31 | 24 | 48 | Present | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR32 | 1 | 0.5 | *Absent* | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR33 | 24 | 32 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR35 | 24 | 48 | Present | V57M | - | A39T |
| KNY_NGAMR36 | 0.19 | 0.25 | *Absent* | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR41 | 12 | 12 | Present | V57M | - | A39T |
| KNY_NGAMR42 | 12 | 24 | Present | V57M | - | T86A, D79N, H105Y |
| KNY_NGAMR45 | NT | NT | Present | V57M | - | - |
| KNY_NGAMR50 | 32 | 16 | Present | V57M | A121G, G120D, -N122 | A39T |
| KNY_NGAMR52 | NT | NT | *Absent* | V57M | A121G, G120N, -N122 | T86A, H105Y |
| KNY_NGAMR53 | 16 | 6 | Present | V57M | A121G, -N122 | A39T |
| KNY_NGAMR54 | 8 | 2 | Present | V57M | - | A39T |

The following indicate: DOX, Doxycycline, TET, tetracycline; (*), Dutch TetM; (-), No changes observed; NT, not tested; -N122, deletion of N at position 122; -A13, Adenine deletion in the *mtrR* promoter; and Pro, promoter mutation




| | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| ***rpsJ*(S10)** | V57M: n=5 | V57M: n=1 | V57M: n=33 |
| **TetM** | - | - | Dutch: n=1<br>American: n=32 |
| **PenB** | I: n=1<br>II: n=4 | - | II: n=7<br>**III**: n=1<br>**IV**: n=3<br>**V**: n=9 |

**Figure 4.21: Association of tetracycline MICs with identified AMR determinants**

### 4.3.3 Fluoroquinolones

The tested fluoroquinolone antibiotics included; ciprofloxacin (CIP) and norfloxacin (NOR). Analysis of the previously determined Antimicrobial Susceptibility Test data (Appendix I) showed that, 38 (95%) of the tested isolates were ciprofloxacin resistant (MICs > 0.06mg/L), none had intermediary resistance (MIC >0.03-0.06 mg/L) while 2 (5%) were ciprofloxacin susceptible (MICs ≤ 0.03mg/L) (Appendix XIII)

Double GyrA amino acid substitutions; S91F and D95G/A were identified in 39 of the 41 isolates (Figure 4.22). Thirty eight of the 39 isolates were ciprofloxacin resistant with MICs above 0.38mg/L (Figure 4.26). The two ciprofloxacin susceptible isolates (KNY_NGAMR2 and KNY_NGAMR4) lacked QRDR mutations and had wild type

GyrA and ParC (Table 4.8, Figure 4.26). Of the 39 isolates with double GyrA amino acid substitutions, 29 had an additional E91G or S87R ParC amino acid substitution (Figure 4.23). E91G amino acid substitution was the most common ParC mutation and was identified in 26 isolates, while S87R was identified in 3 isolates (Figure 4.23).

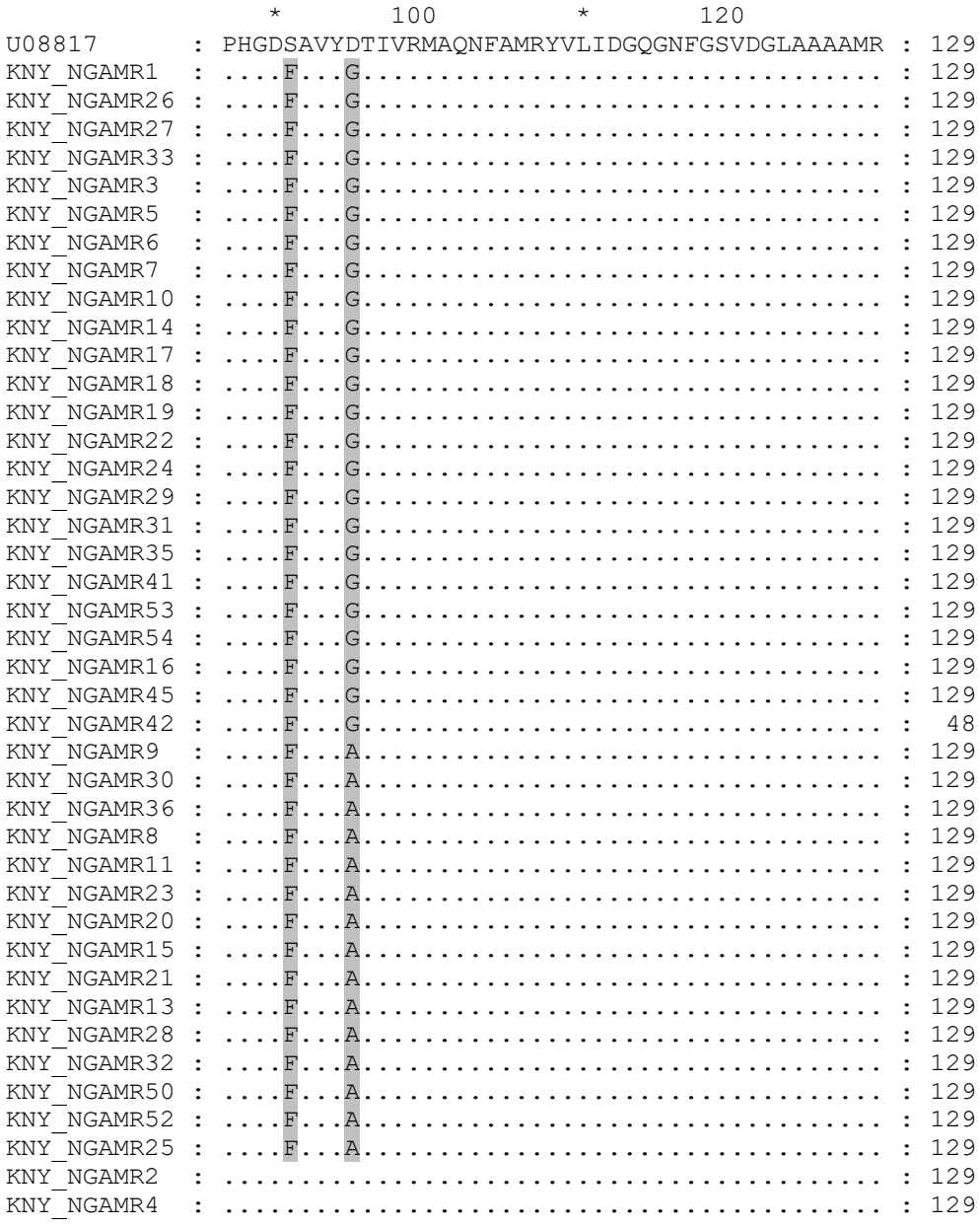



**Figure 4.22: S91F and D95G/A amino acid changes in GyrA.**

A wild type GyrA (accession no. U08817) is included as the control sequence in Figure 4.22.

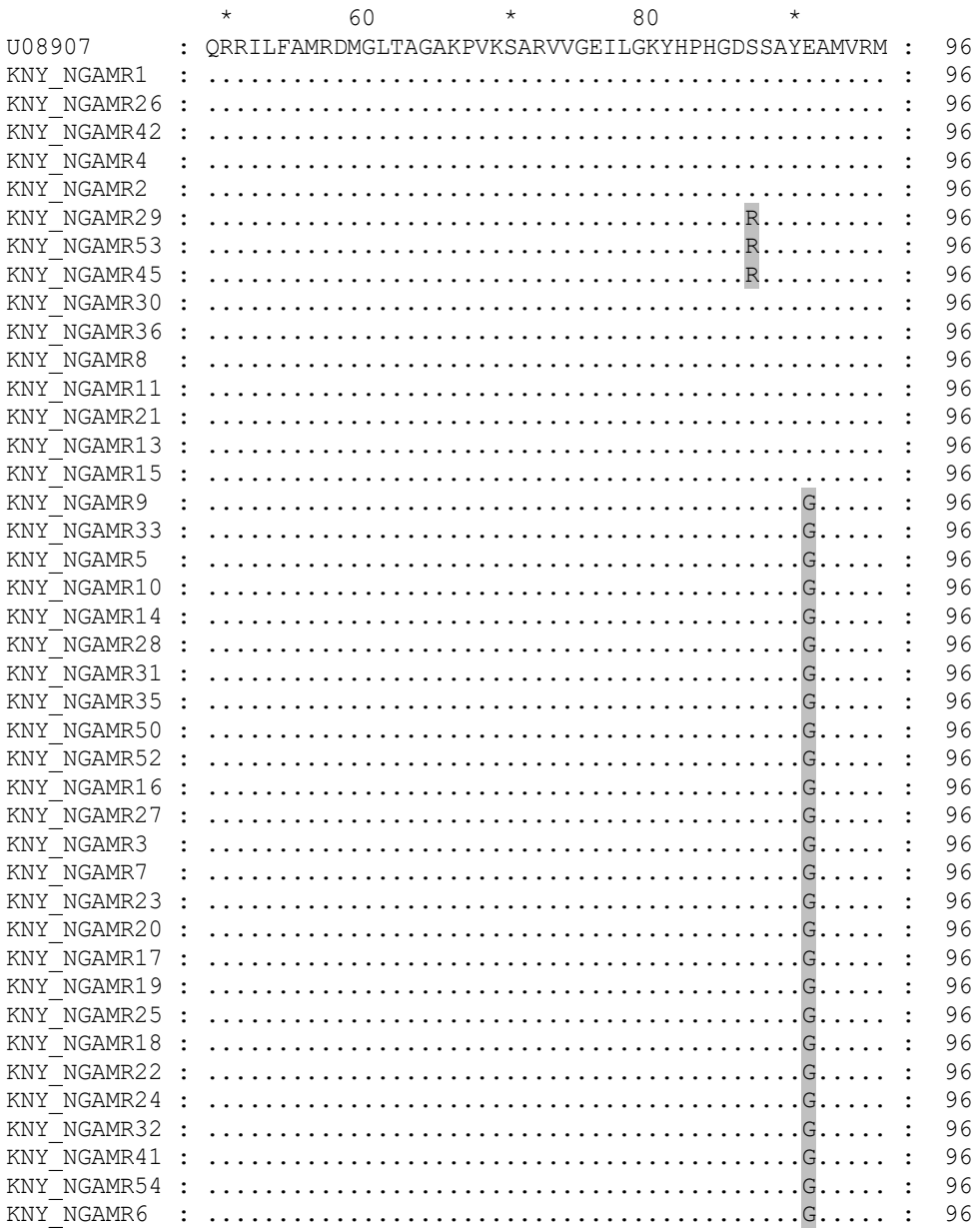



**Figure 4.23: E91G and S87R amino acid changes in ParC.**

A wild type ParC (accession no. U08907) is included as the control sequence in Figure 4.23.

Ciprofloxacin MICs for isolates with double GyrA QRDR mutations (M=3.588, ±SEM = 0.6803) were significantly higher (t (10) = 2.263, $p$ = 0.0472*) compared to MICs of isolates expressing wild-type GyrA and ParC (M=0.0110, ±SEM = 0.0050). Additionally, compared to the ciprofloxacin MICs of isolates with double GyrA QRDR mutations alone (M=3.588, ±SEM = 0.6803), the MICs of isolates with an additional ParC mutation (M=11.00, ±SEM = 1.844) were significantly higher (t (36) = 2.360, $p$ = 0.0238*) (Figure 4.24).

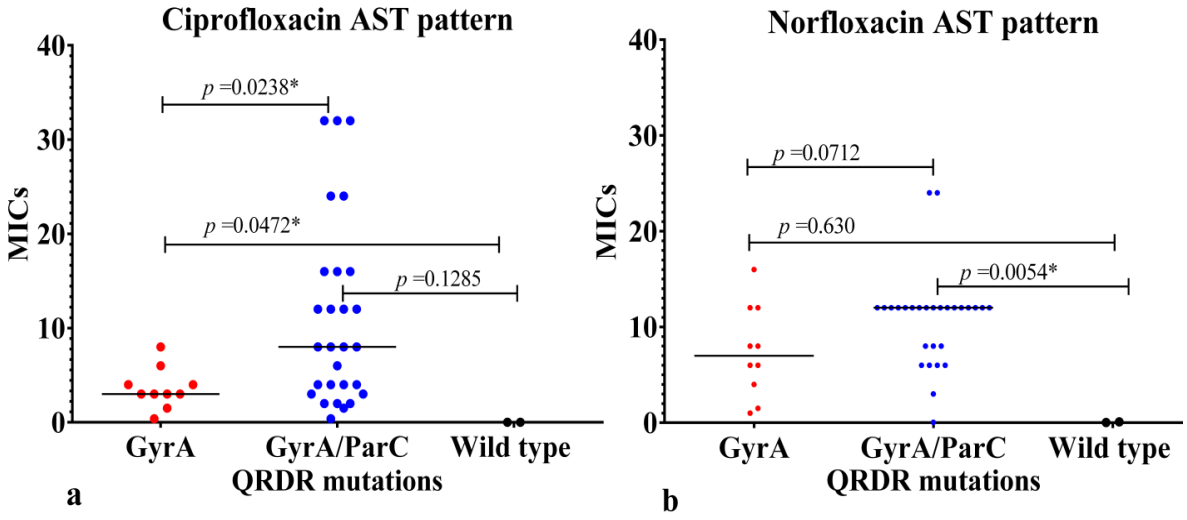



**Figure 4.24: Effects of altered QRDR on ciprofloxacin (a) and norfloxacin (b) susceptibilities.**

In Figure 4.24, the central bars across each group of points locates the mean for that group

Norfloxacin MICs of isolates expressing both GyrA and ParC QRDR mutations (M=10.82, ±SEM = 0.980) were significantly different (t (28) = 3.018, $p$ = 0.0054*) from those of isolates with wild-type QRDR (M=0.0585, ±SEM = 0.0355). There was no significant difference (t (36) = 1.859, $p$ = 0.0712) between norfloxacin MICs of isolates expressing double GyrA QRDR mutations (M=7.450, ±SEM = 1.521) and those expressing both GyrA and ParC QRDR mutations (M=10.82, ±SEM = 0.9380) (Figure 4.24).

None of the isolates had any single GyrA amino acid substitution in the QRDR and there was no isolate containing a ParC mutation without the simultaneous presence of double GyrA mutations. No mutations were found at G85, D86, S87, S88, and R116 of ParC as reported by other studies from other countries (Bodoev & Il'Ina, 2015; Sood et al., 2017). Additional amino acid substitutions found outside the QRDR regions in both GyrA and ParC proteins which have not yet been associated with gonococcal fluoroquinolone resistance include; GyrA (M250I), and ParC (A156T, P289L, V359M, I384V, A435V, F479L, and I596V) (Table 4.12). No significant increase in ciprofloxacin (M=8.477, ±SEM = 1.779, t (38) = 0.1109, $p$ = 0.9123) and norfloxacin MICs (M=9.485, ±SEM = 1.195, t (38) = 0.069, $p$ = 0.9456) was observed in the isolates harbouring PorB amino acid changes (described in sub-section 4.3.1) when compared to ciprofloxacin (M=8.813, ±SEM = 2.474) and norfloxacin MICS (M=9.361, ±SEM = 1.085) of isolates without the described PorB alterations (Figure 4.25).

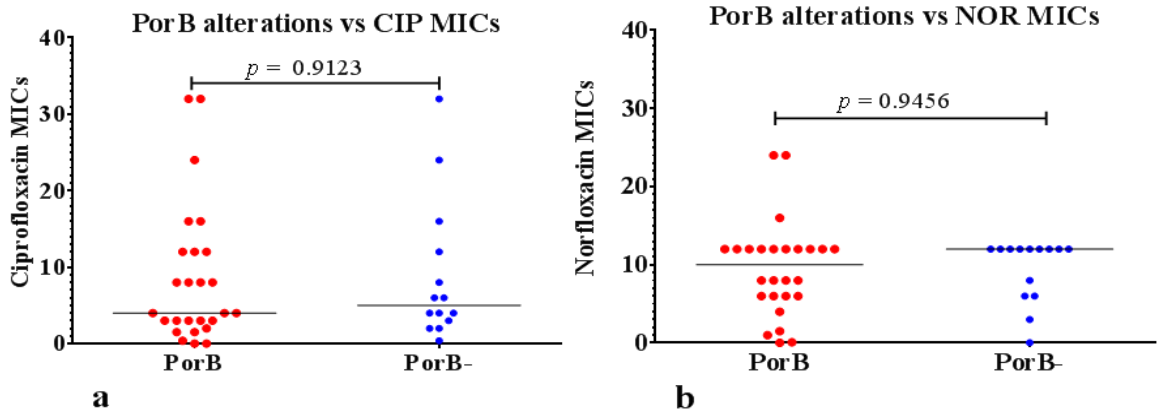



**Figure 4.25: Effects of altered PorB on ciprofloxacin (a) and norfloxacin (b) susceptibilities.**

In Figure 4.25, the central bars across each group of points locate the mean for that group. PorB indicates presence of alterations while PorB- indicates absence of alterations in porinB

ParE G410V amino acid substitution previously associated with quinolone resistance was not identified in any isolate as all isolates had wild type ParE.

**Table 4.8: AMR determinants mediating resistance to fluoroquinolones.**

| | MICs (mg/L) | | Genetic determinants of antibiotic resistance | | | |
|---|---|---|---|---|---|---|
| Sample ID | NOR | CIP | GyrA | ParC | PorB | mtrR |
| KNY_NGAMR1 | 1.5 | 0.38 | S91F,D95G | WT | A121G,G120D,-N122 | - |
| KNY_NGAMR2 | 0.094 | 0.016 | WT | WT | G120D | G45D,-A13(Pro) |
| KNY_NGAMR3 | 6 | 3 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR4 | 0.023 | 0.006 | WT | WT | A121S,N122K | A39T |
| KNY_NGAMR5 | 6 | 4 | S91F,D95G | E91G | A121G,G120N,-N122 | H105Y |
| KNY_NGAMR6 | 12 | 12 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR7 | 12 | 8 | S91F,D95G | E91G | A121S,N122K | - |
| KNY_NGAMR8 | 6 | 3 | S91F,D95A | WT | A121G,G120D,-N122 | A39T |
| KNY_NGAMR9 | 24 | 12 | S91F,D95A | E91G | A121G,-N122 | A39T |
| KNY_NGAMR10 | 12 | 8 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR11 | 8 | 3 | S91F,D95A | WT | A121G,G120D,-N122 | A39T |
| KNY_NGAMR13 | 12 | 3 | S91F,D95A | WT | A121G,G120D,-N122 | A39T |
| KNY_NGAMR14 | 24 | 16 | S91F,D95G | E91G | A121S,N122K | A39T |
| KNY_NGAMR15 | 16 | 4 | S91F,D95A | WT | A121G,-N122 | A39T |
| KNY_NGAMR16 | 6 | 8 | S91F,D95G | E91G | A121G,-N122 | A39T |
| KNY_NGAMR17 | 12 | 6 | S91F,D95G | E91G | - | T86A,D79N,H105Y |
| KNY_NGAMR18 | 0.047 | 0.38 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR19 | 8 | 4 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR20 | 12 | 8 | S91F,D95A | E91G | A121G,G120D,-N122 | A39T |
| KNY_NGAMR21 | 8 | 3 | S91F,D95A | WT | A121G,G120D,-N122 | A39T |
| KNY_NGAMR22 | 12 | 4 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR23 | 12 | 12 | S91F,D95A | E91G | A121G,G120D,-N122 | A39T |
| KNY_NGAMR24 | 12 | 4 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR25 | 12 | 2 | S91F,D95G | E91G | - | A39T,G→A(Pro) |
| KNY_NGAMR26 | 1 | 1.5 | S91F,D95G | WT | A121G,-N122 | A39T |
| KNY_NGAMR27 | 12 | 24 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR28 | 12 | 24 | S91F,D95A | E91G | A121G,N122 | A39T |
| KNY_NGAMR29 | 12 | 12 | S91F,D95G | S87R | A121G,-N122 | A39T |
| KNY_NGAMR30 | 4 | 4 | S91F,D95A | WT | A121G,-N122 | A39T |
| KNY_NGAMR31 | 12 | >32 | S91F,D95G | E91G | A121G,G120D,-N122 | A39T |
| KNY_NGAMR32 | 8 | 16 | S91F,D95A | E91G | A121G,G120D,-N122 | A39T |
| KNY_NGAMR33 | 8 | 2 | S91F,D95G | E91G | A121G,-N122 | A39T |
| KNY_NGAMR35 | 6 | 32 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR36 | 6 | 8 | S91F,D95A | WT | A121G,G120D,-N122 | A39T |
| KNY_NGAMR41 | 12 | 16 | S91F,D95G | E91G | - | A39T |
| KNY_NGAMR42 | 12 | 6 | S91F,D95G | WT | - | T86A,D79N,H105Y |
| KNY_NGAMR45 | NT | NT | S91F,D95G | S87R | - | - |
| KNY_NGAMR50 | 12 | 1.5 | S91F,D95A | E91G | A121G,G120D,-N122 | A39T |
| KNY_NGAMR52 | 12 | >32 | S91F,D95A | E91G | A121G,G120N,-N122 | T86A,H105Y |
| KNY_NGAMR53 | 12 | 3 | S91F,D95G | S87R | A121G,-N122 | A39T |
| KNY_NGAMR54 | 3 | 2 | S91F,D95G | E91G | - | A39T |

The following indicate: CIP, ciprofloxacin; NOR, norfloxacin; (-), No changes observed; NT, not tested; -N122, deletion of N at position 122; -A13, Adenine deletion in the *mtrR* promoter; and Pro, promoter mutation

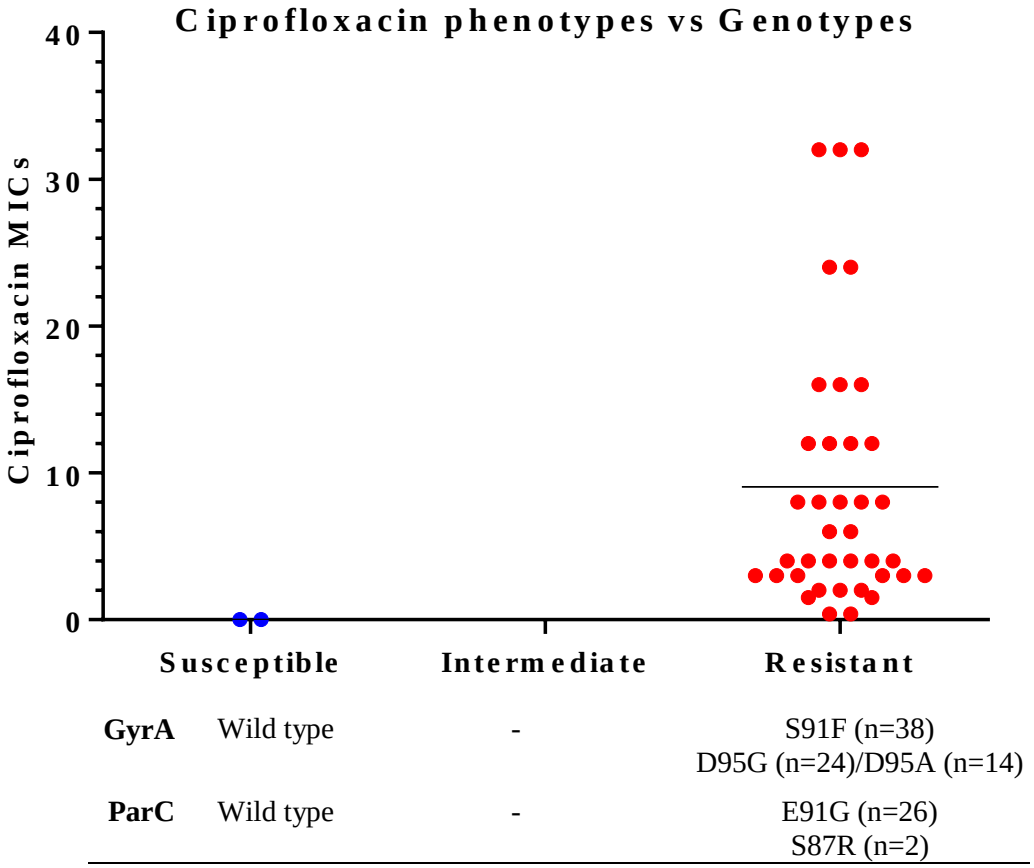



**Figure 4.26: Association of ciprofloxacin MICs with identified AMR determinants.** The central bars across each group of points locate the mean for that group.

### 4.3.4 Macrolides

The tested macrolide antibiotic was azithromycin (AZM). Analysis of the previously determined Antimicrobial Susceptibility Test data (Appendix I) showed that, 3 (7.7%) of the tested isolates had low levels of azithromycin resistance (MICs >0.5mg/L), 8 (20.5%) had intermediary resistance (MIC > 0.25-0.5mg/L) while 28 (71.8%) were azithromycin susceptible (MICs ≤ 0.25mg/L) (Appendix XIV).

Although reduced azithromycin susceptibility was observed, with 3 isolates having low level resistance (MIC range of 1-2mg/L), no genetic determinants specifically associated with azithromycin resistance were identified (Table 4.9). None of the isolates had rRNA

methylase encoding *erm (B/C/F)* genes which are associated with low level azithromycin resistance (Roberts et al., 1999). In addition *mefA/E* genes encoding a membrane bound efflux MefA protein which confers resistance to macrolides (Shortridge et al., 1996; Luna et al., 2000) was not identified any isolate (Table 4.9).

**Table 4.9: Association of azithromycin AST profiles with identified AMR determinants**

| | AST profile | | |
|---|---|---|---|
| AMR determinant | Susceptible | Intermediate | Resistant |
| 23S rRNA (*rrl* alleles) | Wild type | Wild type | Wild type |
| *erm(B/C/F)* | Absent | Absent | Absent |
| *mefA* | Absent | Absent | Absent |
| *rplD* (ribosomal protein L4) | Wild type | Wild type | Wild type |
| *rplV* (ribosomal protein L22) | Wild type | Wild type | Wild type |

The A2143G mutation (corresponding to A2059G in *Escherichia coli* numbering) in the *N. gonorrhoeae* 23S rRNA which is associated with high level azithromycin resistance (MICs >256mg/L - 4,096), and C2599T (corresponding to C2611T *E. coli* numbering) associated with low level azithromycin resistance (MICs > 2mg/L) were not identified in any isolate. All the isolates had wild type *rrl* genes which encode the four 23S rRNA alleles (Ng et al., 2002; Chisholm et al., 2010). Amino acid substitutions in large subunit ribosomal proteins L4 encoded by *rplD* and L22 encoded by *rplV* associated with macrolide resistance in *E. coli* and *Streptococcus pneumoniae* were also not observed in any of the study isolates

The isolate with adenine deletion (-A13) in the 13bp inverted repeat region between the -10 and -35 hexamers of the *mtrR* promoter, and MtrR G45D amino acid substitution (reported in section 0) had an intermediate azithromycin susceptibility. The four isolates harbouring T86A, D79N, and H105Y had both susceptible and intermediate susceptibility to azithromycin (Table 4.11). No significant increase in azithromycin MICs ($t(37) = 0.1588$, $p = 0.1208$) was observed in the isolates harbouring MtrR A39T amino acid substitution (M=0.2863, ±SEM = 0.05116) when compared to isolates without MtrR A39T (M=0.5357, ±SEM = 0.2511) (Figure 4.27).

No significant increase in azithromycin MICs (M=0.2906, ±SEM = 0.0727, t (37) = 0.9279, $p$ = 0.3595) was observed in the isolates harbouring PorB amino acid changes (described in sub-section 4.3.1) when compared to azithromycin MICs (M=0.4118, ±SEM = 0.1141) of isolates without the described PorB alterations (Figure 4.27). G→T mutation in the -10 hexamer (5'TAGAAT3'→5'TATAAT3') of *macAB* operon promoter associated with increased MacAB expression and consequently macrolide resistance in *N. gonorrhoeae* was not observed in any of the current study isolates.

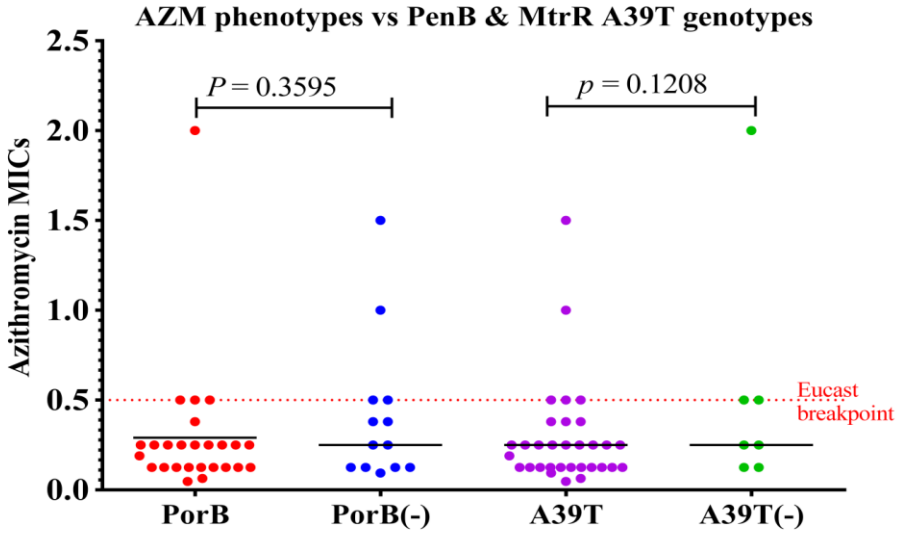



**Figure 4.27: Effects of altered PorB and MtrR (A39T) on azithromycin susceptibility.**

In Figure 4.27, the central bars across each group of points locate the mean for that group. AZM- azithromycin

### 4.3.5 Spectinomycin

Analysis of the previously determined spectinomycin (SPT) Antimicrobial Susceptibility Test data (Appendix I) showed that, all the tested isolates were spectinomycin susceptible (MICs ≤ 64mg/L) (Appendix XV).

Though increased spectinomycin MICs were observed, all isolates were susceptible (Table 4.11). No genetic determinants specifically associated with spectinomycin resistance were identified in any isolate (Table 4.10).

**Table 4.10: Association of spectinomycin MICs with identified AMR determinants**

| | AST profiles | | |
|---|---|---|---|
| AMR determinant | Susceptible | Intermediate | Resistant |
| 16S rRNA (*rrl* alleles) | Wild type | N/A | N/A |
| *rpsE*(ribosomal protein S5) | Wild type | N/A | N/A |

N/A- there are no EUCAST intermediate MIC breakpoints for spectinomycin.

V27 deletion, K28E andT24P amino acid substitutions in ribosomal protein S5 encoded by *rpsE* and associated with spectinomycin resistance (MICs of 128mg/L) (Ilina et al., 2013) were not identified (Table 4.11).

Mutations in position 1192, 1063 - 1066, and 1190 – 1193(*E. coli* numbering) in 16S rRNA *rrls* alleles associated with very high levels of spectinomycin resistance (MICs of ≥1,024 mg/L) (Galimand et al., 2000) were not identified in any isolate.

## Table 4.11: AMR determinants mediating resistance to macrolides and spectinomycin

| Sample ID | AZM | *Erm* &*mefA* | L4, L22, & 23S rRNA | SPT | GEN | 16S rRNA & S5 | PorB | MtrR |
|---|---|---|---|---|---|---|---|---|
| KNY_NGAMR1 | 0.25 | Absent | WT | 8 | 3 | WT | A121G, G120D, -N122 | - |
| KNY_NGAMR2 | 0.5 | Absent | WT | 8 | 2 | WT | G120D | G45D, -A13 (Pro) |
| KNY_NGAMR3 | 0.125 | Absent | WT | 6 | 1.5 | WT | - | A39T |
| KNY_NGAMR4 | 0.125 | Absent | WT | 2 | 2 | WT | A121S, N122K | A39T |
| KNY_NGAMR5 | 0.125 | Absent | WT | 2 | 0.75 | WT | A121G, G120N, -N122 | H105Y |
| KNY_NGAMR6 | 0.25 | Absent | WT | 3 | 2 | WT | - | A39T |
| KNY_NGAMR7 | 2 | Absent | WT | 16 | 6 | WT | A121S, N122K | - |
| KNY_NGAMR8 | 0.25 | Absent | WT | 4 | 3 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR9 | 0.25 | Absent | WT | 6 | 6 | WT | A121G, -N122 | A39T |
| KNY_NGAMR10 | 0.38 | Absent | WT | 16 | 3 | WT | - | A39T |
| KNY_NGAMR11 | 0.25 | Absent | WT | 8 | 4 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR13 | 0.125 | Absent | WT | 24 | 2 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR14 | 0.125 | Absent | WT | 8 | 2 | WT | A121S, N122K | A39T |
| KNY_NGAMR15 | 0.5 | Absent | WT | 12 | 6 | WT | A121G, -N122 | A39T |
| KNY_NGAMR16 | 0.38 | Absent | WT | 4 | 6 | WT | A121G, -N122 | A39T |
| KNY_NGAMR17 | 0.5 | Absent | WT | 6 | 12 | WT | - | T86A, D79N, H105Y |
| KNY_NGAMR18 | 0.5 | Absent | WT | 6 | 6 | WT | - | A39T |
| KNY_NGAMR19 | 1.5 | Absent | WT | 1 | 2 | WT | - | A39T |
| KNY_NGAMR20 | 0.125 | Absent | WT | 12 | 3 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR21 | 0.125 | Absent | WT | 12 | 6 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR22 | 1 | Absent | WT | 12 | 4 | WT | - | A39T |
| KNY_NGAMR23 | 0.125 | Absent | WT | 4 | 2 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR24 | 0.38 | Absent | WT | 8 | 8 | WT | - | A39T |
| KNY_NGAMR25 | 0.094 | Absent | WT | NT | 8 | WT | - | A39T, G→A (Pro) |
| KNY_NGAMR26 | 0.25 | Absent | WT | 8 | 4 | WT | A121G, -N122 | A39T |
| KNY_NGAMR27 | 0.25 | Absent | WT | 8 | 4 | WT | - | A39T |
| KNY_NGAMR28 | 0.5 | Absent | WT | 8 | 4 | WT | A121G, -N122 | A39T |
| KNY_NGAMR29 | 0.25 | Absent | WT | 2 | 4 | WT | A121G, -N122 | A39T |
| KNY_NGAMR30 | 0.125 | Absent | WT | 8 | 4 | WT | A121G, -N122 | A39T |
| KNY_NGAMR31 | 0.25 | Absent | WT | 3 | 1 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR32 | 0.047 | Absent | WT | 1.5 | 3 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR33 | 0.25 | Absent | WT | 4 | 12 | WT | A121G, -N122 | A39T |
| KNY_NGAMR35 | 0.125 | Absent | WT | NT | 8 | WT | - | A39T |
| KNY_NGAMR36 | 0.125 | Absent | WT | 1 | 1.5 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR41 | NT | Absent | WT | 4 | 4 | WT | - | A39T |
| KNY_NGAMR42 | 0.125 | Absent | WT | 8 | 1.5 | WT | - | T86A, D79N, H105Y |
| KNY_NGAMR45 | NT | Absent | WT | NT | NT | WT | - | - |
| KNY_NGAMR50 | 0.064 | Absent | WT | 4 | 3 | WT | A121G, G120D, -N122 | A39T |
| KNY_NGAMR52 | 0.25 | Absent | WT | 3 | 1.5 | WT | A121G, G120N, -N122 | T86A, H105Y |
| KNY_NGAMR53 | 0.19 | Absent | WT | 4 | 2 | WT | A121G, -N122 | A39T |
| KNY_NGAMR54 | 0.125 | Absent | WT | 6 | 4 | WT | - | A39T |

The following indicate: AZM, Azithromycin; SPT, spectinomycin; GEN, gentamycin; (-), No changes observed; NT, not tested; -N122, deletion of N at position 122; -A13, Adenine deletion in the *mtrR* promoter; and Pro, promoter mutation

### 4.3.6 Sulphonamides

Sulphonamide resistance is mainly mediated by mutations in *folP* gene which encode dihydropteroate synthase (DHPS). No antibiotic belonging to the sulphonamide drug class was tested in the STI surveillance program and therefore Antimicrobial Susceptibility Test (AST) data was not available. Nevertheless, amino acid changes in dihydropteroate synthase expressed by the study isolates were analyzed. R228S amino acid substitution in dihydropteroate synthase associated with sulphonamide resistance was identified in 40 (97.6%) of the 41 isolates (Figure 4.28). Other changes identified included T66M, P68S, E76 deletion and A81V in the DHPS protein (Table 4.12).

A summary of the antibiotic resistance determinants and their comparisons with previously described determinants is provided in Table 4.12.

```
                      *       220         *       240         *
FA1090       : QHNIALMRHLPELMAETGLPLLIGVSRKRMIGELTGEADAAARVHGSVAA : 250
KNY_NGAMR9   : ............................S..................... : 249
KNY_NGAMR5   : ............................S..................... : 249
KNY_NGAMR6   : ............................S..................... : 249
KNY_NGAMR1   : ................................................... : 250
KNY_NGAMR27  : ............................S..................... : 250
KNY_NGAMR30  : ............................S..................... : 250
KNY_NGAMR36  : ............................S..................... : 250
KNY_NGAMR45  : ............................S..................... : 250
KNY_NGAMR2   : ............................S..................... : 250
KNY_NGAMR4   : ............................S..................... : 250
KNY_NGAMR8   : ............................S..................... : 250
KNY_NGAMR11  : ............................S..................... : 250
KNY_NGAMR18  : ............................S..................... : 250
KNY_NGAMR15  : ............................S..................... : 250
KNY_NGAMR21  : ............................S..................... : 250
KNY_NGAMR22  : ............................S..................... : 250
KNY_NGAMR13  : ............................S..................... : 250
KNY_NGAMR24  : ............................S..................... : 250
KNY_NGAMR41  : ............................S..................... : 250
KNY_NGAMR42  : ............................S..................... : 250
KNY_NGAMR52  : ............................S..................... : 250
KNY_NGAMR54  : ............................S..................... : 250
KNY_NGAMR19  : ............................S..................... : 250
KNY_NGAMR26  : ............................S..................... : 250
KNY_NGAMR33  : ............................S..................... : 250
KNY_NGAMR3   : ............................S..................... : 250
KNY_NGAMR7   : ............................S..................... : 250
KNY_NGAMR10  : ............................S..................... : 250
KNY_NGAMR23  : ............................S..................... : 250
KNY_NGAMR14  : ............................S..................... : 250
KNY_NGAMR20  : ............................S..................... : 250
KNY_NGAMR17  : ............................S..................... : 250
KNY_NGAMR28  : ............................S..................... : 250
KNY_NGAMR29  : ............................S..................... : 250
KNY_NGAMR31  : ............................S..................... : 250
KNY_NGAMR32  : ............................S..................... : 250
KNY_NGAMR35  : ............................S..................... : 250
KNY_NGAMR50  : ............................S..................... : 250
KNY_NGAMR53  : ............................S..................... : 250
KNY_NGAMR16  : ............................S..................... : 250
KNY_NGAMR25  : ............................S..................... : 250
```

**Figure 4.28: R228S amino acid substitution in DHPS.**

A wild type DHPS from *N. gonorrhoeae* strain FA 1090 is included as the control sequence in Figure 4.28

**Table 4.12: Summary of identified AMR determinants.**

| Drug | Determinant | Known changes | Observed changes | Differences |
|---|---|---|---|---|
| β-lactams | PBP2 (*penA*): Mosaic *penA* alleles | Pattern X and XXIII | Not identified | No new pattern identified |
| | Non-mosaic *penA* alleles | Pattern I –IX, XI-XXII | Patterns; XXII, IX, XIX, XIV, II | |
| | PBP1 (*ponA1*) | L421P | L421P | None |
| | *mtrR* promoter | -A13bp, G→A, +T, +TT, A→C | -A13bp, G→A | None |
| | MtrR (*mtrR*) | A39T, G45D, D79N, T86A, H105Y | A39T, G45D, D79N, T86A, H105Y, A29P, C66Y | A29P, C66Y |
| | PorB (*porB1b*) | G101K, A102G, G120, A121, N122, -A121, -N122 | G120D/N, A121G/S, N122K, -N122 | |
| | PilQ (*PenC*) | E666K | Not identified | — |
| | TEM plasmid type | Asian, African, Toronto | Asian and African | |
| | *blaTEM* allele | TEM-1, TEM-135 | TEM-1, TEM-239 | TEM-239 (Q5 insertion, H6Y) |
| Fluoroquinolones | GyrA (*gyrA*) | S91 | S91F | M250I |
| | | D95 | D95G/A | A156T, P289L, V359M, I384V, A435V, F479L, and I596V |
| | ParC (*parC*) | S87 | S87R | |
| | | E9l | E91G | |
| | | G85, D86, S88, R116 | Not identified | |
| | ParE (*parE*) | G410 | Not identified | — |
| Spectinomycin | 16SrRNA (*rrl*) | bp1192 | WT | — |
| | | bp 1063-1066 | WT | — |
| | | bp 1190-1193 | WT | — |
| | Ribosomal protein S5 (*rpsE*) | T24P, -27, K28E | WT | — |
| Macrolides | 23SrRNA (*rrl*) | bp2059 | WT | — |
| | | bp2611 | WT | — |
| | rRNA methylases *(erm)* | *ermB/C/F* | Not identified | — |
| | MefA/E (Efflux pump) | *mefA/E* | Not identified | — |
| | Ribosomal protein L4 (*rplD*) | G70D, V125A, A147G, R157Q | WT | — |
| | Ribosomal protein L22 (*rplV*) | Multiple point mutations | WT | — |
| | *macAB* promoter | G→T mutation in -10 hexamer | WT | — |
| Tetracyclines | TetM (tetM) | Dutch and American | Both identified | None |
| | S10 (*rpsJ*) | V57M | V57M | None |
| Sulphonamides | DHPS (*folP*) | R228S | R228S | None |

(-) show deletions

# CHAPTER FIVE

# DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

## 5.1 Discussion

### a) Sequence characterization and phylogenetic analyses

Identification and genotypic characterization of antibiotic resistant bacterial strains through molecular typing is a key factor in monitoring the emergence, transmission and spread of gonococcal drug resistance (Unemo & Dillon, 2011). Varied sequence types indicate genetic diversity in bacterial isolates. This study identified a high number of different MLST and NG-MAST STs most of which were novel and widespread across the regions. The findings show the evolution of sequence types and adaptation of the gonococci to the Kenyan region. Most of the isolates formed singular STs indicating genetic diversity among the sampled gonococci. The observed non-regional distribution of both MLST and NG-MAST STs indicate a heterogeneous gonococcal population in Kenya. The observed heterogeneity and lack of clonal spread could possibly have resulted from the introduction of strains from multiple sources. Such genotype information is useful in informing outbreak investigations and in the identification of antimicrobial resistant gonococcal strains. As already reported in previous studies (Ilina et al., 2010), NG-MAST was more discriminatory compared to MLST since more NG-MAST STs were identified in the present study.

MLST 1901 is globally associated with resistance and decreased susceptibility to extended spectrum cephalosporins, ciprofloxacin and Gemifloxacin (Magnus Unemo et al., 2012). Likewise NG-MAST 1407 is globally associated with both azithromycin and extended spectrum cephalosporins resistance (M. Unemo et al., 2012). Neither of these two sequence types was identified in the present study signifying that they have not been introduced into the studied regions.

Analysis of genetic relatedness among the 41 study isolates based on their core genomes did not reveal any MLST or NG-MAST region-based clustering. The five identified clusters comprised isolates from different regions which belonged to varied sequence types. Despite the absence of region based clustering in the study isolates and the genetic diversity portrayed by varied NG MAST an MLST STs, the Kenyan isolates clustered closely together compared with other global isolates. These findings indicate that the Kenyan isolates are closely related and are evolving independently into their own distinct lineages.

In Japan *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone were found to be genetically closely related and belonged to NG-MAST 1407 (Shimuta et al., 2013). In Sweden *N. gonorrhoeae* isolates with reduced susceptibility to third-generation cephalosporins were found to belong to NG-MAST ST326 or ST925 (Lindberg et al., 2007). These finding suggests that gonococci with reduced cephalosporin resistance could be clonal. In the present study, 6 of the 41 isolates were found to have slightly elevated ceftriaxone and cefixime MICs. Unlike the findings by Shimuta et al, (2013) and Lindberg et al, (2007) these six strains belonged to different novel NG MAST STs and phylogenetic clusters.

### b) Characterization of antimicrobial resistance

#### i. Resistance to Beta-lactams

Mutations and recombination in gonococcal *penA* gene give rise to two alleles of the gene; mosaic *penA* and non-mosaic *penA* (Ameyama et al., 2002). Altered *penA* encode PBP2 with a decreased acylation activity and have been associated with resistance to β-lactams. The mosaic *penA* which has at least 60 amino acid changes compared to the wild type results from recombination of gonococcal *penA* genes with those of commensal Neisseria species. Mosaic *penA* and non-mosaic *peA* genes having A501 mutations cause conformational alteration of the β-lactam binding pocket. They are the

main genetic determinants that have specifically been associated with resistance to extended spectrum cephalosporins while the non-mosaic *penA* mutations lead to penicillin resistance (Ito et al., 2005; Osaka et al., 2008). Both mosaic *penA* and non-mosaic *peA* genes having A501 were not identified in any of the present study isolates which corresponds with the phenotypic data where all study isolates were susceptible to ceftriaxone and cefixime. None of the study isolates had a wild type *penA*. Contrary to the association of non-mosaic *penA* with penicillin resistance, all study isolates both penicillin resistant and susceptible had non mosaic *penA* with five different mutation patterns. Four non-mosaic *penA* patterns; XIX, IX, XIV an XXII were identified in six isolates which had slightly elevated ceftriaxone MICs (≥0.004-0.094mg/L). Pattern IX which has previously been associated with reduced cephalosporin susceptibility (Whiley et al., 2007a; Liao et al., 2011) was the only identified pattern which had a P551L alteration. All the isolates expressing this pattern were penicillin resistant. This observation supports previous results by Whiley et al, (2010) which explained the involvement of P551S/L in reduced susceptibility to ceftriaxone and penicillin. On the contrary, patterns XIV and XXII which were observed in isolates with slightly elevated ceftriaxone MICs in this study were previously found only in ceftriaxone susceptible (MIC ≤0.016mg/L) isolates from the Netherlands (de Laat et al., 2019).

In addition to the transpeptidase activity possessed by PBP2, PBP1 has an additional transglycosylase activity. Although both proteins catalyze the formation of peptide cross-links during peptidoglycan synthesis, PBP1 is inhibited by a 10 fold concentration of penicillin compared to PBP2 (Barbour, 1981). Ropp et al, (2000) for the first time reported the involvement of PonA L421P substitution in the development of penicillin resistance. Due to the structural effects of proline, the substitution of leucine with proline at position 421 of PBP1 imparts a structural perturbation which disturbs the three dimensional structure of the active site resulting in a reduced penicillin acylation and consequently resistance (Ropp et al., 2002). However, L421P substitution was not sufficient to cause a high level penicillin resistance but an additional mutated locus

(*penC*) in isolates confer high level penicillin MICs (2-4mg/L) (Ropp et al., 2002). PonA L421P mutation has also been reported in Japan leading to a higher β-lactam MICs in isolates which expressed this altered PBP1 (Shigemura et al., 2005). PonA L421P substitution was identified in the present study. Four of the six isolates which had elevated ceftriaxone MICs had this PonA substitution. This study did not find a significant association between penicillin MICs and L421P substitution. This could possibly have been contributed by the absence of *penC* mutations in the current study isolates.

Beta lactamase production in gonococci has been associated with penicillin resistance (Shaskolskiy et al., 2019). Similarly, high penicillin MICs were observed in isolates which expressed β-lactamase in the present study. Statistically, the association between the penicillin MICs and the presence of a β-lactamase was significantly high indicating that penicillin resistance in the Kenyan strains is mainly mediated by plasmid borne *blaTEM* genes. Although a statistically significant association was observed between penicillin MICs and the presence of TEM, this study also identified two non-PPNG isolates which were penicillin resistant. These two isolates expressed chromosomal modifications in *penA*, *porB* and *mtrR* which have previously been associated with penicillin resistance (Unemo & Shafer, 2014). These findings indicate that in addition to the action of β-lactamases, chromosomal modifications contribute to penicillin resistance Kenyan gonococci. Contrary to the previous findings associating β-lactamase with penicillin resistance, this study also discovered three PPNG isolates which were not resistant to penicillin. All the three isolates expressed wild type *ponA* but expressed altered *mtrR*. One of the three isolates expressed an altered *porB* while the remaining two expressed wild type *porB*. Continued surveillance and monitoring of β-lactamase production in Kenyan gonococci is required in order to better understand the susceptibility pattern observed in these three non-penicillin resistant PPNGs.

Six of the study isolates expressed a unique *blaTEM-239* which was recently described for the first time in Kenyan gonococci from Coastal Kenya and is associated with high

level of penicillin resistance (Cehovin et al., 2018). However, results from this work found one isolate expressing TEM-239 which had intermediate susceptibility to penicillin while the rest were penicillin resistant. Interestingly, this isolate lacked *ponA* and *porB* mutations indicating that high level of penicillin resistance is brought about by synergism between *blaTEM-239* and other chromosomal mutations. The more stable and potent extended spectrum β-lactamases which can breakdown cephalosporins including ceftriaxone (Wang et al., 2002), were not identified in any of the study isolates.

Two β-lactamase plasmid types of different origins (African and Asian) were identified in this study. African β-lactamase plasmid (pDJ5) which was first identified from Africa (Phillips, 1976; Dillon & Yeung, 1989), was found to pre-dominate the Asian type. This finding confirms observations of an earlier study from Coastal Kenya (Cehovin et al., 2018).

**ii. Resistance to tetracyclines**

The American type TetM is predominant in gonococci from the United Kingdom, and eastern and central Africa (Gascoyne-Binzi et al., 1992) but originated from equatorial regions of Africa (Turner et al., 1999). On the contrary, Dutch type TetM is predominant in gonococcal isolates from the Netherlands, Asia and South America (Gascoyne-Binzi et al., 1992). The present study found that in Kenya, the American TetM predominate the Dutch TetM. Kenya being in the equatorial region of Africa, the predominance of the American TetM in the present study confirms the expected epidemiology of this resistance marker. The identification of a Dutch type TetM in one of the study isolates shows introduction of gonococcal isolates expressing Dutch TetM into Kenya.

Chromosomal V57M substitution in S10 protein encoded by *rpsJ* reduces the affinity of tetracycline for its 30S ribosomal target. Together with *mtrR* and *porB* resistance determinants they cause chromosomally mediated tetracycline resistance in gonococci (MIC≥2mg/L) (Hu et al., 2005; Unemo & Shafer, 2014). In the present study all the

Kenyan isolates, both tetracycline resistant and susceptible, had the V57M. A previous study from Coastal Kenya observed similar findings and suggested that V57M substitution had no effect on tetracycline MICs (Cehovin et al., 2018). Tetracycline resistance was observed only in isolates expressing TetM. Contrary to the association of TetM presence with high-level tetracycline resistance (MIC≥16 mg/L) in gonococci (Pitt et al., 2019), this study discovered 10 TetM expressing isolates which had a lower level of tetracycline resistance (MIC range of 2-12mg/L). A high doxycycline MICs in isolates which harboured TetM was also identified. This observation is similar to the findings from a Coastal Kenya study and indicates that TetM is involved in doxycycline resistance in Kenyan gonococci (Cehovin et al., 2018).

The identification of an Asian type β-lactamase plasmid and Dutch TetM determinant in Kenyan gonococci indicates that there is circulation of plasmid mediated penicillin and tetracycline resistance between different *N. gonorrhoeae* isolates from different countries. The circulation of tetracycline resistant isolates harbouring conjugative TetM plasmids needs to be monitored since these plasmids can co-transfer other plasmids carrying β-lactamase encoding plasmids and other plasmids carrying other antimicrobial associated genes. High prevalence of plasmid mediated penicillin and tetracycline resistance observed in this study implies that the drugs are not suitable for gonorrhea treatment in Kenya.

### iii. Resistance to fluoroquinolones

Simultaneous presence of mutations in both GyrA and ParC QRDRs was associated with significantly higher ciprofloxacin and norfloxacin MICs when compared to MICs of isolates with wild-type QRDRs (Figure 4.24). Several studies have shown that GyrA mutations initiate fluoroquinolone resistance in gonococci, while additional accumulation of mutations in the ParC elevates the resistance (Belland et al., 1994; Bodoev & Il'Ina, 2015; Sood et al., 2017). Findings from this study agree with these observations. The ciprofloxacin MICs of isolates which had mutations in both GyrA and

ParC QRDRs were significantly higher compared to both the MICs of isolates which only had GyrA mutations and those which expressed wild-type QRDRs. These findings confirm the observation of a recent study in Kenya (Cehovin et al., 2018) which showed that double GyrA mutations (S91F and D95G/A) are associated with fluoroquinolone resistant gonococci.

There was no significant increase in norfloxacin MICs in isolates expressing both GyrA and ParC QRDR mutations, when compared to MICs of isolates expressing double GyrA mutations alone. The significant difference in norfloxacin MICs observed between isolates expressing both GyrA and ParC QRDR mutations and those expressing wild-type QRDRs indicate that the triple QRDR mutations could be involved in mediating norfloxacin resistance in the Kenyan gonococci (Figure 4.24). Ciprofloxacin and norfloxacin differ at position N1 of the fluoquinolone acid core, where ciprofloxacin has a cyclopropane ring and norfloxacin has an ethyl group (Sarkozy, 2001; Sharma et al., 2009). This structural difference could have contributed to the different MIC patterns observed between the two drugs in this study. G410V amino acid substitution in ParE shown to significantly increase ciprofloxacin MICs in *N. gonorrhoeae* (Lindback et al., 2002) was not observed in any of the study isolates.

Antimicrobial resistance is an expected result of the interaction of many organisms with their environment. Consequently, the observed high ciprofloxacin, penicillin and tetracycline resistance mediated by both chromosomal and plasmid borne genes could have arisen from continued use of the antibiotics. Despite the fact that the use of penicillin and tetracycline for gonorrhea treatment was stopped many years ago, they are widely available to the public and are inappropriately used through self-prescription in many parts of Africa (Kariuki, 1997; Viberg et al., 2009; Attram et al., 2019).

### iv. Resistance to macrolides

This study did not identify any antimicrobial resistance determinants specifically associated with macrolide resistance. These finding explains why a larger proportion of the study isolates were azithromycin susceptible with only 3 isolates having a low level azithromycin resistance (1-2mg/L). The observed low level azithromycin resistance could be mediated by reduced drug accumulation resulting from modified PorB and MtrR which were identified in these isolates. These observations are worrying because these three isolates had elevated ceftriaxone MICs (0.004-0.094mg/L), which together with azithromycin, are the dual therapy recommended by the Kenyan National Guidelines for treatment of gonococcal infections (NASCOP, 2018). Similar findings of an association between elevated ceftriaxone and azithromycin MICs have been reported in Canada (Martin et al., 2016). The co-evolution of cephalosporin and azithromycin resistance ought to be monitored closely since it would probably lead to failure in the treatment of gonorrheal infections in the future.

**v. Resistance to spectinomycin**

Spectinomycin is an antibiotic which is expensive and difficult to obtain. It is not commonly used and consequently resistance to it is rare. It is administered as an intramuscular injection and has poor efficacy against pharyngeal infections (Victor, 2005; Centers for Disease, 2006). However, it is safe and effective in treatment of uncomplicated urogenital and anorectal gonococcal infections (Judson et al., 1985). This study did not identify any genetic determinants of spectinomycin resistance. The results explain the lack of phenotypic resistance to spectinomycin observed in the STI study. The finding is similar to those of other studies from Europe (Cole et al., 2010; Florindo et al., 2010).

**vi. Reduced drug accumulation**

An *in-vitro* study by Tanaka et al, (1998) suggested that reduced drug accumulation in cells contribute to the development of drug resistance in gonococci. Reduced drug

accumulation can result from an active efflux system or reduced drug influx (Tanaka et al., 1996). Mutations in the promoter or encoding region of the *mtrR* gene, which leads to over expression of the MtrCDE efflux pump has been associated with resistance to antibacterial agents (Lucas et al., 1997). Common mutations in *mtrR* implicated in increased drug efflux in *N. gonorrhoeae* include: adenine deletion (A-) in the 13bp inverted repeat region between the -10 and -35 hexamers of the MtrR promoter, and A39T, G45D, and H105Y mutations in the *mtrR* structural gene (Warner et al., 2008). Warner and Shafer, (2008) demonstrated that the location of A39T in MtrR can have significant effect in binding of the repressor protein to its target thereby causing mtrCDE pump de-repression and increased drug efflux.

Inactivation of MtrCDE pump in the extensively drug resistant (XDR) H041gonococcal strain; (the first clinical XDR isolate with high level ceftriaxone resistance) was found to increase its susceptibility to penicillin, ceftriaxone azithromycin, tetracycline and solithromycin (Golparian et al., 2014). Loss of this pump in other clinical isolates led to a significant increase in extended cephalosporins, penicillin, azithromycin, ciprofloxacin and solithromycin susceptibility (Golparian et al., 2014). These results revealed that over expression of MtrCDE pump contribute to antibiotic resistance in gonococci. Although mutations in both the promoter and structural gene of MtrR were identified in this study, all the isolates were susceptible to ceftriaxone and cefixime. Findings from this work support previous studies which reported that resistance to ceftriaxone and cefixime is mainly mediated by mosaic *penA* alleles (Ameyama et al., 2002; Ito et al., 2005) and non-mosaic PenA having A501 alteration (de Laat et al., 2019).

Adenine deletion (-A13) in *mtrR* promoter was shown to decrease azithromycin susceptibility by nearly 10-fold. (Zarantonelli et al., 1999). It has also been reported to be associated with reduced cephalosporin susceptibility in gonococci (Camara et al., 2012; Magnus Unemo et al., 2012). One isolate in the present study expressing -A13 had intermediary azithromycin susceptibility which supports this observation. However, this isolate had very low ceftriaxone MIC (0.002mg/L). This observation is consistent with

prior suggestion that for MtrR mutations to elevate ceftriaxone MICs additional *penA* and *porB* mutations are required (Zhao et al., 2009). A39T amino acid substitution was the predominant mtrR alteration observed in the present study. Although this study did not find a significant association between A39T amino acid substitution and azithromycin MICs, a larger sample size is required to inform on the effect of this substitution towards the development of azithromycin resistance in Kenyan gonococci.

A study by Tanaka et al. (2006) proposed that decreased susceptibility to ceftriaxone might result from presence of a double alteration in the MtrR coding region which may lead to more efficient efflux pump production in *N. gonorrhoeae*. However, whereas double and triple alterations in the MtrR coding region were observed in this study, the isolates were susceptible to ceftriaxone and they did not even have elevated ceftriaxone MICs. Five of the six isolates with elevated ceftriaxone MICs had a single MtrR alteration while one had a wild type MtrR. In a previous study in China, MtrR T86A substitution was identified only in isolates showing reduced susceptibility to ceftriaxone (Liao et al., 2011). Contrary to this observation, the three isolates which had T86A substitution in this study were all ceftriaxone susceptible and did not even have elevated ceftriaxone susceptibility. These observations may indicate polygenic effects which may involve other suppressor mutations.

Reduced drug permeation through the porinB (PorB) protein in gonococci has been associated with substitutions with charged amino acids at G120 and A121, and deletions of A121 and N122 in PorB (Olesky et al., 2002; Olesky et al., 2006). A previous study by Olesky et al, (2002) suggested that alterations at position 120 and 121 of PorB alter its conformation at loop 3 leading to a reduction in the entry of antibiotics into the periplasmic space. Alterations at G120 with negatively charged aspartate or asparagine, A121 with glycine or serine, and N122 deletion or substitution with a positively charged lysine was found in the present study. Additionally, a high variability in the PorBs of the analyzed isolates was found. These identified mutations were expressed in five different combinations ranging from single to triple alterations per isolate.

No clear relation was detected in regard to ceftriaxone MICs since two of the six isolates with elevated ceftriaxone MICs had wild type PorBs while the others had varied *porB* alterations. In addition, *porB* alterations were identified in ceftriaxone susceptible isolates without elevated MICs. This observation contradicts earlier findings which found *porB* alterations in gonococcal isolates with reduced ceftriaxone susceptibility (Demczuk et al., 2015).

Simultaneous occurrence of both altered MtrR and PorB was demonstrated to increase both penicillin and ceftriaxone MIC by x4 and x2.5 'respectively' (Zhao et al., 2009). Such synergy between altered MtrR and PorB in reducing ceftriaxone accumulation was not observed in the present study. Only three of the six isolates with elevated ceftriaxone MICs had both altered MtrR and PorB. The rest including an isolate with highest ceftriaxone MIC (0.094mg/L) had either altered PorB or MtrR but not both. These study findings are similar to those observed in other studies (Tanaka et al., 2006; Liao et al., 2011).

Substitutions at position 120 and 121 with aspartate was shown to only confer intermediate level resistance to both penicillin and tetracycline (Olesky et al., 2002). Contrary to these former findings, several isolates harboring an aspartate substitution at position 120 in the present study had high levels of penicillin and tetracycline resistance. Additionally, this substitution was even observed in tetracycline susceptible isolates in the present study. Interestingly all isolates expressing both A121S substitution in addition to N122K substitution were all penicillin resistant indicating that these alterations could have contributed to reduced penicillin influx into the cells. One of the three isolates with low level azithromycin resistance had both of these mutations too (A121S, N122K), whereas the other two had wild type PorBs. This study did not find a significant relation between the MICs of the tested penicillin, tetracyclines, fluoroquinolones and azithromycin and the occurrence of PorB alterations.

In a previous study, novel PorB deletions at both A121 and N122 positions, were associated with high levels of both chromosomal penicillin (MIC of 4-8 mg/L) and tetracycline (MICs of 4-16 mg/L) resistance (Sun et al., 2010). It is worth noting that the present study identified deletion at only position N122. Additionally, non-tetM and non-PPNG isolates harbouring N122 PorB deletions did not have high penicillin (0.064-3mg/L) and tetracycline (0.064-0.5mg/L) MICs as reported in the previous study. Comparing our findings with those of the previous study suggests that for such high levels of both chromosomal penicillin and tetracycline resistance to occur the deletions at A121 and N122 have to occur concurrently.

## 5.2 Conclusions

This study has demonstrated the circulation of a heterogeneous gonococcal population in Kenya evidenced by non-regional distribution of both MLST and NG-MAST STs. High number of varied MLST and NG-MAST STs indicate genetic diversity in Kenyan gonococci

Kenyan *N. gonorrhoeae* strains are closely related compared to global strains and have evolved into their own distinct lineages devoid of region-specific clustering.

The high penicillin and tetracycline resistance observed in the Kenyan strains is overwhelmingly mediated by plasmid borne *blaTEM1*, a unique class of *blaTEM-239, tetM* genes as well as non-mosaic *penA* alleles and *rpsJ* chromosomal mutations. GyrA S91F, D95G/D95A and ParC E91G or S87R mutations drive the fluoroquinolone resistance in Kenyan gonococci.

The absence of molecular markers specifically associated with high levels of resistance to ceftriaxone and azithromycin (the current recommended dual therapy for treatment of gonococcal infections), shows that these antibiotics are still useful for treatment of gonococcal infections in Kenya while spectinomycin and gentamycin are suitable alternatives

## 5.3 Recommendations

Following the findings of this study it is recommended that;

The ban on use of penicillin, tetracycline and even ciprofloxacin for the treatment of gonococcal infections should be enhanced.

Continued gonococcal antimicrobial susceptibility testing and molecular surveillance is required so as to; a) understand the effects of and relation between both altered porins and efflux pumps on gonococcal AMR in Kenya, b) monitor the emergence and spread of antimicrobial resistance in Kenyan gonococci particularly to ceftriaxone and azithromycin since they are the dual therapy currently recommended by both CDC and the Kenyan National Guidelines for treatment of gonococcal infection

The generated information on circulating gonococcal strains, their MLST and NG MAST sequence types, and antimicrobial resistance determinants is useful in guiding treatment and preparedness of gonococcal infections in Kenya. In addition, the generated whole genome sequences data will be useful in evaluation of other molecular markers.

## REFERENCES


Ahrenfeldt, J., Skaarup, C., Hasman, H., Pedersen, A. G., Aarestrup, F. M., & Lund, O. (2017). Bacterial whole genome-based phylogeny: construction of a new benchmarking dataset and assessment of some existing methods. *BMC Genomics, 18*(1), 19.

Alam, M. A. (2008). The Plasmids in Gonococci. *Bangladesh Journal of Medical Microbiology, 2*(2), 35-47.

Alirol, E., Wi, T. E., Bala, M., Bazzo, M. L., Chen, X. S., Deal, C., . . . Balasegaram, M. (2017). Multidrug-resistant gonorrhea: A research and development roadmap to discover new medicines. *PLoS Medicine, 14*(7), e1002366.

Allan-Blitz, L. T., Leon, S. R., Bristow, C. C., Konda, K. A., Vargas, S. K., Flores, J. A., . . . Klausner, J. D. (2017). High prevalence of extra-genital chlamydial or gonococcal infections among men who have sex with men and transgender women in Lima, Peru. *International Journal of STD & AIDS, 28*(2), 138-144.

Allen, V. G., Mitterni, L., Seah, C., Rebbapragada, A., Martin, I. E., Lee, C., . . . Low, D. E. (2013). *Neisseria gonorrhoeae* treatment failure and susceptibility to cefixime in Toronto, Canada. *Journal of the American Medical Association, 309*(2), 163-170.

Ambler, R. P. (1980). The structure of beta-lactamases. *Philosophical Transactions of The Royal Society B Biological Sciences, 289*(1036), 321-331.

Ameyama, S., Onodera, S., Takahata, M., Minami, S., Maki, N., Endo, K., . . . Oishi, Y. (2002). Mosaic-like structure of penicillin-binding protein 2 Gene (*penA*) in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime. *Antimicrobial Agents and Chemotherapy, 46*(12), 3744-3749.

Amies, C. R. (1967). Development of resistance of gonococci to penicillin: an eight-year study. *Canadian Medical Association Journal, 96*(1), 33-35.

Argimon, S., Abudahab, K., Goater, R. J. E., Fedosejev, A., Bhai, J., Glasner, C., . . . Aanensen, D. M. (2016). Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. *Microbial Genomics, 2*(11), e000093.

Ashford, W., Golash, R., & Hemming, V. (1976). Penicilunase-Producing *Neisseria gonorrhoeae*. *Lancet, 308*(7987), 657-658.

Attram, N., Agbodzi, B., Dela, H., Behene, E., Nyarko, E. O., Kyei, N. N. A., . . . Letizia, A. G. (2019). Antimicrobial resistance (AMR) and molecular characterization of *Neisseria gonorrhoeae* in Ghana, 2012-2015. *PLoS One, 14*(10), e0223598.

Ayala, P., Vasquez, B., Wetzler, L., & So, M. (2002). *Neisseria gonorrhoeae* porin P1.B induces endosome exocytosis and a redistribution of Lamp1 to the plasma membrane. *Infection and Immunity, 70*(11), 5965-5971.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., . . . Kubal, M. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics, 9*(1), 75.

Bachmann, L. H., Johnson, R. E., Cheng, H., Markowitz, L., Papp, J. R., Palella, F. J., Jr., & Hook, E. W. (2010). Nucleic acid amplification tests for diagnosis of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* rectal infections. *Journal of Clinical Microbiology, 48*(5), 1827-1832.

Bailey, J. K., Pinyon, J. L., Anantham, S., & Hall, R. M. (2011). Distribution of the blaTEM gene and blaTEM-containing transposons in commensal *Escherichia coli*. *Journal of Antimicrobial Chemotherapy, 66*(4), 745-751.

Ball, P. (2000). Quinolone generations: natural history or natural selection? *Journal of Antimicrobial Chemotherapy, 46*(Suppl. 3), S17-24.

Balloux, F., Bronstad Brynildsrud, O., van Dorp, L., Shaw, L. P., Chen, H., Harris, K. A., . . . & Eldholm, V. (2018). From Theory to Practice: Translating Whole-Genome Sequencing (WGS) into the Clinic. *Trends in Microbiology, 26*(12), 1035-1048.

Barbour, A. G. (1981). Properties of penicillin-binding proteins in *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy, 19*(2), 316-322.

Barry, P. M., & Klausner, J. D. (2009). The use of cephalosporins for gonorrhea: the impending problem of resistance. *Expert Opinion on Pharmacotherapy, 10*(4), 555-577.

Bearden, D., & Rodvold, K. (1999). Penetration of macrolides into pulmonary sites of infection. *Infections in Medicine, 16*(7), 480-+.

Belland, R. J., Morrison, S. G., Ison, C., & Huang, W. M. (1994). *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. *Molecular Microbiology, 14*(2), 371-380.

Bignell, C., & Fitzgerald, M. (2011). UK national guideline for the management of gonorrhoea in adults, 2011. *International Journal of STD & AIDS, 22*(10), 541-547.

Bignell, C., & Unemo, M. (2013). 2012 European guideline on the diagnosis and treatment of gonorrhoea in adults. *International Journal of STD & AIDS, 24*(2), 85-92.

Bilek, N., Ison, C. A., & Spratt, B. G. (2009). Relative contributions of recombination and mutation to the diversification of the *opa* gene repertoire of *Neisseria gonorrhoeae*. *Journal of Bacteriology, 191*(6), 1878-1890.

Billker, O., Popp, A., Brinkmann, V., Wenig, G., Schneider, J., Caron, E., & Meyer, T. F. (2002). Distinct mechanisms of internalization of *Neisseria gonorrhoeae* by members of the CEACAM receptor family involving Rac1- and Cdc42-dependent and -independent pathways. *EMBO Journal, 21*(4), 560-571.

Bleich, A. T., Sheffield, J. S., Wendel, G. D., Jr., Sigman, A., & Cunningham, F. G. (2012). Disseminated gonococcal infection in women. *Obstetrics & Gynecology, 119*(3), 597-602.

Bodoev, I. N., & Il'Ina, E. N. (2015). Molecular mechanisms of formation of drug resistance in *Neisseria gonorrhoeae*: History and prospects. *Molecular Genetics, Microbiology and Virology, 30*(3), 132-140.

Borovinskaya, M. A., Shoji, S., Holton, J. M., Fredrick, K., & Cate, J. H. D. (2007). A steric block in translation caused by the antibiotic spectinomycin. *ACS Chemical Biology, 2*(8), 545-552.

Bradford, P. A. (2001). Extended-Spectrum, B -Lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews, 14*(4), 933-951.

Brannigan, J. A., Tirodimos, I. A., Zhang, Q. Y., Dowson, C. G., & Spratt, B. G. (1990). Insertion of an extra amino acid is the main cause of the low affinity of penicillin-binding protein 2 in penicillin-resistant strains of *Neisseria gonorrhoeae*. *Molecular Microbiology 4*(6), 913-919.

Brett, M. (1989). A novel gonococcal beta-lactamase plasmid. *Journal of Antimicrobial Chemotherapy*, *23*(4), 653-654.

Brunham, R. C., Fransen, L., Plummer, F., Piot, P., Slaney, L., Bygdeman, S., & Nsanze, H. (1985). Antimicrobial susceptibility testing and phenotyping of *Neisseria gonorrhoeae* isolated from patients with ophthalmia neonatorum in Nairobi, Kenya. *Antimicrobial Agents and Chemotherapy, 28*(3), 393-396.

Brunham, R. C., Gottlieb, S. L., & Paavonen, J. (2015). Pelvic inflammatory disease. *New England Journal of Medicine*, *372*(21), 2039-2048.

Bui, T., & Preuss, C. V. (2020). Cephalosporins. In. Treasure Island (FL): StatPearls Publishing LLC.

Buisine, N., Tang, C. M., & Chalmers, R. (2002). Transposon-like Correia elements: structure, distribution and genetic exchange between pathogenic Neisseria sp. *FEBS Letters, 522*(1-3), 52-58.

Bush, K., & Bradford, P. A. (2016). beta-Lactams and beta-Lactamase Inhibitors: An Overview. *Cold Spring Harbor Perspectives in Medicine 6*(8), a025247.

Camara, J., Serra, J., Ayats, J., Bastida, T., Carnicer-Pont, D., Andreu, A., & Ardanuy, C. (2012). Molecular characterization of two high-level ceftriaxone-resistant *Neisseria gonorrhoeae* isolates detected in Catalonia, Spain. *Journal of Antimicrobial Chemotherapy, 67*(8), 1858-1860.

Campos-Outcalt, D. (2007). Practice alert: CDC no longer recommends quinolones for treatment of gonorrhea: Ceftriaxone is now the recommended treatment. *The Journal of Family Practice, 56*(7), 554-557.

Cehovin, A., Harrison, O. B., Lewis, S. B., Ward, P. N., Ngetsa, C., Graham, S. M., . . . Tang, C. M. (2018). Identification of novel *Neisseria gonorrhoeae* lineages

harbouring resistance plasmids in coastal Kenya. *Journal of Infectious Diseases, 218*(5), 801-808.

Cehovin, A., & Lewis, S. B. (2017). Mobile genetic elements in *Neisseria gonorrhoeae*: movement for change. *Pathogens and Disease, 75*(6).

Centers for Disease, C., Prevention (2006). Notice to readers: discontinuation of spectinomycin. *Morbidity and Mortality Weekly Report, 55*, 370.

Centers for Disease, C., & Prevention. (2007). Update to CDC's sexually transmitted diseases treatment guidelines, 2006: fluoroquinolones no longer recommended for treatment of gonococcal infections. *Morbidity and Mortality Weekly Report, 56*(14), 332-336.

Chapman, J. S., & Georgopapadakou, N. H. (1988). Routes of quinolone permeation in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy, 32*(4), 438-442.

Chisholm, S. A., Dave, J., & Ison, C. A. (2010). High-level azithromycin resistance occurs in *Neisseria gonorrhoeae* as a result of a single point mutation in the 23S rRNA genes. *Antimicrobial Agents and Chemotherapy, 54*(9), 3812-3816.

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews, 65*(2), 232-260.

Cohen, M. S., Hoffman, I. F., Royce, R. A., Kazembe, P., Dyer, J. R., Daly, C. C., . . . Eron, J. J., Jr. (1997). Reduction of concentration of HIV-1 in semen after treatment of urethritis: implications for prevention of sexual transmission of HIV-1. AIDSCAP Malawi Research Group. *Lancet, 349*(9069), 1868-1873.

Cole, M. J., Chisholm, S. A., Hoffmann, S., Stary, A., Lowndes, C. M., & Ison, C. A. (2010). European surveillance of antimicrobial resistance in *Neisseria gonorrhoeae*. *Sexually Transmitted Infections, 86*(6), 427-432.

Committee on Gynecologic, P. (2015). ACOG Committee Opinion No. 645: Dual Therapy for Gonococcal Infections. *Obstetrics & Gynecology, 126*(5), e95-99.

Corander, J., Connor, T. R., O'Dwyer, C. A., Kroll, J. S., & Hanage, W. P. (2012). Population structure in the Neisseria, and the biological significance of fuzzy species. *Journal of the Royal Society Interface, 9*(71), 1208-1215.

Corkill, J. E., Percival, A., & Lind, M. (1991). Reduced uptake of ciprofloxacin in a resistant strain of *Neisseria gonorrhoeae* and transformation of resistance to other strains. *Journal of Antimicrobial Chemotherapy, 28*(4), 601-604.

Cornelissen, C. N. (2011). Molecular pathogenesis of *Neisseria gonorrhoeae*. *Frontiers in Microbiology 2*, 224.

Cornelissen, C. N., Kelley, M., Hobbs, M. M., Anderson, J. E., Cannon, J. G., Cohen, M. S., & Sparling, P. F. (1998). The transferrin receptor expressed by gonococcal strain FA1090 is required for the experimental infection of human male volunteers. *Molecular Microbiology, 27*(3), 611-616.

Costa-Lourenço, A. P. R. d., Barros dos Santos, K. T., Moreira, B. M., Fracalanzza, S. E. L., & Bonelli, R. R. (2017). Antimicrobial resistance in *Neisseria gonorrhoeae*: history, molecular mechanisms and epidemiological aspects of an emerging global threat. *Brazilian Journal of Microbiology, 48*(4), 617-628.

Coulson, A. (1985). ß-Lactamases: Molecular Studies. *Biotechnology and Genetic Engineering Reviews, 3*(1), 219-254.

Cousin Jr, S. L., Whittington, W. L., & Roberts, M. C. (2003). Acquired macrolide resistance genes and the 1 bp deletion in the mtrR promoter in *Neisseria gonorrhoeae*. *Journal of Antimicrobial Chemotherapy, 51*(1), 131-133.

Da Ros, C. T., & Schmitt, S. (2008). Global epidemiology of sexually transmitted diseases. *Asian Journal of Andrology, 10*(1), 110-114.

Dallabetta, G. A., Gerbase, A. C., & Holmes, K. K. (1998). Problems, solutions, and challenges in syndromic management of sexually transmitted diseases. *Sexually Transmitted Infections, 74*(Suppl. 1), S1-11.

Danielsson, D., Faruki, H., Dyer, D., & Sparling, P. F. (1986). Recombination near the antibiotic resistance locus *penB* results in antigenic variation of gonococcal outer membrane protein I. *Infection and Immunity, 52*(2), 529-533.

Davila, S., Wright, V. J., Khor, C. C., Sim, K. S., Binder, A., Breunis, W. B., . . . Hibberd, M. L. (2010). Genome-wide association study identifies variants in the CFH region associated with host susceptibility to meningococcal disease. *Nature Genetics, 42*(9), 772-776.

de Laat, M. M., Wind, C. M., Bruisten, S. M., Dierdorp, M., de Vries, H. J. C., Schim van der Loeff, M. F., & van Dam, A. P. (2019). Ceftriaxone Reduced Susceptible *Neisseria gonorrhoeae* in the Netherlands, 2009 to 2017: From PenA Mosaicism to A501T/V Nonmosaicism. *Sexually Transmitted Diseases, 46*(9), 594-601.

Demczuk, W., Lynch, T., Martin, I., Van Domselaar, G., Graham, M., Bharat, A., . . . Mulvey, M. R. (2015). Whole-genome phylogenomic heterogeneity of *Neisseria gonorrhoeae* isolates with decreased cephalosporin susceptibility collected in Canada between 1989 and 2013. *Journal of Clinical Microbiology, 53*(1), 191-200.

Demczuk, W., Sidhu, S., Unemo, M., Whiley, D. M., Allen, V. G., Dillon, J. R., . . . Martin, I. (2017). *Neisseria gonorrhoeae* Sequence Typing for Antimicrobial Resistance, a Novel Antimicrobial Resistance Multilocus Typing Scheme for Tracking Global Dissemination of *N. gonorrhoeae* Strains. *Journal of Clinical Microbiology, 55*(5), 1454-1468.

Densen, P., & Mandell, G. L. (1978). Gonococcal interactions with polymorphonuclear neutrophils: importance of the phagosome for bactericidal activity. *Journal of Clinical Investigation, 62*(6), 1161-1171.

Dillon, J. A., & Pagotto, F. (1999). Importance of drug resistance in gonococci: from mechanisms to monitoring. *Current Opinion in Infectious Diseases, 12*(1), 35-40.

Dillon, J. A., Ruben, M., Li, H., Borthagaray, G., Marquez, C., Fiorito, S., . . . Prabhakar, P. (2006). Challenges in the control of gonorrhea in South America and the Caribbean: monitoring the development of resistance to antibiotics. *Sexually Transmitted Diseases, 33*(2), 87-95.

Dillon, J. A., Trecker, M. A., & Thakur, S. D. (2013). Two decades of the gonococcal antimicrobial surveillance program in South America and the Caribbean: challenges and opportunities. *Sexually Transmitted Diseases, 89*(Suppl. 4), S36-41.

Dillon, J. A., & Yeung, K. H. (1989). Beta-lactamase plasmids and chromosomally mediated antibiotic resistance in pathogenic Neisseria species. *Clinical Microbiology Reviews*(Suppl. 2), S125-133.

Dinos, G. P. (2017). The macrolide antibiotic renaissance. *British Journal of Pharmacology, 174*(18), 2967-2983.

Dowson, C. G., Jephcott, A. E., Gough, K. R., & Spratt, B. G. (1989). Penicillin-binding protein 2 genes of non-beta-lactamase-producing, penicillin-resistant strains of *Neisseria gonorrhoeae*. *Molecular Microbiology, 3*(1), 35-41.

Dramsi, S., Davison, S., Magnet, S., & Arthur, M. (2008). Surface proteins covalently attached to peptidoglycan: examples from both gram-positive and gram-negative bacteria. *FEMS Microbiology Reviews, 32*(2), 307-320.

Drlica, K. (1999). Mechanism of fluoroquinolone action. *Current Opinion in Microbiology, 2*(5), 504-508.

Duffin Paul, M., & Seifert, H. S. (2010). DNA Uptake Sequence-Mediated Enhancement of Transformation in *Neisseria gonorrhoeae* Is Strain Dependent. *Journal of Bacteriology, 192*(17), 4436-4444.

Duncan, S., Thiong'o, A. N., Macharia, M., Wamuyu, L., Mwarumba, S., Mvera, B., . . . Sanders, E. J. (2011). High prevalence of quinolone resistance in *Neisseria gonorrhoeae* in coastal Kenya. *Sexually Transmitted Infections 87*(3), 231.

Dunlop, E. M. (1949). Gonorrhoea and the sulphonamides. *British Journal of Venereal Diseases, 25*(2), 81-83.

Easmon, C. S., Forster, G. E., Walker, G. D., Ison, C. A., Harris, J. R., & Munday, P. E. (1984). Spectinomycin as initial treatment for gonorrhoea. *British Medical Journal (Clinical research ed.) 289*(6451), 1032-1034.

Edwards, J. L., & Apicella, M. A. (2004). The molecular mechanisms used by *Neisseria gonorrhoeae* to initiate infection differ between men and women. *Clinical Microbiology Reviews, 17*(4), 965-981

Edwards, J. L., & Butler, E. K. (2011). The Pathobiology of *Neisseria gonorrhoeae* Lower Female Genital Tract Infection. *Frontiers in Microbiology, 2*, 102.

Eisenstein, B. I., Sox, T., Biswas, G., Blackman, E., & Sparling, P. F. (1977). Conjugal transfer of the gonococcal penicillinase plasmid. *Science, 195*(4282), 998-1000.

Enright, M. C., & Spratt, B. G. (1999). Multilocus sequence typing. *Trends in Microbiology, 7*(12), 482-487.

Eschenbach, D. A., Buchanan, T. M., Pollock, H. M., Forsyth, P. S., Alexander, E. R., Lin, J. S., . . . Holmes, K. K. (1975). Polymicrobial etiology of acute pelvic inflammatory disease. *New England Journal of Medicine, 293*(4), 166-171.

Etienne, E., Fayemiwo, S. A., & Lewis, D. A. (2011). Characterization of a novel β-lactamase-producing plasmid in *Neisseria gonorrhoeae*: sequence analysis and molecular typing of host gonococci. *Journal of Antimicrobial Chemotherapy, 66*, 1514-1517.

Eyre, D. W., Sanderson, N. D., Lord, E., Regisford-Reimmer, N., Chau, K., Barker, L., . . . Andersson, M. I. (2018). Gonorrhoea treatment failure caused by a *Neisseria gonorrhoeae* strain with combined ceftriaxone and high-level azithromycin resistance, England, February 2018. *Eurosurveillance, 23*(27).

Ezewudo, M. N., Joseph, S. J., Castillo-Ramirez, S., Dean, D., Del Rio, C., Didelot, X., . . . Read, T. D. (2015). Population structure of *Neisseria gonorrhoeae* based on whole genome data and its relationship with antibiotic resistance. *Peer J, 3*, e806.

Fernandes, R., Amador, P., & Prudêncio, C. (2013). β-Lactams. *Reviews in Medical Microbiology, 24*(1), 7-17.

Fiebelkorn, K. R., Crawford, S. A., & Jorgensen, J. H. (2005). Mutations in *folP* associated with elevated sulfonamide MICs for *Neisseria meningitidis* clinical

isolates from five continents. *Antimicrobial Agents and Chemotherapy, 49*(2), 536-540.

Fifer, H., Natarajan, U., Jones, L., Alexander, S., Hughes, G., Golparian, D., & Unemo, M. (2016). Failure of Dual Antimicrobial Therapy in Treatment of Gonorrhea. *New England Journal of Medicine, 374*(25), 2504-2506.

Fleming, A. (1980). On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to Their Use in the Isolation of *B. influenzae*. *Reviews of Infectious Diseases, 2*(1), 129-139.

Florindo, C., Pereira, R., Boura, M., Nunes, B., Paulino, A., Gomes, J. P., & Borrego, M. J. (2010). Genotypes and antimicrobial-resistant phenotypes of *Neisseria gonorrhoeae* in Portugal (2004-2009). *Sexually Transmitted Infections, 86*(6), 449-453.

Forest, K. T., & Tainer, J. A. (1997). Type-4 pilus-structure: outside to inside and top to bottom--a minireview. *Gene, 192*(1), 165-169.

Forsyth, S., Penney, P., & Rooney, G. (2011). Cefixime-resistant *Neisseria gonorrhoeae* in the UK: a time to reflect on practice and recommendations. *International Journal of STD & AIDS, 22*(5), 296-297.

Fox, D. A., Larsson, P., Lo, R. H., Kroncke, B. M., Kasson, P. M., & Columbus, L. (2014). Structure of the Neisserial Outer Membrane Protein Opa60: Loop Flexibility Essential to Receptor Recognition and Bacterial Engulfment. *Journal of the American Chemical Society, 136*(28), 9938-9946.

Francis, F., Ramirez-Arcos, S., Salimnia, H., Victor, C., & Dillon, J. R. (2000). Organization and transcription of the division cell wall (dcw) cluster in *Neisseria gonorrhoeae*. *Gene, 251*(2), 141-151.

Franks, A. G. (1946). Successful combined treatment of penicillin-resistant gonorrhea. *The American Journal of the Medical Sciences, 211*, 553-555.

Freitag, N. E., Seifert, H. S., & Koomey, M. (1995). Characterization of the *pilF-pilD* pilus-assembly locus of *Neisseria gonorrhoeae*. *Molecular Microbiology, 16*(3), 575-586.

Frost, L. S., Leplae, R., Summers, A. O., & Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nature Reviews Microbiology, 3*(9), 722-732.

Galimand, M., Gerbaud, G., & Courvalin, P. (2000). Spectinomycin resistance in Neisseria spp. due to mutations in 16S rRNA. *Antimicrobial Agents and Chemotherapy, 44*(5), 1365-1366.

Gascoyne-Binzi, D. M., Hawkey, P. M., Heritage, J., Turner, A., & Nadarajah, M. (1992). World-wide distribution of high level tetracycline-resistant *Neisseria gonorrhoeae*. *Genitourinary Medicine, 68*(4), 277-278.

Gascoyne-Binzi, D. M., Heritage, J., & Hawkey, P. M. (1993). Nucelotide sequences of the *tet (M)* genes from the American and Dutch type tetracycline resistance plasmids of *Neisseria gonorrhoeae*. *Journal of Antimicrobial Chemotherapy, 32*(5), 667-676.

Gascoyne, D. M., Heritage, J., Hawkey, P. M., Turner, A., & van Klingeren, B. (1991). Molecular evolution of tetracycline-resistance plasmids carrying TetM found in *Neisseria gonorrhoeae* from different countries. *Journal of Antimicrobial Chemotherapy, 28*(2), 173-183.

Georgopapadakou, N. H., & Liu, F. Y. (1980). Penicillin-binding proteins in bacteria. *Antimicrobial Agents and Chemotherapy, 18*(1), 148-157.

Gianecini, R., Oviedo, C., Littvik, A., Mendez, E., Piccoli, L., Montibello, S., & Galarza, P. (2015). Identification of TEM-135 beta-lactamase in *Neisseria gonorrhoeae* strains carrying African and Toronto plasmids in Argentina. *Antimicrobial Agents and Chemotherapy, 59*(1), 717-720.

Gill, M., Simjee, S., Al-Hattawi, K., Robertson, B., Easmon, C., & Ison, C. (1998). Gonococcal Resistance to β-Lactams and Tetracycline Involves Mutation in Loop 3 of the Porin Encoded at the *penB* Locus. *Antimicrobial Agents and Chemotherapy, 42*(11), 2799-2803.

Goire, N., Lahra, M. M., Chen, M., Donovan, B., Fairley, C. K., Guy, R., . . . Whiley, D. M. (2014). Molecular approaches to enhance surveillance of gonococcal antimicrobial resistance. *Nature Reviews Microbiology, 12*(3), 223-229.

Golparian, D., Shafer, W. M., Ohnishi, M., & Unemo, M. (2014). Importance of multidrug efflux pumps in the antimicrobial resistance property of clinical multidrug-resistant isolates of *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy, 58*(6), 3556-3559.

Gouby, A., Bourg, G., & Ramuz, M. (1986). Previously undescribed 6.6-kilobase R plasmid in penicillinase-producing *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy, 29*(6), 1095-1097.

Gransden, W. R., Warren, C. A., Phillips, I., Hodges, M., & Barlow, D. (1990). Decreased susceptibility of *Neisseria gonorrhoeae* to ciprofloxacin. *Lancet, 335*(8680), 51.

Gregory, S. T., & Dahlberg, A. E. (1999). Erythromycin resistance mutations in ribosomal proteins L22 and L4 perturb the higher order structure of 23 S ribosomal RNA. *Journal of Molecular Biology, 289*(4), 827-834.

Griffiss, J. M., Lammel, C. J., Wang, J., Dekker, N. P., & Brooks, G. F. (1999). *Neisseria gonorrhoeae* coordinately uses Pili and Opa to activate HEC-1-B cell microvilli, which causes engulfment of the gonococci. *Infection and Immunity, 67*(7), 3469-3480.

Haas, R., Veit, S., & Meyer, T. F. (1992). Silent pilin genes of *Neisseria gonorrhoeae* MS11 and the occurrence of related hypervariant sequences among other gonococcal isolates. *Molecular Microbiology, 6*(2), 197-208.

Hagblom, P., Korch, C., Jonsson, A. B., & Normark, S. (1986). Intragenic variation by site-specific recombination in the cryptic plasmid of *Neisseria gonorrhoeae*. *Molecular Microbiology, 167*(1), 231-237.

Hall, T. (2011). BioEdit: An important software for molecular biology. *GERF Bulletin of Biosciences, 2*(1), 60-61.

Halter, R., Pohlner, J., & Meyer, T. F. (1984). IgA protease of *Neisseria gonorrhoeae*: isolation and characterization of the gene and its extracellular product. *EMBO Journal, 3*(7), 1595-1601.

Hamilton, H. L., & Dillard, J. P. (2006). Natural transformation of *Neisseria gonorrhoeae*: from DNA donation to homologous recombination. *Molecular Microbiology, 59*(2), 376-385.

Hamilton, H. L., Dominguez, N. M., Schwartz, K. J., Hackett, K. T., & Dillard, J. P. (2005). *Neisseria gonorrhoeae* secretes chromosomal DNA via a novel type IV secretion system. *Molecular Microbiology, 55*(6), 1704-1721.

Handsfield, H. H., Dalu, Z. A., Martin, D. H., Douglas, J. M., Jr., McCarty, J. M., & Schlossberg, D. (1994). Multicenter trial of single-dose azithromycin vs.

ceftriaxone in the treatment of uncomplicated gonorrhea. Azithromycin Gonorrhea Study Group. *Sexually Transmitted Diseases, 21*(2), 107-111.

Handsfield, H. H., Lipman, T. O., Harnisch, J. P., Tronca, E., & Holmes, K. K. (1974). Asymptomatic gonorrhea in men. Diagnosis, natural course, prevalence and significance. *New England Journal of Medicine, 290*(3), 117-123.

Harrison, W. O., Hooper, R. R., Wiesner, P. J., Campbell, A. F., Karney, W. W., Reynolds, G. H., . . . Holmes, K. K. (1979). A trial of minocycline given after exposure to prevent gonorrhea. *New England Journal of Medicine, 300*(19), 1074-1078.

Helm, R. A., Barnhart, M. M., & Seifert, H. S. (2007). *pilQ* Missense mutations have diverse effects on PilQ multimer formation, piliation, and pilus function in *Neisseria gonorrhoeae*. *Journal of Bacteriology, 189*(8), 3198-3207.

Hill, S. A., & Davies, J. K. (2009). Pilin gene variation in *Neisseria gonorrhoeae*: reassessing the old paradigms. *FEMS Microbiology Reviews, 33*(3), 521-530.

Hill, S. A., Masters, T. L., & Wachter, J. (2016). Gonorrhea - an evolving disease of the new millennium. *Microbial Cell, 3*(9), 371-389.

Hirai, K., Aoyama, H., Irikura, T., Iyobe, S., & Mitsuhashi, S. (1986). Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. *Antimicrobial Agents and Chemotherapy, 29*(3), 535-538.

Holmes, K. K., Counts, G. W., & Beaty, H. N. (1971). Disseminated gonococcal infection. *Annals of Internal Medicine, 74*(6), 979-993.

Hooper, D. C. (1999). Mechanisms of fluoroquinolone resistance. *Drug Resistance Updates, 2*(1), 38-55.

Hu, M., Nandi, S., Davies, C., & Nicholas, R. A. (2005). High-level chromosomally mediated tetracycline resistance in *Neisseria gonorrhoeae* results from a point mutation in the *rpsJ* gene encoding ribosomal protein S10 in combination with the *mtrR* and *penB* resistance determinants. *Antimicrobial Agents and Chemotherapy*, *49*(10), 4327-4334.

Hung, M.-C., & Christodoulides, M. (2013). The biology of Neisseria adhesins. *Biology*, *2*(3), 1054-1109.

Ibarz Pavon, A. B., & Maiden, M. C. (2009). Multilocus sequence typing. *Methods in Molecular Biology*, *551*, 129-140.

Ilina, E. N., Malakhova, M. V., Bodoev, I. N., Oparina, N. Y., Filimonova, A. V., & Govorun, V. M. (2013). Mutation in ribosomal protein S5 leads to spectinomycin resistance in *Neisseria gonorrhoeae*. *Frontiers in Microbiology*, *4*, 186.

Ilina, E. N., Oparina, N. Y., Shitikov, E. A., Borovskaya, A. D., & Govorun, V. M. (2010). Molecular surveillance of clinical *Neisseria gonorrhoeae* isolates in Russia. *Journal of clinical microbiology*, *48*(10), 3681-3689.

Irazoki, O., Hernandez, S. B., & Cava, F. (2019). Peptidoglycan Muropeptides: Release, Perception, and Functions as Signaling Molecules. *Frontiers in Microbiology*, *10*(500).

Ison, C. A., Hussey, J., Sankar, K. N., Evans, J., & Alexander, S. (2011). Gonorrhoea treatment failures to cefixime and azithromycin in England, 2010. *Eurosurveillance*, *16*(14), 19833.

Ito, M., Deguchi, T., Mizutani, K. S., Yasuda, M., Yokoi, S., Ito, S., . . . Ezaki, T. (2005). Emergence and spread of *Neisseria gonorrhoeae* clinical isolates

harboring mosaic-like structure of penicillin-binding protein 2 in Central Japan. *Antimicrobial Agents and Chemotherapy, 49*(1), 137-143.

Janda, W. M., Bradna, J. J., & Ruther, P. (1989). Identification of Neisseria spp., Haemophilus spp., and other fastidious gram-negative bacteria with the MicroScan Haemophilus-Neisseria identification panel. *Journal of Clinical Microbiology, 27*(5), 869-873.

Jerse, A. E., Cohen, M. S., Drown, P. M., Whicker, L. G., Isbey, S. F., Seifert, H. S., & Cannon, J. G. (1994). Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. *Journal of Experimental Medicine, 179*(3), 911-920.

Johnson, S. R., & Morse, S. A. (1988). Antibiotic resistance in *Neisseria gonorrhoeae*: genetics and mechanisms of resistance. *Sexually Transmitted Diseases, 15*(4), 217-224.

Jolley, K. A., Bray, J. E., & Maiden, M. C. J. (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Research, 3*, 124.

Jordan, P. W., & Saunders, N. J. (2009). Host iron binding proteins acting as niche indicators for *Neisseria meningitidis*. *PLoS One, 4*(4), e5198.

Judson, F. N., Ehret, J. M., & Handsfield, H. H. (1985). Comparative study of ceftriaxone and spectinomycin for treatment of pharyngeal and anorectal gonorrhea. *Journal of the American Medical Association, 253*(10), 1417-1419.

Juhas, M., van der Meer, J. R., Gaillard, M., Harding, R. M., Hood, D. W., & Crook, D. W. (2009). Genomic islands: tools of bacterial horizontal gene transfer and evolution. *FEMS Microbiology Reviews, 33*(2), 376-393.

Kampmeier, R. H. (1983). Introduction of sulfonamide therapy for gonorrhea. *Sexually Transmitted Diseases, 10*(2), 81-84.

Kannan, K., Kanabar, P., Schryer, D., Florin, T., Oh, E., Bahroos, N., . . . Mankin, A. S. (2014). The general mode of translation inhibition by macrolide antibiotics. *Proceedings of the National Academy of Sciences of the United States of America, 111*(45), 15958-15963.

Kariuki, S. (1997). Kenya: antibiotic resistance. *Lancet, 349*, S9-S10.

Katz, A. R., Effler, P. V., Ohye, R. G., Brouillet, B., Lee, M. V., & Whiticar, P. M. (2004). False-positive gonorrhea test results with a nucleic acid amplification test: the impact of low prevalence on positive predictive value. *Clinical Infectious Diseases, 38*(6), 814-819.

Kawai, M., Uchiyama, I., & Kobayashi, I. (2005). Genome comparison in silico in Neisseria suggests integration of filamentous bacteriophages by their own transposase. *DNA Research, 12*(6), 389-401.

Kivata, M. W., Mbuchi, M., Eyase, F., Bulimo, W. D., Kyanya, C. K., Oundo, V., . . . Distelhorst, J. (2020). Plasmid mediated penicillin and tetracycline resistance among *Neisseria gonorrhoeae* isolates from Kenya. *BMC Infectious Diseases, 20*(1), 703.

Klausner, J. D., Kohn, R., & Kent, C. (2004). Etiology of clinical proctitis among men who have sex with men. *Clinical Infectious Diseases, 38*(2), 300-302.

Knox, J. R. (1995). Extended-spectrum and inhibitor-resistant TEM-type beta-lactamases: mutations, specificity, and three-dimensional structure. *Antimicrobial Agents and Chemotherapy, 39*(12), 2593-2601.

Kong, K. F., Schneper, L., & Mathee, K. (2010). Beta-lactam antibiotics: from antibiosis to resistance and bacteriology. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica, 118*(1), 1-36.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution, 35*(6), 1547-1549.

Lagace-Wiens, P. R., Duncan, S., Kimani, J., Thiong'o, A., Shafi, J., McClelland, S., . . . Mehta, S. D. (2012). Emergence of fluoroquinolone resistance in *Neisseria gonorrhoeae* isolates from four clinics in three regions of Kenya. *Sexually Transmitted Diseases, 39*(5), 332-334.

Lavanya, R. (2017). Sulphonamides: A Pharmaceutical Review. *International Journal of Pharmaceutical Science Invention, 6*(2), 1-3.

Lesher, G. Y., Froelich, E. J., Gruett, M. D., Bailey, J. H., & Brundage, R. P. (1962). 1,8-Naphthyridine Derivatives. A New Class of Chemotherapeutic Agents. *Journal of Medicinal and Pharmaceutical Chemistry, 91*(5), 1063-1065.

Levine, C., Hiasa, H., & Marians, K. J. (1998). DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. *Biochimica et Biophysica Acta, 1400*(1-3), 29-43.

Lewis, D. A. (2010). The Gonococcus fights back: is this time a knock out? *Sexually Transmitted Infections, 86*(6), 415-421.

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. *Boinformatics, 25*(14), 1754-1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., . . . Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics, 25*(16), 2078-2079.

Liao, M., Gu, W. M., Yang, Y., & Dillon, J. A. (2011). Analysis of mutations in multiple loci of *Neisseria gonorrhoeae* isolates reveals effects of PIB, PBP2 and MtrR on reduced susceptibility to ceftriaxone. *Journal of Antimicrobial Chemotherapy, 66*(5), 1016-1023.

Lindback, E., Rahman, M., Jalal, S., & Wretlind, B. (2002). Mutations in *gyrA, gyrB, parC,* and *parE* in quinolone-resistant strains of *Neisseria gonorrhoeae*. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica, 110*(9), 651-657.

Lindberg, R., Fredlund, H., Nicholas, R., & Unemo, M. (2007). *Neisseria gonorrhoeae* isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in *penA, mtrR, porB1b,* and *ponA. Antimicrobial Agents and Chemotherapy, 51*(6), 2117-2122.

Liu, S. V., Saunders, N. J., Jeffries, A., & Rest, R. F. (2002). Genome analysis and strain comparison of correia repeats and correia repeat-enclosed elements in pathogenic Neisseria. *Journal of Bacteriology, 184*(22), 6163-6173.

Lucas, C. E., Balthazar, J. T., Hagman, K. E., & Shafer, W. M. (1997). The MtrR repressor binds the DNA sequence between the *mtrR* and *mtrC* genes of *Neisseria gonorrhoeae*. *Journal of Bacteriology, 179*(13), 4123-4128.

Luna, V. A., Cousin, S., Jr., Whittington, W. L., & Roberts, M. C. (2000). Identification of the conjugative mef gene in clinical *Acinetobacter junii* and *Neisseria gonorrhoeae* isolates. *Antimicrobial Agents and Chemotherapy, 44*(9), 2503-2506.

Mahoney, J., Ferguson, C., Buchholtz, M., & Van Slyke, C. (1943). The Use of Penicillin Sodium in the Treatment of Sulfonamide. Resistant Gonorrhoea in Men. A Preliminary Report. *American Journal of Syphilis, 27*(5), 525-528.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., . . . Spratt, B. G. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America, 95*(6), 3140-3145.

Maier, B., Koomey, M., & Sheetz, M. P. (2004). A force-dependent switch reverses type IV pilus retraction. *Proceedings of the National Academy of Sciences of the United States of America, 101*(30), 10961-10966.

Mandrell, R. E., Griffiss, J. M., Smith, H., & Cole, J. A. (1993). Distribution of a lipooligosaccharide-specific sialyltransferase in pathogenic and non-pathogenic Neisseria. *Microbial Pathogenesis, 14*(4), 315-327.

Marri, P. R., Paniscus, M., Weyand, N. J., Rendon, M. A., Calton, C. M., Hernandez, D. R., . . . So, M. (2010). Genome sequencing reveals widespread virulence gene exchange among human Neisseria species. *PLoS One, 5*(7), e11835.

Marri, P. R., Paniscus, M., Weyand, N. J., Rendón, M. A., Calton, C. M., Hernández, D. R., . . . So, M. (2010). Genome Sequencing Reveals Widespread Virulence Gene Exchange among Human Neisseria Species. *PLOS ONE, 5*(7), e11835.

Martin, I., Sawatzky, P., Liu, G., Allen, V., Lefebvre, B., Hoang, L., . . . Mulvey, M. R. (2016). Decline in Decreased Cephalosporin Susceptibility and Increase in Azithromycin Resistance in *Neisseria gonorrhoeae*, Canada. *Emerging Infectious Diseases, 22*(1), 65-67.

Martin, I. M., Ison, C. A., Aanensen, D. M., Fenton, K. A., & Spratt, B. G. (2004). Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. *Journal of Infectious Diseases, 189*(8), 1497-1505.

Martin Jr, J. E., Lester, A., Price, E. V., & Schmale, J. D. (1970). Comparative study of gonococcal susceptibility to penicillin in the United States, 1955-1969. *Journal of Infectious Diseases*, 459-461.

Mazzei, T., Mini, E., Novelli, A., & Periti, P. (1993). Chemistry and mode of action of macrolides. *Journal of Antimicrobial Chemotherapy, 31*(Suppl C), S1-9.

McAnena, L., Knowles, S. J., Curry, A., & Cassidy, L. (2015). Prevalence of gonococcal conjunctivitis in adults and neonates. *Eye (London, England), 29*(7), 875-880.

McCormack, W. M., Stumacher, R. J., Johnson, K., & Donner, A. (1977). Clinical spectrum of gonococcal infection in women. *Lancet, 1*(8023), 1182-1185.

Mehta, S. D., Maclean, I., Ndinya-Achola, J. O., Moses, S., Martin, I., Ronald, A., . . . Zenilman, J. M. (2011). Emergence of quinolone resistance and cephalosporin MIC creep in *Neisseria gonorrhoeae* isolates from a cohort of young men in Kisumu, Kenya, 2002 to 2009. *Canadian Medical Association Journal, 55*(8), 3882-3888.

Meyer, T. F., & Hill, S. A. (2003). Genetic variation in the Pathogenic Neisseria species. In *Antigenic variation* (pp. 142-164): Elsevier Inc.

Mlisana, K., Naicker, N., Werner, L., Roberts, L., van Loggerenberg, F., Baxter, C., . . . Abdool Karim, S. S. (2012). Symptomatic vaginal discharge is a poor predictor of sexually transmitted infections and genital tract inflammation in high-risk women in South Africa. *Journal of Infectious Diseases 206*(1), 6-14.

Moniot-Ville, N., Guibert, J., Moreau, N., Acar, J. F., Collatz, E., & Gutmann, L. (1991). Mechanisms of quinolone resistance in a clinical isolate of *Escherichia coli* highly resistant to fluoroquinolones but susceptible to nalidixic acid. *Journal of Antimicrobial Chemotherapy, 35*(3), 519-523.

Morse, S. A., Johnson, S. R., Biddle, J. W., & Roberts, M. C. (1986). High-level tetracycline resistance in *Neisseria gonorrhoeae* is result of acquisition of streptococcal *tetM* determinant. *Antimicrobial Agents and Chemotherapy, 30*(5), 664-670.

Mortimer, T. D., & Grad, Y. H. (2019). Applications of genomics to slow the spread of multidrug-resistant *Neisseria gonorrhoeae*. *Annals of the New York Academy of Sciences, 1435*(1), 93-109.

Muhammad, I., Golparian, D., Dillon, J.-A. R., Johansson, Å., Ohnishi, M., Sethi, S., . . . Bala, M. (2014). Characterisation of bla TEM genes and types of β-lactamase plasmids in *Neisseria gonorrhoeae*–the prevalent and conserved bla TEM-135 has not recently evolved and existed in the Toronto plasmid from the origin. *BMC Infectious Diseases, 14*(1), 454.

Muller, A., Gunther, D., Dux, F., Naumann, M., Meyer, T. F., & Rudel, T. (1999). Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. *EMBO Journal, 18*(2), 339-352.

Muller, A., Rassow, J., Grimm, J., Machuy, N., Meyer, T. F., & Rudel, T. (2002). VDAC and the bacterial porin PorB of *Neisseria gonorrhoeae* share mitochondrial import pathways. *EMBO Journal, 21*(8), 1916-1929.

Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiology Spectrum, 4*(2), 10.

Nakayama, S.-i., Tribuddharat, C., Prombhul, S., Shimuta, K., Srifuengfung, S., Unemo, M., & Ohnishi, M. (2012). Molecular analyses of TEM genes and their corresponding penicillinase-producing *Neisseria gonorrhoeae* isolates in Bangkok, Thailand. *Antimicrobial Agents and Chemotherapy, 56*(2), 916-920.

NASCOP. (2018). *Kenya National Guidelines for Prevention, Management and Control of Sexually Transmitted Infections*. Nairobi, Kenya

Nassif, X., Pujol, C., Morand, P., & Eugene, E. (1999). Interactions of pathogenic Neisseria with host cells. Is it possible to assemble the puzzle? *Mol Microbiol, 32*(6), 1124-1132.

Ng, L. K., Martin, I., Liu, G., & Bryden, L. (2002). Mutation in 23S rRNA associated with macrolide resistance in *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy, 46*(9), 3020-3025.

Noinaj, N., Buchanan, S. K., & Cornelissen, C. N. (2012). The transferrin-iron import system from pathogenic Neisseria species. *Mol Microbiol, 86*(2), 246-257.

O'Rourke, M., & Stevens, E. (1993). Genetic structure of *Neisseria gonorrhoeae* populations: a non-clonal pathogen. *Journal of General Microbiology, 139*(11), 2603-2611.

Ohnishi, M., Golparian, D., Shimuta, K., Saika, T., Hoshina, S., Iwasaku, K., . . . Unemo, M. (2011). Is *Neisseria gonorrhoeae* initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. *Antimicrobial Agents and Chemotherapy, 55*(7), 3538-3545.

Ohnishi, M., Ono, E., Shimuta, K., Watanabe, H., & Okamura, N. (2010). Identification of TEM-135 β-lactamase in penicillinase-producing *Neisseria gonorrhoeae* strains in Japan. *Antimicrobial Agents and Chemotherapy, 54*(7), 3021-3023.

Ohnishi, M., Saika, T., Hoshina, S., Iwasaku, K., Nakayama, S., Watanabe, H., & Kitawaki, J. (2011). Ceftriaxone-resistant *Neisseria gonorrhoeae*, Japan. *Emerging Infectious Diseases, 17*(1), 148-149.

Olesky, M., Hobbs, M., & Nicholas, R. A. (2002). Identification and analysis of amino acid mutations in porin IB that mediate intermediate-level resistance to penicillin and tetracycline in *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy, 46*(9), 2811-2820.

Olesky, M., Zhao, S., Rosenberg, R. L., & Nicholas, R. A. (2006). Porin-mediated antibiotic resistance in *Neisseria gonorrhoeae*: ion, solute, and antibiotic permeation through PIB proteins with *penB* mutations. *Journal of Bacteriology, 188*(7), 2300-2308.

Oliphant, C. M., & Green, G. M. (2002). Quinolones: a comprehensive review. *Canadian Medical Association Journal, 65*(3), 455-464.

Omura, S. (2002). *Macrolide antibiotics: chemistry, biology, and practice* (Second Edition ed.). San Diego, California, USA: Elsevier.

Osaka, K., Takakura, T., Narukawa, K., Takahata, M., Endo, K., Kiyota, H., & Onodera, S. (2008). Analysis of amino acid sequences of penicillin-binding protein 2 in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime and ceftriaxone. *Journal of Infection and Chemotherapy, 14*(3), 195-203.

Otto, R. A., & Noorily, A. D. (1994). Cephalosporin antibiotics. *Ear, nose, & throat, 73 12*, 900, 903, 907-913.

Pachulec, E., & van der Does, C. (2010). Conjugative plasmids of *Neisseria gonorrhoeae*. *PLoS One, 5*(4), e9962.

Palafox, S., Jasper, S., Tauber, A. D., & Foster, S. (2011). Ophthalmia neonatorum. *Journal of Clinical & Experimental Ophthalmology*, *2*(1), 24.

Palmer, H. M., Leeming, J. P., & Turner, A. (2000). A multiplex polymerase chain reaction to differentiate beta-lactamase plasmids of *neisseria gonorrhoeae*. *Journal of Antimicrobial Chemotherapy, 45*(6), 777-782.

Pardo, D., & Rosset, R. (1977). Properties of ribosomes from erythromycin resistant mutants of *Escherichia coli*. *Molecular Genetics and Genomics 156*(3), 267-271.

Parkhill, J., Achtman, M., James, K. D., Bentley, S. D., Churcher, C., Klee, S. R., . . . Barrell, B. G. (2000). Complete DNA sequence of a serogroup A strain of *Neisseria meningitidis* Z2491. *Nature, 404*(6777), 502-506.

Parsons, N. J., Andrade, J. R., Patel, P. V., Cole, J. A., & Smith, H. (1989). Sialylation of lipopolysaccharide and loss of absorption of bactericidal antibody during conversion of gonococci to serum resistance by cytidine 5'-monophospho-N-acetyl neuraminic acid. *Microbial Pathogenesis, 7*(1), 63-72.

Patel, A. L., Chaudhry, U., Sachdev, D., Sachdeva, P. N., Bala, M., & Saluja, D. (2011). An insight into the drug resistance profile & mechanism of drug resistance in *Neisseria gonorrhoeae*. *Indian Journal of Medical Research, 134*(4), 419-431.

Perine, P. L., Biddle, J. W., Nsanze, H., D'Costa, L. J., Osaba, A. O., & Widy-Wirski, R. (1980). Gonococcal drug resistance and treatment of gonorrhoea in Nairobi. *East African Medical Journal, 57*(4), 238-246.

Pham, T. D. M., Ziora, Z. M., & Blaskovich, M. A. T. (2019). Quinolone antibiotics. *RSC Medicinal Chemistry, 10*(10), 1719-1739.

Phillips, I. (1976). Beta-lactamase-producing, penicillin-resistant gonococcus. *Lancet, 2*(7987), 656-657.

Piddock, L. J. (1999). Mechanisms of fluoroquinolone resistance: an update 1994–1998. *Drugs, 58*(2), 11-18.

Piekarowicz, A., Klyz, A., Majchrzak, M., Adamczyk-Poplawska, M., Maugel, T. K., & Stein, D. C. (2007). Characterization of the dsDNA prophage sequences in the genome of *Neisseria gonorrhoeae* and visualization of productive bacteriophage. *BMC Microbiology, 7*(1), 66.

Pitt, R., Sadouki, Z., Town, K., Fifer, H., Mohammed, H., Hughes, G., . . . Cole, M. J. (2019). Detection of *tet(M)* in high-level tetracycline-resistant *Neisseria gonorrhoeae*. *Journal of Antimicrobial Chemotherapy, 74*(7), 2115-2116.

Plummer, F. A., Chubb, H., Simonsen, J. N., Bosire, M., Slaney, L., Maclean, I., . . . Brunham, R. C. (1993). Antibody to Rmp (outer membrane protein 3) increases susceptibility to gonococcal infection. *Journal of Clinical Investigation, 91*(1), 339-343.

Poehlsgaard, J., & Douthwaite, S. (2005). The bacterial ribosome as a target for antibiotics. *Nature Reviews Microbiology, 3*(11), 870-881.

Poole, K. (2000). Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. *Antimicrobial Agents and Chemotherapy, 44*(9), 2233-2241.

Powell, A. J., Tomberg, J., Deacon, A. M., Nicholas, R. A., & Davies, C. (2009). Crystal structures of penicillin-binding protein 2 from penicillin-susceptible and -resistant strains of *Neisseria gonorrhoeae r*eveal an unexpectedly subtle mechanism for antibiotic resistance. *Journal of Biological Chemistry, 284*(2), 1202-1212.

Quillin, S. J., & Seifert, H. S. (2018). *Neisseria gonorrhoeae* host adaptation and pathogenesis. *Nature Reviews Microbiology, 16*(4), 226-240.

Rambaut, A. (2012). FigTree v1. 4. In: Molecular Evolution, Phylogenetics and Epidemiology Research Group.

Rambaut, A., Suchard, M., Xie, D., & Drummond, A. (2015). Tracer v1. 6. 2014. In: Molecular Evolution, Phylogenetics and Epidemiology Research Group.

Redgrave, L. S., Sutton, S. B., Webber, M. A., & Piddock, L. J. (2014). Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. *Trends in Microbiology, 22*(8), 438-445.

Reyn, A., Korner, B., & Bentzon, M. W. (1958). Effects of penicillin, streptomycin, and tetracycline on *N. gonorrhoeae* isolated in 1944 and in 1957. *British Journal of Venereal Diseases, 34*(4), 227-239.

Rice, P. A., McQuillen, D. P., Gulati, S., Jani, D. B., Wetzler, L. M., Blake, M. S., & Gotschlich, E. C. (1994). Serum resistance of *Neisseria gonorrhoeae*. Does it thwart the inflammatory response and facilitate the transmission of infection? *Annals of the New York Academy of Sciences, 730*, 7-14.

Richter, M., & Rossello-Mora, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proceedings of the National Academy of Sciences of the United States of America, 106*(45), 19126-19131.

Roberts, M. C., Chung, W. O., Roe, D., Xia, M., Marquez, C., Borthagaray, G., . . . Holmes, K. K. (1999). Erythromycin-resistant *Neisseria gonorrhoeae* and oral commensal Neisseria spp. carry known rRNA methylase genes. *Antimicrobial Agents and Chemotherapy, 43*(6), 1367-1372.

Roberts, M. C., Wagenvoort, J. H., van Klingeren, B., & Knapp, J. S. (1988). tetM- and beta-lactamase-containing *Neisseria gonorrhoeae* (tetracycline resistant and

penicillinase producing) in The Netherlands. *Antimicrobial Agents and Chemotherapy*, *32*(1), 158.

Ropp, P. A., Hu, M., Olesky, M., & Nicholas, R. A. (2002). Mutations in *ponA*, the gene encoding penicillin-binding protein 1, and a novel locus, *penC*, are required for high-level chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy, 46*(3), 769-777.

Rouquette-Loughlin, C. E., Balthazar, J. T., & Shafer, W. M. (2005). Characterization of the MacA-MacB efflux system in *Neisseria gonorrhoeae*. *Journal of Antimicrobial Chemotherapy, 56*(5), 856-860.

Sarkozy, G. (2001). Quinolones: a class of antimicrobial agents. *VETERINARNI MEDICINA-PRAHA-*, *46*(9/10), 257-274.

Sauvage, E., Kerff, F., Terrak, M., Ayala, J. A., & Charlier, P. (2008). The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews*, *32*(2), 234-258.

Schoen, C., Blom, J., Claus, H., Schramm-Gluck, A., Brandt, P., Muller, T., . . . Frosch, M. (2008). Whole-genome comparison of disease and carriage strains provides insights into virulence evolution in *Neisseria meningitidis*. *Proceedings of the National Academy of Sciences of the United States of America, 105*(9), 3473-3478.

Segal, E., Hagblom, P., Seifert, H. S., & So, M. (1986). Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. *Proceedings of the National Academy of Sciences of the United States of America, 83*(7), 2177-2181.

Shafer, W. M. (2018). Mosaic Drug Efflux Gene Sequences from Commensal Neisseria Can Lead to Low-Level Azithromycin Resistance Expressed by *Neisseria gonorrhoeae* Clinical Isolates. *mBio, 9*(5), e01747-01718.

Shafer, W. M., Balthazar, J. T., Hagman, K. E., & Morse, S. A. (1995). Missense mutations that alter the DNA-binding domain of the MtrR protein occur frequently in rectal isolates of *Neisseria gonorrhoeae* that are resistant to faecal lipids. *Microbiology, 141*(4), 907-911.

Shafer, W. M., & Folster, J. P. (2006). Towards an understanding of chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae:* evidence for a porin-efflux pump collaboration. *Journal of Bacteriology, 188*(7), 2297-2299.

Sharma, P. C., Jain, A., & Jain, S. (2009). Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects. *Acta Pol Pharm, 66*(6), 587-604.

Shaskolskiy, B., Dementieva, E., Kandinov, I., Filippova, M., Petrova, N., Plakhova, X., . . . Gryadunov, D. (2019). Resistance of *Neisseria gonorrhoeae* isolates to beta-lactam antibiotics (benzylpenicillin and ceftriaxone) in Russia, 2015-2017. *PLoS One, 14*(7), e0220339.

Shaughnessy, J., Ram, S., & Rice, P. (2019). Biology of the Gonococcus: Disease and Pathogenesis. *Methods in Molecular Biology, 1997*, 1-27.

Shigemura, K., Shirakawa, T., Massi, N., Tanaka, K., Arakawa, S., Gotoh, A., & Fujisawa, M. (2005). Presence of a mutation in ponA1 of *Neisseria gonorrhoeae* in numerous clinical samples resistant to various beta-lactams and other, structurally unrelated, antimicrobials. *Journal of Infection and Chemotherapy, 11*(5), 226-230.

Shimuta, K., Unemo, M., Nakayama, S.-i., Morita-Ishihara, T., Dorin, M., Kawahata, T., & Ohnishi, M. (2013). Antimicrobial Resistance and Molecular Typing of *Neisseria gonorrhoeae* Isolates in Kyoto and Osaka, Japan, 2010 to 2012: Intensified Surveillance after Identification of the First Strain (H041) with High-Level Ceftriaxone Resistance. *Antimicrobial Agents and Chemotherapy, 57*(11), 5225.

Shortridge, V. D., Flamm, R. K., Ramer, N., Beyer, J., & Tanaka, S. K. (1996). Novel mechanism of macrolide resistance in *Streptococcus pneumoniae*. *Diagnostic Microbiology and Infectious Disease, 26*(2), 73-78.

Siguier, P., Gourbeyre, E., & Chandler, M. (2014). Bacterial insertion sequences: their genomic impact and diversity. *FEMS Microbiology Reviews, 38*(5), 865-891.

Sim, J. H., Anikst, V., Lohith, A., Pourmand, N., & Banaei, N. (2015). Optimized Protocol for Simple Extraction of High-Quality Genomic DNA from Clostridium difficile for Whole-Genome Sequencing. *Journal of Clinical Microbiology, 53*(7), 2329-2331.

Simms, A. N., & Jerse, A. E. (2005). Role of Phase and Antigenic Variation in *Neisseria gonorrhoeae* Colonization. In *Colonization of Mucosal Surfaces* (pp. 327-350). Washington, DC: American Society of Microbiology.

Skaar, E. P., Lecuyer, B., Lenich, A. G., Lazio, M. P., Perkins-Balding, D., Seifert, H. S., & Karls, A. C. (2005). Analysis of the Piv recombinase-related gene family of *Neisseria gonorrhoeae*. *Journal of Bacteriology, 187*(4), 1276-1286.

Snyder, L. A., Cole, J. A., & Pallen, M. J. (2009). Comparative analysis of two *Neisseria gonorrhoeae* genome sequences reveals evidence of mobilization of Correia Repeat Enclosed Elements and their role in regulation. *BMC Genomics, 10*(1), 70.

Snyder, L. A., Davies, J. K., & Saunders, N. J. (2004). Microarray genomotyping of key experimental strains of *Neisseria gonorrhoeae* reveals gene complement diversity and five new neisserial genes associated with Minimal Mobile Elements. *BMC Genomics, 5*(1), 23.

Snyder, L. A., McGowan, S., Rogers, M., Duro, E., O'Farrell, E., & Saunders, N. J. (2007). The repertoire of minimal mobile elements in the Neisseria species and evidence that these are involved in horizontal gene transfer in other bacteria. *Mol Biol Evol, 24*(12), 2802-2815.

Snyder, L. A. S., Butcher, S. A., & Saunders, N. J. (2001). Comparative whole-genome analyses reveal over 100 putative phase-variable genes in the pathogenic Neisseria spp. *Microbiology, 147*(Pt 8), 2321-2332.

Snyder, L. A. S., Jarvis, S. A., & Saunders, N. J. (2005). Complete and variant forms of the 'gonococcal genetic island' in *Neisseria meningitidis*. *Microbiology, 151*(Pt 12), 4005-4013.

Sood, S., Agarwal, M., Bala, M., Mahajan, N., Singh, R., Kapil, A., . . . for, I. C. S. (2017). Exploring quinolone resistance-determining region in *Neisseria gonorrhoeae* isolates from across India. *Indian Journal of Medical Research, 146*(Suppl. 1), S64-S69.

Spencer-Smith, R., Varkey, E. M., Fielder, M. D., & Snyder, L. A. (2012). Sequence features contributing to chromosomal rearrangements in *Neisseria gonorrhoeae*. *PLoS One, 7*(9), e46023.

Spratt, B. G. (1978). The mechanism of action of penicillin. *Science Progress, 65*(257), 101-128.

Spratt, B. G., Bowler, L. D., Zhang, Q. Y., Zhou, J., & Smith, J. M. (1992). Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal Neisseria species. *Journal of Molecular Evolution, 34*(2), 115-125.

Spratt, B. G., Zhang, Q. Y., Jones, D. M., Hutchison, A., Brannigan, J. A., & Dowson, C. G. (1989). Recruitment of a penicillin-binding protein gene from *Neisseria flavescens* during the emergence of penicillin resistance in *Neisseria meningitidis*. *Proceedings of the National Academy of Sciences of the United States of America, 86*(22), 8988-8992.

Srifeungfung, S., Roongpisuthipong, A., Asavapiriyanont, S., Lolekha, R., Tribuddharat, C., Lokpichart, S., . . . Tongtep, P. (2009). Prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in HIV-seropositive patients and gonococcal antimicrobial susceptibility: an update in Thailand. *Japanese Journal of Infectious Diseases, 62*(6), 467-470.

Stefanova, M. E., Tomberg, J., Olesky, M., Holtje, J. V., Gutheil, W. G., & Nicholas, R. A. (2003). *Neisseria gonorrhoeae* penicillin-binding protein 3 exhibits exceptionally high carboxypeptidase and beta-lactam binding activities. *Biochemistry, 42*(49), 14614-14625.

Stolz, E., Zwart, H. G., & Michel, M. F. (1975). Activity of eight antimicrobial agents in vitro against *N. gonorrhoeae*. *British Journal of Venereal Diseases, 51*(4), 257-264.

Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J., & Rambaut, A. (2018). Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution, 4*(1), vey016.

Sun, A., Fan, X., Gu, Y., Du, P., Tang, R., Mao, Y., . . . Yan, J. (2010). Predominant *porB1A* and *porB1B* genotypes and correlation of gene mutations with drug resistance in *Neisseria gonorrhoeae* isolates in Eastern China. *BMC Infectious Diseases, 10*, 323.

Swanson, J., Belland, R. J., & Hill, S. A. (1992). Neisserial surface variation: how and why? *Current Opinion in Genetics and Development, 2*(5), 805-811.

Swartley, J. S., McAllister, C. F., Hajjeh, R. A., Heinrich, D. W., & Stephens, D. S. (1993). Deletions of Tn916-like transposons are implicated in tetM-mediated resistance in pathogenic Neisseria. *Mol Microbiol, 10*(2), 299-310.

Swedberg, G., Fermer, C., & Skold, O. (1993). Point mutations in the dihydropteroate synthase gene causing sulfonamide resistance. *Advances in Experimental Medicine and Biology, 338*, 555-558.

Taktikos, J., Lin, Y. T., Stark, H., Biais, N., & Zaburdaev, V. (2015). Pili-Induced Clustering of *N. gonorrhoeae* Bacteria. *PLOS ONE, 10*(9), e0137661.

Tanaka, M., Fukuda, H., Hirai, K., Hosaka, M., Matsumoto, T., & Kumazawa, J. (1994). Reduced uptake and accumulation of norfloxacin in resistant strains of *Neisseria gonorrhoeae* isolated in Japan. *Genitourinary Medicine, 70*(4), 253-255.

Tanaka, M., Nakayama, H., Haraoka, M., & Saika, T. (2000). Antimicrobial resistance of *Neisseria gonorrhoeae* and high prevalence of ciprofloxacin-resistant isolates in Japan, 1993 to 1998. *Journal of Clinical Microbiology, 38*(2), 521-525.

Tanaka, M., Nakayama, H., Huruya, K., Konomi, I., Irie, S., Kanayama, A., . . . Kobayashi, I. (2006). Analysis of mutations within multiple genes associated with resistance in a clinical isolate of *Neisseria gonorrhoeae* with reduced

ceftriaxone susceptibility that shows a multidrug-resistant phenotype. *International Journal of Antimicrobial Agents, 27*(1), 20-26.

Tanaka, M., Otsuki, M., Nishino, T., Kobayashi, I., Matsumoto, T., & Kumazawa, J. (1996). Mutation in DNA gyrase of norfloxacin-resistant clinical isolates of N*eisseria gonorrhoeae*. *Genitourinary Medicine, 72*(4), 295-297.

Tanaka, M., Sakuma, S., Takahashi, K., Nagahuzi, T., Saika, T., Kobayashi, I., & Kumazawa, J. (1998). Analysis of quinolone resistance mechanisms in *Neisseria gonorrhoeae* isolates in vitro. *Sexually Transmitted Infections, 74*(1), 59-62.

Tapsall, J. (2001). *Antimicrobial resistance in Neisseria gonorrhoeae*. Retrieved from

Tapsall, J. W. (2005). Antibiotic resistance in *Neisseria gonorrhoeae*. *Clinical Infectious Diseases, 41*(Suppl. 4), S263-268.

Tapsall, J. W., Ndowa, F., Lewis, D. A., & Unemo, M. (2009). Meeting the public health challenge of multidrug-and extensively drug-resistant *Neisseria gonorrhoeae*. *Expert Review of Anti-infective Therapy, 7*(7), 821-834.

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *American Journal of Infection Control, 34*(Suppl. 1), S3-10.

Tobiason, D. M., & Seifert, H. S. (2006). The obligate human pathogen, *Neisseria gonorrhoeae*, is polyploid. *PLoS Biology, 4*(6), e185.

Tobiason, D. M., & Seifert, H. S. (2010). Genomic content of Neisseria species. *Journal of Bacteriology, 192*(8), 2160-2168.

Tonjum, T. (2005). Neisseria Trevisan 1885, 105 AL. *Bergey's Manual of Systematic Bacteriology*, 777-798.

Trembizki, E., Buckley, C., Lawrence, A., Lahra, M., Whiley, D., & Investigators, G. S. (2014). Characterization of a novel *Neisseria gonorrhoeae* penicillinase-producing plasmid isolated in Australia in 2012. *Antimicrobial Agents and Chemotherapy, 58*(8), 4984-4985.

Turner, A., Gough, K. R., & Leeming, J. P. (1999). Molecular epidemiology of *tetM* genes in *Neisseria gonorrhoeae*. *Sexually Transmitted Diseases, 75*(1), 60-66.

Unemo, M. (2015). Current and future antimicrobial treatment of gonorrhoea - the rapidly evolving *Neisseria gonorrhoeae* continues to challenge. *BMC Infectious Diseases, 15*, 364.

Unemo, M., Ballard, R., Ison, C., Lewis, D., Ndowa, F., & Peeling, R. (2013). *Laboratory diagnosis of sexually transmitted infections, including human immunodeficiency virus*. Geneva, Switzerland: WHO Press.

Unemo, M., Del Rio, C., & Shafer, W. M. (2016). Antimicrobial Resistance Expressed by *Neisseria gonorrhoeae*: A Major Global Public Health Problem in the 21st Century. *Microbiology Spectrum, 4*(3), 10.

Unemo, M., & Dillon, J.-A. R. (2011). Review and International Recommendation of Methods for Typing *Neisseria gonorrhoeae* Isolates and Their Implications for Improved Knowledge of Gonococcal Epidemiology, Treatment, and Biology. *Clinical Microbiology Reviews, 24*(3), 447.

Unemo, M., Fasth, O., Fredlund, H., Limnios, A., & Tapsall, J. (2009). Phenotypic and genetic characterization of the 2008 WHO *Neisseria gonorrhoeae* reference strain panel intended for global quality assurance and quality control of gonococcal antimicrobial resistance surveillance for public health purposes. *Journal of Antimicrobial Chemotherapy, 63*(6), 1142-1151.

Unemo, M., Golparian, D., Nicholas, R., Ohnishi, M., Gallay, A., & Sednaoui, P. (2012). High-level cefixime-and ceftriaxone-resistant *Neisseria gonorrhoeae* in France: novel *penA* mosaic allele in a successful international clone causes treatment failure. *Antimicrobial Agents and Chemotherapy, 56*(3), 1273-1280.

Unemo, M., Golparian, D., Potocnik, M., & Jeverica, S. (2012). Treatment failure of pharyngeal gonorrhoea with internationally recommended first-line ceftriaxone verified in Slovenia, September 2011. *Eurosurveillance, 17*(25), 20200.

Unemo, M., Golparian, D., Skogen, V., Olsen, A. O., Moi, H., Syversen, G., & Hjelmevoll, S. O. (2013). *Neisseria gonorrhoeae* strain with high-level resistance to spectinomycin due to a novel resistance mechanism (mutated ribosomal protein S5) verified in Norway. *Antimicrobial Agents and Chemotherapy, 57*(2), 1057-1061.

Unemo, M., & Nicholas, R. A. (2012). Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhea. *Future Microbiology, 7*(12), 1401-1422.

Unemo, M., & Shafer, W. M. (2011). Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. *Annals of the New York Academy of Sciences, 1230*, E19-28.

Unemo, M., & Shafer, W. M. (2014). Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. *Clinical Microbiology Reviews, 27*(3), 587-613.

van Hall, M. A., Petit, P. L., van Hall, H. N., Mouton, R. P., & Ndinya-Achola, J. O. (1991). Prevalence of resistance of *N. gonorrhoeae* to penicillin and three other antibiotics in a rural area in Kenya. *East African Medical Journal, 68*(11), 853-859.

van Putten, J. P. (1993). Phase variation of lipopolysaccharide directs interconversion of invasive and immuno-resistant phenotypes of *Neisseria gonorrhoeae*. *EMBO Journal, 12*(11), 4043-4051.

Van Slyke, C., Wolcott, R., & Mahoney, J. (1941). The chemotherapy of gonococcic infections: I. Summary of experience with sulfanilamide ii. Investigation of chemotherapeutic effect of sulfapyridine. *Journal of the American Medical Association, 116*(4), 276-280.

Van Slyke, C. J., Arnold, R. C., & Buchholtz, M. (1943). Penicillin Therapy in Sulfonamide-Resistant Gonorrhea in Men. *American Journal of Public Health and the Nation's Health, 33*(12), 1392-1394.

Verhagen, A. R., Van der Ham, M., Heimans, A. L., Kranendonk, O., & Maina, A. N. (1971). Diminished antibiotic sensitivity of *Neisseria gonorrhoeae* in urban and rural areas in Kenya. *Bull World Health Organ, 45*(6), 707-717.

Viberg, N., Mujinja, P., Kalala, W., Kumaranayake, L., Vyas, S., Tomson, G., & Lundborg, C. S. (2009). STI management in Tanzanian private drugstores-practices and roles of drugsellers. *Sexually Transmitted Diseases, 85*(4), 300-307.

Victor, L. Y. (2005). *Antimicrobial Therapy And Vaccines: Microbial Agents* (Vol. 2): Apple Trees Productions.

Virji, M. (2009). Pathogenic neisseriae: surface modulation, pathogenesis and infection control. *Nature Reviews Microbiology, 7*(4), 274-286.

Vollmer, W., Blanot, D., & De Pedro, M. A. (2008). Peptidoglycan structure and architecture. *FEMS Microbiology Reviews, 32*(2), 149-167.

Wang, J., Gray-Owen, S. D., Knorre, A., Meyer, T. F., & Dehio, C. (1998). Opa binding to cellular CD66 receptors mediates the transcellular traversal of *Neisseria gonorrhoeae* across polarized T84 epithelial cell monolayers. *Mol Microbiol, 30*(3), 657-671.

Wang, X., Minasov, G., & Shoichet, B. K. (2002). Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. *Journal of Molecular Biology, 320*(1), 85-95.

Warner, D. M., Shafer, W. M., & Jerse, A. E. (2008). Clinically relevant mutations that cause derepression of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. *Mol Microbiol, 70*(2), 462-478.

Weel, J. F., Hopman, C. T., & van Putten, J. P. (1991). Bacterial entry and intracellular processing of *Neisseria gonorrhoeae* in epithelial cells: immunomorphological evidence for alterations in the major outer membrane protein P.IB. *Journal of Experimental Medicine, 174*(3), 705-715.

Westrom, L., Joesoef, R., Reynolds, G., Hagdu, A., & Thompson, S. E. (1992). Pelvic inflammatory disease and fertility. A cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. *Sexually Transmitted Diseases, 19*(4), 185-192.

Whiley, D. M., Jennison, A., Pearson, J., & Lahra, M. M. (2018). Genetic characterisation of *Neisseria gonorrhoeae* resistant to both ceftriaxone and azithromycin. *Lancet Infectious Diseases, 18*(7), 717-718.

Whiley, D. M., Limnios, E. A., Ray, S., Sloots, T. P., & Tapsall, J. W. (2007a). Diversity of *penA* alterations and subtypes in *Neisseria gonorrhoeae* strains from

Sydney, Australia, that are less susceptible to ceftriaxone. *Antimicrobial Agents and Chemotherapy, 51*(9), 3111-3116.

Whiley, D. M., Limnios, E. A., Ray, S., Sloots, T. P., & Tapsall, J. W. (2007b). Further questions regarding the role of mosaic *penA* sequences in conferring reduced susceptibility to ceftriaxone in *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy, 51*(2), 802-803.

Whiley, D. M., Tapsall, J. W., & Sloots, T. P. (2006). Nucleic acid amplification testing for *Neisseria gonorrhoeae*: an ongoing challenge. *Journal of Molecular Diagnostics, 8*(1), 3-15.

WHO. (1993). 1993 sexually transmitted diseases treatment guidelines. Centers for Disease Control and Prevention. *Morbidity and Mortality Weekly Report, 42*(RR-14), 1-102.

Wi, T., Lahra, M. M., Ndowa, F., Bala, M., Dillon, J. R., Ramon-Pardo, P., . . . Unemo, M. (2017). Antimicrobial resistance in *Neisseria gonorrhoeae*: Global surveillance and a call for international collaborative action. *PLoS Medicine, 14*(7), e1002344.

Wolfgang, M., Lauer, P., Park, H. S., Brossay, L., Hebert, J., & Koomey, M. (1998). PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. *Mol Microbiol, 29*(1), 321-330.

Workowski, K. A., Berman, S. M., & Douglas, J. M., Jr. (2008). Emerging antimicrobial resistance in *Neisseria gonorrhoeae*: urgent need to strengthen prevention strategies. *Annals of Internal Medicine, 148*(8), 606-613.

Workowski, K. A., & Bolan, G. A. (2015). Sexually transmitted diseases treatment guidelines, 2015. *Morbidity and Mortality Weekly Report, 64*(RR-03), 1-137.

Yang, Q. L., & Gotschlich, E. C. (1996). Variation of gonococcal lipooligosaccharide structure is due to alterations in poly-G tracts in lgt genes encoding glycosyl transferases. *Journal of Experimental Medicine, 183*(1), 323-327.

Yang, Y., Liao, M., Gu, W. M., Bell, K., Wu, L., Eng, N. F., . . . Dillon, J. A. (2006). Antimicrobial susceptibility and molecular determinants of quinolone resistance in *Neisseria gonorrhoeae* isolates from Shanghai. *Journal of Antimicrobial Chemotherapy, 58*(4), 868-872.

Yeung, K. H., Dillon, J. R., Pauze, M., & Wallace, E. (1986). A novel 4.9-kilobase plasmid associated with an outbreak of penicillinase-producing *Neisseria gonorrhoeae*. *Journal of Infectious Diseases, 153*(6), 1162-1165.

Zapun, A., Morlot, C., & Taha, M.-K. (2016). Resistance to β-lactams in Neisseria ssp due to chromosomally encoded penicillin-binding proteins. *Antibiotics, 5*(4), 35.

Zarantonelli, L., Borthagaray, G., Lee, E. H., & Shafer, W. M. (1999). Decreased azithromycin susceptibility of *Neisseria gonorrhoeae* due to *mtrR* mutations. *Antimicrobial Agents and Chemotherapy, 43*(10), 2468-2472.

Zhao, S., Duncan, M., Tomberg, J., Davies, C., Unemo, M., & Nicholas, R. A. (2009). Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in *Neisseria gonorrhoeae*. *Antimicrobial Agents and Chemotherapy, 53*(9), 3744-3751.

# APPENDICES

## Appendix I: Antimicrobial susceptibility data for the study isolates.

| Isolate details | | | | MICs (mg/L) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample ID | Region | Year | Host gender | CFX | CRO | PEN | DOX | TET | NOR | CIP | AZM | SPT | GEN |
| KNY_NGAMR1 | Nairobi | 2015 | Male | <0.016 | <0.002 | 0.064 | 0.75 | 0.064 | 1.5 | 0.38 | 0.25 | 8 | 3 |
| KNY_NGAMR2 | Nairobi | 2015 | Male | <0.016 | 0.002 | 64 | 1.5 | 0.5 | 0.094 | 0.016 | 0.5 | 8 | 2 |
| KNY_NGAMR3 | Nairobi | 2015 | Male | <0.016 | NT | 48 | 6 | 16 | 6 | 3 | 0.125 | 6 | 1.5 |
| KNY_NGAMR4 | Coast | 2016 | Male | <0.016 | <0.002 | 8 | 16 | 12 | 0.023 | 0.006 | 0.125 | 2 | 2 |
| KNY_NGAMR5 | Nyanza | 2016 | Male | <0.016 | 0.006 | 3 | 12 | 16 | 6 | 4 | 0.125 | 2 | 0.75 |
| KNY_NGAMR6 | Coast | 2017 | Male | <0.016 | <0.016 | 0.094 | 0.75 | 0.75 | 12 | 12 | 0.25 | 3 | 2 |
| KNY_NGAMR7 | Coast | 2014 | Male | <0.016 | 0.008 | >256 | 12 | 16 | 12 | 8 | 2 | 16 | 6 |
| KNY_NGAMR8 | Nyanza | 2013 | Female | <0.016 | 0.002 | 0.064 | 0.5 | 0.5 | 6 | 3 | 0.25 | 4 | 3 |
| KNY_NGAMR9 | Nyanza | 2016 | Male | <0.016 | 0.004 | 0.094 | 16 | 32 | 24 | 12 | 0.25 | 6 | 6 |
| KNY_NGAMR10 | Nyanza | 2016 | Male | <0.016 | <0.016 | >256 | 24 | 32 | 12 | 8 | 0.38 | 16 | 3 |
| KNY_NGAMR11 | Nyanza | 2016 | Male | <0.016 | <0.016 | 12 | 12 | 12 | 8 | 3 | 0.25 | 8 | 4 |
| KNY_NGAMR13 | Rift valley | 2014 | Male | <0.016 | <0.002 | 8 | 12 | 12 | 12 | 3 | 0.125 | 24 | 2 |
| KNY_NGAMR14 | Rift valley | 2015 | Male | <0.016 | <0.002 | 64 | 8 | 24 | 24 | 16 | 0.125 | 8 | 2 |
| KNY_NGAMR15 | Nyanza | 2014 | Male | <0.016 | <0.016 | 48 | 16 | 32 | 16 | 4 | 0.5 | 12 | 6 |
| KNY_NGAMR16 | Nyanza | 2015 | Male | <0.016 | <0.016 | 0.19 | 8 | 4 | 6 | 8 | 0.38 | 4 | 6 |
| KNY_NGAMR17 | Nyanza | 2015 | Male | <0.016 | <0.016 | 0.38 | 12 | 16 | 12 | 6 | 0.5 | 6 | 12 |
| KNY_NGAMR18 | Nyanza | 2015 | Male | <0.016 | <0.016 | 0.5 | 32 | 24 | 0.047 | 0.38 | 0.5 | 6 | 6 |
| KNY_NGAMR19 | Nyanza | 2015 | Male | <0.016 | 0.094 | 0.19 | 96 | 64 | 8 | 4 | 1.5 | 1 | 2 |
| KNY_NGAMR20 | Nyanza | 2015 | Male | <0.016 | 0.004 | 12 | 16 | 24 | 12 | 8 | 0.125 | 12 | 3 |
| KNY_NGAMR21 | Nyanza | 2016 | Male | <0.016 | <0.016 | 32 | 16 | 24 | 8 | 3 | 0.125 | 12 | 6 |
| KNY_NGAMR22 | Nyanza | 2016 | Male | <0.016 | 0.004 | 2 | 12 | 32 | 12 | 4 | 1 | 12 | 4 |
| KNY_NGAMR23 | Nyanza | 2017 | Female | <0.016 | <0.002 | 12 | 16 | 8 | 12 | 12 | 0.125 | 4 | 2 |
| KNY_NGAMR24 | Nyanza | 2014 | Male | <0.016 | <0.016 | 2 | 16 | 16 | 12 | 4 | 0.38 | 8 | 8 |
| KNY_NGAMR25 | Nyanza | 2014 | Female | NT | NT | 0.094 | 12 | 24 | 12 | 2 | 0.094 | NT | 8 |
| KNY_NGAMR26 | Nyanza | 2016 | Male | <0.016 | <0.016 | 8 | 24 | 32 | 1 | 1.5 | 0.25 | 8 | 4 |
| KNY_NGAMR27 | Nyanza | 2016 | Male | <0.016 | <0.016 | 1 | 16 | 32 | 12 | 24 | 0.25 | 8 | 4 |
| KNY_NGAMR28 | Nyanza | 2017 | Female | <0.016 | <0.002 | 96 | 24 | 32 | 12 | 24 | 0.5 | 8 | 4 |
| KNY_NGAMR29 | Nyanza | 2017 | Male | <0.016 | <0.002 | 0.094 | 4 | 12 | 12 | 12 | 0.25 | 2 | 4 |
| KNY_NGAMR30 | Nyanza | 2017 | Male | <0.016 | <0.002 | 12 | 12 | 12 | 4 | 4 | 0.125 | 8 | 4 |
| KNY_NGAMR31 | Nyanza | 2017 | Male | <0.016 | <0.002 | 0.38 | 24 | 48 | 12 | >32 | 0.25 | 3 | 1 |
| KNY_NGAMR32 | Nyanza | 2017 | Female | <0.016 | <0.002 | 8 | 1 | 0.5 | 8 | 16 | 0.047 | 1.5 | 3 |
| KNY_NGAMR33 | Nyanza | 2016 | Male | <0.016 | <0.016 | 3 | 24 | 32 | 8 | 2 | 0.25 | 4 | 12 |
| KNY_NGAMR35 | Nairobi | 2013 | Female | NT | NT | >256 | 24 | 48 | 6 | 32 | 0.125 | NT | 8 |
| KNY_NGAMR36 | Nairobi | 2013 | Female | <0.016 | <0.016 | NT | 0.19 | 0.25 | 6 | 8 | 0.125 | 1 | 1.5 |
| KNY_NGAMR41 | Nyanza | 2018 | Male | <0.016 | <0.016 | 32 | 12 | 12 | 12 | 16 | NT | 4 | 4 |
| KNY_NGAMR42 | Nyanza | 2018 | Male | <0.016 | <0.016 | 32 | 12 | 24 | 12 | 6 | 0.125 | 8 | 1.5 |
| KNY_NGAMR45 | Nyanza | 2018 | Male | NT | NT | NT | NT | NT | NT | NT | NT | NT | NT |
| KNY_NGAMR50 | Nairobi | 2018 | Male | <0.016 | <0.016 | 16 | 32 | 16 | 12 | 1.5 | 0.064 | 4 | 3 |
| KNY_NGAMR52 | Coast | 2017 | Female | <0.016 | <0.016 | 0.38 | NT | NT | 12 | >32 | 0.25 | 3 | 1.5 |
| KNY_NGAMR53 | Rift valley | 2018 | Male | <0.016 | <0.016 | 6 | 16 | 6 | 12 | 3 | 0.19 | 4 | 2 |
| KNY_NGAMR54 | Rift valley | 2018 | Male | <0.016 | <0.016 | 6 | 8 | 2 | 3 | 2 | 0.125 | 6 | 4 |

*The following indicate: CFX- cefixime, CRO- ceftriaxone, Pen- penicillin, DOX-doxycycline, TET-tetracycline, NOR-norfloxacin, CIP-ciprofloxacin, AZM-azithromycin, SPT-spectinomycin, and GEN- gentamycin*

**Appendix II: EUCAST version 8.0, 2018 standard breakpoints**

| Antibiotic | MIC breakpoint (mg/L)* | |
|---|---|---|
| | Susceptible ≤ | Resistant > |
| Ceftriaxone | 0.125 | 0.125 |
| Cefixime | 0.125 | 0.125 |
| Azithromycin | 0.25 | 0.5 |
| Ciprofloxacin | 0.03 | 0.06 |
| Spectinomycin | 64 | 64 |
| Tetracycline | 0.5 | 1 |
| Penicillin | 0.06 | 1 |

*There are no EUCAST MIC breakpoints for norfloxacin, gentamycin and doxycycline for *N. gonorrhoeae* in 2018 standards.

**Appendix III: KEMRI SERU approval**

20 JAN 2017

# KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

**KEMRI/RES/7/3/1** **January 17, 2017**

**TO:** **MARY WANDIA KIVATA,**
**PRINCIPAL INVESTIGATOR**

**THROUGH:** **DR. LEAH KIRUMBI,**
**ACTING DIRECTOR,CCR,**
**NAIROBI**

20/1/17

Dear Madam,

**RE:** **PROTOCOL NO. KEMRI/SERU/CCR/0053/3385 (*RESUBMISSION OF INITIAL SUBMISSION*): MOLECULAR CHARACTERIZATION OF ATIMICROBIAL RESISTANCE GENES IN NEISSERIA GONORRHEAE ISOLATES FROM KENYA THROUGH GENOME SEQUENCING**

Reference is made to your letter dated 13th December, 2016. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on 12th January, 2017.

This is to inform you that the Committee notes that the issues raised during the 257th Committee B meeting of the KEMRI/SERU held on **16th November, 2016** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **17th January, 2017** for a period of one year. Please note that authorization to conduct this study will automatically expire on **January 16, 2018.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **5th December, 2017**.

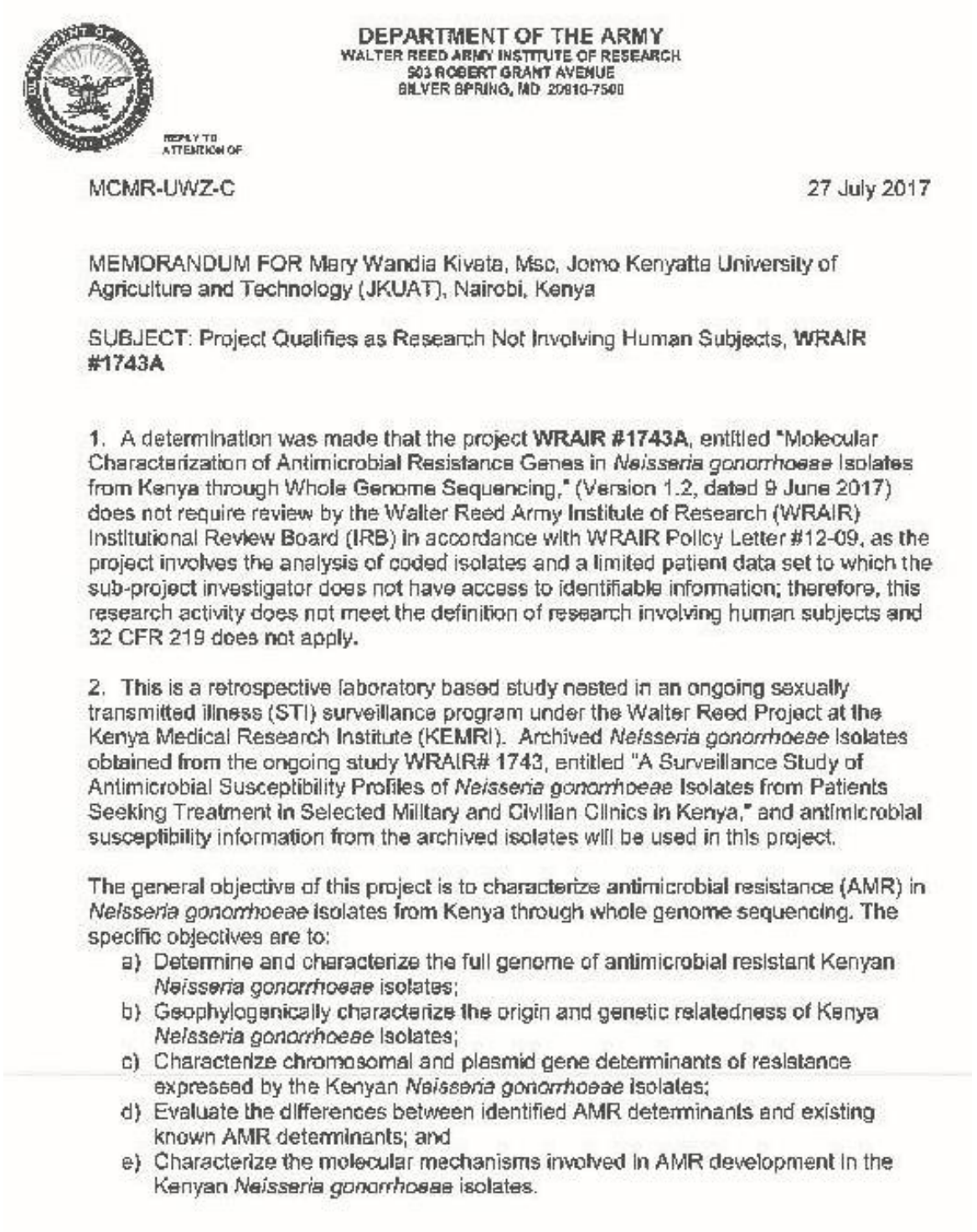
You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

FOR: **DR. EVANS AMUKOYE,**
**ACTING HEAD,**
**KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT**

**Appendix IV: WRAIR approval**



DEPARTMENT OF THE ARMY
WALTER REED ARMY INSTITUTE OF RESEARCH
503 ROBERT GRANT AVENUE
SILVER SPRING, MD 20910-7500

REPLY TO ATTENTION OF

MCMR-UWZ-C

27 July 2017

MEMORANDUM FOR Mary Wandia Kivata, Msc, Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya

SUBJECT: Project Qualifies as Research Not Involving Human Subjects, **WRAIR #1743A**

1. A determination was made that the project **WRAIR #1743A**, entitled "Molecular Characterization of Antimicrobial Resistance Genes in *Neisseria gonorrhoeae* Isolates from Kenya through Whole Genome Sequencing," (Version 1.2, dated 9 June 2017) does not require review by the Walter Reed Army Institute of Research (WRAIR) Institutional Review Board (IRB) in accordance with WRAIR Policy Letter #12-09, as the project involves the analysis of coded isolates and a limited patient data set to which the sub-project investigator does not have access to identifiable information; therefore, this research activity does not meet the definition of research involving human subjects and 32 CFR 219 does not apply.

2. This is a retrospective laboratory based study nested in an ongoing sexually transmitted illness (STI) surveillance program under the Walter Reed Project at the Kenya Medical Research Institute (KEMRI). Archived *Neisseria gonorrhoeae* isolates obtained from the ongoing study WRAIR# 1743, entitled "A Surveillance Study of Antimicrobial Susceptibility Profiles of *Neisseria gonorrhoeae* Isolates from Patients Seeking Treatment in Selected Military and Civilian Clinics in Kenya," and antimicrobial susceptibility information from the archived isolates will be used in this project.

The general objective of this project is to characterize antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* isolates from Kenya through whole genome sequencing. The specific objectives are to:

a) Determine and characterize the full genome of antimicrobial resistant Kenyan *Neisseria gonorrhoeae* isolates;
b) Geophylogenically characterize the origin and genetic relatedness of Kenya *Neisseria gonorrhoeae* isolates;
c) Characterize chromosomal and plasmid gene determinants of resistance expressed by the Kenyan *Neisseria gonorrhoeae* isolates;
d) Evaluate the differences between identified AMR determinants and existing known AMR determinants; and
e) Characterize the molecular mechanisms involved in AMR development in the Kenyan *Neisseria gonorrhoeae* isolates.

MCMR-UWZ-C
SUBJECT: Project Qualifies as Research Not Involving Human Subjects, WRAIR #1743A

The isolates and associated clinical data are recoded with a unique identification (ID), which do not have any subject identifiers. Neither the Principal Investigator (PI) of this project nor the PI of the parent protocol will have access to the code links between the subject identifiers of WRAIR #1743 and the isolates and data for WRAIR# 1743A.

All work will be conducted at the KEMRI Center for Microbiology Research Laboratory. Note that the project PI is a student at the JKUAT. This project is in partial fulfillment for the award of a Doctor of Philosophy (Biotechnology) degree in the Institute for Biotechnology Research, JKUAT.

3. The KEMRI Scientific and Ethics Review Unit reviewed and provisionally approved the subproject on 14 July 2017. This approval also covers the JKUAT. Please provide the ratified approval once it is available.

4. This project is being funded by the Armed Forces Health Surveillance Branch of Global Emerging Infection Surveillance and Response System Program (GEIS).

5. Per the WRAIR Education Policy Letter #11-49, all individuals covered under the WRAIR Human Research Protection Program (HRPP) are required to complete Collaborative Institutional Training Initiative (CITI) training; documentation of completed CITI training has been provided for all research personnel.

6. The PI has the responsibility to obtain all business agreements prior to initiation of any work with partners/collaborators or contracted services. This includes any transfer of data or specimens. Failure to obtain business agreements prior to initiation could result in sanctions or disciplinary actions for both the Detachment Director and PI. The IRB and the HSPB will review business agreements as part of monitoring visits to ensure they were obtained as required and report to the WRAIR Commander as to adherence to this requirement. Please seek clarification from the Office of Research Technology and Applications (ORTA).

Note that a summary of study activities for this project should be included within the continuing review report for the core protocol (WRAIR #1743).

7. No additional information is required at this time. However, should the project investigator or the study personnel gain access to the codes linking the subjects with his/her specimen, should additional assays be used other than those outlined in the project, or should any amendments alter any of the WRAIR's laboratory responsibilities, the submitted project would need an independent determination by the Chair, WRAIR IRB, or the Director, HSPB, as to whether or not the investigator is engaged in human subjects research, and whether or not the WRAIR IRB review and approval are

MCMR-UWZ-C
SUBJECT: Project Qualifies as Research Not Involving Human Subjects, **WRAIR #1743A**

required. No changes, amendments, or addenda may be made to the project without prospective approval/acknowledgement. The WRAIR HSPB reserves the right to review the project records to re-assess the determination of research not involving human subjects. The WRAIR HSPB also reserves the right to review the project records and re-assess the "research not involving human subjects" determination as part of post approval compliance monitoring. The PI is responsible for maintaining records that confirm that the executed activities match the project that was evaluated and found to be "research not involving human subjects."

8. The point of contact for this action is Teresa R. Soderberg, M.A., RAC, at (301) 319-9438 or Teresa.R.Soderberg.civ@mail.mil.

JODY L. PERENCE, M.S., CIP, CCRA, CIM
Director, Human Subjects Protection Branch
Walter Reed Army Institute of Research

CF:
Douglas Shaffer, M.D.
Victor Melendez, LTC, MS
Margaret Mbuchi, M.D.
Josephine Kabutu
ORTA, WRAIR

**Appendix V: Plasmid DNA extraction using QIAprep Spin Miniprep Kit**

Things to do before starting

- AddRNaseAsolutiontoBufferP1.
- Optional: Add Lyse Blue reagent to Buffer P1.
- Add ethanol (96–100%) to Buffer PE.
- Check Buffers P2 and N3 for salt precipitation and re-dissolve at 37°C if necessary.

**Procedure**

1. Label a 1.5 ml microcentrifuge tube with samples identity.
2. Using a sterile inoculation loop pick a distinct colony of grown and confirmed *N. gonorrhoeae* isolates from each culture plate and inoculate a culture of 1–5ml LB medium containing the appropriate selective antibiotic. Incubate for 12–16h at 37°C with vigorous shaking.
3. Harvest the bacterial cells by centrifugation at > 8000rpm (6800 x *g*) in a conventional, table-top microcentrifuge for 3min at room temperature (15–25°C).
4. Re-suspend the pelleted bacterial cells in 250µl Buffer P1 and transfer to a microcentrifuge tube.
5. Add 250µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times.
6. Add 350µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.
7. Centrifuge for 10min at 13,000rpm (~17,900 x g) in a table-top microcentrifuge.
8. Apply 800µl of the supernatant from step 4 to the QIAprep spin column by pipetting.
9. Centrifuge for 30–60s. Discard the flow-through.
10. Wash the QIAprep spin column by adding 0.5ml Buffer PB and centrifuging for 30–60s. Discard the flow-through.

11. Wash QIAprep spin column by adding 0.75ml Buffer PE and centrifuging for 30–60s.
12. Discard the flow-through, and centrifuge at full speed for an additional 1min to remove residual wash buffer.
13. Place the QIAprep column in a clean 1.5ml microcentrifuge tube. To elute DNA, add 50µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of each QIAprep spin column, let stand for 1min, and centrifuge for 1min.
14. Store the DNA at 4°C prior to subsequent analysis

**Appendix VI: Genomic DNA extraction using QIAamp DNA Mini Kit (for bacterial cultures)**

Things to do before starting

- Add the appropriate amount of ethanol (96–100%) as indicated on the bottle to buffers AW1 and AW2 concentrates
- Heat t water bath or heating blocks to 56°C for use in step 3
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 8.
- If a precipitate has formed in Buffer ATL or Buffer AL, dissolve by incubating at 56°C.
- All centrifugation steps should be carried out at room temperature

**Procedure**

1. Label a 1.5ml microcentrifuge tube with samples identity.
2. Using a sterile inoculation loop pick a few distinct colonies of grown and confirmed *N. gonorrhoeae* isolates from culture plate and suspend in180µl of Buffer ATL by vigorous stirring.
3. Add 20µl proteinase K, mix by vortexing for 15s, and incubate at 56°C for 30min until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample.
4. Centrifuge the microcentrifuge tube and its contents briefly to remove drops from the inside of the lid
5. Add 200µl Buffer AL to the sample, mix by pulse-vortexing for 15s, and incubate at 70°C for 10min. Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from inside the lid.
6. Add 200µl ethanol (96–100%) to the sample and mix by pulse-vortexing for 15s. After mixing, briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from inside the lid.
7. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. Close the cap,

and centrifuge at 6000 x g (8000rpm) for 1min. Place the QIAamp Mini spin column in a clean 2ml collection tube (provided), and discard the tube containing

8. Carefully open the QIAamp Mini spin column and add 500µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1min. Place the QIAamp Mini spin column in a clean 2ml collection tube (provided), and discard the collection tube containing the filtrate
9. Carefully open the QIAamp Mini spin column and add 500µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3min.
10. Place the QIAamp Mini spin column in a new 2ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1min.
11. Place the QIAamp Mini spin column in a clean 1.5ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200µl Buffer AE or distilled water. Incubate at room temperature for 1min, and then centrifuge at 6000 x g (8000 rpm) for 1min.
12. Store the DNA at 4°C prior to subsequent analysis

**Appendix VII: DNA quantification using Qubit dsDNA HS Assay Kit**

1. Prepare the Qubit working solution by diluting the Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS Buffer.
2. Label the tube lids. (For both the standards and test samples)
3. Add 190µL of Qubit working solution to each individual assay tubes (For both the standards and test samples)
4. Add 10µL of each Qubit standard and10µL of test sample to the appropriate tube, then mix by vortexing 2-3s.
5. Allow all tubes to incubate at room temperature for 2min

   Proceed to "Reading standards and samples" on Qubit3.0 Fluorometer
6. Insert the tube containing Standard number 1 into the sample chamber, close the lid then press Read standard.
   - When the reading is complete (~3s), remove Standard #1.
7. Insert the tube containing Standard number 2 into the sample chamber, close the lid then press Read standard.
   - When the reading is complete, remove Standard #2.
   - Press Run samples
   - On the assay screen, select the sample volume and units
8. Insert a sample tube into the sample chamber, close the lid then press Read tube. When the reading is complete (~3s), remove the sample tube and record the results.

**Appendix VIII: Drug resistant *Neisseria gonorrhoeae* isolates from Kenya_NGS reads**

https://www.ncbi.nlm.nih.gov/sites/myncbi/1JG2T9uzdYJYK4/collections/60509152/public/

## Appendix IX: Single Nucleotide Polymorphism matrix

| | NGAMR10 | NGAMR11 | NGAMR13 | NGAMR14 | NGAMR15 | NGAMR16 | NGAMR17 | NGAMR18 | NGAMR19 | NGAMR1 | NGAMR20 | NGAMR21 | NGAMR22 | NGAMR23 | NGAMR24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NGAMR10 | 0 | 4735 | 4729 | 1488 | 4757 | 5028 | 4860 | 4632 | 4387 | 5079 | 4827 | 4732 | 4716 | 4826 | 4710 |
| NGAMR11 | 4735 | 0 | 440 | 4796 | 910 | 4199 | 4530 | 3320 | 4011 | 4976 | 1080 | 1245 | 3428 | 1085 | 3398 |
| NGAMR13 | 4729 | 440 | 0 | 4814 | 651 | 4221 | 4521 | 3242 | 4067 | 4963 | 918 | 1153 | 3350 | 923 | 3320 |
| NGAMR14 | 1488 | 4796 | 4814 | 0 | 4813 | 5160 | 4827 | 4905 | 4550 | 5164 | 4802 | 4756 | 4989 | 4801 | 4983 |
| NGAMR15 | 4757 | 910 | 651 | 4813 | 0 | 4086 | 4515 | 3454 | 4101 | 5032 | 1281 | 1495 | 3562 | 1284 | 3532 |
| NGAMR16 | 5028 | 4199 | 4221 | 5160 | 4086 | 0 | 4367 | 4707 | 3991 | 5031 | 4271 | 4447 | 4793 | 4272 | 4785 |
| NGAMR17 | 4860 | 4530 | 4521 | 4827 | 4515 | 4367 | 0 | 4465 | 3352 | 4781 | 4461 | 4356 | 4547 | 4460 | 4543 |
| NGAMR18 | 4632 | 3320 | 3242 | 4905 | 3454 | 4707 | 4465 | 0 | 4028 | 4726 | 3322 | 3315 | 112 | 3321 | 78 |
| NGAMR19 | 4387 | 4011 | 4067 | 4550 | 4101 | 3991 | 3352 | 4028 | 0 | 4497 | 3970 | 4106 | 4112 | 3969 | 4106 |
| NGAMR1 | 5079 | 4976 | 4963 | 5164 | 5032 | 5031 | 4781 | 4726 | 4497 | 0 | 4782 | 5066 | 4824 | 4781 | 4804 |
| NGAMR20 | 4827 | 1080 | 918 | 4802 | 1281 | 4271 | 4461 | 3322 | 3970 | 4782 | 0 | 1720 | 3428 | 5 | 3400 |
| NGAMR21 | 4732 | 1245 | 1153 | 4756 | 1495 | 4447 | 4356 | 3315 | 4106 | 5066 | 1720 | 0 | 3421 | 1719 | 3393 |
| NGAMR22 | 4716 | 3428 | 3350 | 4989 | 3562 | 4793 | 4547 | 112 | 4112 | 4824 | 3428 | 3421 | 0 | 3427 | 190 |
| NGAMR23 | 4826 | 1085 | 923 | 4801 | 1284 | 4272 | 4460 | 3321 | 3969 | 4781 | 5 | 1719 | 3427 | 0 | 3399 |
| NGAMR24 | 4710 | 3398 | 3320 | 4983 | 3532 | 4785 | 4543 | 78 | 4106 | 4804 | 3400 | 3393 | 190 | 3399 | 0 |
| NGAMR25 | 4420 | 3908 | 3960 | 4602 | 4094 | 4215 | 3551 | 3981 | 1673 | 4534 | 3819 | 4100 | 4065 | 3820 | 4059 |
| NGAMR26 | 4985 | 4287 | 4318 | 5019 | 4234 | 1033 | 4210 | 4751 | 3976 | 5017 | 4328 | 4543 | 4837 | 4329 | 4829 |
| NGAMR27 | 4822 | 4381 | 4339 | 4942 | 4244 | 4078 | 4474 | 4300 | 4271 | 4868 | 4220 | 4443 | 4402 | 4219 | 4378 |
| NGAMR28 | 499 | 4608 | 4601 | 1782 | 4693 | 4974 | 4969 | 4678 | 4441 | 5086 | 4695 | 4718 | 4762 | 4694 | 4756 |
| NGAMR29 | 5101 | 4133 | 4185 | 5170 | 4080 | 872 | 4452 | 4621 | 3989 | 5107 | 4216 | 4363 | 4709 | 4211 | 4699 |
| NGAMR2 | 4994 | 4892 | 4905 | 4974 | 4995 | 5167 | 4667 | 5062 | 4299 | 5355 | 4863 | 4872 | 5142 | 4862 | 5140 |
| NGAMR30 | 4668 | 1273 | 1111 | 4801 | 1556 | 3841 | 4544 | 3596 | 3973 | 4954 | 1686 | 1929 | 3704 | 1685 | 3674 |
| NGAMR31 | 879 | 4747 | 4705 | 1818 | 4808 | 4921 | 5141 | 4695 | 4667 | 5150 | 4727 | 4750 | 4779 | 4726 | 4773 |
| NGAMR32 | 4792 | 1127 | 911 | 4789 | 1284 | 4197 | 4489 | 3390 | 4048 | 4783 | 1363 | 1575 | 3498 | 1362 | 3468 |
| NGAMR33 | 4844 | 4292 | 4309 | 4992 | 4211 | 811 | 4255 | 4553 | 3942 | 4863 | 4331 | 4459 | 4635 | 4332 | 4631 |
| NGAMR35 | 309 | 4728 | 4725 | 1669 | 4749 | 5060 | 4916 | 4737 | 4402 | 5123 | 4808 | 4756 | 4821 | 4807 | 4815 |
| NGAMR36 | 4879 | 1415 | 1215 | 4952 | 1580 | 4306 | 4562 | 3352 | 4108 | 4955 | 1730 | 1992 | 3444 | 1735 | 3430 |
| NGAMR3 | 4426 | 4276 | 4292 | 4528 | 4296 | 4000 | 3431 | 4319 | 1316 | 4321 | 3929 | 4354 | 4399 | 3930 | 4397 |
| NGAMR41 | 4700 | 3412 | 3334 | 4973 | 3546 | 4773 | 4553 | 92 | 4116 | 4792 | 3414 | 3403 | 202 | 3413 | 170 |
| NGAMR42 | 4699 | 4490 | 4380 | 4960 | 4323 | 4507 | 4519 | 4454 | 4404 | 4869 | 4681 | 4462 | 4546 | 4681 | 4532 |
| NGAMR45 | 4826 | 4405 | 4363 | 4946 | 4268 | 4092 | 4482 | 4326 | 4277 | 4882 | 4244 | 4465 | 4414 | 4243 | 4404 |
| NGAMR4 | 4648 | 3185 | 3166 | 4841 | 3422 | 4824 | 4587 | 926 | 4338 | 4853 | 3471 | 3211 | 1032 | 3470 | 1004 |
| NGAMR50 | 1077 | 4403 | 4390 | 2070 | 4509 | 4895 | 4801 | 4755 | 4413 | 5040 | 4556 | 4463 | 4839 | 4555 | 4833 |
| NGAMR52 | 4721 | 2338 | 2169 | 4947 | 2520 | 4575 | 4646 | 3308 | 4242 | 4634 | 2525 | 2600 | 3410 | 2530 | 3386 |
| NGAMR53 | 5045 | 4237 | 4301 | 5122 | 4201 | 875 | 4411 | 4719 | 3942 | 5085 | 4272 | 4435 | 4807 | 4273 | 4797 |
| NGAMR54 | 4586 | 3296 | 3265 | 4823 | 3522 | 4801 | 4631 | 1619 | 4346 | 4490 | 3453 | 3507 | 1719 | 3452 | 1697 |
| NGAMR5 | 4301 | 3960 | 3929 | 4824 | 4033 | 4543 | 4894 | 4104 | 3961 | 4794 | 3977 | 4018 | 4202 | 3982 | 4182 |
| NGAMR6 | 4365 | 4520 | 4462 | 4364 | 4500 | 4731 | 4363 | 4366 | 4092 | 4892 | 4369 | 4409 | 4458 | 4368 | 4444 |
| NGAMR7 | 4634 | 4604 | 4561 | 4662 | 4363 | 4235 | 1003 | 4787 | 3216 | 4686 | 4482 | 4544 | 4869 | 4481 | 4865 |
| NGAMR8 | 4724 | 404 | 286 | 4828 | 784 | 4243 | 4522 | 3221 | 4043 | 4918 | 990 | 1161 | 3329 | 989 | 3299 |
| NGAMR9 | 4577 | 4384 | 4316 | 4588 | 4332 | 4719 | 4475 | 4524 | 4517 | 4900 | 4203 | 4233 | 4602 | 4202 | 4602 |

min: 5 max: 5366

| NGAMR25 | NGAMR26 | NGAMR27 | NGAMR28 | NGAMR29 | NGAMR2 | NGAMR30 | NGAMR31 | NGAMR32 | NGAMR33 | NGAMR35 | NGAMR36 | NGAMR3 | NGAMR41 | NGAMR42 | NGAMR45 | NGAMR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4420 | 4985 | 4822 | 499 | 5101 | 4994 | 4668 | 879 | 4792 | 4844 | 309 | 4879 | 4426 | 4700 | 4699 | 4826 | 4648 |
| 3908 | 4287 | 4381 | 4608 | 4133 | 4892 | 1273 | 4747 | 1127 | 4292 | 4728 | 1415 | 4276 | 3412 | 4490 | 4405 | 3185 |
| 3960 | 4318 | 4339 | 4601 | 4185 | 4905 | 1111 | 4705 | 911 | 4309 | 4725 | 1215 | 4292 | 3334 | 4380 | 4363 | 3166 |
| 4602 | 5019 | 4942 | 1782 | 5170 | 4974 | 4801 | 1818 | 4789 | 4992 | 1669 | 4952 | 4528 | 4973 | 4960 | 4946 | 4841 |
| 4094 | 4234 | 4244 | 4693 | 4080 | 4995 | 1556 | 4808 | 1284 | 4211 | 4749 | 1580 | 4296 | 3546 | 4323 | 4268 | 3422 |
| 4215 | 1033 | 4078 | 4974 | 872 | 5167 | 3841 | 4921 | 4197 | 811 | 5060 | 4306 | 4000 | 4773 | 4507 | 4092 | 4824 |
| 3551 | 4210 | 4474 | 4969 | 4452 | 4667 | 4544 | 5141 | 4489 | 4255 | 4916 | 4562 | 3431 | 4553 | 4519 | 4482 | 4587 |
| 3981 | 4751 | 4300 | 4678 | 4621 | 5062 | 3596 | 4695 | 3390 | 4553 | 4737 | 3352 | 4319 | 92 | 4454 | 4326 | 926 |
| 1673 | 3976 | 4271 | 4441 | 3989 | 4299 | 3973 | 4667 | 4048 | 3942 | 4402 | 4108 | 1316 | 4116 | 4404 | 4277 | 4338 |
| 4534 | 5017 | 4868 | 5086 | 5107 | 5355 | 4954 | 5150 | 4783 | 4863 | 5123 | 4955 | 4321 | 4792 | 4869 | 4882 | 4853 |
| 3819 | 4328 | 4220 | 4695 | 4216 | 4863 | 1686 | 4727 | 1363 | 4331 | 4808 | 1730 | 3929 | 3414 | 4681 | 4244 | 3471 |
| 4100 | 4543 | 4443 | 4718 | 4363 | 4872 | 1929 | 4750 | 1575 | 4459 | 4756 | 1992 | 4354 | 3403 | 4462 | 4465 | 3211 |
| 4065 | 4837 | 4402 | 4762 | 4709 | 5142 | 3704 | 4779 | 3498 | 4635 | 4821 | 3444 | 4399 | 202 | 4546 | 4414 | 1032 |
| 3820 | 4329 | 4219 | 4694 | 4211 | 4862 | 1685 | 4726 | 1362 | 4332 | 4807 | 1735 | 3930 | 3413 | 4681 | 4243 | 3470 |
| 4059 | 4829 | 4378 | 4756 | 4699 | 5140 | 3674 | 4773 | 3468 | 4631 | 4815 | 3430 | 4397 | 170 | 4532 | 4404 | 1004 |
| 0 | 4071 | 3919 | 4491 | 4103 | 4556 | 3900 | 4593 | 3942 | 4062 | 4367 | 4069 | 1809 | 4069 | 4426 | 3929 | 4318 |
| 4071 | 0 | 4354 | 4927 | 1106 | 5083 | 3884 | 4898 | 4326 | 1330 | 5072 | 4330 | 4010 | 4815 | 4630 | 4370 | 4898 |
| 3919 | 4354 | 0 | 4920 | 4031 | 5202 | 4345 | 4951 | 4281 | 4017 | 4803 | 4400 | 4456 | 4368 | 3557 | 122 | 4606 |
| 4491 | 4927 | 4920 | 0 | 5043 | 5089 | 4581 | 1042 | 4682 | 4776 | 646 | 4775 | 4495 | 4746 | 4749 | 4923 | 4689 |
| 4103 | 1106 | 4031 | 5043 | 0 | 5102 | 3816 | 4970 | 4156 | 1126 | 5144 | 4233 | 4002 | 4687 | 4529 | 4045 | 4689 |
| 4556 | 5083 | 5202 | 5089 | 5102 | 0 | 5002 | 5250 | 4947 | 5058 | 5008 | 5038 | 4456 | 5152 | 5366 | 5208 | 4992 |
| 3900 | 3884 | 4345 | 4581 | 3816 | 5002 | 0 | 4636 | 1657 | 3888 | 4661 | 1828 | 4259 | 3666 | 4527 | 4403 | 3509 |
| 4593 | 4898 | 4951 | 1042 | 4970 | 5250 | 4636 | 0 | 4786 | 4840 | 1059 | 4820 | 4666 | 4763 | 4950 | 4955 | 4836 |
| 3942 | 4326 | 4281 | 4682 | 4156 | 4947 | 1657 | 4786 | 0 | 4298 | 4794 | 1665 | 4153 | 3482 | 4262 | 4303 | 3380 |
| 4062 | 1330 | 4017 | 4776 | 1126 | 5058 | 3888 | 4840 | 4298 | 0 | 4890 | 4442 | 4083 | 4621 | 4406 | 4023 | 4648 |
| 4367 | 5072 | 4803 | 646 | 5144 | 5008 | 4661 | 1059 | 4794 | 4890 | 0 | 4869 | 4417 | 4805 | 4787 | 4807 | 4780 |
| 4069 | 4330 | 4400 | 4775 | 4233 | 5038 | 1828 | 4820 | 1665 | 4442 | 4869 | 0 | 4334 | 3442 | 4595 | 4456 | 3211 |
| 1809 | 4010 | 4456 | 4495 | 4002 | 4456 | 4259 | 4666 | 4153 | 4083 | 4417 | 4334 | 0 | 4407 | 4634 | 4466 | 4618 |
| 4069 | 4815 | 4368 | 4746 | 4687 | 5152 | 3666 | 4763 | 3482 | 4621 | 4805 | 3442 | 4407 | 0 | 4518 | 4394 | 1018 |
| 4426 | 4630 | 3557 | 4749 | 4529 | 5366 | 4527 | 4950 | 4262 | 4406 | 4787 | 4595 | 4634 | 4518 | 0 | 3583 | 4465 |
| 3929 | 4370 | 122 | 4923 | 4045 | 5208 | 4403 | 4955 | 4303 | 4023 | 4807 | 4456 | 4466 | 4394 | 3583 | 0 | 4628 |
| 4318 | 4898 | 4606 | 4689 | 4689 | 4992 | 3509 | 4836 | 3380 | 4648 | 4780 | 3211 | 4618 | 1018 | 4465 | 4628 | 0 |
| 4449 | 4837 | 4910 | 1234 | 4953 | 5151 | 4463 | 1611 | 4547 | 4716 | 1222 | 4652 | 4521 | 4844 | 4819 | 4914 | 4742 |
| 4343 | 4656 | 4534 | 4617 | 4631 | 5043 | 2691 | 4736 | 2683 | 4495 | 4708 | 2766 | 4420 | 3400 | 4714 | 4560 | 3357 |
| 4084 | 1163 | 4095 | 5047 | 325 | 5119 | 3855 | 4939 | 4272 | 1121 | 5092 | 4307 | 3937 | 4785 | 4619 | 4103 | 4801 |
| 4234 | 4859 | 4648 | 4665 | 4680 | 4973 | 3634 | 4651 | 3509 | 4674 | 4716 | 3485 | 4486 | 1687 | 4528 | 4670 | 1484 |
| 4367 | 4506 | 4867 | 4239 | 4425 | 4634 | 3985 | 4474 | 3990 | 4325 | 4360 | 4197 | 4394 | 4194 | 4919 | 4889 | 4158 |
| 4116 | 4779 | 4847 | 4490 | 4696 | 4929 | 4561 | 4285 | 4511 | 4738 | 4406 | 4447 | 4009 | 4432 | 4992 | 4875 | 4263 |
| 3443 | 4080 | 4487 | 4806 | 4280 | 4671 | 4505 | 5028 | 4432 | 4273 | 4693 | 4584 | 3185 | 4853 | 4476 | 4495 | 4912 |
| 3890 | 4323 | 4321 | 4602 | 4192 | 4938 | 1142 | 4651 | 960 | 4334 | 4720 | 1261 | 4237 | 3313 | 4433 | 4345 | 3186 |
| 4497 | 4737 | 4953 | 4578 | 4612 | 5125 | 4305 | 4329 | 4288 | 4762 | 4675 | 4217 | 4305 | 4588 | 5087 | 4993 | 4505 |

| NGAMR50 | NGAMR52 | NGAMR53 | NGAMR54 | NGAMR5 | NGAMR6 | NGAMR7 | NGAMR8 | NGAMR9 |
|---|---|---|---|---|---|---|---|---|
| 1077 | 4721 | 5045 | 4586 | 4301 | 4365 | 4634 | 4724 | 4577 |
| 4403 | 2338 | 4237 | 3296 | 3960 | 4520 | 4604 | 404 | 4384 |
| 4390 | 2169 | 4301 | 3265 | 3929 | 4462 | 4561 | 286 | 4316 |
| 2070 | 4947 | 5122 | 4823 | 4824 | 4364 | 4662 | 4828 | 4588 |
| 4509 | 2520 | 4201 | 3522 | 4033 | 4500 | 4363 | 784 | 4332 |
| 4895 | 4575 | 875 | 4801 | 4543 | 4731 | 4235 | 4243 | 4719 |
| 4801 | 4646 | 4411 | 4631 | 4894 | 4363 | 1003 | 4522 | 4475 |
| 4755 | 3308 | 4719 | 1619 | 4104 | 4366 | 4787 | 3221 | 4524 |
| 4413 | 4242 | 3942 | 4346 | 3961 | 4092 | 3216 | 4043 | 4517 |
| 5040 | 4634 | 5085 | 4490 | 4794 | 4892 | 4686 | 4918 | 4900 |
| 4556 | 2525 | 4272 | 3453 | 3977 | 4369 | 4482 | 990 | 4203 |
| 4463 | 2600 | 4435 | 3507 | 4018 | 4409 | 4544 | 1161 | 4233 |
| 4839 | 3410 | 4807 | 1719 | 4202 | 4458 | 4869 | 3329 | 4602 |
| 4555 | 2530 | 4273 | 3452 | 3982 | 4368 | 4481 | 989 | 4202 |
| 4833 | 3386 | 4797 | 1697 | 4182 | 4444 | 4865 | 3299 | 4602 |
| 4449 | 4343 | 4084 | 4234 | 4367 | 4116 | 3443 | 3890 | 4497 |
| 4837 | 4656 | 1163 | 4859 | 4506 | 4779 | 4080 | 4323 | 4737 |
| 4910 | 4534 | 4095 | 4648 | 4867 | 4847 | 4487 | 4321 | 4953 |
| 1234 | 4617 | 5047 | 4665 | 4239 | 4490 | 4806 | 4602 | 4578 |
| 4953 | 4631 | 325 | 4680 | 4425 | 4696 | 4280 | 4192 | 4612 |
| 5151 | 5043 | 5119 | 4973 | 4634 | 4929 | 4671 | 4938 | 5125 |
| 4463 | 2691 | 3855 | 3634 | 3985 | 4561 | 4505 | 1142 | 4305 |
| 1611 | 4736 | 4939 | 4651 | 4474 | 4285 | 5028 | 4651 | 4329 |
| 4547 | 2683 | 4272 | 3509 | 3990 | 4511 | 4432 | 960 | 4288 |
| 4716 | 4495 | 1121 | 4674 | 4325 | 4738 | 4273 | 4334 | 4762 |
| 1222 | 4708 | 5092 | 4716 | 4360 | 4406 | 4693 | 4720 | 4675 |
| 4652 | 2766 | 4307 | 3485 | 4197 | 4447 | 4584 | 1261 | 4217 |
| 4521 | 4420 | 3937 | 4486 | 4394 | 4009 | 3185 | 4237 | 4305 |
| 4844 | 3400 | 4785 | 1687 | 4194 | 4432 | 4853 | 3313 | 4588 |
| 4819 | 4714 | 4619 | 4528 | 4919 | 4992 | 4476 | 4433 | 5087 |
| 4914 | 4560 | 4103 | 4670 | 4889 | 4875 | 4495 | 4345 | 4993 |
| 4742 | 3357 | 4801 | 1484 | 4158 | 4263 | 4912 | 3186 | 4505 |
| 0 | 4674 | 4901 | 4748 | 4403 | 4639 | 4626 | 4362 | 4683 |
| 4674 | 0 | 4713 | 3722 | 3353 | 4534 | 4851 | 2251 | 4437 |
| 4901 | 4713 | 0 | 4811 | 4477 | 4681 | 4238 | 4300 | 4649 |
| 4748 | 3722 | 4811 | 0 | 4169 | 4460 | 4790 | 3282 | 4621 |
| 4403 | 3353 | 4477 | 4169 | 0 | 4517 | 4847 | 3943 | 4510 |
| 4639 | 4534 | 4681 | 4460 | 4517 | 0 | 4390 | 4418 | 1649 |
| 4626 | 4851 | 4238 | 4790 | 4847 | 4390 | 0 | 4552 | 4515 |
| 4362 | 2251 | 4300 | 3282 | 3943 | 4418 | 4552 | 0 | 4279 |
| 4683 | 4437 | 4649 | 4621 | 4510 | 1649 | 4515 | 4279 | 0 |

**Appendix X: Summary of β-Lactams antimicrobial susceptibility data**

| | Phenotype: MIC (mg/L) patterns* | | | | |
|---|---|---|---|---|---|
| Drug | Susceptible | Intermediate | Resistant | NT | Total |
| Ceftriaxone | 37 (≤ 0.125) | N/A | 0 (> 0.125) | 4 | 41 |
| Cefixime | 38 (≤ 0.125) | N/A | 0 (> 0.125) | 3 | 41 |
| Penicillin | 2 (≤ 0.06) | 11 (>0.06-1) | 26 (> 1) | 2 | 41 |

*MIC: Minimum Inhibitory Concentration, NT: Not Tested

## Appendix XI: Amino acid changes in the identified PenA and PorB patterns

**Non-mosaic PenA amino acid substitution patterns**

**Pattern XXII;** D346, F505L, A511V, A517G H542N, P553V, K556Q, I557V, I567V, N575, A576V

**Pattern IX;** D346, F505L, A511V, A517G, P552L

**Pattern XIX;** D346, F505L, A511V, A517G, H542N, I567V, N575, A576V

**Pattern XIV;** D346, F505L, A511V, A517G, H542N

**Pattern II;** D346, F505L, A511V, A517G

**PorB amino acid substitution patterns**

**Pattern I;** G120D

**Pattern II;** A121G, G120D, and -N122

**Pattern III;** A121G, G120N, and -N122

**Pattern IV;** A121S, and N122K

**Pattern V;** A121G, and -N122

**Appendix XII: Summary of tetracycline antimicrobial susceptibility data**

| | Phenotype: MIC (mg/L) patterns | | | | |
|---|---|---|---|---|---|
| Drug | Susceptible | Intermediate | Resistant | NT | Total |
| Tetracycline | 5 (≤ 0.5) | 1 (> 0.5-1) | 33 (> 1) | 2 | 41 |

NT: Not Tested

**Appendix XIII: Summary of ciprofloxacin antimicrobial susceptibility data**

| | Phenotype: MIC (mg/L) patterns | | | | |
|---|---|---|---|---|---|
| Drug | Susceptible | Intermediate | Resistant | NT | Total |
| Ciprofloxacin | 2 (≤ 0.03) | 0 (> 0.03-0.06) | 38 (> 0.06) | 1 | 41 |

NT: Not Tested

**Appendix XIV: Summary of azithromycin antimicrobial susceptibility data**

| | Phenotype: MIC (mg/L) patterns | | | | |
|---|---|---|---|---|---|
| Drug | Susceptible | Intermediate | Resistant | NT | Total |
| Azithromycin | 28 (≤ 0.25) | 8 (> 0.25-0.5) | 3 (> 0.5) | 2 | 41 |

NT: Not Tested

**Appendix XV: Summary of spectinomycin antimicrobial susceptibility data**

| | Phenotype: MIC (mg/L) patterns | | | | |
|---|---|---|---|---|---|
| Drug | Susceptible | Intermediate | Resistant | NT | Total |
| Spectinomycin | 38(≤ 64) | N/A | 0 (> 64) | 3 | 41 |

NT: Not Tested

**Appendix XVI: First published research article**

Kivata et al. BMC Microbiology (2019) 19:76
https://doi.org/10.1186/s12866-019-1439-1

BMC Microbiology

RESEARCH ARTICLE Open Access

# *gyrA* and *parC* mutations in fluoroquinolone-resistant *Neisseria gonorrhoeae* isolates from Kenya

Mary Wandia Kivata[2,3*†], Margaret Mbuchi[1,4†], Fredrick Lunyagi Eyase[1,2], Wallace Dimbuson Bulimo[1,6], Cecilia Katunge Kyanya[1], Valerie Oundo[1], Simon Wachira Muriithi[1], Ben Andagalu[1], Wilton Mwema Mbinda[3], Olusegun O. Soge[5], R. Scott McClelland[5], Willy Sang[1,4] and James D. Mancuso[1]

**Abstract**

**Background:** Phenotypic fluoroquinolone resistance was first reported in Western Kenya in 2009 and later in Coastal Kenya and Nairobi. Until recently gonococcal fluoroquinolone resistance mechanisms in Kenya had not been elucidated. The aim of this paper is to analyze mutations in both *gyrA* and *parC* responsible for elevated fluoroquinolone Minimum Inhibitory Concentrations (MICs) in *Neisseria gonorrhoeae* (GC) isolated from heterosexual individuals from different locations in Kenya between 2013 and 2017.

**Methods:** Antimicrobial Susceptibility Tests were done on 84 GC in an ongoing Sexually Transmitted Infections (STI) surveillance program. Of the 84 isolates, 22 resistant to two or more classes of antimicrobials were chosen for analysis. Antimicrobial susceptibility tests were done using E-test (BioMerieux) and the results were interpreted with reference to European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards. The isolates were sub-cultured, and whole genomes were sequenced using Illumina platform. Reads were assembled de novo using Velvet, and mutations in the GC Quinolone Resistant Determining Regions identified using Bioedit sequence alignment editor. Single Nucleotide Polymorphism based phylogeny was inferred using RaxML.

**Results:** Double GyrA amino acid substitutions; S91F and D95G/D95A were identified in 20 isolates. Of these 20 isolates, 14 had an additional E91G ParC substitution and significantly higher ciprofloxacin MICs ($p = 0.0044^{*}$). On the contrary, norfloxacin MICs of isolates expressing both GyrA and ParC QRDR amino acid changes were not significantly high ($p = 0.82$) compared to MICs of isolates expressing GyrA substitutions alone. No single GyrA substitution was found in the analyzed isolates, and no isolate contained a ParC substitution without the simultaneous presence of double GyrA substitutions. Maximum likelihood tree clustered the 22 isolates into 6 distinct clades.

**Conclusion:** Simultaneous presence of amino acid substitutions in ParC and GyrA has been reported to increase gonococcal fluoroquinolone resistance from different regions in the world. Our findings indicate that GyrA S91F, D95G/D95A and ParC E91G amino acid substitutions mediate high fluoroquinolone resistance in the analyzed Kenyan GC.

**Keywords:** Fluoroquinolones, Antimicrobial resistance (AMR), *Neisseria gonorrhoeae*, Mutation, Quinolone resistant determining regions (QRDR)

* Correspondence: marywandia086@gmail.com
†Mary Wandia Kivata and Margaret Mbuchi contributed equally to this work.
[2]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O Box 62,000-00200, Thika, Kenya
[3]Department of Biological and Physical Science, Karatina University (KarU), P.O Box 1957-10101, Karatina, Kenya
Full list of author information is available at the end of the article

## Background

The absence of a gonococcal vaccine reduces preventive measures for gonorrhea and antibiotics remain the only option for management of gonococcal infections. While initially sensitive to new antibiotics, some strains of *Neisseria gonorrhoeae* (GC) develop single or multiple resistance mechanisms to antibiotics. Gonococcal mechanisms of resistance can be plasmid mediated or result from different types of mutations in chromosomal DNA, as well as recombination processes [1–4]. Since the 1970s, a small number of studies have reported both chromosomal and plasmid mediated gonococcal penicillin and tetracycline resistance in Kenya [5–9]. These reports led to the introduction of fluoroquinolones as the first line of drugs for gonorrhea treatment in 1993 [10]. Subsequently, fluoroquinolone resistance was reported in western Kenya in 2009 [11], Coastal Kenya in 2011 and 2012 [12, 13], and Nairobi in 2012 [12]. These findings formed the basis for revision of national treatment guidelines in 2013. In the revised guidelines, ceftriaxone and cefixime replaced fluoroquinolones as first line drugs for gonorrhea treatment. Recently, investigators have begun to explore the molecular mechanisms causing fluoroquinolone resistance in Kenya. A 2018 publication focused on sex workers and Men who have Sex with Men (MSM) from Coastal Kenya found that S91F, D95G/A, and F504 L GyrA amino acid substitutions were associated with fluoroquinolone resistance [14].

Fluoroquinolones block DNA replication by inhibiting the enzymes DNA gyrase (topoisomerase II) and topoisomerase IV [15]. DNA gyrase catalyzes the untwisting of DNA molecules during DNA replication, and consists of two type A subunits and two type B subunits encoded by *gyrA* and *gyrB* genes [16]. Topoisomerase IV consists of two type C subunits and two type E subunits encoded by *parC* and *parE* genes. This enzyme is involved in the decatenation of covalently closed circular DNA molecules during DNA replication [15]. DNA-enzyme-fluoroquinolone-complex inhibits movement of the replication fork; a structure formed by organization of replication proteins and disrupts bacterial DNA replication. Gradual accumulation of point mutations in *gyrA* and *parC* Quinolone Resistant Determining Region (QRDR) leads to amino acid substitutions, which alter the three-dimensional structure of the target protein [17]. Alteration of the target protein structure reduces fluoroquinolone-target enzyme binding affinity, leading to resistance in gonococci [4, 18]. Gonococcal QRDR region lies between amino acids 55–110 and 56–140 in GyrA and ParC respectively [18]. QRDR amino acid substitutions linked to gonococcal fluoroquinolone resistance include substitutions at S91 by F or Y, and D95 in GyrA protein [17, 19]. The GyrA mutations initiate fluoroquinolone resistance in GC, while additional accumulation of substitutions in ParC at G85, D86, S87, S88, Q91 and R116 increases the resistance [4]. In addition to mutations in the QRDR, an in-vitro study by Tanaka et al., [20] suggested that mutations in PorB can cause reduced drug accumulation, which can contribute further to the development of gonococcal fluoroquinolone resistance.

The aim of this study was to analyze amino acid changes in GyrA and ParC QRDRs responsible for gonococcal fluoroquinolone resistance and determine the genetic diversity of multidrug resistant Kenyan GC. In a previous correlation study carried out in Japan, a significant increase in fluoroquinolone MICs ($P < 0.01$ for norfloxacin, $P = 0.058$ for ofloxacin, and $P < 0.05$ for ciprofloxacin) was observed in GC strains which expressed amino acid changes in both GyrA and ParC compared to those of strains expressing GyrA substitutions only [21]. This study therefore sought to further examine the correlation between GyrA and ParC amino acid changes and the level of fluoroquinolone resistance among GC isolated from heterosexual individuals from different geographical regions of Kenya between 2013 and 2017.

## Results

### Clinical and laboratory study population characteristics

The current study is a retrospective laboratory-based study nested in an ongoing Sexually Transmitted Infections (STI) surveillance program under Armed Forces Health Surveillance at the US Army Medical Research Directorate-Africa. A total of 583 symptomatic cases comprising of 332 males and 251 females were enrolled from four geographic locations in Kenya (Nairobi, Coastal Kenya, Nyanza, and Rift Valley) between 2013 and 2017. All presented with urethral discharge (males) or cervical discharge (females) accompanied by symptoms described in Table 1.

Eighty four *Neisseria gonorrhoeae* (GC) isolates were successfully obtained from 73 males and 11 females and their susceptibility to a panel of ten drugs (Ceftriaxone, Cefixime, Azithromycin, Ciprofloxacin, Norfloxacin,

**Table 1** Clinical and laboratory study population characteristics

| | Male | Female | Total |
|---|---|---|---|
| Gender | 332 (56.9%) | 251 (43.1) | 583 |
| Gram stain[a] | 168 (67.7%) | 80 (32.3) | 248 |
| Aptima Combo −2[a,b] | 205 (77.9%) | 58 (22.1) | 263 |
| GC Isolates | 73 (86.9%) | 11 (13.1%) | 84 |
| Symptoms | Positive | Negative | |
| Dysuria | 463 (79.4%) | 120 (20.6%) | 583 |
| Backache | 274 (47%) | 309 (53%) | 583 |
| Lower abdominal pain | 388 (66.6%) | 195 (33.4%) | 583 |
| Fever | 261 (44.8%) | 322 (55.2%) | 583 |
| Itchy genitalia | 342 (58.7%) | 241 (41.3%) | 583 |

[a]Positive for gram negtive diplococci by gram stain
[b]Positive for *Neisseria gonorrhoeae* (GC) by Aptima Combo −2*

Spectinomycin, Tetracycline, Doxycycline, Penicillin and Gentamicin) carried out using the E-test (bioMerieux) method. There was no difference in the prevalence of multidrug resistance among isolates from the different regions. Of the 84, 22 multi-drug resistant GC isolates (2 females and 20 males) were chosen for analysis.

### Antibiotic susceptibilities of studied isolates

Of the 22 isolates selected for sequencing 20 were ciprofloxacin resistant (MICs > 0.06 mg/L), whereas 2 were susceptible. All isolates were penicillin resistant (MICs> 0.06 mg/L) while 16 were tetracycline resistant (MICs> 1 mg/L). Low level azithromycin resistance (MICs> 0.05-2 mg/L) was observed in 7 isolates. None of the isolates were resistant to cefixime, ceftriaxone, or spectinomycin.

### Single nucleotide polymorphisms (SNPs) based phylogeny

Sixty sequences; 22 study genomes and 38 additional comparison genomes obtained from varied geographical regions, were mapped to *N gonorrhoeae* NCCP11945 (GenBank accession number CP001050) genome and SNPs identified through CSI phylogeny pipeline. A total of 24,143 SNPs were detected and were used to create a Maximum Likelihood tree using RaxML (Fig. 1). Among the analyzed Kenya GC, Isolates KNY_NGAMR1 and KNY_NGAMR2 were the most genetically diverse (5569SNPs), while KNY_NGAMR20 and KNY_NGAMR23 were closely related with a SNP difference of 10. Based on SNP phylogenetic tree, the 22 study isolates, and 4 isolates obtained from MSMs from Coastal Kenya in a recent study [14], clustered closely together into six distinct clades (Fig. 1). The close clustering shows that Kenyan isolates have evolved into their own lineages. However, there was no region based clustering observed among the 22 isolates.

### Fluoroquinolone resistance determinants

Of the 22 isolates, 2 had wild-type QRDRs, 6 had only *gyrA* QRDR mutations, and the remaining 14 had both *gyrA* and *parC* QRDR mutations. Double GyrA amino

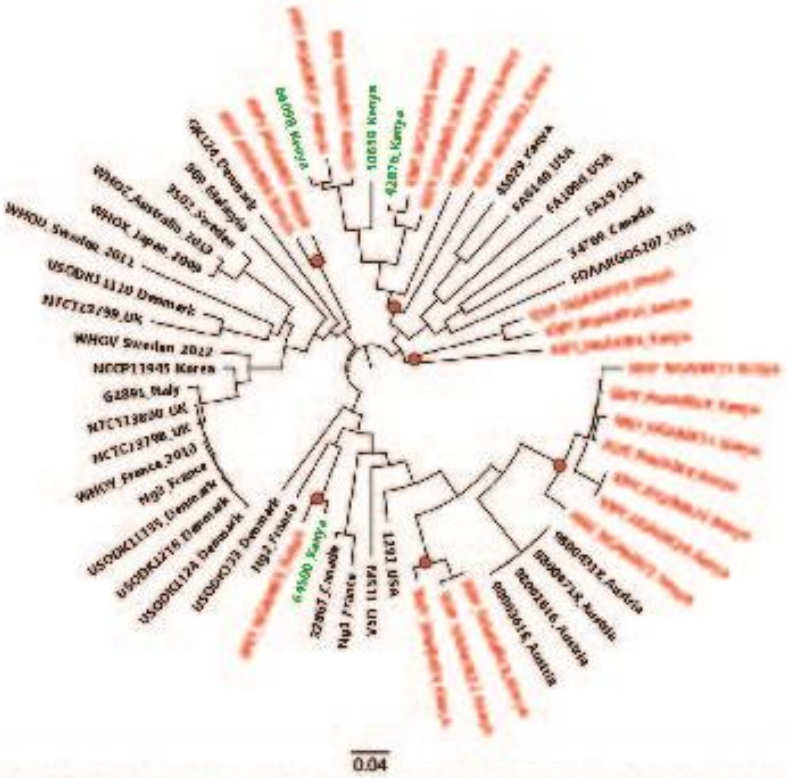



**Fig. 1** Maximum likelihood SNP phylogenetic tree inferred using RaxML. The 22 study isolates (KNY) in red and 4 Kenyan isolates (in green) downloaded from NCBI (64500, 65098, 50659, and 42876) and previously obtained from MSM population clustered closely together into six distinct clades shown in red circles

acid substitutions; S91F and D95G/A (corresponding to C272T, A284G or A284C *gyrA* mutations respectively) were identified in 20 of the 22 isolates. The 20 isolates were all ciprofloxacin-resistant (MICs >0.06 mg/L) (Table 2). Of these 20 isolates, 14 had ParC E91G (corresponding to A272G *parC* mutation) amino acid substitution. The remaining 2 isolates (KNY_NGAMR2 and KNY_NGAMR4) which were susceptible to ciprofloxacin (MICs ≤0.03 mg/L) lacked both *gyrA* and *parC* QRDR mutations (Fig. 2a, and Table 2).

Ciprofloxacin MICs for GC isolates with double GyrA QRDR amino acid substitutions, and for those with both GyrA and ParC QRDR amino acid substitutions were significantly higher compared to MICs of isolates expressing wild-type GyrA and ParC QRDRs ($p = 0.0357$* and $p = 0.0083$* respectively). Additionally, compared to the ciprofloxacin MICs of GC isolates with double GyrA QRDR amino acid substitutions alone, the MICs of GC isolates with an additional ParC amino acid substitution were significantly higher ($p = 0.0044$*) (Fig. 2a). Norfloxacin MICs of GC isolates expressing both GyrA and ParC QRDR amino acid substitutions were significantly different from those of GC isolates with wild-type GyrA and ParC QRDRs ($p = 0.0167$*). There was no significant difference between norfloxacin MICs of GC isolates expressing double GyrA QRDR amino acid substitutions and those expressing both GyrA and ParC QRDR amino acid substitutions ($p = 0.82$) (Fig. 2b).

No single GyrA amino acid substitutions were found in the QRDR of the analyzed isolates and there was no isolate containing a ParC QRDR amino acid substitution without the simultaneous presence of double GyrA QRDR amino acid substitutions. No amino acid changes were found at G85, D86, S87, S88, and R116 of ParC as reported by other studies from other countries [4, 22]. Additional amino acid substitutions found outside the QRDR regions in both GyrA and ParC and which have not yet been associated with gonococcal fluoroquinolone

**Table 2** Ciprofloxacin (CIP) and norfloxacin (NOR) susceptibility data for the 22 GC strains with details of GyrA, ParC, MtrR and PorB amino acid substitutions, and *mtrR* promoter mutation

| Isolate ID | MIC(mg/L) | | Mutations | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *GyrA* | | *ParC* | *MtrR* | | | A13deletion | *PorB* | | |
| | CIP | NOR | S91 | D95 | E91 | A39 | G45 | H105 | | A121 | G120 | N122 |
| KNY_NGAMR1 | 0.38 | 1.5 | F | G | | | | | | | | |
| KNY_NGAMR2 | 0.016* | 0.094 | | | | | D | | A deleted | | D | |
| KNY_NGAMR3 | 3 | 6 | F | G | G | T | | | | | | |
| KNY_NGAMR4 | 0.006* | 0.023 | | | | T | | | | S | | K |
| KNY_NGAMR5 | 4 | 6 | F | G | G | | | Y | | | | |
| KNY_NGAMR6 | 12 | 12 | F | G | G | T | | | | | | |
| KNY_NGAMR7 | 8 | 12 | F | G | G | | | | | S | | K |
| KNY_NGAMR8 | 3 | 6 | F | A | | T | | | | | | |
| KNY_NGAMR9 | 12 | 0.34 | F | A | G | T | | | | | | |
| KNY_NGAMR10 | 8 | 12 | F | G | G | T | | | | | | |
| KNY_NGAMR11 | 3 | 8 | F | A | | T | | | | | | |
| KNY_NGAMR13 | 3 | 12 | F | A | | T | | | | | | |
| KNY_NGAMR14 | 16 | 24 | F | G | G | T | | | | S | | K |
| KNY_NGAMR15 | 4 | 16 | F | A | | T | | | | | | |
| KNY_NGAMR16 | 8 | 6 | F | G | G | T | | | | | | |
| KNY_NGAMR17 | 6 | 12 | F | G | G | | | Y | | | | |
| KNY_NGAMR18 | 0.38 | 0.047 | F | G | G | T | | | | | | |
| KNY_NGAMR19 | 4 | 8 | F | G | G | T | | | | | | |
| KNY_NGAMR20 | 8 | 12 | F | A | G | T | | | | | | |
| KNY_NGAMR21 | 3 | 8 | F | A | | T | | | | | | |
| KNY_NGAMR22 | 4 | 12 | F | G | G | T | | | | | | |
| KNY_NGAMR23 | 12 | 12 | F | A | G | T | | | | | | |

GyrA S91F and D95G/A amino acid substitutions were found in 20 ciprofloxacin resistant isolates. Of the 20 isolates, 14 had an additional ParC E91G substitution. Two ciprofloxacin susceptible isolates lacked the QRDR mutations associated with fluoroquinolone resistance
*- ciprofloxacin susceptible

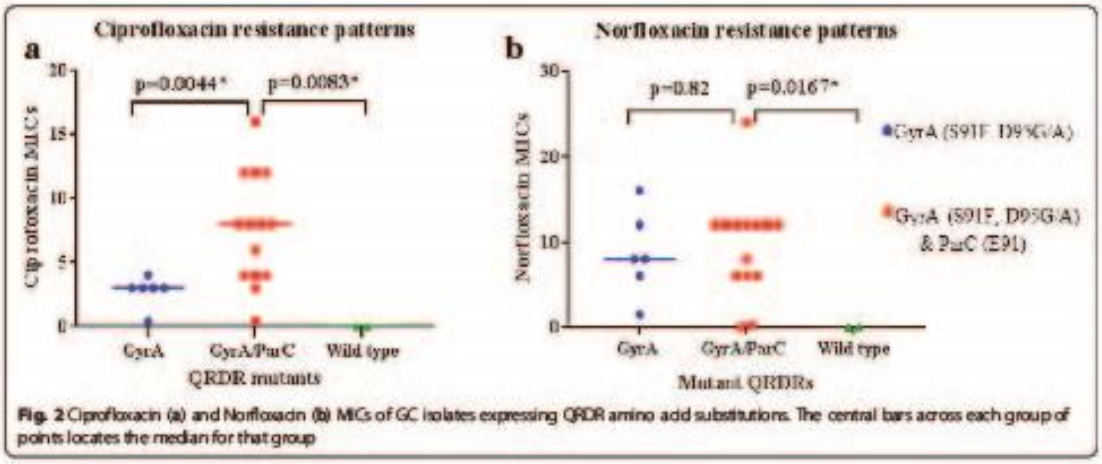



**Fig. 2** Ciprofloxacin (a) and Norfloxacin (b) MICs of GC isolates expressing QRDR amino acid substitutions. The central bars across each group of points locates the median for that group

resistance are; GyrA (M250I), and ParC (A156T, P289L, V359 M, I384V, A435V, F479 L, and I596V).

The *mtrR* gene encodes a repressor protein which represses expression of the *mtrCDE* operon encoding an efflux pump. Mutations in *mtrR* gene and its promoter have been associated with fluoroquonolone resistance in gonococci [23, 24]. Polymorphism analysis of the *mtrR* gene and its promoter showed that; only one isolate (KNY_NGAMR2) had the Adenine deletion in the 13 bp inverted repeat region between the − 10 and − 35 hexamers of the *mtrR* gene promoter while 20 isolates had A39T (17 isolates), G45D (1 isolate) and H105Y (2 isolates) amino acid substitutions in MtrR (Table 2). There were no cases of double amino acid substitutions as each MtrR mutant isolate had only one of the three amino acid substitutions. One isolate (KNY_NGAMR2) which lacked QRDR mutations had very low MICs for both drugs and had both G45D MtrR substitution and Adenine 13 deletion.

PorinB (PorB) is a protein which mediates influx of compounds into bacterial cells. Analysis of PorB protein at positions 120, 121 and 122 showed that 4 isolates (KNY_NGAMR2, KNY_NGAMR4, KNY_NGAMR7, and KNY_NGAMR17) had amino acid substitutions in PorB that have been associated with reduced drug permeability in bacterial cells. There was no observable relation between substitutions in the PorB and fluoroquonolone MICs (Table 2).

## Discussion

High prevalence of ciprofloxacin resistance by EUCAST breakpoints [25] (90.3%, MIC > 0.06 mg/L) was observed in the 84 GC isolates obtained in the STI Surveillance study. Simultaneous presence of amino acid substitutions in both GyrA and ParC QRDRs was associated with significantly higher ciprofloxacin and norfloxacin MICs when compared to MICs of isolates with wild-type QRDRs. Several studies have shown that GyrA amino acid substitutions initiate fluoroquinolone resistance in GC, while additional accumulation of substitutions in the ParC at G85, D86, S87, S88, Q91 and R116 elevate the resistance [4]. Our findings are in agreement with this previous observation as the ciprofloxacin MICs of GC isolates which had amino acid substitutions in both GyrA and ParC QRDRs were significantly higher compared to both the MICs of GC isolates with only double GyrA mutations and those with wild-type QRDRs. Double GyrA amino acid substitutions (S91F and D95G/A) were recently identified and associated with fluoroquinolone resistant GC isolated from MSM in Coastal Kenya [14].

There was no significant increase in norfloxacin MICs in GC isolates expressing both GyrA and ParC QRDR amino acid substitutions, when compared to MICs of isolates expressing double GyrA mutations alone. Ciprofloxacin and norfloxacin differ at position N1 of the fluoquinolone acid core, where ciprofloxacin has a cyclopropane ring and norfloxacin has an ethyl group [26, 27]. This structural difference could have contributed to the different MIC patterns observed between the two drugs in this study. Although our study was limited to the 22 sequenced GC isolates the significant difference in norfloxacin MICs observed between GC isolates expressing both GyrA and ParC QRDR amino acid substitutions and those expressing wild-type QRDRs indicate that the triple QRDR amino acid substitutions could be involved in mediating norfloxacin resistance in the Kenyan GC.

An in-vitro study by Tanaka et al., [20] suggested that reduced drug accumulation in cells contribute to the development of fluoroquinolone resistance in gonococci. Reduced drug accumulation can result from an active efflux system or reduced drug influx [28]. Mutations in the promoter or encoding region of the *mtrR* gene,

which leads to over expression of the *mtrCDE* operon has been associated with resistance to antibacterial agents [29]. Common mutations in *mtrR* implicated in increased drug efflux in GC include the: adenine deletion (A-) in the 13 bp inverted repeat region between the −10 and −35 hexamers of the *mtrR* gene promoter, and A39T, G45D, and H105Y amino acid substitutions in MtrR [30]. Warner and Shafer, [30] highlighted that the location of A39T substitution in MtrR can have significant effect in binding of the repressor protein to its target thereby causing de-repression of *mtrCDE* operon and consequently increased drug efflux. No relationship was observed between MtrR A39T substitution and fluoroquinolone resistance in the present study.

Reduced drug permeation through the porinB protein in GC has been associated with substitutions with charged amino acids at G120 and A121, and deletions of A121 and N122 in the PorB [31]. Although 4 isolates had amino acid substitutions in PorB that have been associated with reduced drug permeability in bacterial cells, this study did not find evidence for the contribution of reduced cell permeability and drug efflux towards fluoroquinolone resistance in analyzed GC isolates.

Mutations in L4 and L22 ribosomal proteins, and in 23 s rRNA genes known to cause high level azithromycin resistance were not identified in any of the study isolates. The low level of azithromycin resistance observed in 7 of the 22 isolates could be as a result of an active efflux MtrCDE pump or from reduced drug influx caused by a mutated PorB.

The 22 isolates obtained from heterosexual population formed 6 phylogenetically distinct clusters, three of which contained GC isolates recovered from MSM population from Coastal Kenya [14].

## Conclusion

Our study reports E91G amino acid substitution in ParC of fluoroquinolone resistant GC isolates from Kenya. The findings indicate that simultaneous presence of GyrA S91F, D95G/D95A, and ParC E91G amino acid substitutions mediate the high fluoroquinolone resistance observed in the analyzed Kenyan GC. Clustering observed in the Kenyan isolates indicate that they are closely related genetically and are evolving into their own distinct lineages.

## Methods

### Study design and study isolates

The current study is a retrospective laboratory-based study nested in an ongoing STI surveillance program under Armed Forces Health Surveillance at the US Army Medical Research Directorate -Africa. Study isolates were obtained as part of the study entitled "A surveillance study of antimicrobial susceptibility profiles of *Neisseria gonorrhoeae* isolates from patients seeking treatment in selected clinics in Kenya" Walter Reed Army Institute of Research (WRAIR) Human Subject Protection Board (HSPB) Protocol #1743/Kenya Medical Research Institute Scientific and Ethics Review Unit (SERU) #1908.

Symptomatic males and females were enrolled from four geographic locations in Kenya (Nairobi, Coastal Kenya, Nyanza, and Rift Valley) between 2013 and 2017. Eighty four *Neisseria gonorrhoeae* (GC) isolates were successfully obtained and their susceptibility to a panel of ten drugs (Ceftriaxone, Cefixime, Azithromycin, Ciprofloxacin, Norfloxacin, Spectinomycin, Tetracycline, Penicillin and Gentamicin) carried out using the E-test (BioMerieux) method. GC isolates found to be resistant to more than two classes of drugs were classified as multidrug resistant. Twenty two archived and viable GC isolates resistant to two or more classes of antimicrobial agents were chosen for analysis (Table 3).MIC breakpoints for ciprofloxacin resistance were set at susceptible ≤0.003 mg/L, and resistant > 0.06 mg/L, based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 8.0, 2018.

### Ethical consideration

Permission to carry out the study was obtained from both Kenya Medical Research Institute Scientific and Ethics Review Unit (SERU) and Walter Reed Army Institute of Research (WRAIR) Human Subject Protection Board (HSPB) (KEMRI/SERU/CCR/0053/3385: WRAIR#1743A).

### GC isolation and species verification

Samples were taken only after the informed consent and assent process was completed and the study subject was enrolled into the parent STI surveillance study. Depending on the sex of the subjects, urethral swabs for males or of cervical swabs for females were collected. Microscopy examination of gram stained swabs followed by laboratory culture of swab material to identify gram negative diplococci was used to screen specimens for further culture, isolation and identification of *Neisseria gonorrhoeae*. Plates containing selective GC medium were inoculated with genital swabs and transported in a $CO_2$ enriched environment to the Centre for Microbiology Research laboratory (CMR), located at the KEMRI headquarter for isolation, identification and confirmation of *N. gonorrhoeae* before antimicrobial susceptibility testing (AST) was undertaken. Once the inoculated transport medium reached the CMR laboratory, it was incubated at 37 °C in an incubator enriched with 5% $CO_2$ for 48 h and examined daily for any growth.

To obtain pure isolates for Antibiotic Susceptibility Testing (AST), gram negative, catalase and oxidase positive *Neisseria* colonies from the transport medium were grown in a newly prepared non selective GC medium

**Table 3** Isolate multi-drug resistance patterns

| Isolate ID | Year of isolation | Resistance patterns | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | CFM | CRO | PEN | SPT | AZM | CIP | TET |
| KNY_NGAMR1 | 2015 | S | S | R | S | S | R | S |
| KNY_NGAMR2 | 2015 | S | S | R | S | R | S | S |
| KNY_NGAMR3 | 2015 | S | S | R | S | S | R | R |
| KNY_NGAMR4 | 2016 | S | S | R | S | S | S | R |
| KNY_NGAMR5 | 2016 | S | S | R | S | S | R | R |
| KNY_NGAMR6 | 2017 | S | S | R | S | S | R | S |
| KNY_NGAMR7 | 2014 | S | S | R | S | R | R | R |
| KNY_NGAMR8 | 2013 | S | S | R | S | S | R | S |
| KNY_NGAMR9 | 2016 | S | S | R | S | S | R | S |
| KNY_NGAMR10 | 2016 | S | S | R | S | S | R | R |
| KNY_NGAMR11 | 2016 | S | S | R | S | S | R | R |
| KNY_NGAMR13 | 2014 | S | S | R | S | S | R | R |
| KNY_NGAMR14 | 2015 | S | S | R | S | S | R | R |
| KNY_NGAMR15 | 2014 | S | S | R | S | R | R | R |
| KNY_NGAMR16 | 2015 | S | S | R | S | S | R | R |
| KNY_NGAMR17 | 2015 | S | S | R | S | R | R | R |
| KNY_NGAMR18 | 2015 | S | S | R | S | R | R | R |
| KNY_NGAMR19 | 2015 | S | S | R | S | R | R | S |
| KNY_NGAMR20 | 2015 | S | S | R | S | S | R | R |
| KNY_NGAMR21 | 2016 | S | S | R | S | S | R | R |
| KNY_NGAMR22 | 2016 | S | S | R | S | R | R | R |
| KNY_NGAMR23 | 2017 | S | S | R | S | S | R | R |

MIC breakpoints were based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 8.0, 2018.CRO: Ceftriaxone CFM: cefixime, PEN: penicillin, SPT: spectinomycin, AZM: azithromycin, CIP: ciprofloxacin, and TET: tetracycline. S: susceptible MIC and R: resistance MIC. There are no EUCAST MIC breakpoints for norfloxacin, gentamycin and doxycycline

base supplemented with IsoVitalex®, BD. Antimicrobial susceptibility testing was done on the pure isolates following confirmation using API NH (Biomerieux). Minimal inhibitory concentrations (MICs) to selected antibiotics were determined using the E-test strip (A B, Biodisk, Sweden) method. Pure isolates were stocked on Trypticase soy broth with 20% glycerol and stored at −80 °C. For genomic characterization of multi-drug resistant GC isolates, the frozen isolates were thawed, inoculated on Modified Thayer-Martin (GC agar, BD containing vancomycin, nystatin, colistin and trimethoprim lactate) and incubated at 37 °C in 5% $CO_2$ for 18 to 24 h. Plates with typical GC growth were further subjected to oxidase and catalase biochemical tests.

### DNA extraction and quantitation

Genomic DNA from confirmed GC positive cultures was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of genomic DNA were determined by Qubit® dsDNA HS Assay using Qubit 3.0 fluorometer, (Thermo Fisher Scientific Inc. Wilmington, Delaware USA) according to the manufacturer's instructions. Quantified DNA was stored in a −20 °C freezer prior to sequencing.

### Whole-genome sequencing and assembly

#### *Library preparation and sequencing*

Libraries were prepared from 5μg of genomic DNA of each sample. The DNA was processed by Illumina Nextera XT sample preparation kit (Illumina Inc. San Diego, CA, USA) and uniquely indexed using Illumina Nextera XT Index Kit (Illumina Inc. San Diego, CA, USA). The libraries were purified and normalized using Agencourt AMPure XP beads (Beckman Coulter, Beverly, Massachusetts). All recovered elutes were pooled in equal volumes. DNA concentration of the pool was determined using Qubit ds DNA HS Assay (Thermo Fisher Scientific Inc. Wilmington, Delaware USA). Paired end reads were generated on Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a paired-end 2 × 250 bp protocol.

### De novo assembly

Sequence raw reads were assembled through the assembly pipeline (version1.2) available from the Center for Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk/services) which is based on the Velvet algorithms for de novo assembly of short reads [32]. Assembled contigs were aligned against FA1090 (GenBank Accession number AE0049969) *N. gonarrhoeae* reference genome using Mauve [33]. Sequence reads for this study are deposited on National Centre for Biotechnology Information (NCBI) SRA Accession SRP154258.

### Genome-wide single nucleotide polymorphism phylogeny

To determine the genetic diversity between the study isolates, additional 38 draft genome sequences of *N. gonorrhoeae* strains obtained from varied geographical regions were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/) and included in the analysis. Single Nucleotide Polymorphisms (SNPs) were identified using the CSI phylogeny pipeline available on Centre for Genome Epidemiology (CGE) (http://www.genomicepidemiology.org) [34]. Raw sequence reads and the additional draft genome sequences were mapped to *Neisseria gonorrhoeae* NCCP11945 (GenBank accession number CP001050) reference genome, using BWA version v.0.7.17 [35]. SNPs from each genome were called using SAMTools v.1.7 [36], and concatenated into a single fasta alignment. The concatenated sequences were used for constructing a maximum likelihood tree using RaxML with generalized time reversible (GTR) model [37]. The tree was visualized by using FigTree version 1.4.3 [38]

### Identification of antimicrobial resistance determinants

Annotation of the whole genome to determine fluoroquinolone resistance was performed with Rapid Annotation using Subsystem Technology (RAST) (http://rast.nmpdr.org) in Pathosystems Resource Integration Center version 3.5.7 (PATRIC) (https://www.patricbrc.org) [39] and different antibiotic determinants were saved into separate feature groups. Reference GyrA (GenBank Accession number U08817) and ParC (GenBank Accession number U08907) were downloaded from the NCBI website https://www.ncbi.nlm.nih.gov. Mutations in the *gyrA*, *parC*, *porB* and *mtrR* genes and proteins were determined using Bioedit sequence alignment editor version 7.0.5 [40].

### Statistical analysis

Wilcoxon Mann-Whitney statistical test was conducted in GraphPad Prism V7.0.4 (GraphPad Software) [41]. Statistical comparisons were two tailed and were performed with the significance level set at $P < 0.05$.

### Abbreviations

AST: Antibiotic Susceptibility Testing; CGE: Center for Genomic Epidemiology; CMR: Centre for Microbiology Research; EUCAST: European Committee on Antimicrobial Susceptibility Testing; GC: *Neisseria gonorrhoeae*; KEMRI: Kenya Medical Research Institute; MIC: Minimum Inhibitory Concentration; MSM: Men who have Sex with Men; QRDR: Quinolone Resistant Determining Region; SRA: Sequence Read Archive; STI: Sexually Transmitted Infection

### Acknowledgments

The authors thank Kimita Gathii and Justin Nyasinga for NGS sequencing guidance and support, as well as field staff working under the STI surveillance study and all KEMRI CMR laboratory staff for their assistance and support with this project.

### Funding

This work was funded by the Global Emerging Infections Surveillance (GEIS)/ Armed Forces Health Surveillance Branch (ProMIS P0080_17_KY_1.1.1). The funding body provided the funds and had no role in the design of the study; collection, analysis, and interpretation of data; or in writing of the manuscript.

### Availability of data and materials

Data supporting the conclusion in this article is included within the article and all of the datasets analyzed in this article are available from the corresponding author. The sequence reads generated and analyzed in the current study are linked to the National Centre for Biotechnology Information (NCBI) SRA Accession SRP154258 Data.

### Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70–25. This paper is published with the approval by Director Kenya Medical Research Institute.

### Authors' contributions

MWK: Experiment design, Bacteria culture and Isolate identification, NGS sequencing and analysis of sequencing data, manuscript writing, MWM: Project conception, experimental and study design, data analysis and manuscript writing, FLE, & WDB: Experiment design, Analysis of sequencing data, OKK: NGS sequencing and analysis of sequencing data, VO, & SWM: Bacteria culture and isolate identification, WMM: manuscript writing and Technical consultation, WS, BA, OOS, & RSM: Technical consultation. All authors read and approved the paper.

### Ethics approval and consent to participate

Permission to carry out the study was obtained from both Kenya Medical Research Institute Scientific and Ethics Review Unit (SERU) and Walter Reed Army Institute of Research (WRAIR) Human Subject Protection Board (HSPB) (KEMRI/SERU/CCR/0053/3385: WRAIR#1748A). Consent to participate is not applicable for this study because it was retrospective laboratory based, used archived samples, and there was no interaction with subjects.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



### Author details

[1]US Army Medical Research Directorate-Africa, P.O Box 606, 00621, Village Market, Nairobi, Kenya. [2]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P.O Box 62,000-00200, Thika, Kenya. [3]Department of Biological and Physical Science, Karatina University (KarU), P.O Box 1957-10101, Karatina, Kenya. [4]Kenya Medical

Research Institute (KEMRI), P. O. Box 54840-00200, Nairobi, Kenya. [5]Department of Global Health and Medicine, University of Washington, 325 9th Avenue, Box 359931, Seattle, WA 98104, USA. [6]Department of Biochemistry, School of Medicine, University of Nairobi, P.O. Box 30197, GPO, 00100, Nairobi, Kenya.

Received: 20 August 2018 Accepted: 20 March 2019
Published online: 08 April 2019

## References

1. Dillon J-AR, Parti RP, Thakur SD. Antibiotic resistance in *Neisseria gonorrhoeae*: will infections be untreatable in the future? Immunodeficiency. 2016;2:5.
2. Unemo M, Shafer WM. Antimicrobial resistance in Neisseria gonorrhoeae in the 21st century: past, evolution, and future. Clin Microbiol Rev. 2014;27(3):587–613.
3. Unemo M, del Rio C, Shafer WM. Antimicrobial resistance expressed by Neisseria gonorrhoeae: a major global public health problem in the 21st century. Microbiology spectrum. 2016;4(3).
4. Bodoev I, Ilina E. Molecular mechanisms of formation of drug resistance in Neisseria gonorrhoeae: history and prospects. Mol Genet Microbiol Virol. 2015;30(3):132–40.
5. Slaney L, Chubb H, Mohammed Z, Ronald A. In-vitro activity of meropenem against Neisseria gonorrhoeae, Haemophilus influenzae and H. Ducreyi from Canada and Kenya. J Antimicrob Chemother. 1989;24(suppl_A):183–6.
6. Verhagen A, Van der Ham M, Heimans A, Kranendonk O, Maina A. Diminished antibiotic sensitivity of Neisseria gonorrhoeae in urban and rural areas in Kenya. Bull World Health Organ. 1971;45(6):707.
7. Perine P, Biddle J, Nsanze H, D'COSTA L, Osaba A, Widy-Wirski R. Gonococcal drug resistance and treatment of gonorrhoea in Nairobi. East Afr Med J. 1980;57(4):238–46.
8. Brunham R, Fransen L, Plummer F, Piot P, Slaney L, Bygdeman S, Nsanze H. Antimicrobial susceptibility testing and phenotyping of Neisseria gonorrhoeae isolated from patients with ophthalmia neonatorum in Nairobi, Kenya. Antimicrob Agents Chemother. 1985;28(3):393–6.
9. Van Hall M, Pettit P, Van Hall H, Mouton R, Ndinya-Achola J. Prevalence of resistance of N. Gonorrhoeae to penicillin and three other antibiotics in a rural area in Kenya. East Afr Med J. 1991;68(1):853–9.
10. WHO. 1993 sexually transmitted diseases treatment guidelines. Centers for Disease Control and Prevention. MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports. 1993; 42(Rr-14):1–102.
11. Mehta SD, Maclean I, Ndinya-Achola JO, Moses S, Martin I, Ronald A, Agunda L, Murugu R, Bailey RC, Melendez J. Emergence of quinolone resistance and cephalosporin MIC creep in Neisseria gonorrhoeae isolates from a cohort of young men in Kisumu, Kenya, 2002 to 2009. Antimicrob Agents Chemother. 2011;55(8):3882–8.
12. Lagace-Wiens PR, Duncan S, Kimani J, Thiong'o A, Shafi J, McClelland S, Sanders EJ, Zhanel G, Maraguri N, Mehta SD. Emergence of fluoroquinolone resistance in Neisseria gonorrhoeae isolates from four clinics in three regions of Kenya. Sex Transm Dis. 2012;39(5):332.
13. Duncan S, Thiong'o AN, Macharia M, Wamuyu L, Mwarumba S, Mvera B, Smith AD, Morpeth S, Graham SM, Sanders EJ. High prevalence of quinolone resistance in Neisseria gonorrhoeae in coastal Kenya. Sex Transm Infect. 2011;87(3):231.
14. Cehovin A, Harrison OB, Lewis SB, Ward PN, Ngetsa C, Graham SM, Sanders EJ, Maiden MC, Tang CM. Identification of novel Neisseria gonorrhoeae lineages harbouring resistance plasmids in coastal Kenya. J Infect Dis. 2018.
15. Levine C, Hiasa H, Marians KJ. DNA gyrase and topoisomerase IV: biochemical activities, physiological roles during chromosome replication, and drug sensitivities. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression. 1998;1400(1):29–43.
16. Drlica K. Mechanism of fluoroquinolone action. Curr Opin Microbiol. 1999; 2(5):504–8.
17. Piddock LJ. Mechanisms of fluoroquindone resistance: an update 1994–1998. Drugs. 1999;58(2):11–8.
18. Belland R, Morrison S, Ison C, Huang W. Neisseria gonorrhoeae acquires mutations in analogous regions of gyrA and parC in fluoroquinolone-resistant isolates. Mol Microbiol. 1994;14(2):371–80.
19. Yang Y, Liao M, Gu WM, Bell K, Wu L, Eng NF, Zhang C-G, Chen Y, Jolly AM, Dillon J-AR. Antimicrobial susceptibility and molecular determinants of quinolone resistance in Neisseria gonorrhoeae isolates from Shanghai. J Antimicrob Chemother. 2006;58(4):868–872.
20. Tanaka M, Sakuma S, Takahashi K, Nagahuzi T, Saika T, Kobayashi I, Kumazawa J. Analysis of quinolone resistance mechanisms in Neisseria gonorrhoeae isolates in vitro. Sex Transm Infect. 1998;74(1):59–62.
21. Deguchi T, Yasuda M, Nakano M, Ozeki S, Ezaki T, Saito I, Kawada Y. Quinolone-resistant Neisseria gonorrhoeae: correlation of alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV with antimicrobial susceptibility profiles. Antimicrob Agents Chemother. 1996; 40(4):1020–3.
22. Sood S, Agarwal M, Bala M, Mahajan N, Singh R, Kapil A, Sreenivas V, Ram R, Kar HK, Sharma VK. Exploring quinolone resistance-determining region in Neisseria gonorrhoeae isolates from across India. Indian J Med Res. 2017; 146(Suppl 1):S64.
23. Cousin SL Jr, Whittington WL, Roberts MC. Acquired macrolide resistance genes and the 1 bp deletion in the mtrR promoter in Neisseria gonorrhoeae. J Antimicrob Chemother. 2003;51(1):131–3.
24. Zarantonelli L, Borthagaray G, Lee E-H, Shafer WM. Decreased azithromycin susceptibility of Neisseria gonorrhoeae due to mtrR Mutations. Antimicrob Agents Chemother. 1999;43(10):2468–72.
25. APRd C-L, KTBd S, Moreira BM, Fracalanzza SEL, Bonelli RR. Antimicrobial resistance in Neisseria gonorrhoeae: history, molecular mechanisms and epidemiological aspects of an emerging global threat. Braz J Microbiol. 2017;48(4):617–28.
26. Sharma PC, Jain A, Jain S. Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects. Acta Pol Pharm. 2009; 66(6):587–604.
27. Sarkozy G. Quinolones: a class of antimicrobial agents. VETERINARNI MEDICINA-PRAHA. 2001;46(9/10):257–74.
28. Tanaka M, Otsuki M, Nishino T, Kobayashi I, Matsumoto T, Kumazawa J. Mutation in DNA gyrase of norfloxacin-resistant clinical isolates of Neisseria gonorrhoeae. Sex Transm Infect. 1998;73(4):295–7.
29. Lucas CE, Balthazar JT, Hagman KE, Shafer WM. The MtrR repressor binds the DNA sequence between the mtrR and mtrC genes of Neisseria gonorrhoeae. J Bacteriol. 1997;179(13):4123–8.
30. Warner DM, Shafer WM, Jerse AE. Clinically relevant mutations that cause derepression of the Neisseria gonorrhoeae MtrC-MtrD-MtrE efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. Mol Microbiol. 2008;70(2):462–78.
31. Olesky M, Hobbs M, Nicholas RA. Identification and analysis of amino acid mutations in porin IB that mediate intermediate-level resistance to penicillin and tetracycline in Neisseria gonorrhoeae. Antimicrob Agents Chemother. 2002;46(9):2811–20.
32. Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 2008;18(5):821–9.
33. Darling AE, Mau B, Perna NT. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One. 2010;5(6):e11147.
34. Ahrenfeldt J, Skaarup C, Hasman H, Pedersen AG, Aarestrup FM, Lund O. Bacterial whole genome-based phylogeny: construction of a new benchmarking dataset and assessment of some existing methods. BMC Genomics. 2017;18(1):19.
35. Li H, Durbin R. Fast and accurate short read alignment with burrows–wheeler transform. Bioinformatics. 2009;25(14):1754–60.
36. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. The sequence alignment/map format and SAMtools. Bioinformatics. 2009;25(16):2078–9.
37. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30(9):1312–3.
38. Rambaut A: FigTree v1. 4. In.; 2012.
39. Wattam AR, Abraham D, Dalay O, Disz TL, Driscoll T, Gabbard JL, Gillespie JJ, Gough R, Hix D, Kenyon R. PATRIC, the bacterial bioinformatics database and analysis resource. Nucleic Acids Res. 2013;43(D1):D581–91.
40. Hall T. BioEdit v. 7.0. 5: biological sequence alignment editor for windows. Ibis Therapeutics a division of Isis pharmaceuticals. 2005; In.; 2016.
41. Prism G. Graphpad software. CA, USA: San Diego; 1994.

**Appendix XVII: Second published research article**

Kivata et al. BMC Infectious Diseases (2020) 20:703
https://doi.org/10.1186/s12879-020-05398-5

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

# Plasmid mediated penicillin and tetracycline resistance among *Neisseria gonorrhoeae* isolates from Kenya

Mary Wandia Kivata[1,2*], Margaret Mbuchi[3,4*], Fredrick Eyase[1,3], Wallace Dimbuson Bulimo[3,5], Cecilia Katunge Kyanya[3], Valerie Oundo[3], Wilton Mwema Mbinda[6], Willy Sang[3,4], Ben Andagalu[3], Olusegun O. Soge[7], Raymond Scott McClelland[8] and John Distelhorst[3]

**Abstract**

**Background:** Treatment of gonorrhea is complicated by the development of antimicrobial resistance in *Neisseria gonorrhoeae* (GC) to the antibiotics recommended for treatment. Knowledge on types of plasmids and the antibiotic resistance genes they harbor is useful in monitoring the emergence and spread of bacterial antibiotic resistance. In Kenya, studies on gonococcal antimicrobial resistance are few and data on plasmid mediated drug resistance is limited. The present study characterizes plasmid mediated resistance in *N. gonorrhoeae* isolates recovered from Kenya between 2013 and 2018.

**Methods:** DNA was extracted from 36 sub-cultured GC isolates exhibiting varying drug resistance profiles. Whole genome sequencing was done on Illumina MiSeq platform and reads assembled *de-novo* using CLC Genomics Workbench. Genome annotation was performed using Rapid Annotation Subsystem Technology. Comparisons in identified antimicrobial resistance determinants were done using Bioedit sequence alignment editor.

**Results:** Twenty-four (66.7%) isolates had both β-lactamase (TEM) and TetM encoding plasmids. 8.3% of the isolates lacked both TEM and TetM plasmids and had intermediate to susceptible penicillin and tetracycline MICs. Twenty-six (72%) isolates harbored TEM encoding plasmids. 25 of the TEM plasmids were of African type while one was an Asian type. Of the 36 isolates, 31 (86.1%) had TetM encoding plasmids, 30 of which harbored American TetM, whereas 1 carried a Dutch TetM. All analyzed isolates had non-mosaic *penA* alleles. All the isolates expressing TetM were tetracycline resistant (MIC> 1 mg/L) and had increased doxycycline MICs (up to 96 mg/L). All the isolates had S10 ribosomal protein V57M amino acid substitution associated with tetracycline resistance. No relation was observed between PenB and MtrR alterations and penicillin and tetracycline MICs.

(Continued on next page)

*Correspondence: marywandia086@gmail.com; Margaret.mbuchi@usamru-kenya.org
[1]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P. O Box 62,000-00200, Thika, Kenya
[3]U.S. Army Medical Research Directorate-Africa, P. O Box 606, Village Market, Nairobi 00621, Kenya
Full list of author information is available at the end of the article

(Continued from previous page)
**Conclusion:** High-level gonococcal penicillin and tetracycline resistance in the sampled Kenyan regions was found to be mediated by plasmid borne *blaTEM* and *tetM* genes. While the African TEM plasmid, TEM1 and American TetM are the dominant genotypes, Asian TEM plasmid, a new TEM239 and Dutch TetM have emerged in the regions.

**Keywords:** *Neisseria gonorrhoeae*, Plasmid, *blaTEM*, *tetM*, Tetracycline, Penicillin

## Background

Gonococcal infections are among the most predominant bacterial sexually transmitted infections (STI) worldwide. Accordingly, gonorrhea remains a major global health concern [1]. *N. gonorrhoeae* has over the years evolved and developed resistance to many of the antibiotics used to treat its infections including the penicillins and tetracyclines [1, 2]. The spread of these antibiotic resistance genes poses a challenge in treatment of gonococcal infections. Penicillins are β-lactam antibiotics that disrupt cell wall formation and integrity by targeting the major penicillin binding proteins (PBPs), mainly PBP1 and PBP2 (encoded by *ponA* and *penA* genes respectively) in gonococci [3, 4]. Tetracyclines inhibit the attachment of aminoacyl tRNA to the acceptor A site in the mRNA-ribosome complex by mainly binding to the 30S ribosomal subunit, and accordingly inhibiting protein synthesis [5]. In *N. gonorrhoeae*, resistance to both penicillin and tetracycline is mediated through two mechanisms: chromosomal mutations and acquisition of plasmid borne genes, mainly *blaTEM-1* for penicillin and *tetM* for tetracycline [6–9].

Seven types of plasmids harboring β-lactamase have been described in penicillinase-producing *Neisseria gonorrhoeae* (PPNG) and named based on geographical areas where they were first described as: Asian; African; Rio/Toronto; Nimes; Johannesburg; New Zealand and Australian [10–15]. The Asian type is the ancestral plasmid from which either deletions or insertions gave rise to the other six plasmid types [12, 16]. These plasmids have been shown to carry *blaTEM-1* encoding TEM1 β-lactamase or its derivatives [17]. TEM1 β-lactamase destroys the activity of β-lactam drugs by hydrolyzing the amide bond in the β-lactam ring but it is not active against extended-spectrum cephalosporins [18]. Single Nucleotide Polymorphisms (SNPs) in *blaTEM-1* resulting in alteration of amino acid configuration around TEM1 β-lactamase active site can convert it to an extended spectrum β-lactamase (ESBL) [19]. The ESBL are more stable and potent and can breakdown cephalosporins including ceftriaxone, the last first line monotherapy for treatment of gonorrhea. *blaTEM-135* encoding a more stable TEM-135 β-lactamase which differs from TEM1 β-lactamase by one amino acid substitution (M182T) has been described in gonococci from several countries [17, 20–22]. It has been described as an intermediate between TEM1 β-lactamase and extended broad spectrum β-lactamase [23]. Both *blaTEM-1* and *blaTEM-135* have mainly been described in Asian, African and Toronto plasmid types and associated with epidemic outbreaks [16, 24, 25].

Chromosomal modifications in at least five different genes including *penA*, *ponA*, *mtrR*, *porB*, and *pilQ* have been implicated in chromosomally mediated gonococcal penicillin resistance [26]. Modifications in *penA*, and *ponA* alter the three dimensional structures of PBP2 and PBP1. This reduces the affinity of PBPs for penicillin and consequently reduces susceptibility to β-lactams [27]. Recombination of gonococcal *penA* with *penA* genes of commensal *Neisseria* species has led to development of a mosaic-like *penA* structure, which has been associated with resistance to cefixime and ceftriaxone in gonococci from different regions [28–31].

The *mtrR* gene encodes MtrR which represses the expression of the multiple transferrable resistance CDE (MtrCDE) efflux pump [26]. In *N. gonorrhoeae*, mutations in the *mtrR* promoter or the MtrR encoding region lead to over expression of the MtrCDE efflux pump and has been associated with resistance to antibacterial agents [32]. Reduced drug permeation resulting from modifications of porinB (PorB also referred to as PenB alterations) encoded by *porB* has also been associated with an intermediate-level resistance to both penicillin and tetracycline in *N. gonorrhoeae* [33].

High tetracycline resistance in gonoccoci is mediated by a transposon-borne (Tn916) class M tetracycline (TetM) resistance determinant. TetM binds to 30S ribosomal subunit thereby blocking tetracycline from binding to its target [5, 34]. There are two different TetM determinants; American and Dutch which are carried by either of two 25.2 MDa conjugative plasmids named "American" and "Dutch" type plasmids found in gonococci [35, 36]. Chromosomal modifications which mediate tetracycline resistance in gonococci include: a) V57M amino acid substitution in S10, a 30S ribosomal protein encoded by *rpsJ* gene. This modification results in an altered tetracycline binding site and consequently reduced binding affinity, and b) modifications in *mtrR* and *porB* which result in reduced drug accumulation [37].

In Kenya a few studies have reported penicillin and tetracycline resistance in *N. gonorrhoeae* since the 1970s [38–41]. Following these reports the use of both penicillin and tetracycline for treatment of gonococcal infections was stopped [42]. Nevertheless, the two drugs are widely available to the public and are inappropriately used through self prescription in many parts of Africa including Kenya [43–45].

Determining the plasmid types and characterizing the antibiotic resistance genes they harbor is significantly important. It helps in monitoring the emergence and spread of antibiotic resistant *N. gonorrhoeae* isolates as well as the spread of plasmid borne genes between different bacteria. Poor surveillance and the fact that both penicillin and tetracycline are neither the first nor the second line drug of choice for treatment of gonorrhea, has limited data on plasmid types and plasmid borne resistance genes in Kenyan gonococci. This study therefore sought to determine the prevalence and identity of TEM plasmids types. We also, characterized both TEM and TetM encoding genes in Kenyan *N. gonorrhoeae* isolates recovered from heterosexual population between 2013 and 2018.

## Methods

### Bacterial isolates and antimicrobial susceptibility testing

Study isolates were obtained as part of an ongoing STI surveillance study (WRAIR#1743, KEMRI#1908) under Armed Forces Health Surveillance at the US Army Medical Research Directorate-Africa (USAMRD-A). The isolates were recovered from both urethral and endocervical samples obtained from male and female patients seeking treatment in selected clinics from four geographic locations in Kenya (Nairobi, Coastal Kenya, Nyanza, and Rift Valley) between 2013 and 2018. Frozen isolates were thawed and inoculated on GC agar base supplemented with vancomycin, nystatin, colistin and trimethoprim lactate, 1% IsoVitaleX (Becton Dickinson, US) and 10% Hemoglobin solution (Becton Dickinson, US) and incubated at 37 °C in 3–5% $CO_2$ for 18–24 h. *N. gonorrhoeae* was confirmed through colony morphology, Gram stain, oxidase, catalase, and APiNH® (Biomerieux) biochemical tests prior to antimicrobial susceptibility testing and DNA extraction. 0.5 MacFarland standard GC inoculums were inoculated on GC agar base medium (Becton Dickinson, US) supplemented with 1% IsoVitaleX (Becton Dickinson, US) and 10% Hemoglobin solution (Becton Dickinson, US). Minimum inhibitory concentrations (MICs) of ceftriaxone; cefixime; azithromycin; ciprofloxacin; norfloxacin; spectinomycin; tetracycline; doxycycline; penicillin and gentamicin were determined using E-test® (Biomerieux) method according to manufacturer's instructions [46, 47]. WHO K and WHO O reference gonococcal strains [48] (antimicrobial susceptibility patterns described in Table 1 below) were used to ensure accuracy of AST data.

MICs breakpoints were interpreted with reference to European Committee on Antimicrobial Susceptibility Testing (EUCAST) version 8.0, 2018 standards (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_8.0_Breakpoint_Tables.pdf) as shown in Table 2. Thirty six viable *N. gonorrhoeae* isolates exhibiting varying antibiotic resistance profiles were chosen for analysis (Table 3).

### DNA extraction

Both Genomic and plasmid DNA were extracted using QIAamp DNA Mini Kit and QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) "respectively" according to the manufacturer's instructions. Qubit dsDNA HS Assay was used to quantitate DNA using Qubit 3.0 fluorometer, (Thermo Fisher Scientific Inc. Wilmington, Delaware USA) according to the manufacturer's instructions, and DNA stored at −20 °C prior to sequencing.

### Whole-genome sequencing and sequence analysis

Illumina Nextera XT kit (Illumina Inc. San Diego, CA, USA) was used to prepare libraries from 1 ng of genomic DNA of each sample as per manufacturer's instructions. Sequence reads were generated on Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a paired-end 2 × 300 bp protocol [49]. The generated reads are linked to NCBI BioProjects: PRJNA481622 and PRJNA590515. Raw reads were trimmed for quality and assembled de novo using CLC Genomics Workbench version 12.0. Blast searches were performed using BLASTN suite in National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov). Assembled genomes were annotated in Pathosystems Resource Integration Center version 3.5.31 (PATRIC) (https://www.patricbrc.org) using Rapid Annotation Subsystem Technology (RAST) [26, 50]. Identified TEM and TetM determinants

**Table 1** Antimicrobial susceptibility patterns of the WHO reference strains (mg/L) [48]

| Antimicrobial | WHO K | WHO O |
|---|---|---|
| Ceftriaxone | 0.064 | 0.032 |
| Cefixime | 0.25 | 0.016 |
| Azithromycin | 0.25 | 0.25 |
| Ciprofloxacin | > 32 | 0.008 |
| Spectinomycin | 16 | > 1024 |
| Tetracycline | 2 | 2 |
| Penicillin | 2 | > 32 |
| β-lactamase (PPNG) | Negative | Positive |

*PPNG* Penicillinase producing *N. gonorrhoeae*. Currently there are no EUCAST MIC breakpoints for norfloxacin, gentamycin and doxycycline for *N. gonorrhoeae*

**Table 2** EUCAST breakpoints used for MIC interpretation for the tested antibiotics

| Antibiotic | MIC breakpoint (mg/L) | |
|---|---|---|
| | Susceptible ≤ | Resistant > |
| Ceftriaxone | 0.125 | 0.125 |
| Cefixime | 0.125 | 0.125 |
| Azithromycin | 0.25 | 0.5 |
| Ciprofloxacin | 0.08 | 0.06 |
| Spectinomycin | 64 | 64 |
| Tetracycline | 0.5 | 1 |
| Penicillin | 0.06 | 1 |

were downloaded and compared with reference TEM1 (GenBank Accession number WP_000027057.1) and TetM (GenBank Accession number WP_047922456.1) downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov). Identification and comparison of amino acid alterations in antimicrobial resistance determinants known to confer drug resistance in *N gonorrhoeae* were done using Bioedit sequence alignment editor version 7.0.5 [51].

### Statistical analysis

Wilcoxon Mann-Whitney statistical tests were conducted in GraphPad Prism version 7.0.4 (www.graphpad.com). Two tailed statistical comparisons were performed with significance level set at $P < 0.05$.

## Results

### Antibiotic susceptibility patterns of the study isolates

Of the 36 analyzed isolates 26 (72.2%) were penicillin resistant (MICs > 1 mg/L), whereas 31 (86.1%) were tetracycline resistant (MICs> 1 mg/L). Thirty-four (94.4%) isolates were ciprofloxacin resistant (MICs > 0.06). Low level of azithromycin resistance (MICs 1-2 mg/L) was observed in 3 (8.3%) isolates. None of the isolates was resistant to cefixime, ceftriaxone, or spectinomycin (Table 3). Twenty-four (66.7%) isolates were resistant to both penicillin and tetracycline, while 23 (63.9%) isolates were resistant to penicillin, tetracycline and ciprofloxacin. Two of the three isolates expressing low level azithromycin resistance were also resistant to penicillin, tetracycline and ciprofloxacin.

### Plasmid types and prevalence

Twenty-four of the 36 (66.7%) isolates had both TEM and TetM encoding plasmids whereas 3 (8.3%) isolates lacked plasmids. Two of these three isolates were penicillin susceptible (0.064 mg/L) while one had intermediate penicillin resistance (0.094 mg/L). The isolates had intermediate to susceptible tetracycline MICs (0.5–0.75 mg/L) (Table 6). Of the 36 isolates, 26 (72.2%) harbored TEM encoding plasmids and were therefore PPNG (Table 4). Twenty-five (96.2%) of the PPNG had the African type plasmid (pDJ5) while 1 (3.8%), had an Asian type plasmid (pDJ4) (Table 5). Thirty one (86.1%) of the 36 isolates harbored TetM encoding plasmids (Table 4). Two (5.5%) PPNG lacked TetM encoding plasmid while seven (19.4%) GC harboring TetM encoding plasmids lacked TEM encoding plasmid (Table 4).

### TEM and TetM genotypes

Of the 26 PPNG, 21 (80.8%) expressed TEM1 β-lactamase encoded by *blaTEM-1* gene, while 5 (19.2%) isolates expressed a β-lactamase encoded by a recently described *blaTEM* allele (NEIS2357 allele 10) (Table 5) [52]. All these new TEM1 alleles were carried by African type TEM plasmids. The sequence of the allele was deposited under GenBank accession number MK497256l and assigned as class A β-lactamase TEM239 (*blaTEM*) gene, *blaTEM-239* allele with a protein accession number QBC36181. American TetM determinant was identified in 30 (96.8%) of the 31 isolates harboring a TetM plasmid, whereas Dutch TetM determinant was identified in only one (3.2%) of those isolates (Table 5).

### Correlation between TEM and TetM presence and penicillin and tetracycline susceptibility

The PPNG had significantly high penicillin MICs (Median 12.00, inter-quartile range (IQR) 44.5), compared to the non-PPNG strains (Median 0.1900, IQR 0.9485, $p = 0.0001^*$) (Fig. 1a). Twenty-four (92.3%) of the 26 PPNG were penicillin resistant with MICs > 1 mg/L, while the remaining two PPNG (KNY_NGAMR18 and KNY_NGAMR27) had intermediary penicillin susceptibility (MICs> 0.06–1 mg/L) (Table 6). Two isolates, KNY_NGAMR33 and KNY_NGAMR54 which were non-PPNG, had penicillin resistant MIC values of 3 and 6 mg/L, respectively (Table 6). All isolates expressing TetM had significantly high tetracycline MICs (Median 16.00, IQR 20) compared to the non-TetM expressing isolates (Median 0.5000, IQR 0.343, $p = 0.0001^*$) and were all tetracycline resistant (MICs> 1 mg/L) (Fig. 1b). Furthermore, all the isolates harboring TetM had higher doxycycline MICs (up to 96 mg/L). The isolate expressing a Dutch TetM had the highest doxycycline and tetracycline MICs (96 mg/L and 64 mg/L respectively) (Table 6).

### Chromosomally encoded antimicrobial resistance determinants in the analyzed isolates

All isolates had non-mosaic *penA* alleles which have been associated with penicillin resistance in gonococci [27]. Five different non-mosaic PenA patterns were identified; patterns XXII, IX, XIX, XIV, and II [53]. Pattern IX was only identified in penicillin resistant isolates.

**Table 3** Antimicrobial susceptibility data of the study isolates

| Isolate details | | MICs (mg/L) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample ID | Year of isolation | CFX | CRO | PEN | TET | CIP | AZM | SPT |
| KNY_NGAMR1 | 2015 | < 0.016 | < 0.002 | 0.064 | 0.064 | 0.38 | 0.25 | 8 |
| KNY_NGAMR2 | 2015 | < 0.016 | < 0.002 | 64 | 0.5 | 0.016 | 0.5 | 8 |
| KNY_NGAMR3 | 2015 | < 0.016 | NT | 48 | 16 | 3 | 0.125 | 6 |
| KNY_NGAMR4 | 2016 | < 0.016 | < 0.002 | 8 | 12 | 0.006 | 0.125 | 2 |
| KNY_NGAMR5 | 2016 | < 0.016 | 0.006 | 3 | 16 | 4 | 0.125 | 2 |
| KNY_NGAMR6 | 2017 | < 0.016 | < 0.016 | 0.094 | 0.75 | 12 | 0.25 | 3 |
| KNY_NGAMR7 | 2014 | < 0.016 | 0.008 | > 256 | 16 | 8 | 2 | 16 |
| KNY_NGAMR8 | 2013 | < 0.016 | 0.002 | 0.064 | 0.5 | 3 | 0.25 | 4 |
| KNY_NGAMR10 | 2016 | < 0.016 | < 0.016 | > 256 | 32 | 8 | 0.38 | 16 |
| KNY_NGAMR11 | 2016 | < 0.016 | < 0.016 | 12 | 12 | 3 | 0.25 | 8 |
| KNY_NGAMR13 | 2014 | < 0.016 | < 0.002 | 8 | 12 | 3 | 0.125 | 24 |
| KNY_NGAMR14 | 2015 | < 0.016 | < 0.002 | 64 | 24 | 16 | 0.125 | 8 |
| KNY_NGAMR15 | 2014 | < 0.016 | < 0.016 | 48 | 32 | 4 | 0.5 | 12 |
| KNY_NGAMR16 | 2015 | < 0.016 | < 0.016 | 0.19 | 4 | 8 | 0.38 | 4 |
| KNY_NGAMR17 | 2015 | < 0.016 | < 0.016 | 0.38 | 16 | 6 | 0.5 | 6 |
| KNY_NGAMR18 | 2015 | < 0.016 | < 0.016 | 0.5 | 24 | 0.38 | 0.5 | 6 |
| KNY_NGAMR19 | 2015 | < 0.016 | 0.094 | 0.19 | 64 | 4 | 1.5 | 1 |
| KNY_NGAMR20 | 2015 | < 0.016 | 0.004 | 12 | 24 | 8 | 0.125 | 12 |
| KNY_NGAMR21 | 2016 | < 0.016 | < 0.016 | 32 | 24 | 3 | 0.125 | 12 |
| KNY_NGAMR22 | 2016 | < 0.016 | 0.004 | 2 | 32 | 4 | 1 | 12 |
| KNY_NGAMR23 | 2017 | < 0.016 | < 0.002 | 12 | 8 | 12 | 0.125 | 4 |
| KNY_NGAMR24 | 2014 | < 0.016 | < 0.016 | 2 | 16 | 4 | 0.38 | 8 |
| KNY_NGAMR26 | 2016 | < 0.016 | < 0.016 | 8 | 32 | 1.5 | 0.25 | 8 |
| KNY_NGAMR27 | 2016 | < 0.016 | < 0.016 | 1 | 32 | 24 | 0.25 | 8 |
| KNY_NGAMR28 | 2017 | < 0.016 | < 0.002 | 96 | 32 | 24 | 0.5 | 8 |
| KNY_NGAMR29 | 2017 | < 0.016 | < 0.002 | 0.094 | 12 | 12 | 0.25 | 2 |
| KNY_NGAMR30 | 2017 | < 0.016 | < 0.002 | 12 | 12 | 4 | 0.125 | 8 |
| KNY_NGAMR31 | 2017 | < 0.016 | < 0.002 | 0.38 | 48 | > 32 | 0.25 | 3 |
| KNY_NGAMR32 | 2017 | < 0.016 | < 0.002 | 8 | 0.5 | 16 | 0.047 | 1.5 |
| KNY_NGAMR33 | 2016 | < 0.016 | < 0.016 | 3 | 32 | 2 | 0.25 | 4 |
| KNY_NGAMR35 | 2013 | NT | NT | > 256 | 48 | 32 | 0.125 | NT |
| KNY_NGAMR41 | 2018 | < 0.016 | < 0.016 | 32 | 12 | 16 | NT | 4 |
| KNY_NGAMR42 | 2018 | < 0.016 | < 0.016 | 32 | 24 | 6 | 0.125 | 8 |
| KNY_NGAMR50 | 2018 | < 0.016 | < 0.016 | 16 | 16 | 1.5 | 0.064 | 4 |
| KNY_NGAMR53 | 2018 | < 0.016 | < 0.016 | 6 | 6 | 3 | 0.19 | 4 |
| KNY_NGAMR54 | 2018 | < 0.016 | < 0.016 | 6 | 2 | 2 | 0.125 | 6 |

*CRO* Ceftriaxone, *CFX* Cefixime, *PEN* Penicillin, *SPT* Spectinomycin, *AZM* Azithromycin, *CIP* Ciprofloxacin, *TET* Tetracycline, *NT* Not tested

**Table 4** β-lactamase encoding and TetM encoding plasmids

| | β-lactamase encoding | Non-β-lactamase encoding | Totals |
|---|---|---|---|
| TetM encoding | 24 (66.7%) | 7 (19.4%) | **31** (86.1%) |
| Non-TetM encoding | 2 (5.5%) | 3 (8.3%) | **5** (13.9%) |
| **Total** | **26** (72.2%) | **10** (27.8%) | **36** (100%) |

**Table 5** TEM and TetM genotypes and Plasmid prevalence

| PPNG plasmid type | African, 25 (96.2%) | Asian, 1 (3.8%) | 26 (100%) |
|---|---|---|---|
| TetM | American, 30 (96.8%) | Dutch, 1 (3.2%) | 31 (100%) |
| TEM genotype | *blaTEM-1*, 21 (81.5%) | *blaTEM-239*, 5 (18.5%) | 27 (100%) |

Pattern XIV which was identified in 16 PPNG was the most prevalent (61.5%) (Table 6).

Reduced drug accumulation resulting from reduced drug influx (due altered or lost porins) or active efflux pump has been shown to contribute/produce additive effects to drug resistance in *N. gonorrhoeae* [54, 55]. Five different patterns of PenB were identified in 23 (63.9%) study isolates: pattern I (G120D); pattern II (A121G, G120D, and -N122); pattern III (A121G, G120N, and -N122); pattern IV (A121S, and N122K) and pattern V (A121G, and -N122) (Table 6). These patterns were formed by alterations in PenB that have been associated with reduced drug accumulation and consequently drug resistance in gonococci [56]. There was no significant increase in both penicillin (Median 8.000, IQR 29, $p = 0.6779$) and tetracycline (Median 12.0000, IQR 18, $p = 0.1203$) MICs observed in isolates harboring the above described PenB amino acid changes when compared to the isolates without the PenB alterations (penicillin; Median 2.000, IQR 39.5600, tetracycline; Median 24.0000, IQR 18).

Modifications in the MtrR promoter and encoding gene which have previously been associated with antibiotic resistance in gonococci were identified in 34 (94.4%) of the 36 analyzed isolates [32]. The modifications included: Deletion of Adenine in the 13 bp (−A13) inverted repeat region between the − 10 and − 35 hexamers of the *mtrR* promoter (1 isolate); G45D (1 isolate); A39T (30 isolates); T86A (2 isolates); D79N (2 isolates) and H105Y (3 isolates) (Table 6). One of the two isolates lacking *mtrR* modifications (KNY_NGAMR7) expressed both TEM239 and TetM and was penicillin and tetracycline resistant. The remaining isolate (KNY_NGAMR1) that lacked MtrR modifications lacked both TEM and TetM. It was both penicillin and tetracycline susceptible. Only two isolates expressed both T86A and D79N substitutions in addition to H105Y. One of these two isolates was a penicillin resistant PPNG while the other was a non-PPNG and had intermediary penicillin susceptibility. They both expressed TetM and were tetracycline resistant. Both -A13 and G45D were expressed by only 1 isolate, KNY_NGAMR2 which was a penicillin resistant PPNG. This isolate lacked TetM and was tetracycline susceptible. A39T the most prevalent modification (83.3%), was not expressed concurrently with any other MtrR modification (Table 6). No significant increase in both penicillin (Median 8.000, IQR 31.1250, $p = 0.8446$ and tetracycline (Median 16.0000, IQR 21, $p = 0.2542$) MICs were observed in the isolates expressing MtrR A39T substitution when compared to the isolates without the MtrR A39T substitution (penicillin; Median 17.5000, IQR 111.6990, tetracycline; Median 16.0000, IQR 17.6090).

L421P amino acid substitution associated with decreased rate of penicillin acylation in gonococci was identified in 17 (47.2%) of the 36 analyzed isolates (Table 6). There was no significant increase in penicillin MICs

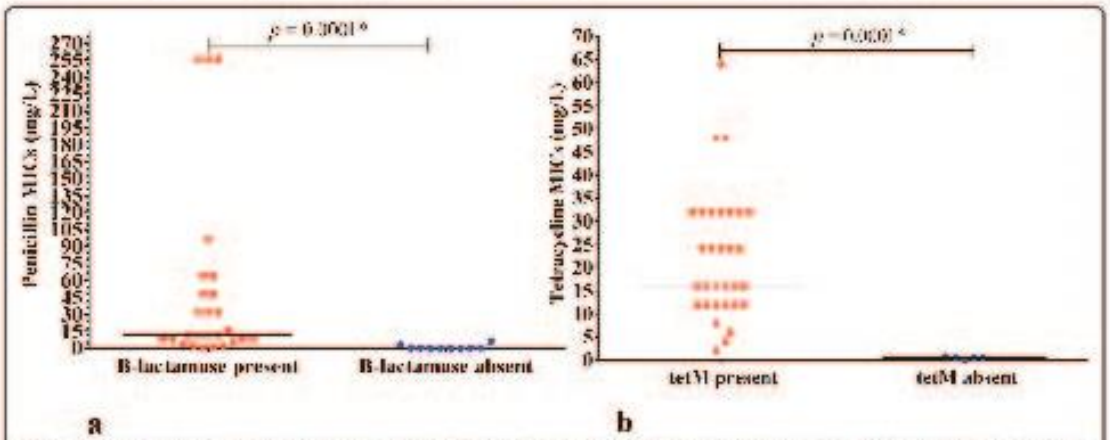



**Fig. 1** This figure shows the relationship between a) penicillin susceptibilities and the presence of β-lactamase, and b) tetracycline susceptibilities and the presence and tetM. The central bar across each group of points locates the median for that group.

**Table 6** Penicillin and tetracycline antimicrobial susceptibility data and identified AMR determinants

| | | Tetracyclines | | | | Penicillin G | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MIC (mg/L) | | tetM Plasmid | S10 | MIC (mg/L) | β-lactamase | *penA* allele | PonA | MtrR | PenB |
| | Sample ID | DOX | TET | | | PEN | | | | | |
| 1 | KNY_NGAMR1 | 0.75 | 0.064 | *Absent* | V57M | 0.064 | NP | Pattern XIV | – | – | A121G, G120D, –N122 |
| 2 | KNY_NGAMR2 | 1.5 | 0.5 | *Absent* | V57M | 64 | TEM1[a] | Pattern I | L421P | G45D, –A13 | G120D |
| 3 | KNY_NGAMR3 | 6 | 16 | Present | V57M | 48 | TEM239 | Pattern IX | L421P | A39T | – |
| 4 | KNY_NGAMR4 | 16 | 12 | Present | V57M | 8 | TEM1 | Pattern XIX | – | A39T | A121S, N122K |
| 5 | KNY_NGAMR5 | 12 | 16 | Present | V57M | 3 | TEM1 | Pattern IX | L421P | H105Y | A121G, G120N, –N122 |
| 6 | KNY_NGAMR6 | 0.75 | 0.75 | *Absent* | V57M | 0.094 | NP | Pattern I | – | A39T | – |
| 7 | KNY_NGAMR7 | 12 | 16 | Present | V57M | > 256 | TEM239 | Pattern XXII | L421P | – | A121S, N122K |
| 8 | KNY_NGAMR8 | 0.5 | 0.5 | *Absent* | V57M | 0.064 | NP | Pattern XIV | L421P | A39T | A121G, G120D, –N122 |
| 9 | KNY_NGAMR10 | 24 | 32 | Present | V57M | > 256 | TEM1 | Pattern I | – | A39T | – |
| 10 | KNY_NGAMR11 | 12 | 12 | Present | V57M | 12 | TEM1 | Pattern XIV | L421P | A39T | A121G, G120D, –N122 |
| 11 | KNY_NGAMR13 | 12 | 12 | Present | V57M | 8 | TEM1 | Pattern XIV | L421P | A39T | A121G, G120D, –N122 |
| 12 | KNY_NGAMR14 | 8 | 24 | Present | V57M | 64 | TEM1 | Pattern I | – | A39T | A121S, N122K |
| 13 | KNY_NGAMR15 | 16 | 32 | Present | V57M | 48 | TEM1 | Pattern XIV | L421P | A39T | A121G, –N122 |
| 14 | KNY_NGAMR16 | 8 | 4 | Present | V57M | 0.19 | NP | Pattern XIV | – | A39T | A121G, –N122 |
| 15 | KNY_NGAMR17 | 12 | 16 | Present | V57M | 0.38 | NP | Pattern XXII | L421P | T86A, D79N, H105Y | – |
| 16 | KNY_NGAMR18 | 32 | 24 | Present | V57M | 0.5 | TEM1 | Pattern XIX | – | A39T | – |
| 17 | KNY_NGAMR19 | 96 | 64 | Present[b] | V57M | 0.19 | NP | Pattern XIV | L421P | A39T | – |
| 18 | KNY_NGAMR20 | 16 | 24 | Present | V57M | 12 | TEM1 | Pattern IX | L421P | A39T | A121G, G120D, –N122 |
| 19 | KNY_NGAMR21 | 16 | 24 | Present | V57M | 32 | TEM1 | Pattern XIV | L421P | A39T | A121G, G120D, –N122 |
| 20 | KNY_NGAMR22 | 12 | 32 | Present | V57M | 2 | TEM1 | Pattern XIX | – | A39T | – |
| 21 | KNY_NGAMR23 | 16 | 8 | Present | V57M | 12 | TEM1 | Pattern IX | L421P | A39T | A121G, G120D, –N122 |
| 22 | KNY_NGAMR24 | 16 | 16 | Present | V57M | 2 | TEM1 | Pattern XIX | – | A39T | – |
| 23 | KNY_NGAMR26 | 24 | 32 | Present | V57M | 8 | TEM239 | Pattern XIV | – | A39T | A121G, –N122 |
| 24 | KNY_NGAMR27 | 16 | 32 | Present | V57M | 1 | TEM239 | Pattern XIV | – | A39T | – |
| 25 | KNY_NGAMR28 | 24 | 32 | Present | V57M | 96 | TEM1 | Pattern I | – | A39T | A121G, –N122 |
| 26 | KNY_NGAMR29 | 4 | 12 | Present | V57M | 0.094 | NP | Pattern XIV | L421P | A39T | A121G, –N122 |
| 27 | KNY_NGAMR30 | 12 | 12 | Present | V57M | 12 | TEM1 | pattern XIV | L421P | A39T | A121G, –N122 |
| 28 | KNY_NGAMR31 | 24 | 48 | Present | V57M | 0.38 | NP | Pattern I | – | A39T | A121G, G120D, –N122 |
| 29 | KNY_NGAMR32 | 1 | 0.5 | *Absent* | V57M | 8 | TEM1 | Pattern XIV | L421P | A39T | A121G, G120D, –N122 |
| 30 | KNY_NGAMR33 | 24 | 32 | Present | V57M | 3 | NP | Pattern XIV | – | A39T | A121G, –N122 |
| 31 | KNY_NGAMR35 | 24 | 48 | Present | V57M | > 256 | TEM1 | Pattern I | – | A39T | – |
| 32 | KNY_NGAMR41 | 12 | 12 | Present | V57M | 32 | TEM1 | Pattern XIX | – | A39T | – |
| 33 | KNY_NGAMR42 | 12 | 24 | Present | V57M | 32 | TEM1 | Pattern XIV | – | T86A, D79N, H105Y | – |
| 34 | KNY_NGAMR50 | 32 | 16 | Present | V57M | 16 | TEM1 | Pattern I | – | A39T | A121G, G120D, –N122 |
| 35 | KNY_NGAMR53 | 16 | 6 | Present | V57M | 6 | TEM239 | Pattern XIV | L421P | A39T | A121G, –N122 |
| 36 | KNY_NGAMR54 | 8 | 2 | Present | V57M | 6 | NP | Pattern XXII | – | A39T | – |

***DOX*** **Doxycycline,** ***TET*** **Tetracycline,** ***PEN*** **Penicillin** [a]**β-lactamase encoding plasmid with an Asian plasmid backbone,** [b]**Dutch TetM determinant,** ***NP*** **Not present, "-" no mutation identified, (–N122)- deletion of N at position 122 of PenB, (–A13)- Adenine deletion in the 13 bp inverted repeat region between the – 10 and – 35 hexamers of the *mtrR* promoter**

**Pattern XXII:** D346, F505L, A511V, A517G H542N, P553V, I556Q, I557V, I567V, N575, A576V

**Pattern IX:** D346, F505L, A511V, A517G, P552L

**Pattern XIX:** D346, F505L, A511V, A517G, H542N, I567V, N575, A576V

**Pattern XIV:** D346, F505L, A511V, A517G, H542N

**Pattern I:** D346, F505L, A511V, A517G

observed in the isolates harboring PonA L421P amino acid changes (Median 12.00, IQR 38.31, $p = 0.7124$) when compared to isolates expressing wild type PonA (Median 6.000, IQR 31.5000). PilQ E666K substitution associated with penicillin resistance was not observed in the present study [27].

One of the two penicillin resistant non-PPNGs, KNY_NGAMR33 expressed altered PenA, MtrR and PenB while KNY_NGAMR54 expressed altered PenA, and MtrR (Table 6). The two PPNG isolates which had intermediary penicillin susceptibility both lacked PonA and PenB alterations but harbored A39T MtrR substitution.

V57M substitution in S10 ribosomal proten, together with *mtrR* and *penB* mutations have been shown to increase tetracycline resistance [37]. S10 V57M was identified in all isolates both tetracycline susceptible and resistant. Three of five isolates with susceptible to intermediary tetracycline susceptibility had both MtrR and PenB amino acid changes while two had either MtrR or PenB amino acid changes each (Table 6). Other chromosomally encoded antimicrobial determinants identified in the analyzed isolates included previously reported altered GyrA (S91F and D95G/A) and ParC (E91G and S87R) which confer resistance to fluoroquinolones [57]. *mefA/E* genes encoding a membrane bound efflux MefA protein, rRNA methylase encoding *erm (B/C/F)* genes, and mutations in 23S ribosomal RNA and large subunit ribosomal proteins L4 encoded by *rplD* and L22 encoded by *rplV* all known to confer resistance to macrolides were not identified in any isolate [58–61]. Two of the three isolates which had a low level azithromycin resistance, expressed A39T MtrR modification, while one expressed an altered PenB (Table 6). Mutations in 16S ribosomal RNA and small subunit ribosomal protein S5 encoded by *rpsE* which confer resistance to spectinomycin [62, 63] were also not found in any of the isolates were also not found in any of the isolates were also not found in any of the isolates. Mosaic *penA* alleles, associated with increased and resistant cefixime and ceftriaxone MICs in gonococci were not identified in the present study.

## Discussion

Two β-lactamase plasmid types of different origins (African and Asian) were identified in this study. African β-lactamase plasmid (pDJ5) which was first identified from Africa [16, 64] was predominant. These findings are similar to observations of a previous study from Coastal Kenya [52]. The Asian type β-lactamase plasmid (pDJ4) initially described in Asia has been associated with epidemic outbreaks in Asian countries [12]. Five of the PPNG harbored a unique *blaTEM-239* allele which has only been reported in Kenya by a previous study [52]. In the study that unraveled *blaTEM-239*, high level penicillin resistance was associated with the allele [52]. However, from our findings, one isolate expressing TEM239 had an MIC suggesting intermediate susceptibility to penicillin while four were resistant.

Although a significant association was observed between penicillin MICs and the presence of TEM, this study also found two non-PPNG isolates which were penicillin resistant. These two isolates expressed chromosomal modifications which have previously been associated with penicillin resistance. These findings indicate that resistance to penicillin in the analyzed Kenyan gonococci is also mediated by chromosomal modifications mechanisms in addition to the plasmid borne TEM. β-lactamase production in gonococci has been associated with resistant penicillin MICs [65] [66]. Contrary to these previous findings, this study identified two PPNG isolates which were not resistant to penicillin. They both lacked PonA and PenB alterations but expressed A39T MtrR substitution known to mediate penicillin resistance in gonococci. Continued surveillance and monitoring of β-lactamase production is required in order to understand the susceptibility pattern observed in these two non-penicillin resistant PPNGs.

The predominance of the American TetM in the present study confirms the expected epidemiology of this resistance marker. In a study by Turner et al., the American TetM was identified in 14 GC isolates with a Kenyan origin [67], suggesting that the American TetM originated from equatorial regions of Africa. The American type was also found to be predominant in GC isolates from the United Kingdom, and eastern and central Africa [68]. Thus, the findings of this study agree with these previous studies. Dutch type TetM is predominant in GC isolates from the Netherlands, Asia and South America [68]. In the present study, identification of a Dutch type TetM in one of the study isolate shows that there is an introduction of the GC expressing Dutch TetM determinant into the sampled Kenyan regions.

Chromosomal V57M substitution in S10 modulates the affinity of tetracycline for its 30S ribosomal target and together with *mtrR* and *penB* mutations, have been shown to cause chromosomally mediated tetracycline resistance in gonococci (MIC≥2 mg/L) [26, 37]. A study from Coastal Kenya observed S10 V57M substitution in both tetracycline resistant and susceptible gonococcal isolates and suggested that this substitution had no effect on the observed low or high level tetracycline resistance [52]. In the present study all the isolates both tetracycline resistant and susceptible had the S10 V57M substitution. Increased and tetracycline resistant MICs were observed only in isolates expressing TetM protein, indicating that resistance to tetracycline in the analyzed Kenyan gonococci is mainly plasmid mediated. Contrary to the association of TetM with high-level tetracycline resistance (MIC≥16 mg/L) in

gonococci, we observed ten tetM expressing isolates which had a lower level of tetracycline resistance (MIC range of 2-12 mg/L) (Table 6) [69]. High doxycycline MICs were also observed in isolates which harbored TetM. This observation is similar to the findings of the former Coastal Kenya study and indicates that TetM is involved in mediating doxycycline resistance in the analyzed Kenyan gonococci [52].

A previous study by Sun et al., (2010) reported novel PorB deletions at both A121 and N122 positions, and associated these changes with high levels of both chromosomal penicillin (MIC of 4–8 mg/L) and tetracycline (MICs of 4–16 mg/L) resistance [56]. It is worth noting that the present study identified deletion at only position N122. Additionally, non-tetM and non-PPNG isolates harboring N122 PorB deletions did not have high penicillin (0.064-3 mg/L) and tetracycline (0.064–0.5 mg/L) MICs as reported in the previous study. Comparing our findings with those of the previous study suggests that for such high levels of both chromosomal penicillin and tetracycline resistance to occur the deletions at A121 and N122 have to occur concurrently.

The identification of an Asian type β-lactamase plasmid and Dutch TetM determinant in Kenyan *N. gonorrhoeae* isolates indicates that there is circulation of plasmid mediated antibiotic resistance between different *N. gonorrhoeae* isolates from different countries. Although the use of penicillin and tetracycline for gonorrhea treatment was stopped many years ago, they are widely available to the public and are inappropriately used through self prescription in many parts of Africa [43, 44]. The observed high prevalence of plasmid mediated penicillin and tetracycline resistance indicates that these drugs are not suitable for gonorrhea treatment.

Mosaic *penA* alleles shown to confer resistance to extended-spectrum cephalosporins in gonococci were not observed in the present study [29–31]. These findings correlate with the observed phenotypic patterns as all the analyzed isolates were susceptible to both ceftriaxone and cefixime. This study did not identify any antimicrobial resistance determinants specifically associated with macrolide resistance. These finding explains the azithromycin phenotypes observed in the study isolates where a larger proportion of the study isolates were azithromycin susceptible with only 8.3% of the isolates having a low level azithromycin resistance. The observed low level azithromycin resistance could be mediated by reduced drug accumulation resulting from modified PenB and MtrR which were identified in these isolates. Absence of molecular markers specifically associated with high levels of azithromycin, cefixime and ceftriaxone in the present study, shows that these antibiotics are still useful for treatment of gonococcal infections in Kenya. Continued molecular surveillance based on larger sample size is required so as to: a) monitor the emergence and spread of ceftriaxone and azithromycin resistance since both drugs are the dual therapy currently recommended by the Kenyan National Guidelines for treatment of gonococcal infections, b) understand the effects of both efflux pumps and altered porins on antibiotic resistance in Kenyan *N. gonorrhoeae* isolates.

## Conclusion

The observed high penicillin and tetracycline resistance in the analyzed Kenyan gonococci is mainly mediated by plasmid-borne *blaTEM*, and *tetM* genes in addition to chromosomal modifications with the African type PPNG, TEM1 β-lactamase and American TetM determinants being the most prevalent. Consequently the ban on the use of these antibiotics for the treatment of gonococcal infections should continue. Asian type PPNG and Dutch TetM determinant, which are less described in the African gonococci, are present in gonococci from the studied Kenyan regions.

### Abbreviations

β-lactamase: Beta lactamase; EUCAST: European committee on antimicrobial susceptibility testing; ESBL: Extended broad spectrum β-lactamase; GC: *Neisseria gonorrhoeae*; KEMRI: Kenya medical research institute; SERU: Scientific and ethics review unit,; WRAIR: Walter reed army institute of research; HSPB: Human subject protection board,; MIC: Minimum inhibitory concentration; NCBI: National centre for biotechnology information; PPNG: Penicillinase Producing *Neisseria gonorrhoeae*; RAST: Rapid Annotation using subsystem technology

### Acknowledgements

The authors thank STI surveillance field staff and KEMRI CMR laboratory staff for their assistance with this project. We also thank Moses Kimita Gathii for assistance with NGS sequencing. We acknowledge Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI SERU) and Walter Reed Army Institute of Research (WRAIR) Human Subject Protection Board (HSPB) for reviewing and approving protocols under which this work was carried out.

### Disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. The Investigators have adhered to the policies for protection of human subjects as prescribed in AR 70–25. This paper is published with the approval by Director Kenya Medical Research Institute.

### Authors' contributions

MWK: study and experimental design; bacteria culture; isolate identification; NGS sequencing; sequencing data analysis; manuscript development and writing, MM: project conception; study and experimental design; data analysis; and manuscript development, FLE, & WDB; experimental design and sequence data analysis, CKK; NGS sequencing and sequencing data analysis, VO; bacteria culture and isolate identification, WMM, WS, BA, COS, & RSM; technical consultation and manuscript review, JD; technical consultation. The manuscript was read and approved by all the authors.

### Funding

This work was funded by the Global Emerging Infections Surveillance (GEIS)/ Armed Forces Health Surveillance Branch (ProMIS P0119_18_KY_012.06). The funding body had no role in the study design; data analysis and interpretation or in the manuscript writing.

**Availability of data and materials**
The datasets supporting the conclusions made in this article are included within the document. The datasets are also available from the corresponding author on reasonable request. Sequence reads generated in this study are linked to NCBI BioProjects: PRJNA481622 and PRJNA590515.

**Ethics approval and consent to participate**
Ethical approval was granted by both Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (KEMRI#3385) and Walter Reed Army Institute of Research Institutional Review Board (WRAIR#1743A). This was a retrospective laboratory based study which analyzed archived samples. Consent to participate was not applicable since there was no interaction with subjects.

**Consent for publication**
Not applicable.

**Competing interests**
Authors declare no conflict of interest in this work.

**Author details**
[1]Institute for Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology (JKUAT), P. O Box 62,000-00200, Thika, Kenya. [2]Department of Biological and Physical Science, Karatina University (KarU), P. O Box 1957-10101, Karatina, Kenya. [3]U.S. Army Medical Research Directorate-Africa, P. O Box 606, Village Market, Nairobi 00621, Kenya. [4]Kenya Medical Research Institute (KEMRI), P. O Box 54840-00200, Nairobi, Kenya. [5]School of Medicine, Department of Biochemistry, University of Nairobi, P. O Box 30197, GPO, Nairobi 00100, Kenya. [6]Department of Chemistry and Biochemistry, Pwani University, P. O Box 195-80108, Mombasa, Kenya. [7]Departments of Global Health and Medicine, University of Washington, 325 9th Avenue, Box 359931, Seattle, WA 98104, USA. [8]Departments of Medicine, Epidemiology, and Global Health, University of Washington, 325 9th Avenue, Box 359931, Seattle, WA 98104, USA.

**Received: 6 February 2020 Accepted: 6 September 2020**
Published online: 15 September 2020

**References**

1. Unemo M, Del Rio C, Shafer WM. Antimicrobial resistance expressed by *Neisseria gonorrhoeae*: a major global public health problem in the 21st century. Microbiol Spectr. 2016;4(3):10.
2. Tapsall JW, Ndowa F, Lewis DA, Unemo M. Meeting the public health challenge of multidrug- and extensively drug-resistant *Neisseria gonorrhoeae*. Expert Rev Anti Infect Ther. 2009;7(7):821–34.
3. Barbour AG. Properties of penicillin-binding proteins in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 1981;19:316–22.
4. Stefanova ME, Tomberg J, Olesky M, Höltje J-V, Gutheil WG, Nicholas RA. *Neisseria gonorrhoeae* penicillin-binding protein 3 exhibits exceptionally high Carboxypeptidase and β-lactam binding activities. Biochemistry. 2003;42(49):14614–25.
5. Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol Mol Biol Rev. 2001;65(2):232–60.
6. Zapun A, Morlot C, Taha MK. Resistance to β-lactams in Neisseria spp due to chromosomally encoded penicillin-binding proteins. Antibiotics. 2016;5(4):35.
7. Bodoev IN, Ilina EN. Molecular mechanisms of formation of drug resistance in *Neisseria gonorrhoeae*: history and prospects. Mol Genet Microbiol Virol. 2015;30(2):132–40.
8. Unemo M, Shafer WM. Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. Ann N Y Acad Sci. 2011;1230(1):E19–28.
9. Lind I. Antimicrobial resistance in *Neisseria gonorrhoeae*. Clin Infect Dis. 1997;24(Supplement_1):S93–7.
10. Alam MA. The plasmids in gonococci. Bangladesh J Med Microbiol. 2008;2(2):35–47.
11. Trembizki E, Buckley C, Lawrence A, Lahra M, Whiley D. Characterization of a novel *Neisseria gonorrhoeae* penicillinase-producing plasmid isolated in Australia in 2012. Antimicrob Agents Chemother. 2014;58(8):4984–5.
12. Ashford W, Golash R, Hemming V. PENICILLINASE-PRODUCING *NEISSERIA GONORRHOEAE*. Lancet. 1976;308(7987):657–8.
13. Brett M. A novel gonococcal β-lactamase plasmid. J Antimicrob Chemother. 1989;23(4):653–4.
14. Gouby A, Bourg G, Ramuz M. Previously undescribed 6.6-kilobase R plasmid in penicillinase-producing *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 1986;29(6):1095–7.
15. Etienne E, Fayemiwo SA, Lewis DA. Characterization of a novel β-lactamase-producing plasmid in *Neisseria gonorrhoeae*: sequence analysis and molecular typing of host gonococci. J Antimicrob Chemother. 2011;66:1514–7.
16. Dillon J, Yeung K. Beta-lactamase plasmids and chromosomally mediated antibiotic resistance in pathogenic Neisseria species. Clin Microbiol Rev. 1989;2(Suppl):S125.
17. Muhammad I, Golparian D, Dillon J-AR, Johansson Å, Ohnishi M, Sethi S, S-c C, S-l N, Sundqvist M, Bala M. Characterisation of bla TEM genes and types of β-lactamase plasmids in *Neisseria gonorrhoeae*–the prevalent and conserved bla TEM-135 has not recently evolved and existed in the Toronto plasmid from the origin. BMC Infect Dis. 2014;14(1):454.
18. Coulson A. β-Lactamases: Molecular Studies. Biotechnol Genet Eng Rev. 1985;3(1):219–54.
19. Knox JR. Extended-spectrum and inhibitor-resistant TEM-type beta-lactamases: mutations, specificity, and three-dimensional structure. Antimicrob Agents Chemother. 1995;39(12):2593.
20. Srifeungfung S, Roongpisuthipong A, Asavapiriyanont S, Lolekha R, Tribuddharat C, Lokpichart S, Sungthong P, Tongtep P. Prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in HIV-seropositive patients and gonococcal antimicrobial susceptibility: an update in Thailand. Jpn J Infect Dis. 2009;63(6):467–70.
21. Wang X, Minasov G, Shoichet BK. Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. J Mol Biol. 2002;320(1):85–95.
22. Ohnishi M, Cho E, Shimuta K, Watanabe H, Okamura N. Identification of TEM-135 β-lactamase in penicillinase-producing *Neisseria gonorrhoeae* strains in Japan. Antimicrob Agents Chemother. 2010;54(7):3021–3.
23. Sri N, Tribuddharat C, Prombhul S, Shimuta K, Srifeungfung S, Unemo M, Ohnishi M. Molecular analyses of TEM genes and their corresponding penicillinase-producing *Neisseria gonorrhoeae* isolates in Bangkok, Thailand. Antimicrob Agents Chemother. 2012;56(2):916–20.
24. Gianecini R, Oviedo C, Litvik A, Mendez E, Picoli L, Montibello S, Galarza P. Identification of TEM-135 β-lactamase in *Neisseria gonorrhoeae* strains carrying African and Toronto plasmids in Argentina. Antimicrob Agents Chemother. 2011;55(1):717–20.
25. Yeung K-H, Dillon J, Pauze M, Wallace E. A novel 49-kilobase plasmid associated with an outbreak of penicillinase-producing *Neisseria gonorrhoeae*. J Infect Dis. 1986;153(6):1162–5.
26. Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. Clin Microbiol Rev. 2014;27(3):587–613.
27. Ropp PA, Hu M, Olesky M, Nicholas RA. Mutations in ponA, the gene encoding penicillin-binding protein 1, and a novel locus, penC, are required for high-level chromosomally mediated penicillin resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 2002;46(3):769–77.
28. Spratt BG, Bowler LD, Zhang QY, Zhou J, Smith JM. Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal Neisseria species. J Mol Evol. 1992;34(2):115–25.
29. Ito M, Deguchi T, Mizutani KS, Yasuda M, Yokoi S, Ito S, Takahashi Y, Ishihara S, Kawamura Y, Ezaki T. Emergence and spread of *Neisseria gonorrhoeae* clinical isolates harboring mosaic-like structure of penicillin-binding protein 2 in Central Japan. Antimicrob Agents Chemother. 2005;49(1):137–43.
30. Ameyama S, Onodera S, Takahata M, Minami S, Maki N, Endo K, Goto H, Suzuki H, Oishi Y. Mosaic-like structure of penicillin-binding protein 2 gene (*penA*) in clinical isolates of *Neisseria gonorrhoeae* with reduced susceptibility to cefixime. Antimicrob Agents Chemother. 2002;46(12):3744–9.
31. Ohnishi M, Saika T, Hoshina S, Iwasaku K, Nakayama S, Watanabe H, Kitawaki J. Ceftriaxone-resistant *Neisseria gonorrhoeae*, Japan. Emerg Infect Dis. 2011;17(1):148–9.
32. Lucas CE, Balthazar JT, Hagman KE, Shafer WM. The *MtrR* repressor binds the DNA sequence between the *mtrF* and *mtrC* genes of *Neisseria gonorrhoeae*. J Bacteriol. 1997;179(13):4123–8.

33. Olesky M, Hobbs M, Nicholas RA. Identification and analysis of amino acid mutations in porin IB that mediate intermediate-level resistance to penicillin and tetracycline in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 2002;46(9):2811–20.
34. Swartley J, McAllister C, Hajjeh R, Heinrich D, Stephens D. Deletions of Tn916-like transposons are implicated in tetM-mediated resistance in pathogenic Neisseria. Mol Microbiol. 1993;10(2):299–310.
35. Gascoyne DM, Heritage J, Hawkey PM, Turner A, van Klingeren B. Molecular evolution of tetracycline-resistance plasmids carrying TetM found in *Neisseria gonorrhoeae* from different countries. J Antimicrob Chemother. 1991;28(2):173–83.
36. Gascoyne-Binzi DM, Heritage J, Hawkey PM. Nucelotide sequences of the tet (M) genes from the American and Dutch type tetracycline resistance plasmids of *Neisseria gonorrhoeae*. J Antimicrob Chemother. 1993;32(5):667–76.
37. Hu M, Nandi S, Davies C, Nicholas RA. High-level chromosomally mediated tetracycline resistance in *Neisseria gonorrhoeae* results from a point mutation in the *rpsJ* gene encoding ribosomal protein S10 in combination with the *mtrR* and *penB* resistance determinants. Antimicrob Agents Chemother. 2005;49(10):4327–34.
38. Verhagen A, Van der Ham M, Heimans A, Kranendonk O, Maina A. Diminished antibiotic sensitivity of *Neisseria gonorrhoeae* in urban and rural areas in Kenya. Bull World Health Organ. 1971;45(6):707.
39. Perine P, Biddle J, Nsanze H, D'COSTA L, Osaba A, Widy-Wirski R. Gonococcal drug resistance and treatment of gonorrhoea in Nairobi. East Afr Med J. 1980;57(4):238–46.
40. Brunham R, Fransen L, Plummer F, Piot P, Slaney L, Bygdeman S, Nsanze H. Antimicrobial susceptibility testing and phenotyping of *Neisseria gonorrhoeae* isolated from patients with ophthalmia neonatorum in Nairobi, Kenya. Antimicrob Agents Chemother. 1985;28(3):393–6.
41. Van Hall M, Petit P, Van Hall H, Mouton R, Ndinya-Achola J. Prevalence of resistance of *N. gonorrhoeae* to penicillin and three other antibiotics in a rural area in Kenya. East Afr Med J. 1991;68(01):853–9.
42. WHO. 1998 sexually transmitted diseases treatment guidelines. Centers for Disease Control and Prevention. MMWR Recomm Rep. 1998;47(RR-1):1–102.
43. Viberg N, Mujinja P, Kalala W, Kumaranayake L, Vyas S, Tomson G, Lundborg CS. STI management in Tanzanian private drugstores-practices and roles of drugsellers. Sex Transm Infect. 2009.
44. Kariuki S. Kenya: antibiotic resistance. Lancet. 1997;349:59–510.
45. Ackram N, Agbodzi B, Dela H, Behene E, Nyarko EO, Kyei NNA, Larbi JA, Lawson BW., Addo KK, Newman MJ, et al. Antimicrobial resistance (AMR) and molecular characterization of *Neisseria gonorrhoeae* in Ghana, 2012-2015. PLoS One. 2019;14(10):e0223598.
46. Adeyemi-Coro FA, Lyon DJ, Ling TK, Cheng AF. E-test method of antimicrobial susceptibility testing of *Neisseria gonorrhoeae* for routine diagnostic service. Afr J Med Med Sci. 2000;29(2):171–3.
47. Liu H, Taylor TH Jr, Pettus K, Trees D. Assessment of Etest as an alternative to agar dilution for antimicrobial susceptibility testing of *Neisseria gonorrhoeae*. J Clin Microbiol. 2014;52(5):1435–40.
48. Unemo M, Golparian D, Sánchez-Busó L, Grad Y, Jacobsson S, Ohnishi M, Lahra MM, Limnios A, Sikora AE, Wi T, et al. The novel 2016 WHO *Neisseria gonorrhoeae* reference strains for global quality assurance of laboratory investigations: phenotypic, genetic and reference genome characterization. J Antimicrob Chemother. 2016;71(11):3096–108.
49. Sim JHC, Aniket V, Lchith A, Pourmand N, Banaei N. Optimized protocol for simple extraction of high-quality genomic DNA from Clostridium difficile for whole-genome sequencing. J Clin Microbiol. 2015;53(7):2329.
50. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Big (2008). The RAST Server: rapid annotations using subsystems technology. 9(1):75.
51. Hall T. BioEdit v. 7.0. 5: Biological sequence alignment editor for Windows. Ibis Therapeutics a division of Isis pharmaceuticals; 2005. In.; 2016.
52. Cehovin A, Harrison OB, Lewis SB, Ward PN, Ngetsa C, Graham SM, Sanders EJ, Maiden MC, Tang CM. Identification of novel *Neisseria gonorrhoeae* lineages harbouring resistance plasmids in coastal Kenya. J Infect Dis. 2018;218(6):801–8.
53. Whiley DM, Limnios EA, Ray S, Sloots TP, Tapsall JW. Diversity of penA alterations and subtypes in *Neisseria gonorrhoeae* strains from Sydney, Australia, That Are Less Susceptible to Ceftriaxone. Antimicrob Agents Chemother. 2007;51(9):3111.
54. Warner DM, Shafer WM, Jerse AE. Clinically relevant mutations that cause derepression of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. Mol Microbiol. 2008;70(2):462–78.
55. Olesky M, Zhao S, Rosenberg RL, Nicholas RA. Porin-mediated antibiotic resistance in *Neisseria gonorrhoeae*: ion, solute, and antibiotic permeation through PIB proteins with penB mutations. J Bacteriol. 2006;188(7):2300–8.
56. Sun A, Fan X, Gu Y, Du P, Tang R, Mao Y, Lin X, Yan J. Predominant *porB1A* and *porB1B* genotypes and correlation of gene mutations with drug resistance in *Neisseria gonorrhoeae* isolates in eastern China. BMC Infect Dis. 2010;10:323.
57. Kivata MW, Mbuchi M, Eyase FL, Bulimo WD, Kyanya CK, Oundo V, Murithi SW, Andagalu B, Mbinda WM, Soge OO, et al. *gyrA* and *parC* mutations in fluoroquinolone-resistant *Neisseria gonorrhoeae* isolates from Kenya. BMC Microbiol. 2019;19(1):76.
58. Ng LK, Martin I, Liu G, Bryden L. Mutation in 23S rRNA associated with macrolide resistance in *Neisseria gonorrhoeae*. Antimicrob Agents Chemother. 2002;46(9):3020–5.
59. Chisholm SA, Dave J, Ison CA. High-level azithromycin resistance occurs in *Neisseria gonorrhoeae* as a result of a single point mutation in the 23S rRNA genes. Antimicrob Agents Chemother. 2010;54(9):3812–6.
60. Luna VA, Cousin S Jr, Whittington WL, Roberts MC. Identification of the conjugative mef gene in clinical *Acinetobacter junii* and *Neisseria gonorrhoeae* isolates. Antimicrob Agents Chemother. 2000;44(6):2503–6.
61. Roberts MC, Chung WO, Roe D, Xia M, Marquez C, Borthagaray G, Whittington WL, Holmes KK. Erythromycin-resistant *Neisseria gonorrhoeae* and oral commensal Neisseria spp. carry known rRNA methylase genes. Antimicrob Agents Chemother. 1999;43(6):1367–72.
62. Gallimand M, Gerbaud G, Courvalin P. Spectinomycin resistance in Neisseria spp. due to mutations in 16S rRNA. Antimicrob Agents Chemother. 2000;44(5):1365–6.
63. Ilina EN, Malakhova MV, Bodoev IN, Oparina NY, Filimonova AV, Govorun VM. Mutation in ribosomal protein S5 leads to spectinomycin resistance in *Neisseria gonorrhoeae*. Front Microbiol. 2013;4:186.
64. Phillips I. β-lactamase-producing, penicillin-resistant gonococcus. Lancet. 1976;308(7987):656–7.
65. Ashford W, Golash R, Hemming V. Penicillinase-producing *Neisseria gonorrhoeae*. Lancet (London, England). 1976;308(7987):657–8.
66. Shaskolskiy B, Dementeva E, Kandinov I, Filippova M, Petrova N, Plakhova X, Chestkov A, Kubanov A, Deryabin D, Gryadunov D. Resistance of *Neisseria gonorrhoeae* isolates to beta-lactam antibiotics (benzylpenicillin and ceftriaxone) in Russia, 2015-2017. PLoS One. 2019;14(7):e0220339.
67. Turner A, Gough K, Leeming J. Molecular epidemiology of *tetM* genes in *Neisseria gonorrhoeae*. Sex Transm Infect. 1999;75(1):60–6.
68. Gascoyne-Binzi D, Hawkey P, Heritage J, Turner A, Nadarajah M. World-wide distribution of high level tetracycline-resistant *Neisseria gonorrhoeae*. Genitourin Med. 1992;68(4):277.
69. Pitt R, Sadouki Z, Town K, Rifer H, Mohammed H, Hughes G, Woodford N, Cole MJ. Detection of tet(M) in high-level tetracycline-resistant *Neisseria gonorrhoeae*. J Antimicrob Chemother. 2019;74(7):2115–6.


## Publisher's Note