UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

PATHOGENOMICS AND ANTIMICROBIAL RESISTANCE ANALYSIS IN *NEISSERIA GONORRHOEAE*

BY

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This thesis is submitted to the University of Ghana, Legon in partial fulfillment of the requirement for the award of **MPHIL in MOLECULAR BIOLOGY Degree.**

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DECLARATION

I hereby declare that this is the product of my own research undertaken under the supervision of Doctor Samuel Duodu, Doctor Samuel Kojo Kwofie and Doctor Naiki Attram and that references made to other people's work have been duly acknowledged. I also declare that this work has neither been presented in whole nor in part for another degree elsewhere.

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ABSTRACT

Gonorrhoea is a poorly controlled public health problem. With the global emergence of resistance to first line antibiotic treatment options, the infection has been predicted to be untreatable in the near future. This emerging trend highlights the need for constant genetic surveillance to unravel the mechanisms of resistance and inform therapy. This study therefore, sought to perform whole genome characterization of *N. gonorrhoeae* collected in Ghana to identify lineages of circulating strains, their antimicrobial resistance (AMR) and some virulence determinants. Gonococci isolates were cultured on gonococcal (GC) medium and identified using the API NH kit (Biomerieux, France). Genomic DNA was extracted from *N. gonorrhoeae* isolates using the QIAamp® DNeasy Ultraclean Microbial kit (Qiagen, Hilden, Germany). Whole genome sequencing (WGS) was performed on 56 isolates using both the Oxford Nanopore MinION and Illumina MiSeq sequencing platforms. The Comprehensive Antimicrobial Resistance Database (CARD) and PubMLST *Neisseria* database were used to catalogue chromosomal and plasmid genes implicated in AMR and assign sequence types (STs). The core genome MLST (cgMLST) approach was used for comparative genomics. The Virulence Factors of Pathogenic Bacteria Database (VFDB) was used to annotate virulence factors. *In vitro* resistance measured by disc diffusion revealed that (56)100%, (51)91% and (50)89.3% of the isolates were resistant to tetracycline, penicillin and ciprofloxacin respectively, while for the E-test method, (54)96.4%, (51)91% and (49)87.5% respectively were recorded. Four isolates exhibited reduced susceptibility to both cefixime and ceftriaxone as measured by disc diffusion. For these isolates, MIC ranges of 0.004 – 0.016 µg/ml and 0.016 - 0.75 µg/ml for ceftriaxone and cefixime respectively were recorded. No spectinomycin and azithromycin resistance was recorded using the E-test method. A total of 22 STs were identified by Multi-Locus Sequence Typing (MLST), with ST-14422 (n=10), ST-1927 (n=8) and ST-11210 (n=7) being the most prevalent. Six novel STs were also identified and submitted for the

assignment of new sequence types (**ST-15634-115641**). Seven clusters of isolates with distinct AMR genotypes were identified after the cgMLST analysis, highlighting the presence of genome wide genetic variation. All isolates harboured chromosomal AMR determinants that confer resistance to beta-lactam antimicrobials and tetracycline. A total of (49)87.5% and (13)23% isolates contained fluoroquinolone and macrolide resistance markers respectively. Plasmids were highly prevalent: p*TetM* and *pBlaTEM* were found in 96%, and 92% of isolates, respectively. All isolates possessed the PI (B) variant of the *porB* gene which is associated with localized infection while high antigenic variations in the pillin genes was also detected. The study highlighted the need for constant genomic surveillance with the looming possible emergence of cephalosporin resistant isolates and isolates with highly variable antigens which could severely impact disease treatment.

DEDICATION

This work is dedicated to my mother, Juliana Amma Adwubi and my entire family.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment length polymorphism
ARDRA	Amplified rRNA gene restriction analysis
AMR	Antimicrobial Resistance
API	Analytical Profile Index
APP	Adhesion and Penetration Protein
AST	Antimicrobial Sensitivity Tests
ATTC	American Type Culture Collection
BIGSdb	Bacterial Isolate Genome Sequence Database
β-lactam	Beta-lactam
CA	Chocolate agar
CARD	Comprehensive Antibiotic Resistance Database
CDC	Centers for Disease and Control
cgMLST	Core genome MLST
CLSI	Clinical and Laboratory Standards Institute
CMTR	Chromosomally mediated tetracycline resistance
CO_2	Carbon dioxide
CSW	Commercial sex workers
DNA	Deoxyribonucleic Acid
DHPS	Dihydropteroate synthase
ESBL	Extended Spectrum beta-lactamase
ESCs	Extended spectrum cephalosporins
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GASP	Global Gonococcal Antimicrobial Surveillance Program
GISP	Gonococcal Isolate Surveillance Project

GGI	Gonococcal genetic island
GHS-ERC	Ghana Health Service Ethics Review Committee
GS	Genetic Systems
LB	Loading beads
LMICs	Low and middle-income countries
LST	LOS sialylation
MAbs	Monoclonal antibodies
MDR	Multi-drug Resistant
MIC	Minimum Inhibition Concentration
MLPPST	Multi-locus predicted proteins sequence typing
MLST	Multi-locus Sequence Typing
MLVA	Multilocus Variable-Number Tandem Repeat analysis
MSMs	Men who have sex with men
MTM	Modified Thayer-Martin media
NAMRU-3 IRB	Naval Medical Research Unit Number Three Institutional Review Board
MAST	Multi-Antigen Sequence Typing
NGS	Next Generation Sequencing
NMIMR	Noguchi Memorial Institute for Medical Research
NMIMR-IRB	Noguchi Memorial Institute for Medical Research Institutional Review Board
NMRC-IRB	Naval Medical Research Center Institutional Review Board
ONT	Oxford Nanopore Technology
PBP	Penicillin binding protein
PPNG	Penicillinase producing gonococci

PBS	Phosphate Buffered Saline
Ph	Pharmacia
PCR	Polymerase Chain Reaction
PFGE	Pulse-field Gel Electrophoresis
PID	Pelvic Inflammatory Disease
QC	Quality control
qPCR	Quantitative Polymerase Chain Reaction
QRDR	Quinolone Resistant Determining Region
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
Rmlst	Ribosomal MLST
SQB	Sequencing Buffer
SMRT	Single-molecule real-time sequencing
STIs	Sexually transmitted infections
ST	Sequence Type
TAE	Tris-Acetate EDTA
T4SS	Type IV secretory element
TSB	Tryptic soy broth
UK	United Kingdom
USA	United States of America
VNTR	Variable-Number Tandem Repeat
WGS	Whole genome sequencing
wgMLST	Whole genome Multilocus Sequence typing
2MRS	2 Military Reception Station

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CHAPTER ONE

1.0. INTRODUCTION

1.1. Background

Sexually transmitted infections (STIs) are a major public health concern globally as they lower the quality of life of affected individuals. Globally, an estimated 376.4 million new cases of curable STIs are recorded yearly, of which gonorrhoea contribute about 23% (87 million) (Rowley *et al*., 2019). Gonorrhoea is an STI caused by the obligate human pathogen *Neisseria. gonorrhoeae*. The disease is among the most reported infectious diseases and remains a global health issue with high morbidity that translates into low productivity and economic losses (Rowley *et al*., 2019; Wi *et al*., 2017). Transmission is highest among sexually active age range of 15-49 years (Rowley *et al*., 2019) and skewed towards individuals with unusual sexual behaviours such as bisexuals, gays and sex workers, as well as ethnic and racial minorities (Newman *et al*., 2015). Urethral gonococci infections lead to early symptoms and could be easily detected and treated. In contrast, cervical, rectal and pharyngeal infections usually present with no symptoms and might require laboratory screening for detection (Grad *et al*., 2014). Women have higher risk of infection since the likelihood of penile-to-vaginal transmission has been estimated at approximately 50% per sexual act as opposed to the 20% chance in vaginal-to-penile transmission (Kirkcaldy *et al*., 2019). Although gonorrhoea is usually not fatal, the asymptomatic nature of the disease in females usually leads to severe scaring of several reproductive tissues and could result in complications such as pelvic inflammatory disease, ectopic pregnancy and infertility (Jabeen *et al*., 2016; Tsevat *et al*., 2017). Maternal infections have been associated with conditions such as premature rupture of membranes, preterm birth, Intra-amniotic infection, spontaneous abortions and low birthweight (Donders *et al*., 1993). Infants born to infected mothers are at risk of contracting neonatal conjunctivitis which can result in blindness if not treated (Wi *et al*., 2017b). It also increases

the risk of acquiring other sexually transmitted infections such as HIV (Workowski *et al*., 2008).

Antibiotics treatment of gonorrhoea dates back to the mid-1930s but the pathogen has developed resistance rendering most of these antimicrobials ineffective. Indeed, the emergence of antibiotic resistant *N. gonorrhoeae* limits the treatment options available for the disease with antibiotics such as sulphonamides, penicillins, tetracyclines, macrolides, fluoroquinolones, and early generation cephalosporins (Unemo, 2015; Wi *et al*., 2017b). After the pathogen became resistant to ciprofloxacin in the 80s, extended spectrum cephalosporins (ESCs) became the only effective antimicrobials for treatment of the infection (Unemo & Shafer, 2014; Unemo, *et al*., 2016). Nonetheless, by the early 2000s, resistance to cefixime emerged in Asia, with the first being reported in Japan (Unemo & Nicholas, 2012; Unemo & Shafer, 2014b). By the mid-2000s, widespread ESC resistance was confirmed in many European countries, North-America and Southern Africa (Deguchi *et al*., 2003; Lewis *et al*., 2013; Ohnishi *et al*., 2011a; Unemo, del Rio, *et al*., 2016). The future of gonorrhoea treatment has become very blurry at this moment, leading the WHO to predict that the disease could become untreatable in the near future. The global widespread nature of cefixime resistance led to the CDC discouraging its use for routine treatment in 2012 (Maldonado & Takhar, 2013). Consequently, ceftriaxone monotherapy became the only empirical first line antimicrobial treatment option, however, a quick decline in its susceptibility was observed globally few years later (Cole *et al*., 2017; Unemo, *et al*., 2016). The rising fears of the potential unavailability of antimicrobial therapy for gonorrhoea led to the introduction of ceftriaxone-azithromycin dual therapy. Although this was rapidly adopted in Europe and the USA, treatment failure to the dual therapy was reported in England in 2018 (Eyre *et al*., 2018). The current situation makes gonorrhoea treatment a major public health problem that needs urgent attention. To address the problem, global concerted efforts including regional and international response plans were initiated in 2012

(Lee *et al*., 2019a; Unemo *et al*., 2019). The WHO spearheaded this intervention with the Global Gonococcal Antimicrobial Surveillance Program (GASP) which was intended to monitor global gonococcal AMR trends and provide effective treatment guidelines and gonococcus public health policy (Omolo *et al*., 2017; Unemo *et al*., 2019; Wi *et al*., 2017a). The effectiveness of these efforts is however, contingent on accurate and timely assessment of the AMR landscape at the various national/regional levels.

Although, there have been good strides made in the surveillance of the emergence and spread of AMR in the West, the situation is far from being controlled in Africa (Wi *et al*., 2017a). Currently, only 7 African countries are active contributory members to the GASP program (Unemo *et al*., 2019). On the local level, countries like Ivory Coast, Uganda, Kenya, Zimbabwe and South Africa (Latif *et al*., 2018; Omolo *et al*., 2017; Workneh *et al*., 2020; Yeo *et al*., 2019) have made strides in establishing surveillance programs based on WHO recommendations recently. However, not much success has been achieved in bringing all the regional programs under one umbrella. Although, information for effecting public health policy on the treatment options in Africa has been scarce, there was enough to declare quinolones ineffective in the African region by 2013, due to high level resistance (Ndowa *et al*., 2013). Resistance to ESC, as well as low sensitivity to gentamycin and azithromycin has also been reported in South Africa (Lewis 2012). With South Africa having the best surveillance system in Africa, it not surprising to have more visibility about their situation. The main problem, however, lies in the many unknowns in the other parts of the continent due to lack of information.

Ghana has no national surveillance program in place, rendering the country's efforts in controlling gonococcal AMR emergence and spread very ineffective and hopeless. Generally, information about gonorrhoea resistance patterns in Ghana has been limited. Newman and colleagues reported that about 12% of *N. gonorrhoeae* isolates collected in 2006 in Ghana were multidrug resistant (MDR) (Newman *et al*., 2011). Following this work, it was after a decade

that Duplessis and colleagues gave a more comprehensive account of gonococci drug resistance profiles in Ghana. In their work, they reported isolates resistant to ciprofloxacin, penicillin, and tetracycline but sensitive to ceftriaxone and cefixime which were the first line treatment options at that time (Duplessis *et al.*, 2013). In the most recent of the reported works, Attram and colleagues identified one isolate with reduced susceptibility to cefixime which raised the concern for the possible emergence of untreatable strains in Ghana (Attram *et al.*, 2019). The emerging trend highlights the need for constant genomic surveillance in order monitor the emergence and possible spread of resistant gonococci strains. With microbial genomics being the hope of future exploits into solving problems like AMR, there is the need to look in this direction. The genome-wide approach is able to bring to bear the full complement of all genomic element responsible for resistance and virulence as well as aid in effective characterization of strains. Currently, genomic information about *N. gonorrhoeae* strains from the African region is very limited with genomic data available only for a few isolates from Kenya (Cehovin *et al.*, 2018). The current situation highlights the need for extensive work from the genomic point of view.

1.2. Rationale

The fight against the emergence and spread of AMR is a global problem that can only be overcome through constant surveillance and timely interventions. With the advent of globalization and international travel, any form of negligence poses not only a local, but a global public health threat. With the lack of proper stewardship and implementation of regulations, antibiotic use in Ghana is very random and uncontrolled, with the consequence of resistance very glaring. The few studies which tried to give an account of the state of *N. gonorrhoeae* antibiotic resistance in Ghana relied on conventional phenotypic/gene-based AMR characterization, which is limited in giving a detailed resolution about pathogen evolution and AMR. In recent times, genome wide study of pathogens has revolutionized

infectious disease research. Next Generation Sequencing (NGS) has been applied in gonococcal lineage identification (Cehovin *et al*., 2018), identification of AMR associated genes (Zhao et al., 2019), transmission of AMR (Kwong *et al*., 2018) and prediction of resistance (Golparia *et al*., 2018). Currently, there is limited genomic data, including details on AMR gene content, virulence factors and lineages of circulating *N. gonorrhoeae* strains in Ghana. In this present study, *N. gonorrhoeae* isolates were collected for almost a decade, thus, providing the opportunity to investigate the dynamics in the trends of AMR and the evolution of these strains. Data from this study regarding AMR gene identification, AMR prediction, and lineage identification as well as information on some virulence factors could be used to shape therapeutic guidelines by matching genomic data to phenotypic resistance data. Such data which can inform public health policy is needed to help curb the menace of the disease.

1.3. Aim

To perform whole genome characterization of *N. gonorrhoeae* and identify lineages of circulating strains, their antimicrobial resistance and some virulence determinants

1.4. Specific objectives

1. To determine the antimicrobial resistance (AMR) trends in *N. gonorrhoeae* isolates collected at two different time points
2. To perform genome characterization and annotation of markers of AMR in *N. gonorrhoeae* isolates
3. To detect plasmids, their associated AMR and identify some virulence determinants in *N. gonorrhoeae* isolates

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. *Neisseria gonorrhoeae*, the Bacterium

Neisseria gonorrhoeae (*N. gonorrhoeae*) is a gram-negative bacterium that causes the genitourinary infection gonorrhoea as well as other gonococcal infections. It is observed under the microscope as gram negative diplococci and was first isolated in 1879 by Albert Neisser (Ligon, 2005). *N. gonorrhoeae* is catalase and oxidase positive, that is, it can convert hydrogen peroxide to oxygen and possesses cytochrome C oxidase respectively. Recommended growth media for *N. gonorrhoeae* include selective Modified Thayer Martin media, Martin Lewis agar and New York City agar as well as non- selective chocolate agar (Bennett *et al*., 2007). The ability of *N. gonorrhoeae* to reduce nitrites, use only glucose to produce acid and grow on selective media distinguishes it from other *Neisseria species* (Bennett *et al*., 2007).The organism bears on its surface pili, proteins and lipooligosaccharides which have a host of functions such as facilitation of movement, adherence to the host, exchange of genetic material and eliciting immune responses from the host (Cehovin & Lewis, 2017; Patel, 2005).

2.1.1. *Neisseria gonorrhoeae* Genome Characteristics

N. gonorrhoeae has a single circular genome which is approximately 2.2Mb in size (Lu *et al*., 2019; Unemo, Golparian, *et al*., 2016). The genome contains approximately 2069 genes which code for about 2002 proteins. There are 67 structural RNAs present (Unemo, Golparian, *et al*., 2016). The organism may carry plasmids, most of which are responsible for antibiotic resistance or for conjugation. Some detected plasmids include pCryptic plasmid, 4.2kb; *pBlaTEM*, β-lactamase-producing plasmid, 7.5kb and pConjugative plasmid, 42kb (Nakayama *et al*., 2012a; Unemo & Shafer, 2014b). Some strains contain a 57kb DNA sequence which is known as the gonococcal genomic island (GGI) (Cehovin & Lewis, 2017; Harrison *et al*., 2016). The GGI codes for a type IV secretory element (T4SS) which is a multiprotein complex

made up of effector and conjugation systems that translocate DNA or proteins (Cehovin & Lewis, 2017; Snyder *et al*., 2005).

2.1.2. Epidemiology of *N. gonorrhoeae*

Gonorrhoea is one of the most common Sexually Transmitted Infections (STIs) across the globe with an estimated 78 million new cases in the year 2012 alone (Yin *et al*., 2018). Countries with proper surveillance systems in place report increase in incidence of gonorrhoea, for example in the United Kingdom (UK), there was an 11% increase between 2014 and 2015. It was the second most reported notifiable infectious disease in USA in 2015 (Alirol, Wi, Bala, Bazzo, Chen, Deal, *et al*., 2017). In France also, cases of gonorrhoea among men who have sex with men (MSMs) doubled between 2013 and 2015 and in the United States of America (USA), there was a 5% increase in the same time period. Almost all states in Australia recorded close to a 30% increment in number of cases between 2010 and 2014 (Costa-Lourenço *et al*., 2017). Declining condom use rates, urbanization, poor detection and inadequate treatment or treatment failure are responsible for this increase in number of gonorrhoea cases (Kularatne *et al*., 2018; Rowley *et al*., 2019). The global incidence of the infection recorded in 2016 was 26 per 1000 men and 20 per 1000 women. High, middle and low-income countries are all affected by gonorrhoea although the WHO African Region and the Americas have the highest infection rates worldwide (50 new infections per 1000 women and 100 new infections per 1000 men annually). Globally, there has been an increase in case rates in recent times. In some recent updates, increased case rates of 75.2% from 2009 to 2017 in the USA, 33.5 per 100 000 in 2010 to 55.4 per 100 000 in 2015 in Canada and 65.5 per 100 000 in 2013 to 118.0 per 100 000 in 2017 in Australia have been reported (Kirkcaldy *et al*., 2019).

2.1.3. Symptoms of Gonorrhoea

Although urogenital gonorrhoea may go unnoticed among 40% of men and more than 50% of women, it usually manifests as urethritis. When left untreated, urethritis may lead to

epididymitis, decreases fertility and urethral stricture (Kirkcaldy *et al*., 2019; Wi *et al*., 2017a). Symptoms are usually non-specific in females when the infection is present and may include abdominal pain, abnormal vaginal discharge, dysuria and dyspareunia (Kirkcaldy *et al*., 2019; Yeo *et al*., 2019). The lack of identifiable symptoms often leads to the infection going unnoticed and untreated, which may in turn lead to serious complications. For example, about 10% of female patients develop Pelvic Inflammatory Disease (PID) and as a result, are at a higher risk of infertility (Alirol, Wi, Bala, Bazzo, Chen, Dillon, *et al*., 2017; Tsevat *et al*., 2017). There are also a host of pregnancy complications associated with untreated gonorrhoea such as premature births, premature rupture of membranes, ectopic pregnancies and spontaneous miscarriages (Unemo, 2015). Perinatal transmission happens in about 40% of gonorrhoea cases usually in low and middle-income countries (LMICs). Mothers with gonorrhoea infections can pass it on to their infants at birth, resulting in neonatal conjunctivitis. If left untreated, this may lead to scarring and blindness (Wi *et al*., 2017a). In both sexes, extra genital infections commonly occur. Rectal and pharyngeal infections are among the most prevalent extra genital infections. Although mostly asymptomatic, rectal infections may manifest in anal discharge and pain, while pharyngeal infections may manifest as mild sore throat and pharyngitis (Tsevat *et al*., 2017). The pharynx has been found to be a suitable site for antibiotic resistance to emerge since it is a suitable site for commensal *Neisseria spp*. to confer resistance to *N. gonorrhoeae* (Deguchi *et al*., 2012). Scattered infections of gonococcal arthritis also occur. Due to their asymptomatic nature however, extra genital infections are rarely treated though they play a key role in gonorrhoea transmission. Gonorrhoea coinfections commonly exist with other major STIs like HIV, Chlamydia trachomatis, Herpes Simplex Virus and Mycoplasma genitalium (Callander *et al*., 2018). These often provide a collaborative effect on the transmission of the disease and its severity.

2.1.4. Transmission

Though it is a well-established paradigm that *N. gonorrhoeae* attaches to sperm aiding its easy transmission from men to women, the efficiency of transmission from women to men has been a bit elusive (Isabella & Clark, 2011; Lewis *et al*., 2015; Quillin & Seifert, 2018; Serruto *et al*., 2003). After transmission, the organism establishes contact with the mucosal epithelium of the host to replicate and eventually transmits to new host. Virulence factors such as Adhesion and Penetration Protein (App), pili, LOS sialylation (*lst*), LOS synthesis (IgtA-H), Protein 1 (*porB)* play major roles in adhesion to establish an infection (Cehovin & Lewis, 2017). The main virulent factors of *N. gonorrhoeae* is the Pili and App and *porB* (Virji, 2009). This pili a hair-like appendage involved in DNA transformation, twitching motility, adherence to epithelial cells, and protection from polymorphonuclear leukocytes killing (Cehovin & Lewis, 2017; Virji, 2009). The Type IV pilus is primarily composed of repeating units of the pilin protein *pilE* encoded by chromosomal locus *pil* (Cehovin *et al*., 2017). During the initial process of infection, it adheres to the host epithelial cells (Bergstrom *et al*., 1986; Klee *et al*., 2000; Lambden *et al*., 1979).

2.1.5. Pathogenesis and Virulence

Pathogenicity is a complex multi-factorial process which is controlled by specific genomic regions of the pathogen including virulence and resistance determinants (Wilson *et al*., 2002) . *N. gonorrhoeae* is a known obligate human pathogen, highly adapted to evading and modulating both the innate and adaptive immune systems to benefit its replication and survival (Hill & Davies, 2009). Hence, as it progresses through various stages of pathogenesis, it expresses a repertoire of virulent factors that promote its survival and replication inside the host. When *N. gonorrhoeae* enters the host, it colonizes the mucosa membrane of the reproductive tracts by expressing a repertoire of virulent factors such as the Pilin and a major porin, Protein 1 (P1) encoded by a single locus, *porB*. The *porB* is essential for iron and nutrient

uptake, regulating apoptosis pathways and targets host mitochondria to promote infections (Deo *et al*., 2018). The antigenic expression of the other membrane protein *porB* within a strain is stable. However, in *N. gonorrhoeae* strains, *por B* alleles occurs in one of two allelic forms, PI(A) or PI(B), based on immunological and structural similarities. The organism expresses either one or other of these porin homologous groups but never both, and the antigenic reactions of these highly diverse. Strains that express the outer membrane PI(A) tend to be associated with disseminated disease, whereas PI(B) expressing isolates typically cause localized urogenital infections (Deo *et al*., 2018; Ducey *et al*., 2005; Isabella & Clark, 2011). The inherent ability/mechanisms through which gonococci evade host defenses and adverse environmental conditions include natural competence, efficient transformation, variable surface structures and the propensity for horizontal gene transfer which has culminated in rapid developing resistance to every major class of antibiotics used in gonococci treatment (Quillin & Seifert, 2018). Some strains of *N. gonorrhoeae* harbour the gonococcal genetic island (GGI) that codes for a type IV secretory element (T4SS) which is a multi-protein complex made up of effector and conjugation systems that translocate DNA or proteins (Ramsey *et al*., 2011) . The GGI has been shown to increase the rate of recombination which tends to influence the resistance to several antibiotics (Harrison *et al*, 2016).

2.2. *N. gonorrhoeae* Typing

Typing methods are aimed at grouping organisms based on certain unique features that aids in easy characterization. Molecular typing is a way of identifying and categorising specific strains of microorganisms by investigating their genetic material. Over the years, these methods have been crucial in STI cases playing a valuable role in the biological confirmation of sexual contacts in epidemiological surveillance studies (Tapsall, 2006). Additionally, molecular typing can be used as an infection control tool within a healthcare institution to detect whether infections are related, especially during outbreak investigations (Barry & Klausner, 2009). The

understanding of these mechanisms can be used to design targeted therapeutics and help inform public health interventions strategies to control transmission and the spread of circulating strains.

N. gonorrhoeae strains have until recently been distinguished by auxotyping, serotyping, plasmid profiling and many other phenotypic typing methods (Abrams & Trees, 2017) . Consequently, the reproducibility and their discriminatory powers have always been a great concern and a need for more improved typing methods (Abrams & Trees, 2017). For instance, it can be technically challenging to perform phage and bacteriocin typing, also, Serotyping is not available for all bacterial species and can be labour intensive and very costly depending on the number of isolates (Hill, Masters & Wachter, 2016). Moreover, phenotypic markers when expressed under certain environmental or culture conditions are always not stable. This makes it a daunting typing method to employ for the characterization of various *N. gonorrhoeae* strains ("*Neisseria gonorrhoea* genome statistics" Broad Institute, Retrieved: 30th January 2020)

Molecular typing methods developed to categorise the causative organisms of gonorrhoea includes sequencing of overlapping *por* gene fragments, Whole Genome Sequencing, DNA fingerprinting, Restriction Fragment Length Polymorphism of rRNA genes, Restriction Endonuclease Analysis, Pulse-Field Gel Electrophoresis, Multi-Antigen Sequence Typing (NG-MAST) and Multi-Locus Sequence Typing (MLST) (OLSEN *et al*., 2008). Amongst these, the broadly used sequence types are Multi-Antigen Sequence Typing (NG-MAST) and Multi-Locus Sequence Typing (MLST) (Graham *et al*., 2017; Viscidi & Demma, 2003). These two methods have played important roles in investigating the mechanisms of gonococcal infections and antimicrobial-resistant gonococcal strains. Additionally, ribotyping, Arbitrarily Primed PCR, Amplified Fragment length polymorphism (AFLP), *Opa*-typing, are the other

molecular genetic methods developed for the characterization of gonococcal strains. (Abrams & Trees, 2017b)

2.2.1. Non-DNA-Based Typing Methods

These methods have been widely used to characterise *N. gonorrhoeae* for many years. Non-DNA based methods employ the use of phenotypic characteristics such as antimicrobial susceptibility profile, serovar determination and auxotyping. However, due to inherent insensitivity of these methods to discriminate isolates correctly, conclusions and inferences about strain types and distribution acquired cannot be confidently reproduced under different environmental conditions (Ilina E. *et al*., 2010).

2.2.1.1. Auxotyping

Auxotyping and Serovar determination were combined to determine the A/S classes to type gonococci isolates. Auxotyping classifies isolates by profiling the different nutritional requirements. Typically, the bacterial requirements for amino acids, vitamins, pyrimidines and purines are profiled. Although this method showed relatively higher discriminatory power at the time of its invention, the laborious and time-consuming nature of the process prompted the search for better options.

2.2.1.2. Serovar Typing

The outer membrane Porin (PorB, encoded by *porB* gene) protein of *N. gonorrhoeae* can be used as a typing and diagnostic method to identify varying strains. This is possible due an antigenic heterogeneity of these outer membrane proteins. The principle is based on the agglutination as a result of interactions between gonococcal antigens in the outer membrane and panels of specific monoclonal antibodies (MAbs) (Fudyk *et al*., 1999). Two major schemes have been developed. These are the Genetic Systems (GS) and the Pharmacia (Ph) panels. However, the widely used MAbs of the GS panel are no longer available. While the Ph panel

of MAbs is still commercially available. The serovar typing methods have been extensively used as controls to evaluate the new genotyping method used to differentiate *N. gonorrhoeae* isolates (Unemo *et al*., 2014). Moreover, the use of the serovar typing method provides a higher discriminatory ability than auxotyping. It is fast, easy to perform, and relatively cost-effective. It does not require sophisticated equipment and provides information on the antigenicity of expressed *porB* (Unemo & Dillon, 2011). However, the major drawback to this method includes less discriminatory power compared to DNA based methods. Also, subjective interpretation makes it difficult to reproduce results, coupled with low specificity of some MAbs, resulted in non-serotypeable strains. Serovars variants due to the evolution of the *porB* gene makes this method not as reliable as it used to be.

2.2.2. DNA-Based Typing Methods

The DNA-based typing methods involves the use DNA sequence data to characterize plasmids, determine nucleotide or amino acid polymorphisms in a single locus or multiple loci using several methods and more recently and whole genome sequencing (WGS). These methods can be broadly divided into two groups: gel electrophoresis (gel-based DNA-based typing methods) and DNA sequence analysis (DNA sequence-based typing methods). These methods are better for the discrimination of strains and have since become increasingly more cost-effective and reproducible (Martin *et al*., 2004).

Gel-based DNA-based typing methods involve analysis of DNA bands using techniques like Restriction Fragment Length Polymorphism (RFLP) resolved using pulsed-field gel electrophoresis (PFGE), *Opa* typing and Ribotyping. Using DNA sequence-based typing methods specific typing schemes which include full- or extended-length *porB* sequence analysis, *N. gonorrhoeae* Multi-antigen Sequence Typing (NG-MAST), and Multi-locus Sequence Typing (MLST) (Town *et al*., 2018) for gonococci have been developed.

2.2.3. Multi-locus Sequence Typing

The process of characterising multiple loci of an organism is known as Multi-locus sequence typing (MLST). MLST categorises isolates using internal fragments of multiple (usually 7) housekeeping genes sequences. The analysis of *N. gonorrhoeae* isolates provides the necessary typing scheme for differentiating various isolates based on the following seven housekeeping genes: *abcZ, adk, fumC, gdh, glnA, gnd*, and *pyrD*. The procedure involves PCR amplification of targeted genes followed by DNA sequencing. Variations in a set of housekeeping genes are used to characterise and differentiate between strains by their unique allelic profiles(Abrams & Trees, 2017b; Shimuta *et al*., 2013).

The selection of these genes is based on the fact that they are relatively conserved, evolutionarily more neutral, slowly evolving, , and are relatively evenly distributed throughout the genome (Donà *et al*., 2017; Unemo *et al*., 2014). Every unique sequence present within a bacterium strain is assigned as distinct allele and, for each strain, the alleles at each of the loci define the allelic profile or sequence type (ST) (O'Rourke & Stevens, 1993) Hence, different sequences for each locus are assigned divergent allele numbers, and the combination of alleles at the seven loci defines an allelic profile.

2.2.4. *N. gonorrhoeae* Multi-Antigen Locus Typing (NG-MAST)

Neisseria gonorrhoeae strains can also be typed molecularly via multi-antigen sequence typing (NG-MAST) system. NG-MAST is the most widely used tool in molecular epidemiological surveillance of gonorrhoea (Buono *et al*., 2012). The method explores the different internal fragments of two highly polymorphic loci of *N. gonorrhoeae*: *porB* (490 bp) and *tbpB* (390 bp). The genes encode the β-subunit of the transferrin binding protein (Martin *et al*., 2004)). NG-MAST can be accessed at the public database (http://www.ng-mast.net) for the assignment of discrete allele numbers and sequence types (STs).

NG-MAST has been vital in defining gonococcal populations and clusters of infection identification and particular strains for investigating treatment failures and in medico-legal cases (Buono *et al*., 2012; Martin *et al*., 2004). NG-MAST has also been as a tool for predicting specific antimicrobial resistance phenotypes in *N. gonorrhoeae* isolates (Martin *et al*., 2004).

2.2.5. PCR-Based Typing Methods

PCR-based typing methods of *N. gonorrhoeae* include Multi-locus Variable-Number Tandem Repeat (VNTR) analysis (MLVA), Amplified Fragment Length Polymorphism (AFLP), Whole-Cell Repetitive Element Sequence-Based PCR (rep-PCR) analysis, arbitrarily primed PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD) typing and Amplified rRNA gene restriction analysis (ARDRA)- a variant of ribotyping (Bennett *et al*., 2012). These methods categorise various species depending on variations in the genome of the organism (Unemo & Dillon, 2011). PCR amplification methods like Amplified RNA gene restriction analysis explores the use of a ribosomal gene fragments such as parts of the 16S rRNA gene, part of the 23S rRNA gene and parts the 16S-23S rRNA spacer region followed by restriction enzyme digestion and subsequent gel electrophoresis analysis (Demczuk *et al*., 2016; Heymans *et al*., 2012).

2.2.5.1. *Opa*-Typing

Gonococci strains possesses a family of 11 distinct and highly variable *opa* genes, making opa genes ideal for typing purposes. The extensive variation and rapid evolving nature of the *opa* gene makes it the ideal marker to exploit for short-term transmission of gonorrhoea (Khaki *et al*., 2009). To conduct *opa* typing, the 11 *opa* genes primer-amplified by the polymerase chain reaction, followed by restriction enzymes digestion, and fragment separation based on mass-to-charge on polyacrylamide to provide an *opa*-type. Opa typing is highly discriminatory as the *opa*-types of gonococci isolated worldwide for some decades now have been unique (Unemo & Dillon, 2011).

2.2.5.2. Ribotyping

Ribotyping uses Southern blot to detect polymorphisms that are present in the ribosomal RNA regions. This typing method is based on RFLP analysis of rRNA genes. The method has appeared to show good reproducibility, stability, and type-ability for characterization of gonococcal isolates (Khaki *et al*., 2009). Gonococci chromosomal DNA is subjected to the action of restriction enzymes, resulting` in widely segregated ribosomal genes, followed by identification of restriction fragments by hybridization to a specific rRNA probe (Khaki *et al*., 2009). However, because of the low discriminatory ability, ribotyping has little applicability for *N. gonorrhoeae* strains (Khaki *et al*., 2009; Sethi *et al*., 2013).

2.3. Evolution of Sequencing Technologies

The development of the chain termination method for determining the sequence of DNA fragments was a major breakthrough in biology (Sanger *et al*., 1977), making possible analysis of genetic variability. Two decades after, its discovery, the method was employed to sequence the reference genomes of two bacteria pathogens (Maniloff, 1996). The introduction of next generation sequencing (NGS), pioneered by the whole genome shot-gun approach opened new dimensions to the through-put and ease of data generation (Weber & Myers, 1997). With emergence of new technologies, the main limitations of short read sequencing, which is the lack of genome contiguity due to the its inability to resolve repetitive positions in the genome led to development of long read approaches like the nanopore sequencing by Oxford Nanopore Technology Inc. (ONT) and Single-molecule real-time sequencing (SMRT) by Pacific Biosciences (PacBio) which have complemented the highly accurate short read approaches (Clarke *et al*., 2009; Eid *et al*., 2009). Like research into most pathogens, *Neisseria* research has benefited from genomics as the first *Neisseria* genomes, which were *Neisseria meningitidis* were published in 2000 (Parkhill *et al*., 2000; Tettelin *et al*., 2000). Three years

later, the first *N. gonorrhoeae* FA1090 (http://www.genome.ou.edu/gono.html, NC 0 02946) was published.

2.4. Molecular Epidemiology in the era of Whole Genome Sequencing

Classification of microbial isolates is mainly aimed at providing answers in the areas of diagnosis: investigating transmissions within/between populations; outbreak detection and monitoring to track local or regional spread; evolutionary analysis to determine origins of characteristic strains and assessing vaccine therapeutic potential (Jolley *et al*., 2012). To achieve each of these different levels of characterization requires a different level of typing resolution. In the pre-WGS era, conventional DNA typing methods were used to characterize bacterial. The most frequently used approach is the multi-locus sequence typing (MLST) method which is based on 7 house-keeping genes. MLST has proven to be extremely useful as it has been used in different ways like analysing between species evolutionary changes over time (Pannekoek *et al*., 2008) and assessing intra-taxa variability (Dean *et al*., 2009). Multi-locus sequence typing which is based on core genes addresses some of the problems of phylogenetics which include the effects of lateral gene flow and the constant recombination that occurs in many bacterial species (Jolley & Maiden, 2014). With continual decrease in the cost of WGS, there is an imminent prospect of incorporating it into real-time and routine bacterial epidemiological studies. Whole genome sequencing, thus, provides a benchmark for the characterization of microbes to resolutions that answers our specific questions, however, the task herein, lies in efficiently exploiting the large amounts of data generated to meet these purposes. With the advent of WGS, bacterial epidemiological characterization has been extended to the whole genome level.

2.5. Bacterial Whole Genome Typing Methods

In the advent of microbial genomics, the idea of MLST has been extended to the genomics level. Approaches like whole genome MLST (wgMLST); core genome MLST (cgMLST);

coreSNP typing and ribosomal MLST (rMLST) have proven to show more discriminatory power and resolution than conventional approaches and as such, are very useful for epidemiological investigations (Gona *et al*., 2020; Henri *et al*., 2017). The core genome consists of a set of homologous genes that are present in all the species of an organism while the pan genome comprise the core genome together with dispensable genes that may be present or absent in a particular strain and may provide a selective advantage under specific conditions (Wu *et al*., 2018). Core genome MLST is based on core genes while wgMLST encompasses both core and accessory genes. Also, the idea of cgMLST and wgMLST can be extended to the absence or presence of predicted proteins; multi-locus predicted proteins sequence typing (MLPPST), thus we can look at wgMLPPST and cgMLPPST (Leekitcharoenphon *et al*., 2014). The single nucleotide polymorphism (SNP) approach involves extracting SNPs from both genes and intergenic regions done by mapping of raw sequence reads to a well characterized reference (Henri *et al*., 2017). The ribosomal multi-locus sequence typing (rMLST) approach focuses on combing bacterial genealogy and typing through the indexing of variations harboured in the bacterial ribosome protein subunits (*rps* genes) encoded by 53 genes (Jolley *et al*., 2012). Just like the SNP typing, rMLST relies on a curated reference. Ribosomal multi-locus typing schemes are seen to be ideal for developing a universal characterization scheme because the *rps* genes are functionally conserved; present in all bacteria; well distributed across the chromosome and encode proteins which are stable even under selection pressure (Jolley *et al*., 2012).

2.6. Whole Genome Sequencing Data Analysis

The demanding task of processing the large amounts of data generated from WGS projects has been met with numerous approaches that try extract data from the reconstructed genomes without alterations due to computational deficiencies. Typically, whole genome reconstruction has been achieved using two main methods: mapping of genomic fragments to a reference

sequence or performing *de novo* assembly of the short reads into longer contiguous sequences. The former approach however, because of the complete reliance on the reference sequence has many drawbacks when dealing with bacterial genomes. The problems that may arise when conducting reference-based assembly on bacterial genomes stem from the high recombination or the presence of many insertion sequences. Also, novel variation which are not present in the reference may remain undetected (Maiden *et al*., 2015). On the other hand, *de novo* assembly presents a more powerful approach that only relies on the date present in a particular sample. Most *de novo* assembly tools are based on the use of de Brujin graphing to effectively assembly short reads into contigs that contains the majority of the genome segments (Ronen *et al*., 2012). Once assembled, the reconstructed genome must be correctly annotated using known genes or genome databases that have catalogued thousands of genes, many of which have known and unknown functionalities. One of such databases is the Bacterial Isolate Genome Sequence Database (BIGSdb) platform (Jolley & Maiden, 2010).

2.7 The Bacterial Isolate Genome Sequence Database (BIGSdb)

The Bacterial Isolate Genome Sequence Database which is hosted on PubMLST.org (http://pubmlst.org/software/database/bigsdb/) is an open source, web-accessible database that combines data storage, retrieval, and analysis of linked phenotypic and genotypic information in an accessible, scalable and computationally efficient fashion. The BIGSDB database architecture was built on the already existing mlstdbNet (Jolley *et al*., 2004) software which was built to store and distribute MLST data. Aside from the identification of conventional loci, the software is able define and identify genetic variants and number of loci available in a query nucleotide sequence. Further indexing of the characterized loci into organised schemes enables efficient evolutionary and functional characterization of individual strains. Individual strains and loci can be further indexed to accommodate alternate schemes that enables accessible cross referencing of similar studies (Jolley & Maiden, 2010).

PubMLST.org/Neisseria hosted on the (PubMLST.org) website currently archives and annotates data from: 68,910 isolates; 30,978 genomes and 1,519,661 allele from *Neisseria spp.* An exciting feature of the database is the link between antibiotic resistance and antigen typing databases, enabling easy antigen typing and automated allele assignment. The database has been used by several studies to characterize the two pathogenic *Neisseria spp., N. gonorrhoeae and N. meningitidis* (Harrison *et al*., 2016; Jolley, Hill, *et al*., 2012; Le *et al*., 2020; Lewis *et al*., 2013; Maiden *et al*., 2015).

2.8 AMR databases

The genomics revolution is taking infectious disease research into a new dimension and just like any other pathogen, application of genomics in the study of *N. gonorrhoeae* will help refine our understanding about various pathogen adaptive mechanisms to emerging therapies and evolution. The volume of data generated by whole WGS projects present a computational challenge to Scientists because these large amounts of data must be processed to make information readily available. In order to address this issue, several databases have been developed to catalogue such information. Some of the AMR databases include: AMRFinderPlus(https://www.ncbi.nlm.nih.gov/pathogens/antimicrobialresistance/AMRFinder/) (Feldgarden *et al*., 2019), hosted by the National Center for Biotechnology Information (NCBI) to characterize genes associated with beta-lactam resistance; as well as ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) , which is maintained by the Center for Genomic Epidemiology, and identifies acquired genes and/ or mutations that mediate antibiotic resistance phenotype. Perhaps the most comprehensive of AMR databases today is the Comprehensive Antibiotic Resistance Database (CARD: https://card.mcmaster.ca) (Alcock *et al*., 2020), which is a peer-reviewed, curated catalogue of AMR determinants and their associated phenotypes. Data is organized using AMR gene detection models and antibiotics resistance ontology. Resistome prediction is based on SNP models and homology. At the time

of this write up, CARD contained 3103 AMR detection models; data from 88 pathogens, 9560 chromosomes, 21362 plasmids, 102181 WGS assemblies and 222011 alleles.

2.9. Application of WGS in *N. gonorrhoeae* Characterization

In the past decade, several studies have used genome sequencing approaches to investigate *N. gonorrhoeae* antimicrobial resistance in different parts of the world (Al Suwayyid *et al*., 2018; Cehovin *et al*., 2018; Golparian *et al*., 2020; Nicol *et al*., 2015; Peng *et al*., 2019). In 2016, the WHO published a list of well characterized reference strains for both phenotypic and genotypic AMR determinants (Unemo, Golparian, *et al*., 2016). The strains characterized included 8 previously existing strains and 6 novel strains. The 14 reference strains are comprehensively characterized for molecular AMR determinants, plasmids, sequence types, serovar; antibiograms and prolyliminopeptidase production. The result of this is a universal standard for quality control and reproducibility. In a recent study, WGS analysis of *N. gonorrhoeae* spanning a century has revealed the evolution of molecular AMR determinants (Golparian *et al*., 2020). This comprehensive analysis brought to bear the impact of antimicrobial use, and that *pen B* was the only AMR determinant that was in detectable frequency prior to the gonococci antimicrobial therapeutics. The study revealed that multidrug resistant (MDR) gonococci emerged between 1950s-1970s. Their genomic approach also revealed that AMR emerged in one MDR clade which has a 3-times higher genomic mutation rate, confirming the versatility of the clade in adapting to the ever-changing antimicrobials.

2.10. Mechanisms of Antimicrobial Resistance in *N. gonorrhoeae*

The natural competency of *N. gonorrhoeae* gives it an extraordinary capacity to alter its genetic material. Through transformation (transfer of part of or whole genes), the pathogen is able to efficiently adjust its genome through all types of mutations. *N. gonorrhoeae* employs the use of these mechanisms to promptly adapt to and thrive in the often-adverse conditions at different sites in the human host. The highly evolving nature of the pathogen has led to the development

and acquisition of nearly all known physiological mechanisms of AMR to all antimicrobials recommended and/or used for treatment. Some these mechanisms include: enzymatic antimicrobial modification or destruction; antimicrobial target modification or protection which reduces affinity and antimicrobial influx/efflux regulation (Ohnishi *et al*., 2011b; Tapsall, 2001; Unemo & Shafer, 2014a). Most *N. gonorrhoeae* genetic AMR determinants are chromosomally situated, however the *bla*TEM gene and the *tetM* gene, which are responsible for high-level resistance to penicillin and tetracycline, respectively, are known to be plasmid borne (Nakayama *et al*., 2012b; Unemo & Shafer, 2014a). Although, certain AMR determinants alone can result in high-level resistance for the antimicrobial, the presence of a single AMR determinant only confers a marginal increase in AMR that is of less clinical significance, thus, the cumulative effects of several AMR determinants and their complex interactions are essential in the development of clinically significant AMR levels (Tapsall, 2001, 2006)

In gonococci, chromosomal mediated resistance is through transformation. Commensal *Neisseria spp*. frequently inhabit the pharynx and are often exposed to antimicrobials. Continual uncontrolled exposure to antimicrobials may eventually lead development of resistance in commensal *Neisseria spp* and consequently acting as a reservoir of AMR genes, which can be horizontally transferred to gonococci through transformation (Unemo & Shafer, 2014; Sánchez-Busó *et al*., 2019a). Gonococci extra-chromosomal (Plasmid)-mediated AMR is through conjugation and has only been identified in penicillins and tetracyclines (Tapsall, 2011). A conjugative plasmid is therefore, essential to achieve the process.

2.10.1. Sulphonamide Resistance

Sulphonamides act by targeting the bacterial dihydropteroate synthase (DHPS) enzymes. The incapacitation of DHPS inhibits bacterial folic acid synthesis which has detrimental consequences. To salvage the situation, gonococci react by over-synthesizing *p*-aminobenzoic

acid, which produces a dilution effect on the antimicrobial agent. Also, alterations in the *folP* gene as a result point mutations or the presence of a mosaic gene containing DNA sequences from commensal *Neisseria spp* which encodes the drug target, DHPS results in sulphonamide resistance (Costa-Lourenço *et al*., 2017; Unemo & Shafer, 2014).

2.10.2. Penicillin Resistance

β-lactam antimicrobial agents work by inhibiting peptidoglycan cross-linking in the bacterial cell wall. The target of β-lactam antimicrobial agents are penicillin-binding proteins (PBPs) which are enzymes located in the cell envelope and are responsible for cell wall metabolism. Binding of the β-lactam ring to transpeptidase enzymes PBPs, results in the hydrolyses of the cyclic amide bond of β-lactamase susceptible penicillins, resulting in bactericidal activity (Chen *et al*., 2019; Nakayama *et al*., 2012b).

2.10.2.1. Chromosomally Mediated Penicillin Resistance.

Over the course time, there has been a stepwise accrual of chromosomal changes which have contributed significantly to the penicillin resistance (Tapsall, 2001). Chromosomally mediated penicillin resistance in gonococci arise from mutations that alter the penicillin binding proteins (PBPs). Alterations in PBP-1 and PBP-2 usually results in decreased affinity for penicillins (Tomberg *et al*., 2010). The most important target of β-lactam antimicrobials is the PBP-2 which is encoded by the *penA* locus (Ohnishi *et al*., 2011b). In penicillin-resistant gonococci, there are usually, 5 to 9 *penA* gene mutations which consequently leads to a decrease in the PBP-2 acylation rates that results in the phenotypic manifestation of penicillin resistance (about 6-8 fold decrease in susceptibility), (Powell *et al*., 2009). Acquisition of resistant *penA* mutations were as a result of transformation by *Neisseria spp* with reduced PBP2 acylation rates (Spratt *et al*., 1992). Traditionally, an aspartate insertion at positions 345 of PBP2 (Asp345a) is the most frequently observed mutation in penicillin-resistant gonococcus, alongside other mutations which lie in the carboxyl terminal (Unemo, Golparian, *et al*., 2016).

Although the Asp345a and the C-terminal mutations are close to the active site of PBP2, C-terminal mutations do not have an effect on the crystal structure of the protein, however, they affect the rates of acylation by penicillin significantly (Powell *et al*., 2009). With the unprecedented emergence of resistance in the past few decades, several *penA* genes with mosaic stricture have been described (De Silva *et al*., 2016; Igawa *et al*., 2018). Typically, they contain about 60-70 amino acid substitutions from the wild-type *pen A* gene and can confer resistance to penicillins and/or extended-spectrum cephalosporins (ESCs) (Unemo *et al*., 2012). Although, primarily, chromosomally mediated penicillin resistance mechanisms are as a result of a changes in PBP2, a single missense mutation in the *ponA* (ponA1 allele) is required for high level penicillin resistance (Ropp *et al*., 2002). In *ponA* the amino acid substitution L421P, is responsible for about 3-to-4 fold decrease in penicillin acylation in PBP1 (Ropp *et al*., 2002). Although the reversion *ponA1* to wild-type *ponA* produced a phenotype with a 2-to-4 fold decrease in penicillin MIC, the introduction of *ponA1* into a strain containing *penB, penA* and *mtrR* resistance markers had no effect on penicillin MIC (Ropp *et al*., 2002), suggesting the presence of an unknown resistance determinant or epistatic effect.

Another penicillin resistance determinant is the *mtrCDE* efflux pump system (*mtrR* resistance determinant). Mutations in the efflux pump system results in its overexpression which increases efflux of penicillin out of the cell (Barbour, 1981; Unemo & Nicholas, 2012). On the other hand, the *porB* resistance determinant mediated by mutations in the outer membrane porin channel regulates outer membrane permeability by reducing influx of penicillin and thereby enhancing resistance (Olesky *et al*., 2002; Unemo & Nicholas, 2012). Furthermore, alterations in the pore forming secretin *pilQ* type IV pili has been associated with penicillin resistance in laboratory strains, however, these strains must also harbour the *penA, penB* and *mtrR* resistance determinants (Unemo & Nicholas, 2012; Zhao *et al*., 2005). The existence of *pilQ* mutations

in clinical isolates are not likely to occur due to the fact that they affect essential processes responsible for pathogenesis by disrupting type IV pili formation (Zhao *et al*., 2005)

2.10.2.2. Plasmid Mediated Penicillin Resistance

Penicillinase producing gonococci (PPNG) has been associated with emergence and spread of high-level penicillin resistance in gonococci globally (Nakayama *et al*., 2012b). Traditionally, gonococci exhibiting plasmid-mediated penicillin resistance contain the plasmid harbouring the blaTEM-1 gene that encodes the TEM-1-type beta-lactamase enzyme (Muhammad *et al*., 2014; Nakayama *et al*., 2012b). This enzyme is responsible for the hydrolysis of the cyclic amide bond in the beta-lactam ring and eventually rendering the drug infective (Muhammad *et al*., 2014; Yan *et al*., 2019). The TEM-1 allele has evolved with a single nucleotide change at position 539, resulting in an M182T amino acid substitution leading to the TEM-135 allele type (Nakayama *et al*., 2012b; Yan *et al*., 2019). The β-lactamase producing plasmids in gonococci have further been classified based on epidemiological origin. The geographically characterized plasmids include: the Asian (7,426 bp); African (5,599 bp); Toronto/Rio (5,153 bp) (Brett, 1989; Gouby *et al*., 1986; Unemo & Shafer, 2014Unemo & Shafer, 2014). Other epidemiologically characterized plasmids types include the Johannesburg, New Zealand and the Nîmes plasmid types (Unemo & Shafer, 2014Unemo & Shafer, 2014; Muhammad *et al*., 2014). The Asian plasmid is the ancestral plasmid from which the other plasmids emerged either through insertions or deletions. Deletion derivatives include: the African, Toronto/ Rio, and Johannesburg plasmids whiles insertion derivatives New Zealand, and the Nîmes plasmids (Unemo & Shafer, 2014; Muhammad *et al*., 2014). The TEM-135 allele type has been of much interest because only a single amino acid substitution is required to produce an extended-spectrum β-lactamase (ESBL) gonococci which will render cephalosporins infective, leading to a drawback in efforts to combat gonococci AMR. Also, blaTEM-135 strains have been reported to exhibit the highest penicillinase activity that also translates to having higher MICs

(Yan *et al*., 2019). Furthermore have been reported to be carried by the Rio/Toronto and Asian plasmids predominantly (Muhammad *et al*., 2014; Yan *et al*., 2019)

2.10.3. Tetracycline Resistance

Tetracycline resistance is mediated by both chromosomal and plasmid-based determinants. Chromosomally mediated resistance markers associated with tetracycline resistance include the *rpsJ mtrR* and *penB* genes whiles the plasmid mediated-resistance in conferred by the presence *tetM* determinant carried by p*tetM* conjugative plasmid (Hu *et al*., 2005; Zheng *et al*., 2015).

2.10.3.1. Chromosomally Mediated Tetracycline Resistance (CMTR)

Chromosomally mediated tetracycline resistance has been associated with three main gene targets. Primarily the *tet-2* gene (*rpsJ*) which encodes the ribosomal protein is the main CMTR determinant (Hu *et al*., 2005). The occurrence of a Val-57-to-Met (V57M) amino acid substitution in the *rpsJ* is responsible for the resistance phenotype. Val 57 is positioned at the rRNA binding site for tetracycline, however, protein structural analysis revealed that the V57M substitution alters the structure of the rRNA leading to a reduced tetracycline affinity to the ribosome and hence the observation (Hu *et al*., 2005; Unemo & Shafer, 2014). The other two determinants which are the *mtrR* and *penB* genes contribute to tetracycline resistance by controlling intracellular antibiotic concentrations through efflux and influx mechanisms (Hu *et al*., 2005).

2.10.3.2. Plasmid Mediated Tetracycline Resistance

Plasmid mediated tetracycline resistance is conferred *tetM* gene carried by the 25.2 MDa conjugative plasmid. The *tetM* determinant mediates high-level tetracycline resistance (MICs > 16 mg/L) in gonococci (Morse *et al*., 1986). Two types of the conjugative plasmid have been described. These include the original 25.2 MDa and 24.5 MDa variant which subsequently

named the "American" and "Dutch" types respectively based on their epidemiological origins (Unemo & Shafer, 2014; Zheng *et al*., 2015). The mechanism by which *tetM* confers tetracycline resistance is by directly dislodging tetracycline from the ribosome to prevent the inhibition of bacterial protein synthesis (Dönhöfer *et al*., 2012; Unemo & Shafer, 2014b).

2.10.4. Quinolone Resistance

Fluoroquinolones act by inhibiting DNA replication by binding to DNA gyrase and consequently preventing the enzyme from forming DNA supercoils (Belland *et al*., 1994; Knapp *et al*., 1997). Quinolone resistance in *N. gonorrhoeae* have been associated with point mutations in the chromosomal *gyrA* and *parC* genes, which encode for DNA gyrase and topoisomerase IV, respectively (Kivata *et al*., 2019a; Knapp *et al*., 1997). Accumulation of mutations in the Quinolone Resistant Determining Region (QRDR) in *gyrA* and *parC* eventually leads to alterations in the three-dimensional structure of the protein and its associated functional consequences (Knapp *et al*., 1997). The changes in fluoroquinolones target protein structure results in low enzyme binding affinity and hence the development of resistance. The QRDR region is located between amino acids 56–140 and 55–110 in *parC* and *gyrA* respectively (Kivata *et al*., 2019b). In *gyrA*, the amino acid substitutions at S91F or Y, or D95N are associated with ciprofloxacin resistance. The synergistic effects of mutations in *parC* (S88P and E91K) further increases ciprofloxacin resistance in gonococci (Kivata *et al*., 2019a; Unemo & Shafer, 2014).

2.10.5. Macrolide Resistance

Macrolides act to prevent the translocation of the peptidyl-tRNA, thereby, preventing peptides from leaving the 50 ribosomal RNA units through interactions with the *23S rRNA* and consequently inhibiting protein synthesis (Douthwaite & Champney, 2001). Macrolide resistance may result from ribosomal target modification that may arise from mutations in *23S rRNA*, rRNA enzymatic associated modification of 23S rRNA and/or antimicrobial efflux

systems (Unemo & Shafer, 2014). The amino acid substitutions C2611T and A2059G in the *23S rRNA* have associated with erythromycin and azithromycin resistance in gonococci. Furthermore, the overexpression of the *MtrCDE* efflux pump and/or the *MacAB* efflux systems have also been identified to contribute macrolide resistance in gonococci (Chen *et al*., 2019; Martin *et al*., 2012)

2.10.6. Spectinomycin Resistance

Spectinomycin act by causing a bacteriostatic effect through the inhibition of protein synthesis by binding to the 30S ribosomal subunit of gonococci. The interaction of spectinomycin with 16S rRNA blocks the catalysis of the peptidyl-tRNA from the aminoacyl site to the peptidyl site leading the inhibition of protein translation (Allen *et al*., 2011; Unemo & Shafer, 2014). Spectinomycin resistance in gonococci have be associated with the C1192U mutation which occurs in the spectinomycin-binding region of the *16s rRNA* (Unemo *et al*., 2014). In recent times, K26E substitution and Val25 deletion in the *rpsE* gene have also been implicated in spectinomycin resistance (Unemo *et al*., 2013).

2.10.7. Extended Spectrum Cephalosporin (ESC) Resistance

Cephalosporins which are beta-lactam antimicrobials act by binding to penicillin binding proteins (PBPs) which eventually, leads to the inhibition of cross-linking in the peptidoglycan of the bacterial cell wall and subsequent bactericidal activity (Unemo & Shafer, 2014; Thakur *et al*., 2017). Mutations in the PBPs that modify cephalosporin targets as well as antibiotic efflux/influx systems are the known causes of cephalosporin resistance in gonococci (Unemo & Shafer, 2014). The *penA* gene which encodes the penicillin binding protein 2 (PBP2) is the main cephalosporin target. Cephalosporin resistant gonococci strains typically contains the mosaic *penA* a gene that harbours between 60 to 70 amino acid substitutions (Ohnishi *et al*., 2011a; Unemo *et al*., 2012). Strains with the mosaic *penA* allele lack the penA-Asp345a insertion which is typical of penicillin resistant strains (Unemo & Shafer, 2014). The mosaic

penA allele originated from DNA transformation and recombination with penA gene fragments that were derived from commensal *Neisseria species* which may include *Neisseria flavescens, Neisseria cinerea, Neisseria polysaccharea, Neisseria perflava* and *Neisseria sicca* (Unemo & Shafer, 2014; Unemo *et al*., 2012). The presence of several non-synonymous amino-acid substitutions (I312M, V316T A501, F504L, A510V, N512Y, A516G, G545S, P551S and P551L) (Liao *et al*., 2011; Thakur *et al*., 2014; Thakur *et al*., 2017; Unemo, Golparian, *et al*., 2016; Whiley *et al*., 2007) have been associated with ESC resistance. The mutations G545S, I312M, and V316T are located on the active site of beta-active site, thereby, having the potential to decrease acylation by the enzymes (Unemo & Shafer, 2014). Although, PBP2 amino acid substitutions is the primary factor underlying ESC resistance, specific mutations in *mtrR* and *porB* genes have also been identified to affect increased efflux and decreased influx of cephalosporins, thereby contributing to resistance (Lahra *et al*., 2018; S. D. Thakur *et al*., 2014).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study Design

The study had a retrospective cross-sectional design and was part of a larger project that enrolled participants with suspected Sexually Transmitted Infection (STI) from the period of 2012 to 2019. Archived *N. gonorrhoeae* isolates collected from this study was used in this work.

3.2. Study Sites and Description

N. gonorrhoeae isolates were collected from individuals presenting with STI symptoms enrolled at 5 healthcare facilities (37 Military Hospital-5.5886 N, 0.1841 W and Adabraka Polyclinic STI clinic-5.5616 N, 0.21307 W, Accra; Naval Health Center-4.93994 N,1.70496 W, Airforce Medical Center-4.9016 N, 1.7831 W and 2 Military Reception Station Army clinic-4.90807 N,1.79805 W all at Sekondi/Takoradi). These sites were selected based on their location; number of patients seen daily with STIs symptoms as well as the site population. All sites were located in southern Ghana, 2 from Greater Accra and 3 from Western region of Ghana.

The 37 Military Hospital is one of the largest hospitals in Ghana, located in the Cantonments area of the Accra metropolis. It was established in 1941 to provide care for troops during the World War II and is one of the major referral hospitals in Accra which sees a diversity of patient population, including both civilian and military personnel. It treats patients from all around Accra has a 500-bed capacity and sees 50-80 thousand patients annually. About 20 STI cases is estimated to present at 37 Military Hospital weekly.

The Adabraka Polyclinic is located in the West Ridge area of the Accra Metropolitan district in the Greater Accra Region. It houses a Ghana Health Service accredited Sexually Transmitted Infection (STI) Clinic that see patients from within and around the Metropolis, especially high-risk groups like commercial sex workers (CSW), homosexuals and sexually active youths. Numerous STI research has been conducted at the clinic, hence the staff have experience in such research. The clinic has been recorded to see approximately five to ten patients with STI symptoms weekly.

The 2 Garrison military clinics are located at the Western Region, in the cities of Sekondi/Takoradi. The Naval Health facilities is located at the Sekondi fishing harbour, the Airforce Medical Centre is located at the Airforce base by the major Takoradi transport yard and the 2 Military Reception Station (2MRS) (Army Clinic) is location at Barracks at Apremdo, in Takoradi. The recently established off shore oil fields accessed through Sekondi/Takoradi have led to the migration of different population, thereby potentially introducing new strains from all over the world.

3.3. Study Population and Recruitment

3.3.1. Recruitment of Study Participants

All individuals who sought healthcare at the afore-mentioned clinics, diagnosed with STI by a clinician and were introduced to the study were recruited. Symptoms included inflammation of the urethra (urethritis) or inflammation of the cervix (cervicitis), abnormal bleeding and discharge in both men and women. Written informed consent was obtained prior to enrolling each patient. Individuals who consented to participate in the study were asked to provide a urine sample and two (urethra or endo-cervical) swabs.

3.3.2. Inclusion Criteria:

a) Individuals presenting with symptoms of urethral/vaginal discharge, pain during urination, pain in genitals, dysuria and intermenstrual bleeding in women, as well as abdominal pain and vaginal pruritus.

b) Individuals aged ≥ 12 and willing to provide parental/guidance written permission as well as assent for sample collection and testing

d) Ability to complete the consent and /or assent form

3.3.3. Exclusion Criteria:

a) Individuals < 12 years of age

b) Individuals unwilling to provide consent or assent

c) Individuals presenting without an STI syndrome or suspicion of Gonorrhoea

3.3.4. Sample Size

56 *N. gonorrhoeae* isolates collected from consented individuals with STI symptoms between 2012 and 2015 and 2018-2019 were analysed.

3.4. Ethical Consideration

Ethical approval was obtained from the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB), the 37 Military Hospital Institutional Review Board, the Ghana Health Service Ethics Review Committee (GHS-ERC), the US Naval Medical Research Unit Number Three Institutional Review Board (NAMRU-3 IRB) and US Naval Medical Research Center Institutional Review Board (NMRC-IRB). All specimen analysed in this study were de-identified to protect the identity of the study participants.

3.5. Sample Collection and Transportation

3.5.1. Urethral Swabs

Specimen were collected by qualified health personnel, from volunteers who provided written informed consent or assent with parental permission. Mucopurulent discharge specimen was

collected for males using flocked urethral swabs with a mini tip without full insertion into the urethra. Two swab specimens, one at a time were collected from males. It was impossible to collect any specimen by inserting the swab into the male urethra, as study volunteers rejected this procedure out of fear of discomfort/pain.

3.5.2. Endocervical Swabs

With the use of a sterile speculum the vaginal canal and the cervix was opened and examined with the help of an examination lamp. After examination, two swabs, one at a time, were inserted and rotated against the cervical opening and wall of the endocervical canal several times for 20-30 seconds and withdrawn without touching the vaginal surface.

3.5.3. Specimen Transportation and Storage

The endocervical/urethral swabs were streaked on Modified Thayer-Martin (MTM) media and kept in a 5-10% CO_2 pouch (Becton Dickinson® gas pack EZ-CO_2). The incubated media in the CO_2 pouch were transported to NMIMR under ambient temperature within a maximum of six hours. At NMIMR, the plates were incubated at 37˚C. The endocervical/urethral swabs were kept in vials, and transported on ice to the NMIMR laboratory and stored at the lab at -20˚C. *N. gonorrhoeae* isolates obtained from study sites outside Accra were stored in 2ml cryovial containing tryptic soy broth (TSB) with 20% glycerol and stored at -70˚C and transported to the NMIMR laboratory with a mobile -70˚C freezer. At NMIMR all isolates were cryopreserved at -70˚C after confirmatory identification.

3.6. Laboratory Procedures

3.6.1. Microbial Culture

The endocervical/urethral swab was rolled firmly on MTM agar to make a large Z mark and using a sterile loop, streaks are made on the plate and incubated at 37°C. The plates were observed after 18-24 hours, 48 hours and finally 72 hours for growth. When pure growth was detected, single greyish translucent small colonies were picked and sub-cultured on a fresh

chocolate agar plate which was incubated in a 5-10% CO_2 atmosphere at 35-37°C for 18-24 hours.

3.6.2. Presumptive *N. gonorrhoeae* identification

Isolates obtained from sites outside Accra (Sekondi/Takoradi laboratories) were presumptively identified by Gram staining, catalase and oxidase tests.

3.6.2.1. Gram Stain

For Gram staining one or two pure colonies of the isolate were emulsified with a drop of saline (0.85% NaCl) on a dry alcohol cleaned microscope slide. The slide with the emulsified bacterial isolates was air dried, heat fixed and then stained. Gram staining was performed using the Becton Dickinson® Gram stain kit and reagents. The slide was allowed to cool before it was flooded with crystal violet solution (3.0g Crystal violet, 50ml Isopropanol, 50ml ethanol/methanol, in 900ml distilled water) for 1 minute and rinsed gently with running water. The slide was then flooded with Gram's iodine (mordant) solution (3.3g of Iodine crystals, 6.6g of Potassium Iodide in 1000ml distilled water) to form the crystal violet-iodine complex after which the iodine mordant was left to act for 1 minute and then washed with running water. The smear was decolorized in less than 20 seconds until no violet colour washed off, by carefully rinsing the slide with the Gram's decolourizer, which is made up of acetone/isopropanol (25:75) solution. This step was done with extreme caution, because the smear could easily be washed off. The slide was then flooded with the counterstain, Safranin (Safranin O powder, 4.0g, ethanol/methanol 200ml in 800ml distilled water) for 1minute and then gently rinsed with water and allowed to air dry in an upright position.

Quality control (QC) of the Gram stain kit was routinely performed with *Staphylococcus aureus* ATCC 25923 (Gram-positive cocci) and *Escherichia coli* ATCC 25922 (Gram-negative rods).

Microscopy was done using a light microscope with the oil immersion lens (x100). Typical *N. gonorrhoeae* colonies from lab cultures were seen as pink (taking the colour of the counter stain, Safranin) diplococci joined together by flattened sides.

3.6.2.2. Catalase Test

Catalase activity was tested using freshly grown isolates by smearing colonies on a sterile slide with a loop and placing a drop of superoxol (30% hydrogen peroxide) on the colonies. Presumptive *N. gonorrhoeae* isolates produced a strong explosive bubbling reaction. This indicates the conversion of the hydrogen peroxide to oxygen and water.

3.6.2.3. Oxidase Test

The oxidase activity of the isolates was tested using the Becton Dickinson® oxidase dropper containing dimethylparaphenylene diamine (DMPD) or tetramethylparaphenylene diamine (TMPD). With the use of a sterile swab stick, 2 or 3 colonies of freshly grown isolates were picked and a drop of the oxidase reagent was placed on the spot on the swab where the isolates were smeared. An immediate colour change from colourless to purple coloration was an indication that the bacterium was oxidase positive.

3.6.3. Confirmatory Identification of *N. gonorrhoeae* using Analytical Profile Index NH (API-NH)

The API-NH (Biomerieux, France) was used to confirm and identify *N. gonorrhoeae*, which uses dehydrated miniaturized tests as well as a specialized database. The kit can undertake 10 biochemical identification tests including penicillinase activity, carbohydrate (glucose-GLU, fructose-FRU, maltose-MAL and saccharose-SAC) and metabolism; ornithine decarboxylase-ODC, urease-URE, lipase-LIP, alkaline phosphatase-PAL, β- galactosidase- βGAL, proline arylamidase-ProA, gamma glutamyl transferase-GGT and indole-IND activities. The API NH test was performed according to manufactures' instructions, as follows:

Freshly grown (18-24hrs) pure cultures, which were Gram negative diplococci, as well as catalase and oxidase positive were used for this test. Using a sterile swab, few isolated colonies were picked and mixed in 0.85% NaCl solution, making a suspension turbidity equivalent to 4 McFarland standard. A sterile Pasteur pipette was used to distribute the suspension, avoiding the formation of bubbles. For the 7 underlined tests namely; PEN, GLU, FRU, MAL, SAC, ODC and URE, only the tube part of the microtubes were filled whiles the remaining 3 tests; LIP/ProA, PAL/GGT and βGAL/IND, the microtubes were completely filled.; PEN to URE tests were then filled to the brim with mineral oil to create an anaerobic condition. After which the test strip was placed in a humid chamber and incubated aerobically at 36°C ± 2°C for 2 to 2½ hours. After incubation period, the reaction was read by referring to the Reading Table in the package insert. Some tests were read spontaneously and some read after the addition of reagents. Three microtubes are bifunctional and able to perform 2 reactions in the same tube:

1. LIP (Spontaneous reaction) / ProA (Shows reaction after addition of ZYM B reagent)
2. PAL (Spontaneous reaction) / GGT (Shows reaction after addition of ZYM B reagent)
3. βGAL (Spontaneous reaction) / IND (Shows reaction after addition of JAMES reagent)

The results were recorded on the recording table provided with the kit (1, 2, or 4 points for positive (+) reaction, 0 points for negative (-) reaction). Three test reactions are added together at a time to give a 4-digit number, which can then be looked up in the bacterial identification catalogue or API database to confirm the identity of the organism.

N. gonorrhoeae is penicillinase variable, produces acid from only glucose and is proline arylamidase positive by the API-NH test.

API-NH confirmed *N. gonorrhoeae* isolates were archived after subculturing on non-selective media (Chocolate agar), in a minimum of two and a maximum of four 1.5ml vials containing 20% glycerol/Tryptic Soy Broth (TSB) and stored at -70°C. This was used for further tests, such as, Antimicrobial Sensitivity Tests (AST) and molecular assays including sequencing.

3.6.4. Antimicrobial Sensitivity Test (AST); Disc Diffusion Method (Kirby-Bauer)

API-NH confirmed *N. gonorrhoeae* isolates that were not more than 24 hours old, grown on a non-selective agar (chocolate agar (CA) were used for AST. Four to five pure colonies were picked and suspended in 0.85% saline to achieve a suspension equivalent to 0.5 McFarland standard. The bacterial suspension was diluted or supplemented with more organisms or saline as necessary. A sterile cotton swab was then soaked in the suspension and excess fluid squeezed out by pressing the swab against the side of the tube and applied to the surface of a GC agar (Becton Dickinson®) plate supplemented with 1% isovitalex (BBL®, Becton Dickinson®, New Jersey, USA). The agar plate was inoculated evenly by swabbing the surface in a forward and backward manner and then rotating the plate to approximately 60^{o} to ensure complete coverage. Different antibiotic discs (Becton Dickinson®, New Jersey, USA) with various concentrations (Appendix 1) were then placed on the surface of the agar plate, properly spaced to minimize inhibition zone overlap. A maximum of five antibiotic discs were placed on a 100mm agar plate. The plates were inverted and incubated at 35°C-37°C in an aerobic environment for 18-24 hours before reading. The diameter of the zones of inhibition were measured in millimetres using a digital calliper and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines of 2018. Quality Control (QC) for AST was performed with the *N. gonorrhoeae* ATCC strain 49226.

Outlined in appendix 1 are the CLSI values as well as interpretations. Isolates that were resistant by the disk diffusion method were further confirmed by the E-test method as described below.

3.6.5. E-test ® Procedure

After testing isolates for resistance by the disk diffusion method, the Minimum Inhibition Concentrations (MICs) of the resistant isolates were further assessed using the E-test® (BioMerieux) strips. Briefly, bacterial suspensions prepared as described in the Kirby-Bauer

procedure above were spread on a GC agar (Becton Dickinson®, New Jersey, USA) plate supplemented with 1% isovitalex (BBL, Becton Dickinson). E-test strips were placed radially on the surface of each inoculated agar plate and incubated under the same conditions as described above. Only two E-test strips were placed on a 100mm agar plate, to avoid overlap of the ellipse formed by inhibition of bacterial growth. *N. gonorrhoeae* ATCC 49226 was used as the standard strain (QC strain) for the antibiotic susceptibility testing

3.6.6. Antimicrobial Agents

Antimicrobial agents used for AST included antibiotics currently recommended for the treatment of gonorrhoea in Ghana as well as those regularly tested under the Gonococcal Isolate Surveillance Project (GISP), by the US Department of Health and Human Services and the Centers for Disease Control and Prevention (CDC) for the surveillance and rapid detection of resistant *N gonorrhoeae* in the United States. The Minimum Inhibition Concentration (MIC) Alert Value of isolates, as listed under the GISP protocol were re-tested to confirm the high MICs. Retesting of these isolates was done with each batch of isolates, provided this was no longer than one month after the initial test. Antimicrobial agents used for testing and their alert values as described under the GISP protocol are listed below:

Ciprofloxacin MIC ≥ 1.0 µg/ml

Penicillin MIC ≥ 2 µg/ml

Ceftriaxone MIC ≥ 0.125 µg/ml

Azithromycin MIC ≥ 2.0 µg/ml

Spectinomycin MIC >128 µg/ml

Cefixime MIC ≥ 0.25 µg/ml

Tetracycline MIC ≥ 2 µg/ml).

3.6.7. DNA Extraction of *N. gonorrhoeae* Isolates

Genomic DNA was extracted from pure freshly grown *N. gonorrhoeae* isolates or archived isolates from non-viable cells using the QIAamp® DNeasy Ultraclean Microbial kit (Qiagen, Hilden, Germany) following manufacturer's procedures described below:

Briefly, approximately 3/4 of 10 µL inoculation loop full of bacterial colonies from a freshly grown *N. gonorrhoeae* isolate on chocolate agar were picked and suspended in 300µl of PowerBead solution in a labelled tube. For non-viable isolates, 1mL of the bacterial suspension stored in tryptic soy broth was centrifuged at 14000rpm to pellet the bacterial cells before transferring the pellet to the PowerBead solution. The solution and bacterial cells were resuspended by vortexing until the sample homogenized completely. The homogenized isolate in the Powerbead solution was then transferred to a labelled Powerbead tube (containing beads) and 50µl of solution SL was then added. The solution in the Powerbead tube was then briefly vortexed and incubated for 20-25 minutes at 65°C. The Powerbead tube was then secured in the Mini-beadBeater-96 (Biospec Products Inc, USA) and beaten at a speed 6.0m/s for 35 seconds for 3 cycles. After bead beating, the Powerbeads were then centrifuged at 10,000xg for 2 minutes. The supernatant was then transferred into a new labelled 2ml collection tube, after which 100µL of solution IRS was added. The mixture was then aggressively vortexed for about 5seconds and then incubated for 10minutes at 4°C or on ice for 5minutes. Following incubation, the tube was centrifuged at 12,000xg for 2minutes, after which the entire volume was transferred in a new labelled 2ml tube avoiding transfer of any precipitate. An evenly mixed SB solution of 900µl was added to the supernatant and then vortexed for about 5minutes. About 650µl of the mixture was transferred to a labelled Spin Filter and centrifuged at 12,000xg for 2minutes. The flow through was discarded and the remaining solution added to the Spin Filter and centrifuged at 12,000xg for an additional 2 minutes. The Spin Filter was then washed by adding 300µl solution CB and centrifuged at 12,000xg for 2 minutes and the flow through

discarded. The tube was centrifuged for an additional 2 minutes at 12,000xg to remove any residual CB solution. The Spin Filter was placed in a new labelled 2ml collection tube and 50µl of Solution EB added to the centre of the white filter membrane, followed by incubation for 1minute. It was then centrifuged at 12,000xg for 2minutes, after which the eluted DNA was collected in the tube and Spin Filter discarded.

The extracted DNA was quantified using the Qubit dsHs kit (Invitrogen, Malaysia) as described below. The DNA was stored at -20°C until sequencing was done.

3.6.8. Nucleic Acid Quantification

To determine the concentration of double stranded DNA of each isolate extracted, the Qubit ds high sensitivity kit was used. This was done following manufacturer's instructions as described below.

The standards (S1 and S2) for the test were first determined, this was used to estimate the concentration of the samples. This was done by adding 20µl of S1 and S2 to individual transparent 0.5ml tubes containing 180µl of Qubit buffer. Each sample was quantified by adding 1µl of sample to 199µl of Qubit buffer, after which they were incubated for 2minutes before reading in the Qubit 3.0 instrument (Invitrogen, Malaysia).

The readings obtained for each sample were used to determine the starting concentration and volume for whole genome library preparation.

3.7. Whole Genome Sequencing of *N. gonorrhoeae*

3.7.1. Library Preparation and Sequencing on the Illumina MiSeq

3.7.1.1. Library Preparation.

Libraries were prepared using the Kapa HyperPlus Library Prep Kit (Kapa Biosystems, Massachusetts, USA) which is a non-PCR kit. The process includes of library preparation enzymatic fragmentation of high-quality DNA, end repairing, A-tailing and adaptor ligation.

The concentrations of the quantified samples are taken into consideration to determine the starting volume of each sample. Approximately 1µg of DNA needed in a starting volume of 35µL.

The following formula was used to determine the input material for library preparation:

Starting Volume [SV] (µl) = 1000/Qubit result (ng/µl)

The result for SV was then subtracted from 35µl and topped up with the equivalent of 10mM Tris-HCL (pH 8.0-8.5) also known as resuspension buffer (RSB) to make up the starting volume of 35µl. For samples with very low concentration the total volume of 35µl of the sample was used.

a. Fragmentation

On a clean 96 well plate 35µl DNA of samples were added to individual wells of the plate. The fragmentation master mix of 5µl of Kapa Fragment buffer (10X) and 10µl of Kapa enzyme was made on ice. A total volume of 15µl of the master mix was added to each well containing samples. The plate was then sealed and ran on the Eppendorf Mastercycler X50s (Eppendorf, Hamburg, Germany) using the following program:

Heated lid: Off

4°C hold (pre-cool)

37°C for 5mins

4°C hold

b. End Repair and A-Tailing

After fragmentation the plate was spun and placed immediately on ice, while on ice a master mix of 7µl of End Repair and A-Tailing buffer and 3µl of End Repair and A-Tailing Enzyme

mix was made. A total volume of 10µl of the prepared master mix was added to each well and mixed well by pipetting. The plate was then sealed, spun and the following program ran on the Eppendorf Mastercycler X50s (Eppendorf, Hamburg, Germany).

Heated Lid: 85°C

4°C hold (pre-cool)

65°C for 30 mins

4°C hold

c. Adapter Ligation

Following the above program, the plate was immediately placed on ice. Adaptors were removed from -20°C freezer and allowed to thaw on ice and the following master mix was made:

30µl Ligation buffer

10µl Ligase buffer

5µl PCR-grade water

2.5µl RSB

A total of 47.5µl of the master mix was added to each well and the reaction was mixed well by pipetting several times. Following this, 2.5µl of 15µM unique paired Truseq UD adapters (IDT, New Jersey, USA) were added to each well and mixed thoroughly by pipetting. The plate was then sealed, spun and immediately incubated on the Eppendorf Mastercycler X50s (Eppendorf, Hamburg, Germany) at 20°C for 15mins and held at 4°C. The reaction can be held at -20°C for week or overnight at 4°C.

d. Post-Ligation Clean-up

A post-ligation clean-up was done immediately after adapter ligation or after overnight storage. Clean-up was done by adding 88µl of AMPure XP beads (Beckman Coulter, Indianapolis, USA) which had equilibrated to room temperature and mixing well by pipetting. The reaction was then incubated for 10 minutes at room temperature. The plate was placed on a magnetic rack for 5 minutes or until the reaction completely cleared. The supernatant was removed and discarded and with the plate still on the magnet 300µl of 80% ethanol was added to each well. The plate was then incubated for 30seconds at room temperature and the supernatant discarded without disturbing the beads. With the plate still on the magnet, another 300µl of 80% ethanol was added to each well, and then incubated for 30seconds at room temperature. The supernatant was discarded, ensuring all ethanol residue was removed by using a new smaller pipette tip. The plate was then dried for 5minutes by allowing all ethanol to evaporate, making sure the bead did not over dry. After drying, the plate was taken off the magnet and the beads were thoroughly resuspended in 103µl of RSB and incubated for an additional 5minutes at room temperature. The plate was then placed on the magnetic stand and incubated for 5minutes, after which 100µl of supernatant was transferred into a new 96 well plate. This was followed by size selection clean-up.

e. Size Selection Bead Clean-up

A master mix of 92 µL of AMPure XP bead (Beckman Coulter, Indianapolis, USA) and 92µl PCR-grade water was made, of which 160µl of the master mix was added to each well of the plate containing the post ligated cleaned libraries. The reaction was then mixed thoroughly by pipetting, after which, it was incubated for 10 minutes at room temperature. The plate was placed on the magnetic stand for 5minutes. The Supernatant (~250µl) was then transferred into new 96 well plate and 30µl of AMPure XP bead (Beckman Coulter, Indianapolis, USA) added to each well containing the supernatant. The reaction was mixed thoroughly by pipetting up and down and incubated for 10minutes at room temperature. The plate was placed on a

magnetic rack for 5minutes. The supernatant was discarded and whilst the plate was still on the magnetic rack, 300µl of 80% ethanol was added to each well. The plate was then incubated for 30seconds at room temperature and the supernatant discarded without disturbing the beads. With the plate still on the magnet, another 300µl of 80% ethanol was added to each well, then incubated for 30seconds at room temperature. The supernatant was discarded, ensuring all ethanol residue was removed by the use of a new smaller pipette tips. The plate was then dried for 5minutes by allowing all ethanol to evaporate, making sure the bead did not over dry. After drying, the plate was taken off the magnet and the beads were thoroughly resuspended with 23µl of 10mM Tris-HCl (pH 8.0-8.5) and incubated for 5minutes at room temperature. The plate was placed on the magnetic rack for 5minutes or until reaction cleared, after which a minimum of 20µl of the final libraries was transferred into new plate. Libraries were then quantified and later stored at -20°C for downstream analysis.

3.7.1.2. Library Quantification and Size Determination

The Agilent High Sensitivity DNA kit (Agilent Technologies, USA) was used to determine the size and quantify fragmented DNA libraries from above. The kit was taken out of the fridge to equilibrate to room temperature (a minimum of 30minutes) before analysis was done following manufacturer's instructions as described below:

a. Setting up the Chip Priming Station

The syringe clip on the priming station was adjusted by releasing the lever of the clip and sliding it down to the lowest position.

b. Preparing the Gel-Dye Mix

The High Sensitivity DNA dye concentrate (blue) and High Sensitivity DNA gel matrix (red) were allowed to equilibrate to room temperature for 30 min. After that, 15µL of High Sensitivity DNA dye concentrate (blue) was added to a High Sensitivity DNA gel matrix vial

(red). The solution was then vortexed thoroughly, spun down and transferred to the spin filter. The tube was centrifuged at 2240 g ± 20 for 15 minutes. The gel-dye mix was stored at 4 °C and used within 6 weeks of preparation.

c. Loading the Gel-Dye Mix

The gel-dye mix was allowed to equilibrate to room temperature for 30 min before use. A new High Sensitivity DNA chip was placed on the chip priming station and 9µl of gel-dye mix was pipetted into the well-marked big **G**. The plunger was positioned at 1 mL and the chip priming station was closed while the plunger pressed until it was held by the clip. The clip on the syringe was released after waiting exactly 60seconds. After the release the plunger was slowly pulled back to the 1 mL position after waiting for 5seconds. Then the chip priming station was opened and 9 µl of gel-dye mix was pipetted in the wells marked G.

d. Loading the Marker and Samples

In all sample and ladder wells, 5µL of marker (green) was pipetted, no well was left empty. 1 µl of High Sensitivity DNA ladder (yellow) was pipetted into the well-marked Ladder#. In each of the 11 sample wells, 1µL of sample was pipetted and the chip was horizontally placed in the adapter and vortexed for 1minute at the indicated setting (2400 rpm). The chip was run on the Agilent 2100 Bioanalyzer instrument (Agilent Technologies, USA). The average fragment length (bp) of the library and concentration was recorded for downstream applications.

e. Absolute Quantification of Libraries

The Kapa SYBR® FAST quantification kit (Kapa Biosystems, Massachusetts, USA) was used to quantify the prepared libraries. Based on concentrations recorded from the Bioanalyzer, samples which have concentrations greater than 10nM are diluted to get concentrations ranging between 2-10nM. Finally, the libraries were diluted into picomolar concentrations (to the same

units of the standards) by performing a 1:1000 dilution in nuclease-free water before the qPCR assay. The qPCR was then performed using the following reagent volumes and cycling conditions:

a. Reagents and volumes

Component	10µL reaction
Nuclease-free water	1.8- 2µL
2X KAPA SYBR FAST qPCR Master Mix with primers	6µL
Diluted 1:1000 pooled libraries	2µL
50X ROX High	0.2µL

b. Concentration of standards

Standard	Conc. (pM)
DNA Standard 1	20
DNA Standard 2	2
DNA Standard 3	0.2
DNA Standard 4	0.02
DNA Standard 5	0.002
DNA Standard 6	0.0002

c. Cycling Conditions

Step	Temperature	Duration	Cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	35 cycles
Annealing/Extension/ data acquisition	60 °C	45 sec	

3.7.1.3. Library Pooling and Normalization

The results from the bioanalyzer (estimated base pair size) and qPCR concentrations were used to determine the volume of library to add to the loading pool. The quantified libraries were normalized to a concentration of 1 to 2nM (based on the average qPCR concentration).

Calculation of the parameters used calculate the volume of sample to pool:

Average. qPCR conc. (pM) = qPCR conc.

Size adjusted conc (pM) = $\frac{\text{qPCR conc X Illumina fragment length constant (452)}}{\text{Average fragment length (from Bioanalyer)}}$

Concentration of undiluted lib stock (pM) = Conc of undiluted library stock X 1000

Conc of undiluted lib stock (nM) = $\frac{\text{Concentration of undiluted lib stock (pM)}}{1000}$

Average fragment length = base pair (bp) size from bioanalyzer

Dilution (1/x) = 1000

Vol of sample to add to pool = $\frac{\text{Chosen normalized conc. (1 to 2 nM)}}{\text{Total Vol (5 to 10µl)}}$

RSB vol to add = Total Vol (5 to 10) – Vol of sample pooled

Example of tabular presentation of results:

#	Avg. qPCR conc. (pM)	Size adjusted conc (pM)	Conc of undiluted lib stock (pM)	Conc of undiluted lib stock (nM)	Avg fragment length	Dilution (1/x)	Vol of sample to add to pool	RSB vol to add
1	5.1	4.97	4,968	4.97	464	1,000	2.01	2.99

All sample libraries were calculated with the formula provided above and base on the results obtained, individual libraries were pooled. A maximum of 30 bacterial isolates were pooled at a time. The final pool was quantified by qPCR to determine the exact concentration of the pool before diluting for sequencing.

3.7.1.4. Final Library Preparation for Sequencing

The MiSeq V3 cartridge (Illumina, USA) was thawed either at 4°C overnight or in cool water for about an hour or until regents in the cartridge completely thawed. After sample pooling, the following reagents were used to prepare the library for sequencing:

PhiX control – Sequencing control, it has evenly distributed nucleotide bases and helps obtain uniform clustering during sequencing. The 10nm PhiX concentration was diluted 1 to 2nm (depending on the concentration of library) using resuspension buffer in a new 1.5ml tube. A final volume of 5µL was sufficient (1µL PhiX + 4µL resuspension buffer).

Sodium Hydroxide (NaOH) – used to denature double stranded library DNA. NaOH was diluted to 0.1N NaOH from a 2N NaOH concentration using nuclease-free water. A final volume of 10µL was sufficient.

HT1 buffer – provided in the sequencing kit, used to dilute the library to the appropriate concentration in picomol (pM).

In a new 1.5ml tube, a 2% ratio of the diluted PhiX to the library was made (i.e. 9.8µl of library and 0.2µl of diluted PhiX control). The reaction was vortexed and briefly spun. 10µl of the prepared 0.1N NaOH was added to the tube containing pooled library and PhiX, making a total volume of 20µl. The reaction was again briefly vortexed and spun. The reaction was then incubated for 5minutes at room temperature. The denatured library was diluted to 20pM, by adding 980µl of HT1 buffer, which was then briefly vortexed spun and kept on ice.

Thereafter, the 20pM denatured libraries were further diluted to the desired concentration (12 – 14pM), to obtain a recommended cluster density of 1,200 – 1,400 K/mm^2

e. Sequencing on an Illumina MiSeq at NMIMR.

3.7.1.5. Whole Genome Sequencing (Oxford Nanopore Technology)

Sequencing libraries for Oxford Nanopore Technology (ONT) were prepared using the Rapid Barcoding kit (SQK-RBK004) (Oxford Nanopore Technologies, UK) by following the manufactures protocol. The details of the procedure is described below.

1. The DNA to be sequenced was first quantified on the Qubit fluorometer (Thermo Fischer Scientific, USA).

2. Approximately 400ng of total DNA was adjusted is a volume of 7.5 µL as starting concentration.

3. DNA fragmentation and barcoding were done by adding 2.5 µl of an appropriate Fragmentation mix, RB01-12 to each sample and mixed by flicking the tube, and spinning down.

4. The samples were then incubated at 30° C for 1 minute and then at 80° C for 1 minute. Followed by a brief cooldown on ice.

5. All barcoded samples were pooled into one tube, approximately, 10 µL for each sample.

6. To the entire pooled barcoded sample from Step 5, an equal volume of resuspended AMPure XP beads was added and mixed by flicking the tub

7. The mixture was incubated at room temperature for 5 minutes with intermittent flicking.

8. The sample was span down, pelleted on a magnet and the supernatant pipetted off.

9. Keeping the tube on the magnet the beads were washed 2 times with 200 µl freshly prepared 70% ethanol without disturbing the pellet. All residual ethanol was removed and the beads were allowed to dry for 2 minutes.

10. The tube was removed from the magnetic rack and the pellet was resuspended in 10 µl of 10 mM Tris-HCl pH 7.5-8.0. The mixture was incubated for 2 minutes at room temperature.

11. The beads were pelleted on a magnet until the eluate is clear and colourless.

12.10 µL of the eluate which contains the DNA was transferred into in a clean 1.5 ml Eppendorf tube.

13. 1 µl of RAP was added to 10 µl of barcoded DNA, mixed, span down and incubated at room temperature for 5 minutes.

14. The MinION flowcell was allowed to equilibrate to room temperature and primed using flow cell priming mix.

15. In a new tube, the library for loading was prepared by combining the following:

- 34 µl Sequencing Buffer (SQB)
- 25.5 µl Loading Beads (LB), mixed immediately before use
- 4.5 µl Nuclease-free water
- 11 µl DNA library

16. 75 µl of the library was loaded to the flow cell via the SpotON sample port in a dropwise fashion.

17. The experiment was run for 48 hours to acquire sequencing data

3.7.2. Whole Genome Sequence Analysis

3.7.2.1. Illumina Assembly

The raw fastq files were uploaded into Geneious Prime, v.2020.2.4 for analysis. The reads 1 and 2 were paired using "Set Paired Reads" with an insert size of 550 bp. The paired fastq files were quality filtered to Phred score ≥ 20, filtered for minimum read length of 50bp and adaptor trimmed using BBDuk (BBMap – Bushnell B. – sourceforge.net/projects/bbmap/). Read quality was confirmed using the FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The resultant high-quality reads were used for *de novo* Illumina-only genome assembly using the SPAdes assembler v 3.13.0. The generated contig files were scanned to manually remove contigs consisting entirely of homonucleotide bases.

3.7.2.2. Oxford Nanopore Assembly

Raw reads were base-called and demultiplexed in real-time via the MinKNOW software (Oxford Nanopore Technologies, Oxford, UK). Only reads designated as pass were included for further processing. The pass reads were trimmed off adapter sequences using Porechop (v0.2.1, https://github.com/rrwick/Porechop). Nanofilt (v.1.0.5, available from, https://github.com/wdecoster/nanofilt) was used to quality-filter trimmed reads to remove reads with average quality <9 and length shorter than 500 bp. The resultant reads were used for draft *de novo* ONT-only genome assemblies using Flye (v.2.8.1, available at https://github.com/fenderglass/Flye) with the suggested parameters for ONT sequencing "correctedErrorRate = 0.120 -nanopore-raw" and an estimated genome size of 2.3 Mb.

3.7.2.3. Illumina-ONT Hybrid Assembly

Quality filtered and trimmed raw fastq files from both sequencing technologies described above were used as input files to perform Illumina-ONT hybrid assemblies using SPAdes v 3.13.0.

3.7.2.4. Genome Annotation and Typing

The data generated from the three assemblies were submitted to PubMLST (www. pubmlst.org/Neisseria) for annotation. The BIGsdb software at PubMLST automatically identifies and annotates defined loci with ≥98% sequence identity to alleles in the database. The BIGsdb annotation enabled assignment of multi-locus sequence typing (MLST) sequence types (STs), NG-MAST, NG-STAR types, identification of resistance markers, plasmids, the presence of gonococcal genomic islands, etc. The novel sequence types were submitted to the curators of the databases of the various typing schemes for assignment of new types.

3.7.2.5. Annotation of AMR Determinants

The data generated from the three assemblies were submitted to the Comprehensive Antimicrobial Resistance Database (https://card.mcmaster.ca/home) for annotation and analysed for AMR determinants using the Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi) tool. The results designated in the 'perfect' and 'strict' categories were considered for evaluation. The presence of point mutations that were implicated in resistance were manually confirmed by performing alignments in Geneious Prime, v.2020.2.4.

3.7.2.6. Comparative Genomics

To understand how the isolates from Ghana compare to the rest of the world, a whole genome phylogeny based on concatenated core loci (Core genome MLST, cgMLST) was explored. Using the Genome Comparator tool on the BIGsdb platform at https://pubmlst.org, the 56 gonococcal genomes were compared alongside 3 reference genomes, 14 WHO AMR reference strains and 70 other genomes originating from Africa, USA, UK and China. The sequence data of these isolates were retrieved from https://pubmlst.org/organisms/neisseria-spp . Two groups of isolates were selected for the analysis: the first was made up of isolates that belonged to

MLST type present in our isolates (ST-11366, ST-1588, ST-1893, ST-1599, ST-1603, ST-1931, ST-1579, ST-11422, ST-1583 and ST-1596). The second group of isolates where those of MLST types which have been associated with highly resistant clones that are internationally disseminated, but were not present in our isolates (ST-1901, ST-9363, ST-7363 and ST-7367) (Lee *et al*., 2019a; Lefebvre *et al*., 2018; Unemo *et al*., 2012; Unemo, Golparian, *et al*., 2016). The *N. gonorrhoeae* cgMLST v1.0 scheme was implemented with a core genome threshold of loci present in 95 % of all genomes. The generated core genome alignments were used to construct maximum likelihood phylogenies using RaxML v. 4.0 under the General Time Reversible (GTR) GAMMA nucleotide substitution model, and computing 1000 bootstrap replicates. The resultant trees were visualised and annotated with iTOL (v.5.6.3, available at https://itol.embl.de/). Using the same approach described above the Ghanaian isolates alone were also compared.

3.7.2.7. Analysis of Virulence Factors

The sequence data from the isolates were uploaded to the Virulence Factors of Pathogenic Bacteria (VFDB) database (http://www.mgc.ac.cn) and compared to the virulent factors present in the *N. gonorrhoeae* reference FA1090 (GenBank accession number AE004969) Sequence alignment was done with the Seaview software and using the Maximum-likelihood tree reconstruction with PhyML available in Seaview software (Gouy *et al*., 2010). The genome comparator in the BIGSdb platform (https://pubmlst.org) was also used to detect the presence of the gonococcal genomic island.

CHAPTER FOUR

4.0. Results

4.1. Sociodemographic of Study Participants

A total of 56 isolates were available for analysis, 31(55.3%) from 2012-2015 and 25(44.7%) from 2018-2019. Out of these, 55(98%) were from male participants. The age group 25-31 produced the highest number of isolates (29) representing about 52% of the isolates. For marital status, single patients produced the highest number of isolates 44(78%). The isolates were fairly distributed between the study sites, with 22(39%) from 37 Military Hospital whiles the Adabraka Polyclinic and 3 Garrison Clinics, Takoradi produced 17(30%) of the isolates each. The small nature of the sample size did not permit drawing meaningful statistical inferences from the demographic data. A summary of isolate distribution among study participants is presented in table 1.

Table 1: Distribution of isolates among study participants

	2012-2015 Isolate n (%)	2018-2019 Isolate n (%)	Total N (%)
Gender			
Male	31(100)	24(96.4)	55(98.2)
Female	0(0.0)	1(4.0)	1(1.8)
Age(years)			
18-24	10(32.3)	5(20.0)	15(26.8)
25-31	14(45.2)	15(60.0)	29(51.8)
32-38	4(12.9)	5(20.0)	9(14.1)
39-45	0(0.0)	0(0.0)	0(0.0)
Above 46	3(9.7)	0(0.0)	3(5.4)
Marital status			
Single	24(77.4)	20(80.0)	44(78.6)
Married	7(22.6)	4(16.0)	11(19.6)
Divorced	0(0.0)	1(4.0)	1(1.8)
Site			
Adabraka Polyclinic	9(29.0)	8(32.0)	17(30.4)
37 Military Hospital	12(38.7)	10(40.0)	22(39.3)
3 Garrison Clinic, Takoradi	10(32.2)	7(28.0)	17(30.4)

Total	**31**	**25**	**56**

4.2. Antimicrobial Susceptibility Testing

In vitro resistance measured by disc diffusion revealed that 100%, 91% and 89.3% of the isolates were resistant to tetracycline, penicillin and ciprofloxacin respectively, while for the E-test method, 96.4%, 91% and 87.5% respectively were recorded. The MICs for the resistant isolates ranged from 2 to 192 µg/ml for tetracycline, 3 to >32 µg/ml for penicillin and 3 to >256 µg/ml for ciprofloxacin. Four isolates exhibited reduced susceptibility to both cefixime and ceftriaxone as measured by disc diffusion. For these isolates, MIC ranges of 0.004 – 0.016 µg/ml and 0.016 - 0.75 µg/ml for ceftriaxone and cefixime respectively were recorded. The proportion of azithromycin resistant isolates as measured by disc diffusion was 23.3%, however, no azithromycin resistant isolate was recorded via the E-test method. MIC range of 0.064 – 0.75 µg/ml recorded. No spectinomycin resistance was recorded using the two methods. The percentage of resistant isolates and MIC ranges for antibiotics (tetracycline, penicillin and ciprofloxacin) that showed wide difference in MIC values for the two time points of collection are presented in figures 1-4.

4.3. AMR Dynamics between the Two Time Points

Generally, there were no significant changes in the percentage of resistant isolates observed for all the antibiotics between the two time points, (Fig. 1). A further look at antibiotics that showed wide ranges of MICs (penicillin, tetracycline and ciprofloxacin) gave a clearer picture about the dynamics in resistance between the two time points (2012-2015: phase1; 2018-20119: phase 2), [Fig.2-3]. For penicillin, about 80% of the phase 1 isolates had MICs greater that the measurable limit (32 µg/ml), compared to 16% observed in phase 2. This could suggest that penicillin MICs are gradually declining down the years. For tetracycline, MICs were fairly distributed across various ranges, suggesting marginal change in the tetracycline MICs between

the two time points (Fig. 1). Ciprofloxacin MICs ranges between the time points remained comparably stable for most of the ranges except for MICs greater than the measurable limit, of which the phase 2 isolates were twice as much the numbers recorded in phase 1. This could suggest a marginal increase in ciprofloxacin MICs down the years. This could be so because 18.9% of the phase 1 isolates did not have any chromosomal ciprofloxacin determinant compared to phase 2 isolates in which all of them had at least one ciprofloxacin determinant.

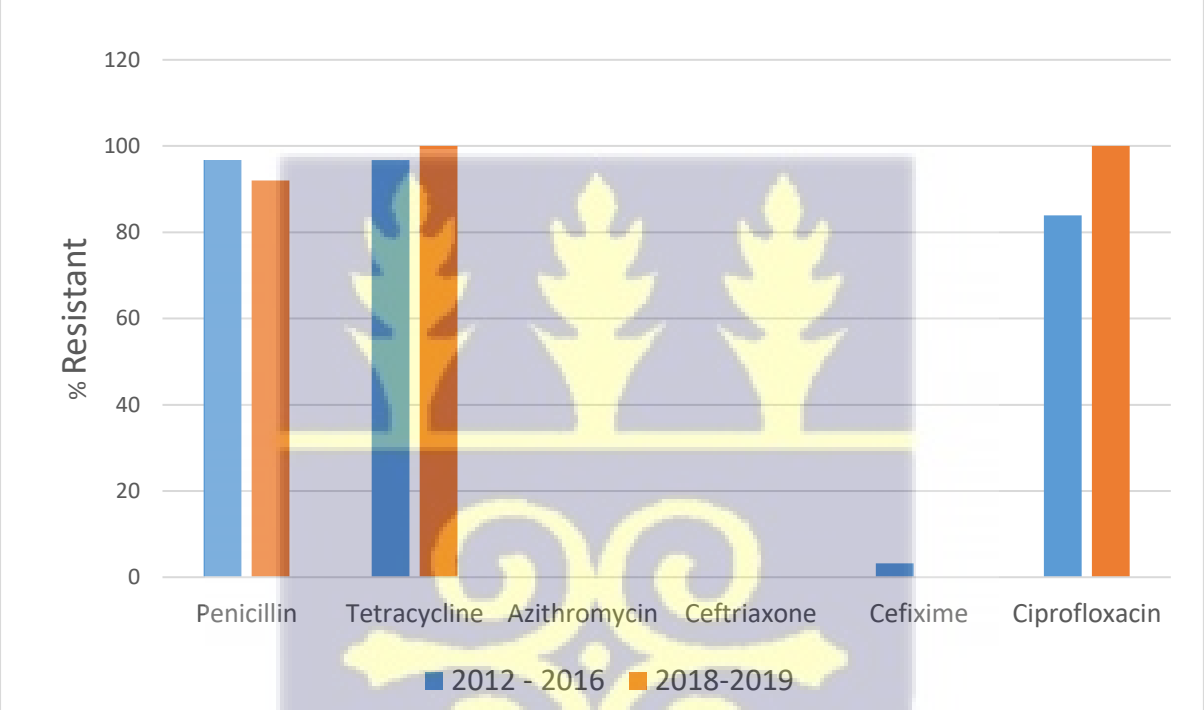

Figure 1: Percentage of resistant isolates between the two time points (2012-2016/2018-2019)

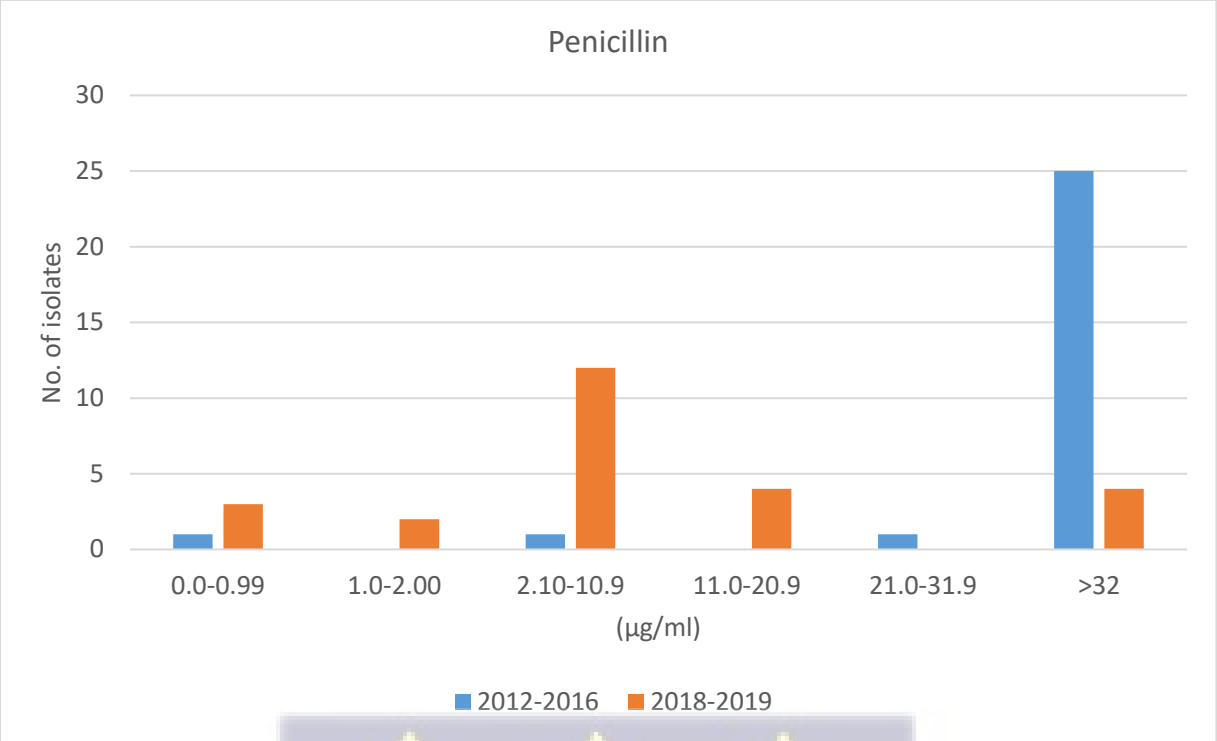

Figure 2: MIC ranges recorded for penicillin between the two time points (2012-2016/2018-2019)

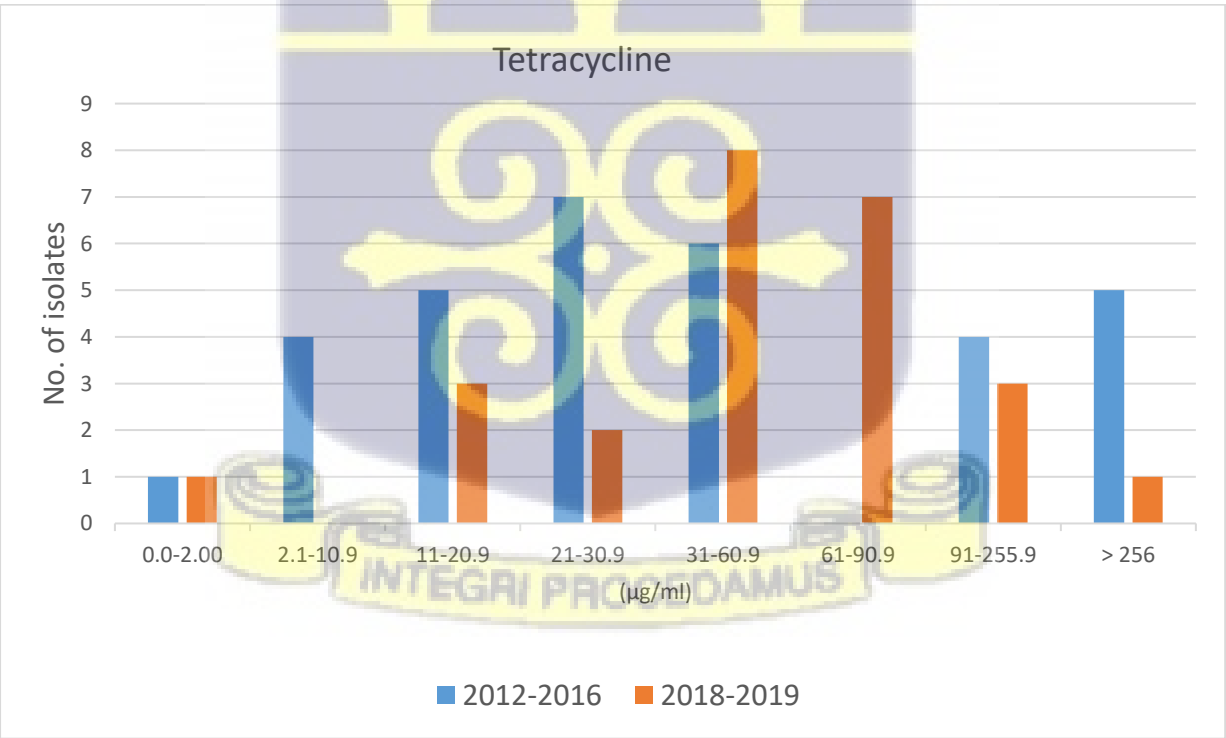

Figure 3: MIC ranges recorded for tetracycline between the two time points (2012-2016/2018-2019)

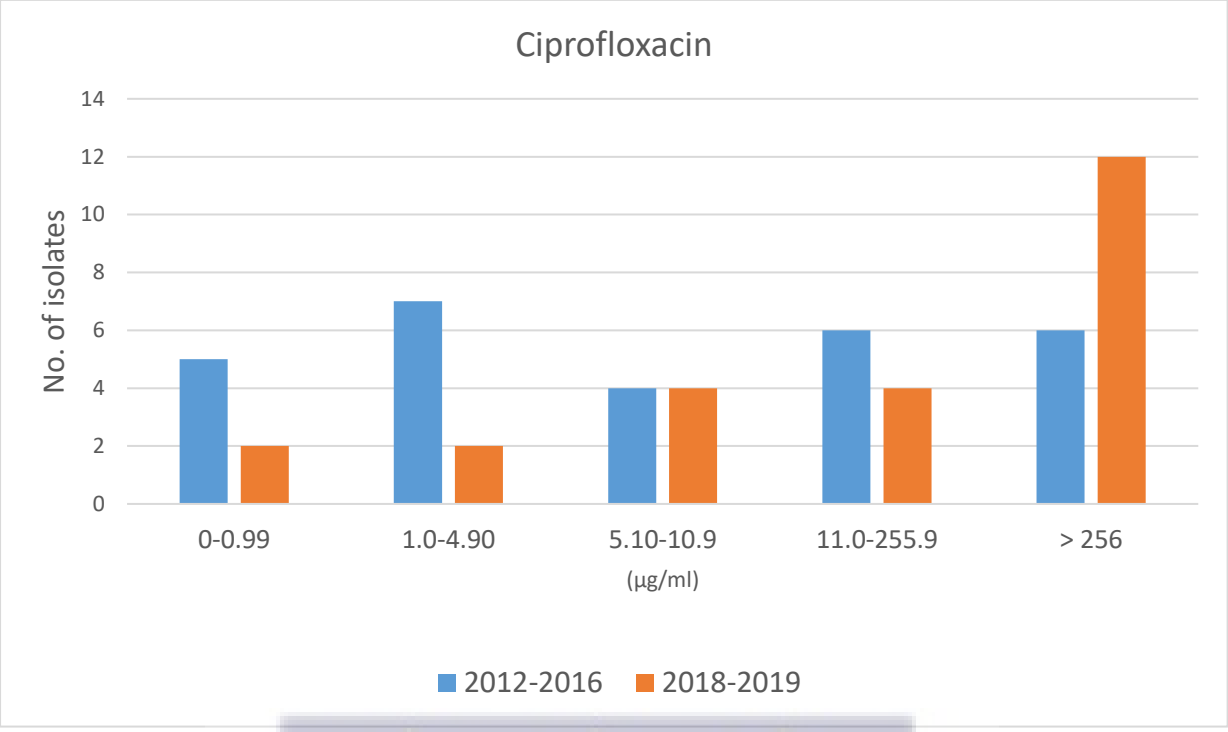

Figure 4: MIC ranges recorded for ciprofloxacin between the two time points (201-2016/2018-2019)

4.4. Whole Genome Sequencing

A total 60 gonococcal isolates were sequenced using both the Illumina MiSeq and the Oxford Nanopore MinION platforms. Assembly of the two datasets resulted in 56 samples passing assemblies for both Illumina and ONT platforms. This resulted in the generation of 56 Illumina-ONT assemblies from the two datasets. Averagely, the assemblies consisted of 2,180,100 bp and 188 contigs; 2,230,900 bp and 3 contigs and 2,222,948 bp and 43 contigs for Illumina-only, ONT-only and Illumina-ONT hybrid assemblies respectively. The average coverage across the genome was 120X, 440 X and 420X for Illumina-only, ONT-only and Illumina-ONT hybrid assemblies respectively. A summary of the assembly parameters is presented in Table 2.

Table 2: Sequencing and assembly statistics

Sequencing assembly statistics					Plasmid assembly statistics		
Platform	Average no. of reads	Average coverage (X)	Average no. of contigs	Average size of assembly	Plasmids	Average no. of reads	Average coverage (X)
Illumina	1,325,000	120	188	2,180,100	pteM	152,100	460
ONT	165,505	440	3	2,230,900	*pBlaTEM*	63,280	980
Illumina-ONT	2,619,000	420	43	2,222,948			

4.5. Molecular Epidemiology/AMR Typing

A total of 22 STs were identified by MLST, with ST-14422 (n=10), ST-1927 (n=8) and ST-11210 (n=7) being the most prevalent. Six novel STs were also identified and submitted for the assignment of new sequence types (**ST-15634-115641**). NG-MAST typing produced 36 sequence types with ST8948 (n=7), ST12791 (n=6) and ST10251 (n=4) being the most prevalent. Notably, one ST1407 which has been associated with elevated MICs in cephalosporins was identified. Thirteen novel sequence types were also identified and submitted for the assignment of new sequence types (**ST-19707-19719**). NG-STAR AMR typing identified 29 unique sequence types with ST-464 (n=8) and the novel ST-3366 (n=8) being the most prevalent. Notably, 20 of the 29 STs were novel. The novel sequence types were submitted for the assignment of new sequence types (**ST-3352-3353**).

Table 3: Results from molecular epidemiology and AMR typing schemes

Molecular epidemiological typing				AMR typing	
MLST		NG-MAST		NG-STAR	
ST	No. of isolates	ST	No. of isolates	ST	No. of isolates
14422	10	8948	7	464	8
1927	8	12791	6	**3366**	8
11210	7	10251	4	**3361**	5
1588	5	16222	2	1215	5
1603	4	3370	2	567	3
15641	3	355	2	**3363**	2
15639	2	16226	2	**3352**	2
11365	2	16227	2	**3359**	2
1596	2	20346	1	**3353**	1
15638	1	16221	1	**3354**	1
15637	1	1737	1	**3355**	1
15636	1	16217	1	**3356**	1
15634	1	211	1	**3357**	1
1591	1	16218	1	**3358**	1
1583	1	16219	1	**3360**	1
11241	1	3178	1	**3362**	1
1579	1	1407	1	**3364**	1
1893	1	16225	1	**3365**	1
1931	1	16228	1	**3367**	1
14789	1	16231	1	**3368**	1
13766	1	9523	1	**3370**	1
11976	1	16232	1	**3371**	1
		2025	1	**3372**	1
		15597	1	438	1
		19707-19719	(1 of each)	157	1
				567	1
				308	1
				893	1
				891	1
Novel	**6**	**Novel**	**13**	**Novel**	**20**
Total	**22**	**Total**	**36**	**Total**	**29**

4.6. Comparative Genomics

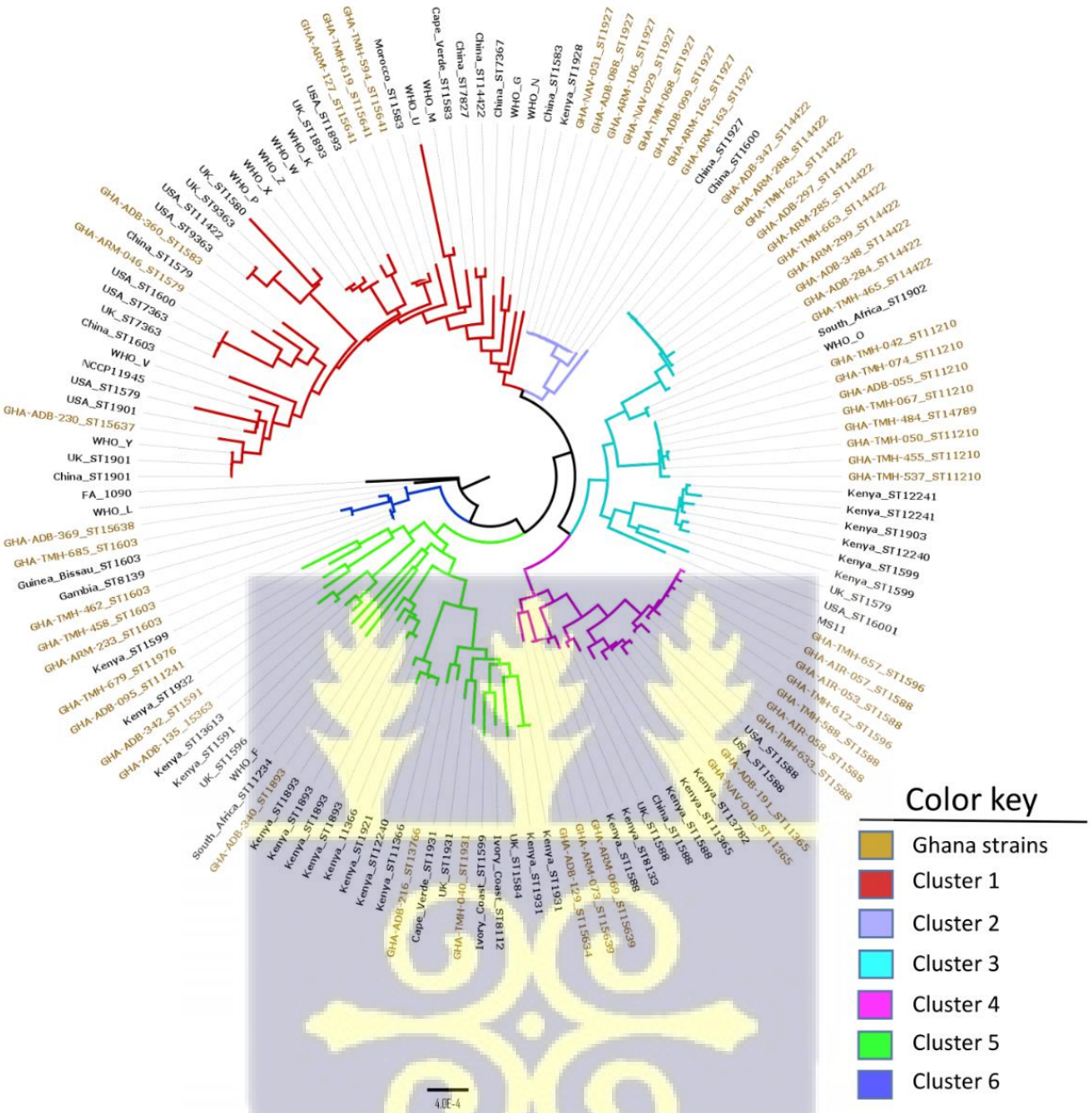

Figure 5: Whole genome genealogy of *N. gonorrhoeae* strains from Ghana compared to strains obtained from different parts of the world, as well as WHO AMR reference strains. The phylogeny was based on concatenated core loci present in at least 95% of the isolates and was constructed using the Genome Comparator tool on the BIGsdb platform at PubMLST.org. The strains are labelled with their MLST STs and country of origin. The phylogenetic tree was constructed using RaXML v8.2.11 and visualized in FigTree v1.4.4.

4.7 Core Genome MLST (cgMLST) Clusters AMR Phenotypes

The core genome loci were resolved into a maximum likelihood phylogenetic tree that separated the isolates into 7 main clusters. Cluster 1 consist of 4 ST-1603 isolates and 1 from one of the novel STs, ST-15638. All isolates in the cluster lack the GGI. Cluster 2 is made up of 4 different STs: ST-15637, ST-15641(3) which are both novel and ST-1579 and ST-1583. All with the exception of the isolate with ST-15637 lack the GGI. The third cluster consist of 8 isolates, all of which belong to the ST-1927 and possessed GGI. The Fourth cluster is made of 12 isolates of diverse STs: novel ST-15634, ST-15639(2); ST-1588(5), ST-1596(2) and ST-11365(2). All isolates in this cluster lack the GGI. Cluster 5 is one of the all GGI-harbouring clades and also contained the most diverse of STs, having all isolates in the cluster belonging to different STs which included: ST-11976, ST-11241, ST-15363, ST-13766, ST-1893, ST-1591 and ST-1931. Cluster 6 consists of non-GGI harbouring clade and two STs: ST-11210(7) and ST-14789. Cluster 7 isolates did not contain GGI and was made up of 10 isolates all of which belonged to the ST-14422.

Matching the AMR phenotypes to specific clusters, Clusters 3 and 7 presented with identical AMR phenotypes, where all isolates in these cluster were resistant to penicillin, tetracycline and ciprofloxacin but susceptible to cefixime, ceftriaxone, azithromycin and spectinomycin. Clusters 1 and 6 also showed similar phenotypic AMR profile, having isolates presenting with both resistant and non-resistant phenotypes for penicillin, resistance to tetracycline and ciprofloxacin but susceptible to cefixime, ceftriaxone, azithromycin and spectinomycin. Clusters 4 and 5 showed unique resistance phenotypes while cluster 2 was the only group which had an isolate which was resistant to a cephalosporin (cefixime).

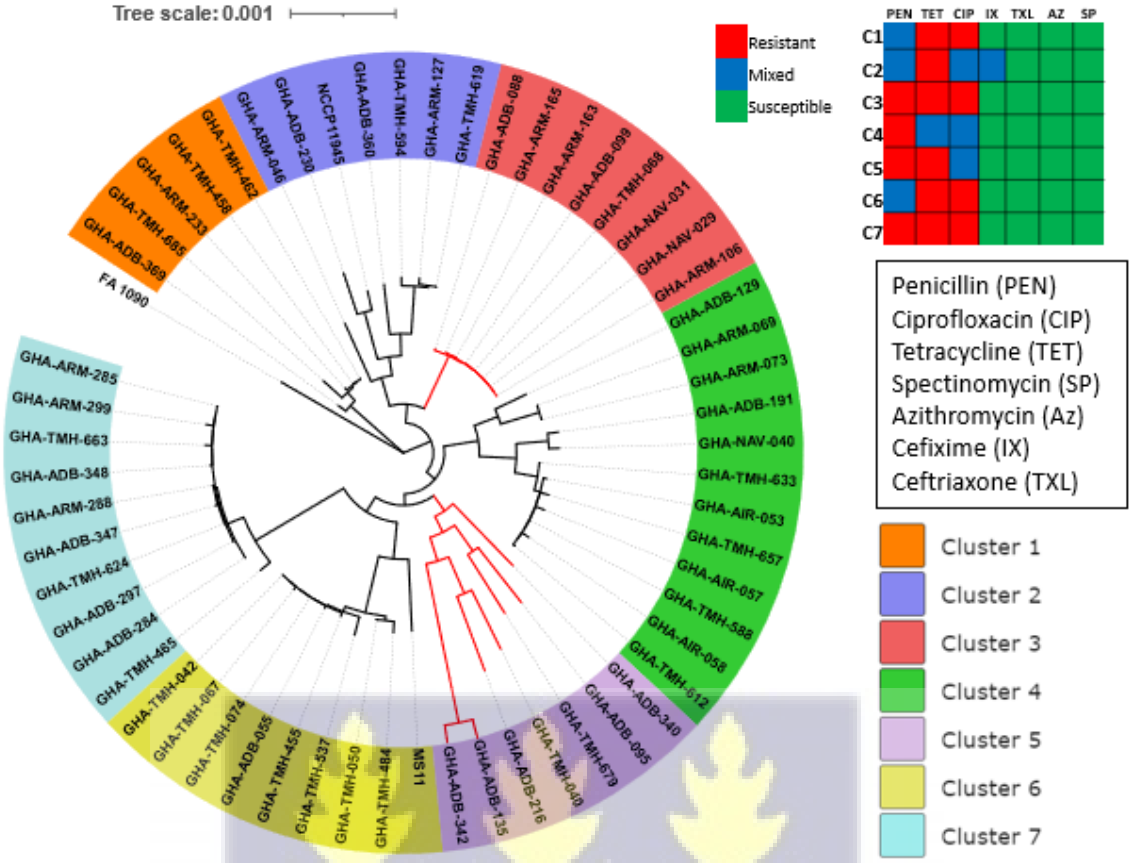

Figure 6: Core genome MLST clustering of the 56 isolates and their associated phenotypic AMR profile. The phylogenetic tree was based on concatenated core genome loci. Seven distinct clusters were obtained with each cluster defined by a colour key represented above. The clusters that contain GGI have their branches coloured in red. The AMR phenotypes of the clusters were mapped and defined using keys represented above. Red: resistant phenotype; blue: a mix of both resistant and susceptible phenotypes; green: susceptible phenotype. The phylogenetic tree was constructed using RaXML and visualized in iTOL.

4.8. AMR Analysis Gene(s) Annotation

4.8.1. Chromosomally Mediated AMR

All isolates harboured chromosomal AMR determinants that confer resistance to beta-lactam antimicrobials and tetracycline. Isolates exhibited the occurrence one of the 4 different *penA* AA substitution patterns (P) in (P1: F504L, A510V, A516G; P2: A501T, G542S, F504L, A510V, A516G; P3: F504L, A510V, A516G, P551S and P4: F504L, A510V). Seven different *penA* alleles were identified, with non-mosaic allele II being the most prevalent. The only mosaic *penA* allele was found in the isolate with reduced susceptibility to cefixime. Amino acid substitution L421P in *ponA* which is also associated with resistance in beta-lactams was identified in 35 (62.5%) of the isolates. Mutations in *porB* which is associated with resistance to tetracycline and beta-lactams was identified in 16 (28.5%). All the 3 characterized mutations in *porB* (G120D, G120K, and A121D) were present. Fluoroquinolone resistance determinants present in the *gyrA* and *parC* gene segments were identified. *gyrA* mutations were present in 51 (91%) of the isolates. Both the S91F 49 (87.5%) and D95G 20 (36%) were present in these proportions. Mutations in the other fluoroquinolone resistance marker, *parC* were identified in 16 (28.5%) of the isolates, with the E91G 9 (16) and S87R 7 (12.5) present. *mtrR* mutations A39T 17 (30%) or G45D 9 (16%) which have been associated with azithromycin and beta-lactam antimicrobials resistance were present. The point mutation V57M in the *rpsJ* gene which has been associated with high-level chromosomally mediated tetracycline resistance was present in all isolates, 56(100%). The amino acid substitution G70D was present in *rpld* and the *macB* resistance markers associated with macrolide resistance were present in 4(7%) and 13(23%) of the isolates respectively. A catalogue of all genotypic AMR determinants is presented in Table 4

Table 4: Annotated chromosomal AMR gene(s)

Chromosomally encoded AMR determinants			
Gene	AMR-Associated AA substitutions	Prevalence n (%)	Antibiotic groups
penA	A501T	8(14)	Beta-lactams: Penicillins & Cephalosporins
	G542S	8(14)	
	F504L	56 (100)	
	A510V	56 (100)	
	A516G	55 (98)	
	P551S	10 (18)	
penB	L421P	35 (62.5)	
gyrA	S91F	49 (87.5)	Fluoroquinolones: Ciprofloxacin
	D95G	20 (36)	
parC	E91G	9 (16)	
	S87R	7 (12.5)	
mtrR	A39T	17 (30)	Beta-lactams & Azithromycin
	G45D	9 (16)	
porB	G120D	11 (20)	Beta-lactams and Tetracyclines
	G120K	4 (7)	
	A121D	1 (2)	
rpsJ	V57M	56 (100)	Tetracyclines
rpld	G70D	4 (7)	Macrolides
macB	None	13 (23)	

Table 5: Concordance between AMR gene presence and resistance phenotype

Antibiotic	**Concordance**
Penicillin	53/56(94.6%)
Tetracycline	55/56(98.2%)
Ciprofloxacin	52/56(92.9%)
Cephalosporins	1/56(1.79%)
Azithromycin	34/56(60.7)

4.8.2. Plasmid Mediated AMR

The p*TetM* conjugative plasmid was present in 54(96%) of the isolates. However, 5(9%) of these plasmids did not contain the *tetM* determinant which is responsible for high-level plasmid mediated tetracycline resistance. Both the Dutch (allele 1) and American (allele 2) types were present. 13 Dutch type and 41 American types were present. Four of the American type plasmids were highly divergent and could have a different ancestry. The non-conjugative plasmid *pBlaTEM* was present in 52(92%) of the isolates. Thirty-six of the *blaTEM* plasmids were of the TEM-1 allele type while the remaining 16 were of the TEM-135 allele type. The prevalence of *blaTEM* plasmids types classified based on geographical origin were as follows: 28 Johannesburg, 16 Australian, 6 African, 1 Asian and a highly divergent plasmid which could be a novel type.

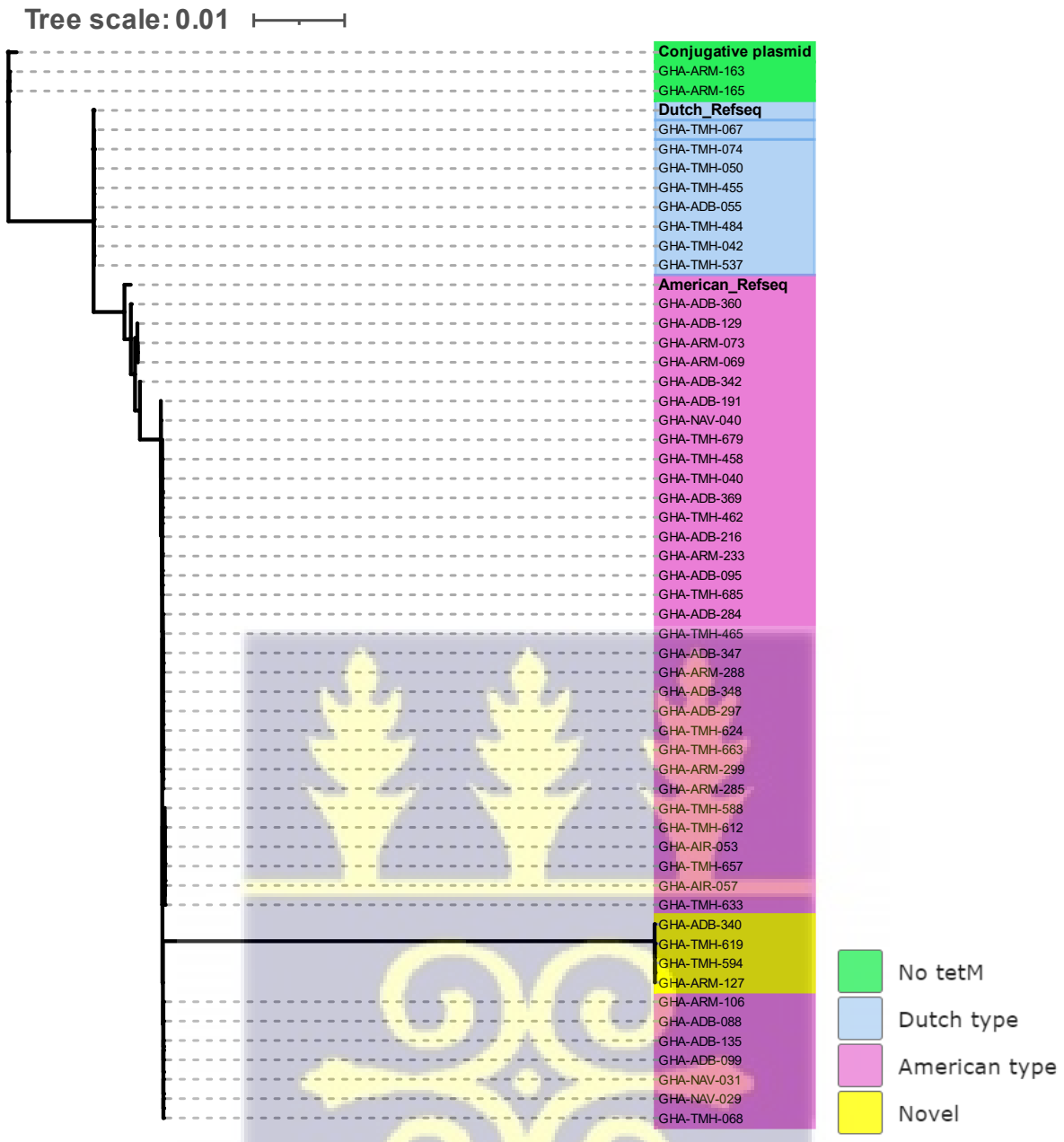

Figure 7: Maximum-likelihood phylogeny of *ptetM* plasmids harboured by the isolates. The various allele types are defined in the colour key. Plasmids that did not harbour *tetM* determinant are coloured green while the highly divergent types are coloured in yellow. The phylogenetic tree was constructed using RaXML and visualized in iTOL.

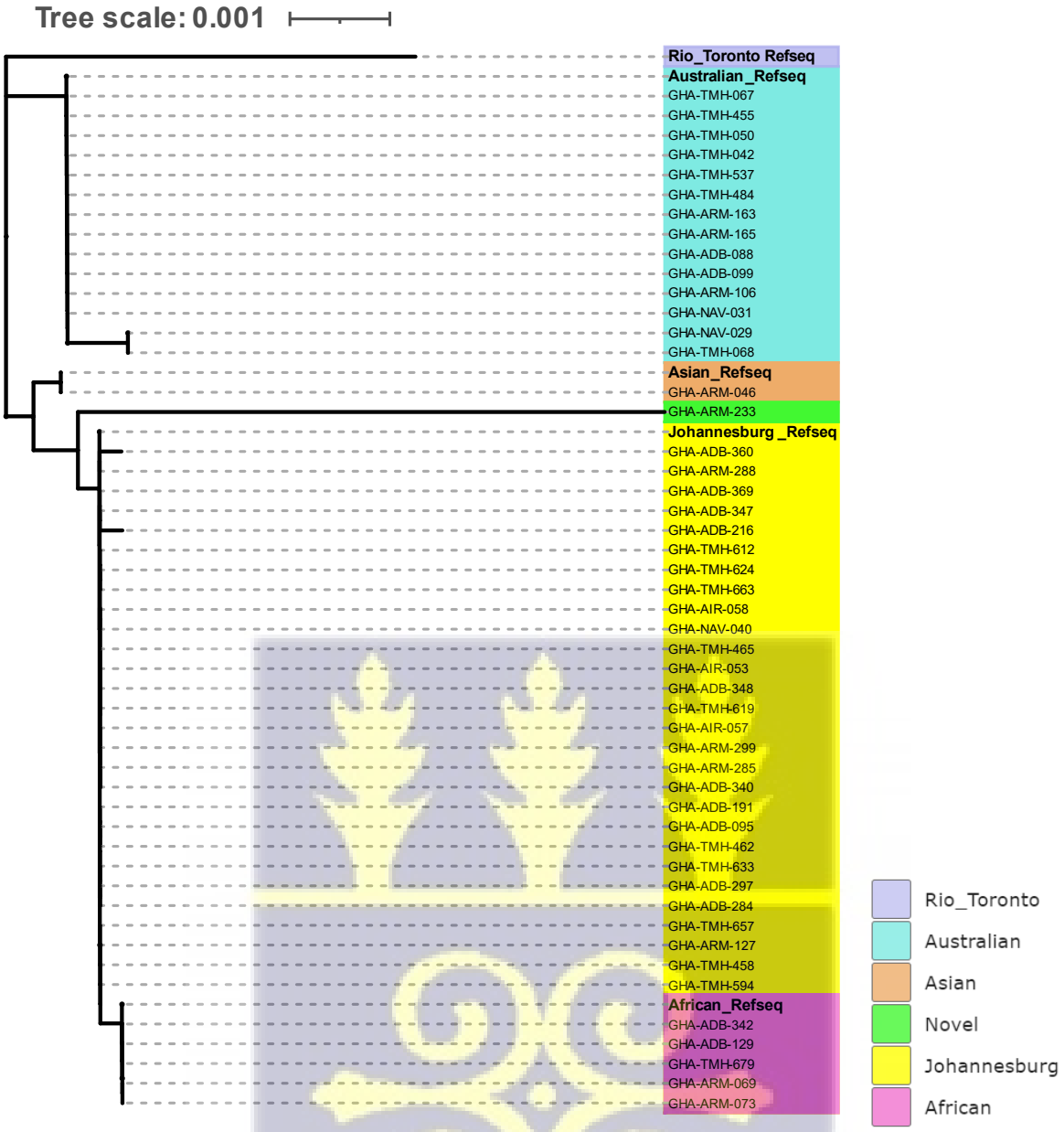

Figure 8: Maximum-likelihood phylogeny of *pBlaTEM* plasmids harboured by the isolates. The various geographical types of the plasmids are coloured coded and defined in the key above. The phylogenetic tree was constructed using RaXML and visualized in iTOL.

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4.9. Virulence Factors

The results showed that all fifty-six (56) strains harbored all the known virulent factors of *N. gonorrhoeae.* These virulent factors include but not limited the Adhesion genes [Adhesion and Penetration protein (App)], Type IV pili genes, Immune evasion genes like (Capsule genes), Invasion genes like the *PorB, PorA* and opacity proteins, as well as several Efflux pump genes (*FarAB, MtrCDE*).

All the isolates harboured the PI (B) allele gene for dissemination. Phylogenetic analysis results showed two major clades, (Fig 9). The closest sequence with homology to the reference strains were GHA-ARM-679, GHA-TMH-050, GHA-ADB-135, GHA-ADB-342. The phylogeny also revealed that no sequence was localized to a particular region. In the pil loci, the *pilE* and *pilS* loci were the most variable amongst the isolates. Compared to the reference, 8 and 4 *pilE and pilS* variants were identified respectively. In the LOS synthesis Loci, the *IgtA* and *IgtH* loci showed variations compared to FA 1090. All capsular proteins responsible for immune evasion were different from that of the reference. Amino acid sequence alignment of showing mutations in the porB gene identified in study samples is presented in appendix III

The genome comparator analysis revealed the presence of gonococcal genomic island (GGI) in 16 (28%) of the isolates. The *ydhB and ydcA* toxin genes which have been associated with cellular survival and biofilm formation were present in the GGI.

Table 6: Major virulent factors in study strains compared to *N. gonorrhoeae* reference FA 1090

VFclass	**Virulence factors**	**Related genes**	**N gonorrhoeae (Prediction)**	**N.gonorrhoeae FA 1090**
			Samples Virulent factors (Predicted)	**chromosome (NC_002946)**
Adherence	Adhesion and penetration protein	app	orf02518	NGO2105
	LOS sialylation	lst	orf01364	NGO1081
	LOS synthesis	kdtA/waaA	orf02279	NGO1915
		lgtA	orf02573	-
		lgtB	orf02574	NGO2156
		lgtC	orf02575	XNG2047*
		lgtD	orf02577	NGO2158
		lgtE	orf02578	NGO2159
		lgtF	orf01526	NGO1353
		lgtG	orf02480	NGO2072
		lgtH	-	-
		rfaC	orf02258	NGO1934
		rfaF	orf01244	NGO0987
		rfaK	orf01527	NGO1354
	Neisseria adhesion A	nadA	-	-
	Phosphoethanolamine modification	lptA	orf01440	-
	Type IV pili	pilC	orf00069; orf02282	NGO0055
		pilD	orf01998	NGO1670
		pilE	orf01436; orf01733; orf02105; orf02106; orf02108; orf02139; orf02141; orf02142	-
		pilF	orf02001	NGO1673
		pilG	orf01997	NGO1669
		pilH	-	NGO0452
		pilI	orf00622	NGO0453
		pilJ	orf00623	NGO0454
		pilK	orf00624	NGO0455
		pilM	orf00133	NGO0098
		pilN	orf00132	NGO0097
		pilO	orf00131	NGO0096
		pilP	orf00130	NGO0095
		pilQ	orf00129	NGO0094
		pilS	orf00093; orf01201; orf02110; orf02138	-
		pilT2	orf00499	NGO0346
		pilT	orf02289	NGO1908
		pilU	orf02288	NGO1909
		pilV	orf01641	NGO1441
		pilW	orf00784	NGO0595
		pilX	orf00625	NGO0456
		pilZ	orf00501	NGO0348
Efflux pump	FarAB	farA	orf02014	NGO1683
		farB	orf02013	NGO1682
	MtrCDE	mtrC	orf01540	NGO1365
		mtrD	orf01539	NGO1364
		mtrE	orf01538	NGO1363
Immune evasion	Capsule	ctrA	-	-
		ctrB	-	-
		ctrC	-	-
		ctrD	-	-
		lipA	-	-
		lipB	-	-
		mynA/sacA	-	-
		mynB/sacB	-	-
		mynC/sacC	-	-
		mynD/sacD	-	-
		siaA/synA	-	-
		siaB/synB	-	-
		siaC/synC	-	-
		siaD/synD	-	-
		synE	-	-
Immune modulator	Factor H binding protein	fHbp	orf00039	NGO0033
	Neisserial surface protein A	nspA	orf00367	NGO0233
	Class 5 outer membrane protein	opc	-	-

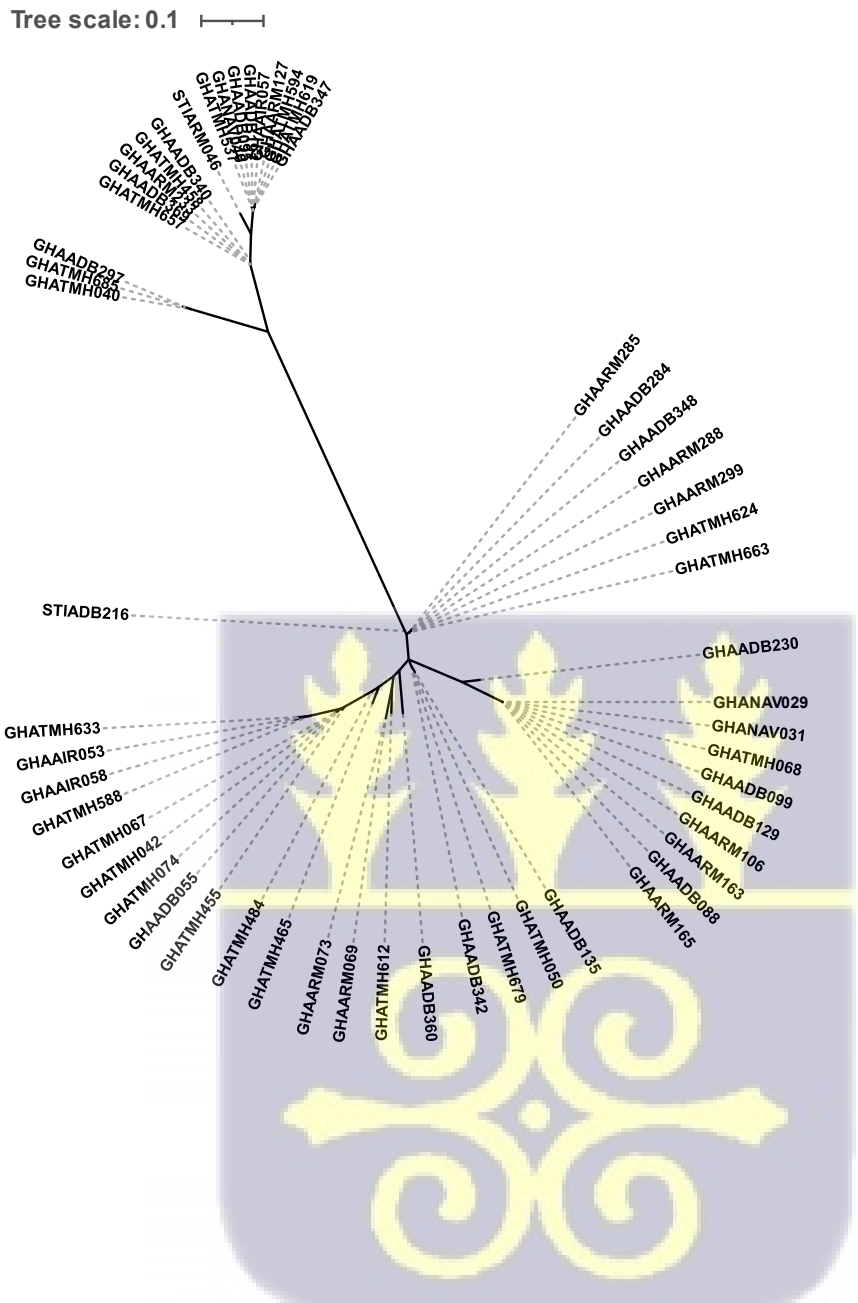

Figure 9: Maximum-Likelihood phylogeny describing the evolutionary relationships of *porB* gene sequences that code for the mature porin of clinical *N. gonorrhoeae* isolates (n = 56). The isolates were compared with the reference genome (FA 1090) with the accession number YP_208842

CHAPTER FIVE

5.0. Discussion

5.1. Molecular Epidemiology

N. gonorrhoeae epidemiological investigations have benefited from the introduction of DNA sequencing techniques. The most widely used molecular epidemiological characterization schemes before the advent of WGS were the MLST and NG-MAST. Overall, the MLST data revealed that the isolates collected in this study were diverse with 22 different sequence types of which six were novel. The most prevalent STs included ST-14422, ST-1927, ST-11210 and ST-1588. ST-1588 is the most globally distributed of these STs, having been previously reported in Africa (Cehovin *et al*., 2018; Harrison *et al*., 2016; Lee *et al*., 2019a), Europe, America (Costa-Lourenço *et al*., 2017) and Asia (Nakayama *et al*., 2012b). ST-1927, is geographically restricted, and mostly identified in Asia (Liu *et al*., 2018; Yan *et al*., 2019) and Russia (Ilina *et al*., 2010). ST-14422 and ST-11210 are less common with ST-14422 traced back to China and USA as sequence origin (https://pubmlst.org/) (Gernert *et al*., 2020). The remaining 12 STs are highly diverse and globally disseminated while 6 are unique to this study.

NG-MAST characterization of the isolates resulted in identification of 36 STs, including 13 that were found to be unique. Of note is the single ST1407 strain which has been characterized to contain the mosaic *penA* gene of the allele type XXXIV associated with widespread cephalosporin resistance (Sánchez-Busó *et al*., 2019b; Unemo *et al*., 2012). Although ST1407 isolates usually belong to MLST ST-7363 and ST-1901(Shimuta *et al*., 2015) the ST1407 isolate recovered from this study was novel, ST-15637 suggesting the possibility of clonal diversity. Comparing the isolates to others isolated in Africa, UK, USA and China it was observed that even for same MLST STs, the Ghana isolates are more clonal and cluster closely together. Generally, the isolates from this study a clustered together and barely mixed with the

globally distributed strain. The Ghanaian strains could therefore be endemic to the region and may be locally transmitted.

5.2. Antimicrobial Resistance

Multi-drug resistant (MDR) gonococci have been a recurrent problem in the treatment and management of gonorrhoeae (Unemo & Shafer, 2014). Comparing the phenotypic AMR data between the two time-points of collection showed that generally, there was no significant changes in the dynamics of AMR between the two collection points (Fig. 1). However, further look at MIC the ranges for individual antibiotics between the two time points revealed a slight decline in penicillin MICs down the years (Fig. 2). This could suggest that penicillin abuse might have reduced since it is no longer effective for the population. If indeed, this is the situation, it presents some hope for future treatment using previously abandoned antibiotics or their modified derivatives, as was the case for chloroquine resistance in *plasmodium* (Balikagala *et al*., 2020; Dagnogo *et al*., 2018). On the other hand, tetracycline and ciprofloxacin MICs between the two time points were fairly distributed across the various MICs ranges although a slight increase was observed in ciprofloxacin down the years (Fig.3 & 4). The slight increase in ciprofloxacin MICs might suggest continual unregulated usage and hence increased antibiotic pressure to enhance resistance (Ventola, 2015).

The severity of AMR has been exacerbated by the complexity of multiple genomic determinants which synergistically contribute to resistance of various antibiotics (Unemo, 2011; Unemo *et al*., 2012; Unemo & Shafer, 2014). NG-STAR characterization which is based of 7 known loci implicated in gonococci AMR identified 29 distinct STs. Interestingly, 20 of these STs were novel, indicating the unique nature of the assembly of genotypic AMR determinants and their possible phenotypic manifestations. The origin of the most prevalent ST, ST464 could not be traced while ST3366 which appears at the same frequency was unique to this study. Of the 7 loci, the 23sRNA gene, associated with azithromycin resistance was the

most conserved, with all isolates exhibiting the wild-type allele 100. One the other hand, the *mtrR* gene, associated with beta-lactam and macrolide resistance had the most diverse array of 15 allele types present in the isolates. Only 1 mosaic *penA* possessing isolate was identified and expectedly, it is the only isolate with reduced susceptibility to the ESC cefixime (Shimuta *et al*., 2015).

To put our samples in the global context, the cgMLST revealed how the Ghanaian isolates cluster with international some strains (Fig.5). It was evident that the Ghanaian isolates were more clonal and are likely endemic to the region. In order to better resolve the lineages of the Ghanaian strains into more compact groups and their associated AMR phenotypes, a genome-wide approach was used. The gene-by-gene approach which characterizes all loci core to the genomes analysed leading to a cgMLST, (Fig.6). Seven phylogenetically distinct clusters were generated by cgMLST. Generally, the individual clusters revealed distinct genotypic AMR profiles.

PBP2 encoded by the *penA* gene is the main target for beta-lactam drugs. Primarily, the insertion of an aspartic acid after the 345 position (D345a) in PBP2 is associated with penicillin resistance (Ito *et al*., 2005; Lindberg *et al*., 2007; Unemo, Golparian, *et al*., 2016). This mutation was present in all isolates, corroborating the high phenotypic resistance observed in this study. The other main penicillin resistance determinant, L421P in *ponA* was significantly represented in about two-thirds of the isolates (Table 4). Other chromosomally encoded penicillin resistance determinants (*mtrR* and *porB*) were also fairly represented to support the the level of phenotypic resistance expressed.

The main problem with the treatment of gonorrhoeae currently has to do with the emergence of resistance to the ESCs which are the last-line empirical treatment options. (Lee *et al*., 2019b; Unemo & Shafer, 2014). Mutations in the transpeptidase domain of PBP2 (AA340–570) have

been the main focus in deciphering the mechanisms responsible for ESCs resistance. Reduced susceptibility to ESCs have been linked to the possession of mosaic-like *penA* allele or the presence of several non-synonymous amino-acid substitutions (I312M, V316T A501, F504L, A510V, A516G, G545S, P551S and P551) (Liao *et al*., 2011; Thakur *et al*., 2014; Thakur *et al*., 2017; Unemo, Golparian, *et al*., 2016; Whiley *et al*., 2007). In this study, GHA-ADB-230 (NG-MAST ST1407) was the only strain that contained a mosaic *penA* allele. This isolate also lacked the D345a insertion reported elsewhere (Calado *et al*., 2019). The GHA-ADB-230 isolate possessed the , F504L, A510V, N512Y and G545S substitutions, which are present in well characterized ESC resistant strains (Ito *et al*., 2005; Thakur *et al*., 2014; Thakur *et al*., 2017; Unemo, Golparian, *et al*., 2016). Unsurprisingly, it was the only isolate with elevated MIC to an ESC, showing a cefixime MIC of 0.75 µg/ml. This isolate also contained the A39T and G120K mutation in *mtrR* and *porB* respectively which have been associated with ESCs resistance(Thakur *et al*., 2014). All the remaining isolates harboured at least 3 amino-acid substitution implicated in reduced susceptibility to ESCs. Although, none of the isolates expressed resistance phenotypically, a few isolates exhibited reduced susceptibility to either cefixime or ceftriaxone by the disc diffusion method.

Tetracycline resistance was observed in all isolates. Chromosomally encoded tetracycline determinants associated with high-level tetracycline resistance including the *rpsJ* gene encoding ribosomal Protein S10 and *porB* (Hu *et al*., 2005; Unemo, Golparian, *et al*., 2016) were present. The amino-acid substitution V57M in *rpsJ* responsible for tetracycline resistance was also present in all the isolates.

Just like penicillin and tetracycline, fluoroquinolone resistance was phenotypically confirmed in almost all isolates. The *gyrA* and *parC* genes are the main known molecular fluoroquinolone resistance determinants (Zhao & Zhao, 2013). Analysis of these genes revealed the presence of the four main characterized mutations in *gyrA* and *parC* known for fluoroquinolone

resistance. Notably, the S91F in *gyrA* was the most prevalent, occurring in 87% of the isolates. This is very high compared to the 26% which was recently observed in Kenya (Kivata *et al*., 2019b) but comparable to the 100% observed in Portugal (Calado *et al*., 2019). Phenotypic macrolide resistance was not observed in this study. However, mutations in the *mtrR* (Gernert *et al*., 2020) gene and presence of *macB* resistance determinant associated with macrolide resistance (Unemo & Shafer, 2014a) was observed in about 20-30% of the isolates indicating that the presence of gene mutations does not always translate into phenotypic resistance.

5.3. Plasmid Encoded AMR Determinants

Plasmids have played an integral role in emergence and spread of high-level resistance to penicillin and tetracycline in gonococcus (Nakayama *et al*., 2012b; Unemo, Golparian, *et al*., 2016). High prevalence (96%) of the conjugative plasmid p*tetM* was observed, a situation identical to the one observed in Kenya and South Africa where 97% and 73% prevalence were recorded respectively (Cehovin *et al*., 2018; Fayemiwo *et al*., 2011). Comparing the phenotypic AMR data between isolates to decipher the effects of p*tetM* revealed that all isolates which had the p*tetM* plasmid carrying the *tetM* determinant showed MICs ranging between 4 and >256 µg/ml, which translates to resistant phenotype. There were isolates that possessed the p*tetM* plasmid without the *tetM* determinant (MIC of 3µg/ml) which also translates to the resistant phenotype. The exhibition of tetracycline resistant phenotype even in the absence of the *tetM* determinant could be attributed to the fact that chromosomally encoded tetracycline resistance determinants (mutations in *mtrR, penB, and rpsJ* genes) contributing to high-level tetracycline resistance (Hu *et al*., 2005), were present in these isolates. A second group of isolates which had the p*tetM* plasmid that did not carry the *tetM* determinant but rather the *tet (W/N/W)* tetracycline resistance determinant showed MICs ranging from 12 to 64 µg/ml, which also translates to the resistant phenotype. The *tet (W/N/W)* determinant, although less common among gonococcus had, a prevalence of 7.79% in *N. gonorrhoeae* plasmid data in the NCBI

database (https://card.mcmaster.ca/ontology/41634). The isolates that did not have the p*tetM* plasmid showed MICs between 1.5 to 2µg/ml, translating to intermediate resistance phenotype. This is consistent with the fact that the p*tetM* plasmid is very important for gonococci to achieve high level tetracycline resistance (Chen *et al*., 2016; Hu *et al*., 2005).

The *pBlaTEM* plasmid plays a key role in resistance to beta-lactam antibiotics including benzyl penicillin, ampicillin and cephaloridine (Cehovin *et al*., 2017). The *blaTEM* gene encodes a β-lactamase responsible for the hydrolysis of the cyclic amide bond in the β-lactam ring and eventually rendering the antibiotics ineffective (Unemo & Shafer, 2014). Both TEM-1 and TEM -135 allele types were detected in this study. The effects of possession of *pBlaTEM* on penicillin AMR phenotypes revealed that nearly all isolates which harboured the *pBlaTEM* plasmid had MICs ranging between 1 to >32 µg/ml, (MIC >2 for 96%) translating to intermediate/resistant phenotype. The single penicillin susceptible isolate which contained the highly divergent *pBlaTEM* plasmid (93.3% match in GenBank) had penicillin MIC of 0.06. This suggests that the genetic changes in this plasmid did not confer a positive selective advantage in the production of penicinillase enzymes required for high-level penicillin resistance. The identification of TEM-135 in some isolates (28.5%) is of concern because further changes to specific amino-acids might lead to the production of a stable extended-spectrum β-lactamase which could result in complete cephalosporin resistance (Nakayama *et al*., 2012a). Although the TEM-135 have been reported to be carried by the Rio/Toronto and Asian plasmids predominantly (Muhammad *et al*., 2014; Yan *et al*., 2019), all TEM-135 alleles identified in this study were carried by the Australian plasmid type. The observed MICs greater than the measurable limit (32 µg/ml) for the *blaTEM-135* possessing strains is in agreement with the observation by Yan and colleagues (Yan *et al*., 2019) who reported that blaTEM-135 strains in their study exhibited the highest penicinillase activity and MICs.

5.4. Virulence Factors

Analysis of the major virulence factors revealed major variations compared to the reference genome, whiles many of these variations were shared among the study strains. The presence of the PI (B) allele confirms that these strains are adapted to causing localised but not disseminated infection (Deo *et al*., 2018; Isabella & Clark, 2011). The identification of several *pilE/pilS* variants suggests the existence of high antigenic variation between the isolates (Rotman *et al*., 2016). The high antigenic variation suggests that the strains may have adopted many strategies for immune evasion. Notably, the different variants of virulence factors identified were distributed across the study sites. The presence of GGI in some isolates suggests that these group isolates will be more competent in initiating infections and have increased propensity for AMR (Cehovin & Lewis, 2017; Harrison *et al*., 2016). Although association between GGI and AMR has been established elsewhere (Harrison *et al*., 2016), this was not the case in our situation. Further works are required to functionally characterize the different virulence factors identified to better understand the pathogenicity of the pathogen.

CHAPTER SIX

6.0. CONCLUSIONS, RECOMMENDATIONS AND LIMITATIONS

6.1. Conclusion

Generally, gonococci phenotypic AMR patterns and genotypic AMR determinants have remained fairly unchanged in almost a decade. The results from this study suggests that cefixime and ceftriaxone which are the drugs for empirical treatment of gonorrhea should be effective against circulating strains. This is likely due to the fact that these drugs are less available to the public and therefore, there is minimal selective pressure to drive cephalosporin resistance at the moment. This notwithstanding, the high prevalence of cephalosporin resistance determinants is a cause for concern with the daring possibility of emergence and spread of cephalosporin resistance in the near future. On the other hand, the readily available and often abused antibiotics (such as penicillin, tetracycline and ciprofloxacin) are practically not effective in gonorrhea treatment at the moment. The high prevalence of plasmids suggests that horizontal transfer of resistance and virulence genes is very widespread and must be monitored.

6.2. Recommendations

1. Further genomics and transcriptomics studies using lager sample sizes is required to make conclusive assertions
2. There should be a national surveillance program which will give a real-time picture of the resistance profiles of circulating strains
3. Public and health workers must be educated on the practice of informed treatment

6.3. Limitations

1. The samples used were from only two regions in southern Ghana and therefore, the results cannot be over emphasized as a true reflection of the situation in Ghana.

2. The small sample size made it difficult to draw associations between observations.

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Appendices

Appendix I: Zone diameter and minimal inhibitory concentration (MIC) interpretive standards of antibiotics used in phenotypic AMR characterization [source: Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards M02, M07, and M11-28th edition, 2018. Clinical and Laboratory Science Institute (CLSI)]

Antimicrobial agent	Disc content/MIC	Zone diameter breakpoints, nearest whole mm			MIC Interpretive Standard µg/ml		
		R	I	S	R	I	S
Cefixime	5ug	-	-	≥31	-	-	≤0.25
Ceftriaxone	30ug	-	-	≥35	-	-	≤0.25
Ciprofloxacin	5ug	≤27	28-40	≥41	≥1.0	0.125-0.5	≤0.06
Tetracycline	30ug	≤30	31-37	≥38	≥2.0	0.5-1.0	≤0.25
Spectinomycin	100ug	≤14	15-17	≥18	≥128	64.0	≤32.0
Penicillin/benzyl penicillin	10units	≤26	27-46	≥47	≥2.0	0.125-1.0	≤0.06
Azithromycin	15ug	≤30	-	-	≥1.0		

S= Sensitive; **I**= Intermediate; **R**= Resistant

Appendix II: The percentage of resistant isolates and MIC ranges for antibiotics (tetracycline, penicillin and ciprofloxacin) that showed wide difference MICs presented for the two time points of collection.

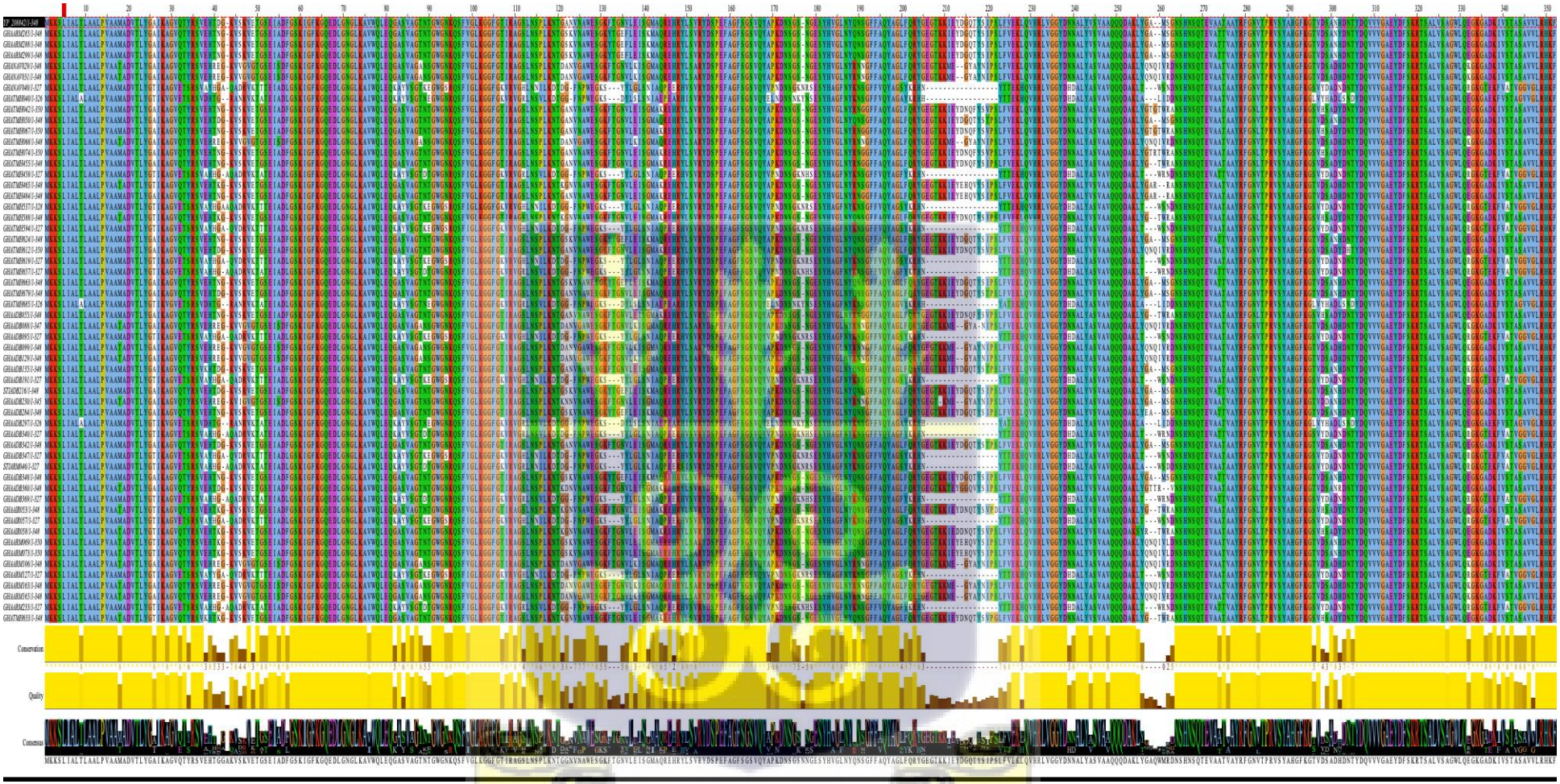
Percentage of resistant isolates between the two collection points

Antibiotic	2012-2016	2018-2019
Penicillin	96.8	92
Tetracycline	96.8	100
Azithromycin	0	0
Ceftriaxone	0	0
Cefixime	3.2	0
Ciprofloxacin	83.9	100

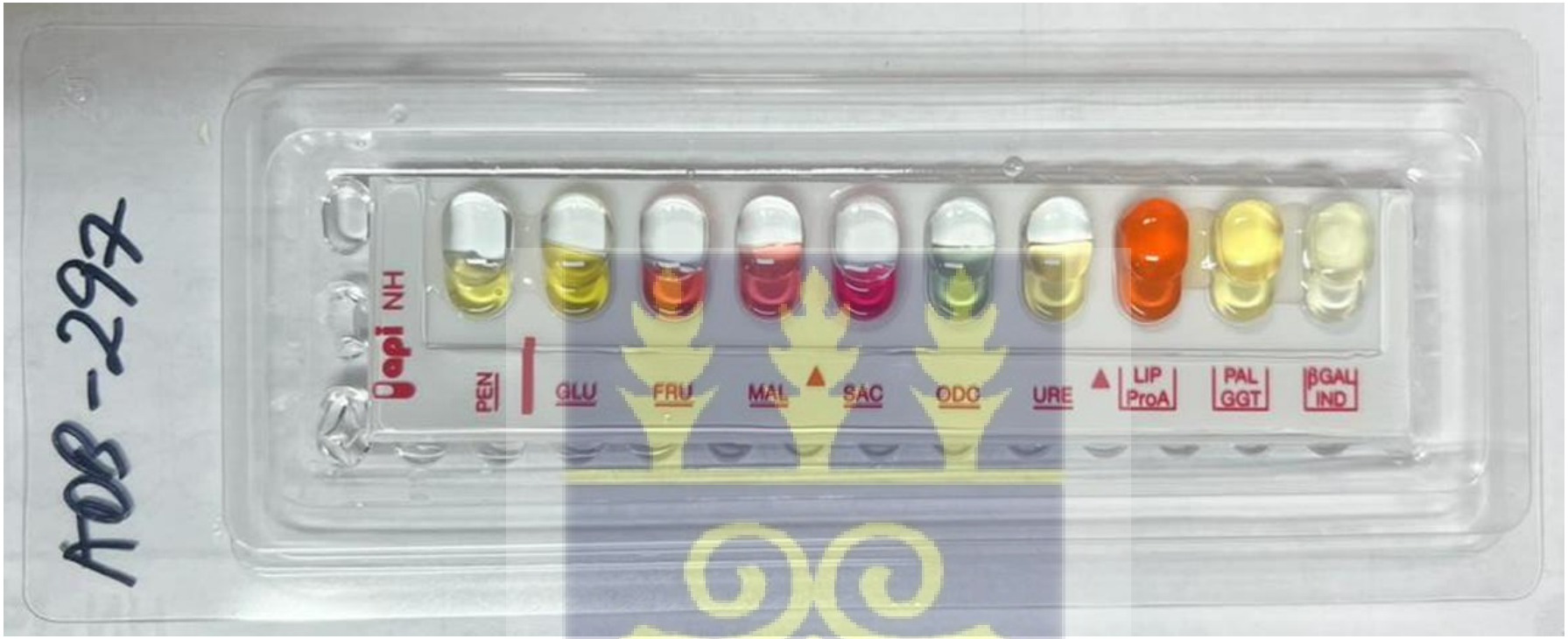
MIC ranges for antibiotics (tetracycline, penicillin and ciprofloxacin)

Penicillin			Tetracycline			Ciprofloxacin		
MIC (µg/ml)	2012-2016	2018-2019	MIC (µg/ml)	2012-2016	2018-2019	MIC (µg/ml)	2012-2016	2018-2019
0.0-0.99	1	3	0.0-2.00	1	1	0-0.99	5	2
1.0-2.00	0	2	2.1-10.9	4	0	1.0-4.90	7	2
2.10-10.9	1	12	11-20.9	5	3	5.10-10.9	4	4
11.0-20.9	0	4	21-30.9	7	2	11.0-255.9	6	4
21.0-31.9	1	0	31-60.9	6	8	> 256	6	12
>32	25	4	61-90.9	0	7			
			91-255.9	4	3			
			> 256	5	1			

Appendix III: Amino acid sequence alignment of showing mutations in the porB gene identified in study samples.The samples (n=56) were compared to por B from reference FA 1090. The alignment was created with Seaview



Appendix IV: Sample of API-NH kit



Appendix V: Noguchi Memorial Institute for Medical Research council (NMIMR)

Scientific and Technical Committee (STC) approval letter

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E-mail: Director@noguchi..ug.edu.gh

Post Office Box LG 581
Legon, Accra
GHANA

My Reference: DF5

Your Reference:

14th January, 2020

Mrs. Naiki Attram
NAMRU–3 GD
University of Ghana
NMINR
Legon

Dear Madam,

APPROVAL OF PROTOCOL

The Scientific and Technical Committee of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, at its meeting on December 3, 2019, reviewed the protocol titled: "Next Generation Sequencing to Assess Antimicrobial Resistance in Neisseria Gonorrhoeae Isolates", STC Paper 1(2) 2019-20, which was submitted by Mrs. Naiki Attram.

The Committee after the review approved the protocol on Tuesday, 14th January, 2020.

The Scientific and Technical Committee avails to you the assurances of its highest consideration.

Thank you.

Yours faithfully,

Professor Abraham Kwabena Anang, PhD
DIRECTOR

Appendix VI: Naval Medical Research council (NMRC) IRB approval letter

DEPARTMENT OF THE NAVY
U.S. NAVAL MEDICAL RESEARCH UNIT No. 3
PSC 824 BOX 23
FPO AE 09623

IN REPLY REFER TO

3900
Ser 00/0210
03 June 2020

From: Commanding Officer, U.S. Naval Medical Research Unit No.3
To: LCDR Terrel Sanders

Subj: APPROVAL ON DETERMINATION OF HUMAN SUBJECT RESEARCH – INITIAL REVIEW FOR PROJECT # NAMRU3.PJT.2019.0007 TITLED "NEXT GENERATION SEQUENCING TO ASSESS ANTIMICROBIAL RESISTANCE IN STORED *NEISSERIA GONORRHOEAE* ISOLATES", PI LCDR TERREL SANDERS

Encl: (1) NMRC IRB Approval on Determination of Human Subject Research – Initial Review

1. Enclosure (1) includes NMRC IRB Chair's approval on your request of Determination of Human Subject Research for the Initial Review of project # NAMRU3.PJT.2019.0007. The NMRC IRB Chair has reviewed your request and determined that the proposed project, as submitted, does not meet the definition of a human subject research and therefore; may proceed without further review by the NMRC IRB. However, if the nature of the activity changes such that it may meet the definition of human subject research, you must report such changes to the NMRC IRB Chair or Vice-Chair for further verification.

2. Any agreement required to secure the samples is your responsibility to obtain prior to initiating this project.

3. Once the project is complete, it is your responsibility to notify the NMRC Office of Research Administration (ORA).

4. Giving the authority granted to me via CNO ltr 3900 Ser N093/19U0086 of 2 Oct 2019, I reviewed Enclosure (1) and I concur with the NMRC IRB Chair's determination as I approve the initiation of subject project.

MONTEVILLE.MARSHALL.RENO.1179872869

M. R. MONTEVILLE

Copy to:
Study file
NMRC ORA