Molecular characterization of the *tetM* gene in clinical isolates of *Neisseria gonorrhoeae* in KwaZulu-Natal, South Africa

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DECLARATION

I, Miss Santhuri Rambaran, declare as follows:

1. That the work described in this dissertation has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.
2. That my contribution to the project was as follows: collection of specimens from the patients and all routine and experimental work (growth of specimens from patients on solid media, biochemical tests for identification, storage of isolates, antimicrobial susceptibility testing (MIC), isolation of DNA and PCR for the genotypic component as well as all the media required) described in this dissertation was carried out by myself in the Department of Infection Prevention Control and Medical Microbiology, Nelson R Mandela School of Medicine under the supervision of Professor P. Moodley.

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PRESENTATIONS

Oral Presentations

1. **Tetracycline resistance in *Neisseria gonorrhoeae***

   Santhuri Rambaran, A. Willem Sturm and Prashini Moodley

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2. **Tetracycline resistance in *Neisseria gonorrhoeae***

   Santhuri Rambaran, A. Willem Sturm and Prashini Moodley

   6th FIDSSA Conference 2015: Emerging Threats, Drakensberg, KwaZulu-Natal

3. **Increased MICs of Ceftriaxone and Cefixime in *Neisseria gonorrhoeae* in KwaZulu-Natal, South Africa**

   Santhuri Rambaran, A. Willem Sturm and Prashini Moodley

   6th FIDSSA Conference 2015: Emerging Threats, Drakensberg, KwaZulu-Natal

DEDICATION

For you,

Dad, Mum, Shaveer and Santrika

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To my creator, for providing me with this opportunity. ***"Sada Bhavani Dahini, Sanmukha Rahe Ganesh Panch Deo Mil Raksha Karahin, Brahma Vishnu Mahesh, Pratah Kal. Twameva Mata cha Pita Twameva, Twameva Bandhu cha Sakha Twameva, Twameva Vidya Dravinam Twameva, Twameva Sravam Mama Deva Deva"***

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AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
Bps	Base pairs
CDC	Centers for Disease Control and Prevention
CHC	Community Health Centre
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CT	*Chlamydia trachomatis*
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetra-acetic Acid
ESCs	Extended Spectrum Cephalosporins
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FWD	Forward
g	Grams
GASP	Gonococcal Antimicrobial Surveillance Programme
GC	Gonococcus
HIV	Human Immunodeficiency Virus

IM	Intramuscular
IPC	Infection Prevention Control
KZN	KwaZulu-Natal
LCAT	Lincomycin, Colistin, Amphotericin and Trimethoprim
MDR	Multi-Drug Resistant
MDa	Megadalton
MIC	Minimum Inhibitory Concentration
mL	Millilitres
mM	milli-Molar
MSM	Men who have sex with men
NAATs	Nucleic Acid Amplification Tests
NG	*Neisseria gonorrhoeae*
Nm	Nanometers
NYC	New York City
PABA	p-aminobenzoic acid
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
Pm	Picomoles
PMB	Pietermaritzburg
PPNG	Penicillinase Producing *Neisseria gonorrhoeae*

REV	Reverse
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
SA	South Africa
SNPs	Single Nucleotide Polymorphisms
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
TBE	Tris-Borate-EDTA
TRNG	Tetracycline Resistant *Neisseria gonorrhoeae*
UK	United Kingdom
UKZN	University of KwaZulu-Natal
µl	micro-liter
USA	United States of America
V	Volts
WHO	World Health Organization
XDR	Extensively- Drug Resistant

ABSTRACT

Neisseria gonorrhoeae, a Gram negative bacterium, causes the sexually transmitted infection, gonorrhoea. This organism is known to rapidly develop resistance and has over the years evolved into a highly drug resistant species. Treatment options for gonorrhoea are now limited as *N.gonorrhoeae* has developed resistance to previously and currently recommended antimicrobial drugs. Effective treatment has become a challenge in many parts of the world including KwaZulu-Natal, South Africa. Resistance to third generation cephalosporins that are one of the remaining options for monotherapy are being reported globally. In this study, an evaluation of susceptibility of previously (ciprofloxacin, ofloxacin, penicillin and tetracycline) and currently (azithromycin, cefixime and ceftriaxone) used antimicrobials to currently circulating strains of *N.gonorrhoeae* was undertaken. MICs were determined using the agar dilution with multipoint inoculation method. Further, characterization of resistance mechanisms amongst tetracycline resistant isolates was done by PCR and gel electrophoresis. The *tetM* gene is known to confer high level tetracycline resistance and there are 2 known variants of this gene which are the American and Dutch respectively.

A total of 1220 patients were recruited from male and female patients presenting to 2 clinics: Boom Street Clinic in Pietermaritzburg, Umgundundlovu and Umlazi Section D Clinic in Umlazi, eThekwini. Three hundred and nineteen isolates of *N.gonorrhoeae* was obtained from both clinics. The results from this study have shown that despite the previously used antimicrobials not being in the current treatment management of sexually transmitted infections (STIs), *N.gonorrhoeae* isolates remain resistant. All isolates (100%) showed high level tetracycline resistance with MIC values ranging from 16 mg/L to ≥32mg/L. Ninety eight (98 %) percent of isolates displayed MIC values in the intermediate and resistant range for penicillin, 70% were resistant to the fluoroquinolones and 68% were resistant to azithromycin.

Two isolates were resistant to cefixime, but not to ceftriaxone. The American and Dutch variants of the *tetM* gene was found in 264 and 29 isolates respectively. Twenty six isolates did not harbour any *tetM* gene. This suggests alternative mechanisms of resistance and requires further investigation.

CHAPTER 1: INTRODUCTION

1.1 Background

Gonorrhoea, a sexually transmitted infection (STI) has become a significant public health concern amid growing antimicrobial resistance (Unemo & Shafer 2015). *Neisseria gonorrhoeae,* the aetiologic agent of the infection has been referenced in biblical times, making it one of the oldest diseases to have plagued mankind (Edwards & Apicella 2004). *N.gonorrhoeae,* was first identified by Albert Neisser in 1879 and cultured in the laboratory in 1882 (Edwards & Apicella 2004; Lewis 2007a). Sulfonamide was the first antimicrobial agent used to treat the disease in 1936, followed by penicillin in 1943 which resulted in a decrease in the prevalence of *N.gonorrhoeae* infections globally (Unemo & Shafer 2014; Edwards & Apicella 2004).

The dawn of the 21st century was met with global increase in the prevalence of *N.gonorrhoeae* infection. This was due the emergence of drug resistant strains of the organism. Recent literature has reported *N.gonorrhoeae* strains with documented resistance to almost all classes of antimicrobials including sulfonamides, cephalosporins, tetracyclines, penicillins, macrolides and fluoroquinolones (Unemo & Shafer 2015). Resistance is mediated via numerous mechanisms including drug inactivation, alteration of the drug target, expulsion of the drug via efflux mechanisms and changes in permeability of the bacterial cell (Patel *et al*. 2011).

Antimicrobial resistance is a major concern in South Africa (SA). The first penicillinase producing *N.gonorrhoeae* (PPNG) was reported in Durban and Johannesburg in 1977 (Robins-Browne *et al*. 1977; Hallett *et al*. 1977). Further concerns regarding resistance to gonorrhoea drug therapy in KwaZulu-Natal (KZN) were highlighted in 2006 following a study that

reported that 60% of patients attending the Prince Cyril Zulu Centre for Communicable Diseases in Durban, which houses one of the largest STI clinics in the province. and 29% of patients attending a Johannesburg clinic that was monitoring urethral culture from men with MUS attending Esselen Street Clinic in central Johannesburg and Alexandra Health Centre which are primary health care facilities displayed resistance to ciprofloxacin (Moodley & Sturm 2005; Lewis 2007.; Kahn 2006). One of the factors driving antimicrobial drug resistance is the inappropriate utilization of antibiotics. Surveillance systems to monitor drug susceptibility of the organism responsible do not feature on the national STI control program in South Africa. Screening of drug resistance occurs only in research settings (Moodley & Sturm 2005).

Mismanagement of *N.gonorrhoeae* infection can lead to severe complications including pelvic inflammatory disease, infertility, neonatal conjunctivitis and blindness, male infertility and disseminated gonococcal infections (Edwards & Apicella 2004). The interaction of gonorrhoea infections with the human immunodeficiency virus (HIV) epidemic is a further concern in South Africa. The inflammatory response and compromised mucosal integrity associated with *N.gonorrhoeae* infection has been reported to increase acquisition of HIV subtype 1 (Edwards & Apicella 2004).

Treatment of resistant strains of *N.gonorrhoeae* is currently limited to the extended spectrum cephalosporins (ESCs) viz: cefixime and ceftriaxone (Unemo & Shafer 2014). These drugs form the first line empirical regime in most countries. However, susceptibility to these agents have decreased globally (Cámara *et al*. 2012; Ohnishi *et al*. 2011; Unemo *et al*. 2012). Recently, multi-drug therapy has been advocated in an effort to delay the emergence of resistance (CDC 2015). However, these might not be affordable in less-resourced settings and

may not be sufficient to alleviate the emergence of resistance and spread of drug resistance (Unemo & Shafer 2014).

1.2 Epidemiology

Gonorrhoea ranks as the most common bacterial STI in the world. In its 2008 global STI report, the World Health Organization (WHO) estimated that more than 498 million incident cases of STI's are diagnosed annually (WHO 2012). Of these cases, 106 million cases were attributed to *N. gonorrhoeae* infection. A substantial increase in the number of cases of gonorrhoea infections were reported from 2005 to 2008 with 87 million cases in 2005 to 106 million cases in 2008. An overall increase of 21% in the global incidence of *N.gonorrhoeae* infections were reported (WHO 2012). The reported prevalence of *N. gonorrhoeae* infections was 36 million adult infections at any point during 2008. The WHO estimate of incident STI cases for the African Region was 93 million. Of these cases, 21 million were attributed to *N. gonorrhoeae* infections. Accurate reporting systems for STI infections outside Western Europe and North America are lacking. This includes South Africa, were the incidence of STIs including *N. gonorrhoeae* infections is unknown. The data on STI's in South Africa are sourced from surveys of women attending antenatal and family planning clinics (Lewis & Marumo 2009). The incidence of *N.gonorrhoeae* in the African region in 2008 was 18.5 and 27.6 per 1000 in females and males respectively (WHO 2012). The incidence rate of STIs among young women in KZN, SA was reported to be 20/100 women-years while the prevalence of STIs was 13% (Naidoo & Wand 2014). Other studies in SA have also reported similar STI incidence rates (Kapiga *et al*. 2009; Mlisana *et al*. 2012; Feldblum *et al*. 2010). Within South Africa, *N.gonorrhoeae* is known to cause 70-80% of male urethritis syndrome and accounts for approximately 15% of vaginal discharge cases (Lewis 2009).

1.3 Physical Characteristics, transmission and clinical manifestations of *Neisseria gonorrhoeae*

N.gonorrhoeae is a Gram-negative diplococcus that has the ability to colonise and invade the mucosal surfaces of the endocervix, urethra, anus, conjunctiva and pharynx. *N.gonorrhoeae* belongs to the order Neisseriales, family Neisseriaceae and genus *Neisseria*. Gonococci are non-sporulating and non-flagellated bacteria. *N.gonorrhoeae* has components such as outer membrane proteins, lipopolysaccharides and pili that assist in the binding and attachment of bacteria to epithelial cells which makes it a very successful pathogen (Tapsall 2001). *N.gonorrhoeae* is able to undergo genetic variation whereby the microbe is able to incorporate new genetic material by either transformation or conjugation (Patel *et al*. 2011). This process has allowed *N.gonorrhoeae* to acquire penicillinase producing plasmids (Patel *et al*. 2011). *Neisseria gonorrhoeae* has also been able to acquire genetic material from related organisms and this is antigenic variability (Patel *et al*. 2011).

There are two species in the genus *Neisseria* that are known to be pathogenic to humans namely; *N.gonorrhoeae* and *N.meningitidis*. There are roughly 30 nonpathogenic species which include; *N.lactamica, N.sicca, N.cinerea, N.flavescens, N.subflava*, and *N.mucosa* commonly found in the nasopharynx of young children and upper respiratory tract which may exist as part of the normal flora (Barth *et al*. 2009).

Close sexual contact with an individual harboring the bacteria is the primary mode of transmission of the disease (Patel *et al*. 2011). Transmission from mother to child during childbirth is also a mode of transmitting the disease. Women are disproportionately affected by the disease. The disease is more effectively transmitted from men to women with a probability of 50-70% compared to transmission from women to men which records a probability of 20-35% (Edwards & Apicella 2004). This may be attributed to anatomical differences in the in

male and female genitalia as well as the duration of contact of the bacteria with the target membranes after exposure (Edwards & Apicella 2004).

Clinical presentation of the disease is affected by the nature of the infecting strain and the site of infection. Most gonococcal infections in women result in asymptomatic infection or mild symptoms. These include mild irritation and discharge. These symptoms may only become significant once infection spreads to the upper genital tract. In men, the symptoms of urethritis and discharge are more evident than in women. The disease has been reported to disseminate leading to chronic joint infection and sepsis. Clinical manifestation in neonates include conjunctivitis, blindness, sepsis and joint infection (Edwards & Apicella 2004).

1.4 Diagnosis of *N.gonorrhoeae*

The diagnosis of *N.gonorrhoeae* is established by the visualization of the organism on light microscopy, culture or nucleic acid amplification tests (NAATs). *N.gonorrhoeae* can be detected by Gram- and methylene blue staining techniques utilizing light microscopy (Unemo *et al* 2013). Microscopy is not recommended as the only method for diagnosis due to its low sensitivity, especially in female patients. To date, culture remains as the gold standard for the diagnosis of *N.gonorrhoeae*. This method has high sensitivity and specificity compared to microscopy and remains the only established method for antimicrobial susceptibility testing (Unemo *et al* 2013). However the sensitivity and specificity of these tests are highly dependent on the specimen. For symptomatic patient specimens, microscopy is highly specific and sensitive however in asymptomatic patient specimens, microscopy sometimes fails to detect the presence of the organism. In resourced settings, NAATs are rapidly replacing culture techniques. This method has a higher sensitivity than the preceding diagnostic techniques; however its ability to detect non-viable organisms leads to false positives (Unemo *et al* 2013).

A disadvantage of NAATs is that isolates for susceptibility testing are not available. The sensitivity of detection for pharyngeal and rectal specimens is greatly increased NAAT testing. Cervical, vaginal and urethral swabs can be used for GC NAAT testing, not only urine.

1.5 Antimicrobial Susceptibility Testing

The global dissemination of antimicrobial resistant strains of *N.gonorrhoeae* highlights the urgent need for periodic surveillance and antimicrobial susceptibility testing. The disk diffusion method differentiates between drug resistant and drug susceptible strains but does not determine the actual MIC of the infecting strain. This technique utilizes discs containing known antimicrobial concentrations. Growth around the discs determines the level of resistance of the organism. The minimum inhibitory concentration (MIC) for the various antimicrobials used to treat *N.gonorrhoeae* infection is obtained using the agar dilution, or E-test® methods. The agar dilution method is the gold standard for MIC determination of *N.gonorrhoeae.* The test is done on Gonococcus (GC) media supplemented with yeast autolysate and horse blood. The E-test® method of MIC determination makes use of an encoded plastic strip the with the antimicrobial drug concentration on a decreasing gradient. (Unemo *et al* 2013). Incubation of the strip with the bacterial isolate produces an ellipse. The MIC is recorded at the point of intersection of the ellipse and the gradient scale marked on the strip. (Unemo *et al* 2013).

1.6 Treatment of *N.gonorrhoeae*

Sexually Transmitted Diseases (STDs) in South Africa are managed syndromically in the public sector. In the private sector, often an attempt at laboratory diagnosis is made. The aim of syndromic management is to ensure that patients presenting with signs and symptoms are treated at the first point of contact with high quality care. This eliminates the delays of laboratory testing. Easy-to-follow algorithms enables primary healthcare workers to manage

the STDs (Unemo *et al* 2013). The approach of syndromic management of symptomatic STIs has been practiced in South Africa for close to 20 years. Some of the antibiotics that are listed in sections 1.6.1 are still used for mono- or dual therapy in remote communities such as Western Australia where the prevalence is not high (Lahra *et al* 2016).

1.6.1 Previously used Antimicrobials to treat infection with *N.gonorrhoeae*

1.6.1.1 Sulfonamides

Sulfonamides were the earliest class of antimicrobials used to treat gonorrhoea infections (Dillon *et al*. 2015). Sulfanilamide was discovered by Gerhard Gomagk in 1935 (Oriel 1994; Lewis 2010). The first studies that made use of sulphanilamide for the treatment of gonococci were in 1937 in USA, UK and Germany (Kampmeier 1983). In 1940-41, sulfapyridine became available for treatment. Resistance to sulphonamides emerged in 1944 and resulted in treatment failure in over 30% of patients (Kampmeier 1983). By the end of that decade widespread resistance *in vitro* was documented in approximately 90% of gonococcal isolates (Lewis 2007).

1.6.1.2 Penicillin

Penicillin was the drug of choice for gonorrhoea treatment following its introduction in 1944 (Patel *et al*. 2011). Penicillin was discovered by Alexander Fleming in 1929 (Fleming 1980) and was known as the "wonder drug" as it was used to treat numerous infectious diseases (Sternberg & Turner 1944; Mahoney *et al*. 1943). Penicillin remained the treatment option for gonorrhoea for decades with increasing drug dosages being applied to treat the infection until resistance to the drug was documented. (Patel *et al*. 2011). Penicillin was first used to treat urethritis in 1943 (Dillon *et al*. 2015). Treatment failure was reported in the early 1950s, associated with the emergence of penicillinase producing *Neisseria gonorrhoeae* (PPNG) in

1976 and the emergence of two types of β-lactamase encoding plasmids (Ashford *et al*. 1975). These plasmids originated in Southeast Asia and sub-Saharan West Africa and result in high-level penicillin resistance (Ashford *et al*. 1975; Percival *et al*. 1976; Phillips 1976). Penicillin was discontinued as the first line antimicrobial drug in many parts of the world following the emergence of chromosomally mediated resistant gonorrhoea (Unemo & Shafer 2014). This resulted in worldwide discontinuation of penicillin for gonorrhoea treatment by 1985 (Dillon *et al*. 2015).

1.6.1.3 Tetracycline

Tetracycline was discovered in 1945 by Benjamin Minge Duggar (Zakeri & Wright 2008). Tetracycline became the alternate treatment option amid increasing penicillin resistance. (Patel *et al*. 2011). It is also utilized in patients that are allergic to penicillin (Reyn *et al*. 1958). The use of this drug is not recommended as several doses are required over an extended period. This brings into play the issue of compliance and exerts selective pressure on *N.gonorrhoeae* leading to the development of chromosomally mediated resistance to tetracycline (Dangor *et al*. 2010; Dillon *et al*. 2015). The drug is not administered to pregnant women and small children as it is associated with discolouration of the teeth in children and suppression of skeletal growth in premature infants (Speer *et al*. 1992). Tetracycline was one of the most extensively used antimicrobials during the 1950s and 1960s in the United States as it was relatively cheap, had few side effects and displayed broad spectrum activity (Speer *et al*. 1992). In 1985, reports of plasmid mediated resistance to tetracycline emerged in the United States of America (USA) (Roberts *et al*. 1988). This was the first report of the *tetM* plasmid and was designated the American *tetM* plasmid (Morse *et al*. 1986). The Dutch variant of the *tetM* plasmid was first described in 1991 (Gascoyne *et al*. 1991). Tetracycline was excluded from

the treatment guidelines in the USA following the emergence of the *tetM* determinant that was responsible for high-level tetracycline resistance (Morse *et al*. 1986). In South Africa, high-level tetracycline resistance was first reported in Bloemfontein(Chalkley *et al*. 1997).

1.6.1.4 Spectinomycin

Spectinomycin, discovered in 1961, was recommended for the management of *N.gonorrhoeae* infections following the emergence of penicillinase producing *N.gonorrhoeae* cases (Lewis and Lukehart 2011). Reports of resistance to spectinomycin emerged from the Netherlands in 1967 (Stolz *et al*. 1975), Philippines in 1981 (Ashford *et al*. 1981) and London, United Kingdom in 1983 (Ison *et al*. 1983) . This led to the worldwide discontinuation of the drug as first-line monotherapy treatment for gonorrhoea (Unemo & Shafer 2014). A recent study in the province of Eastern Cape of South Africa showed that spectinomycin is an effective treatment option for patients with gonococcal infections (Faye *et al*. 2015). However in South Africa, due to the high cost and the lack of availability of spectinomycin, its use is limited (Lewis & Lukehart 2011)

1.6.1.5 Quinolones

In the 1960s synthetic quinolone antimicrobials were discovered by George Lesher and coworkers (Lesher *et al*. 1962). Fluoroquinolones used to treat gonorrhoea included ciprofloxacin and ofloxacin. From mid to late 1980s ciprofloxacin was widely used to treat gonococcal infections. Reports of clinical failures emerged in the 1990s (Gransden *et al*. 1990; Unemo & Shafer 2014). Resistance to ciprofloxacin has been noted in the Far East, South-East Asia, Australia, UK and USA (Dangor *et al*. 2010). Resistance to fluoroquinolones was first reported in the late 1980s in the Asia-Pacific regions which led to discontinuation of the use of fluoroquinolones for gonorrhoea treatment in the mid-2000s (Dillon *et al*. 2015). The United

States (US) Centers for Disease Control and Prevention (CDC) removed this class of antibiotics from its treatment guidelines in 2007 (Unemo & Shafer 2014). Up until 2002 in KZN, SA, isolates were shown to be still susceptible to ciprofloxacin and only in 2003 treatment failures were reported with ciprofloxacin resistance (Moodley & Sturm 2005). In KZN, the prevalence of ciprofloxacin resistant *N.gonorrhoeae* increased from 24% in 2004 to 42% in 2005 (Moodley & Sturm 2005). Ciprofloxacin resistant *N.gonorrhoeae* exceeded 40% in Durban by 2005 and approximately 30% in Gauteng which led to use being discontinued in 2008 (Lewis 2011). In 2004 the prevalence of ciprofloxacin resistance was determined by the National STI Surveillance Programme which reported 24% in Durban, 11% in Johannesburg, 10% in Umtata and 8 and 7% in Pietermaritzburg and Cape Town respectively (Lewis 2007a). The National STI Surveillance Programme is run by the National Institute of Communicable Diseases STI Reference Centre. Data is collected from sentinel sites across the country from primary health care facilities, as well as microbiological surveillance of STIs from microbiology departments based at different universities. In 2008 the National Department of Health released new guidelines for the treatment of STIs in which oral cefixime became the first line treatment of gonococcal infections (Lewis 2011).

1.6.1.6 Macrolides

This class of antimicrobials was discovered in 1952. Erythromycin, the first drug in this class was isolated from *Streptomyces erythraeus,* a soil microorganism (Unemo & Shafer 2014). The synthetic derivative of erythromycin, azithromycin was developed in 1980 (Unemo *et al* 2013). However, this derivative displayed enhanced activity against *N.gonorrhoeae* in comparison to erythromycin (Unemo *et al* 2013). Bignell *et al.* conducted a study to determine the effectiveness of azithromycin as a single dose/ monotherapy in the treatment of gonorrhoea. The results of this study support the use of this drug in combination with a third generation

cephalosporin rather than monotherapy (Bignell & Garley 2010). This subsequently led to the introduction of the drug in dual-antimicrobial therapy regimens in the USA and Europe (Bignell & Unemo 2013; Unemo & Shafer 2014).

1.6.2 Current antimicrobials used to treat *N.gonorrhoeae*

1.6.2.1 Cephalosporins

Cephalosporins were discovered by Guiseppe Brotzu in 1948. Cephalosporin compounds were isolated from cultures of the fungus *Cephalosporium acremonium* (CDC 2005). Cefixime (oral) and ceftriaxone (injectable) are common cephalosporins recommended for treatment of *N.gonorrhoeae* globally (Unemo & Shafer 2014). Cephalosporins are used in combination with metronidazole and doxycycline for the management of discharge causing STIs (National Department of Health South Africa 2015) . Reports of resistance to these agents emerged as early as 2001 in Japan and there were approximately 30% of *N.gonorrhoeae* isolates displaying reduced susceptibility to cefixime (Ohnishi *et al.* 2010; Lewis and Lukehart 2011; Ochiai *et al.* 2008). Treatment failures to cefixime has been reported in Japan, Australia, European countries, South America and Canada (Unemo *et al*. 2012; Deguchi *et al*. 2003; Yokoi *et al*. 2007; Allen *et al*. 2013; Ison *et al*. 2011; Lewis *et al*. 2013; Unemo *et al*. 2011; Unemo *et al*. 2010; Ohnishi *et al*. 2011). Ceftriaxone treatment failures for pharyngeal gonorrhoea have been reported in Japan, Australia and some European countries (Ohnishi *et al*. 2011; Chen *et al*. 2013; Read *et al*. 2013; Unemo *et al*. 2011; Unemo *et al*. 2012). Due to the reports of increased MIC's to oral cephalosporins in 2006, cefixime was discontinued as the first line therapy for gonorrhoea in Japan (Barry & Klausner 2010). Ceftriaxone is the only remaining agent that may be utilized as first line monotherapy. However, due to the emergence of highly resistant strains of *N.gonorrhoeae,* there are concerns of transmission of these resistant strains. This may

render monotherapy ineffective (Unemo & Shafer 2014). In 2011, an untreatable strain of *N.gonorrhoeae* was reported in Japan (Shimuta *et al*. 2013) . This strain was named the HO41 strain and documented resistance to all known antibiotics and was the first extensively drug resistant (XDR) strain to be reported (Shimuta *et al*. 2013). In South Africa, the first report of cefixime resistance was identified in 2013 in Gauteng. There were two cases reported among men who have sex with men (MSM) patients (Lewis *et al*. 2013). A study in Eastern Cape recently reported decreased susceptibility of *N.gonorrhoeae* isolates to third generation cephalosporins (Faye *et al*. 2015).

1.6.3 Future management of *N.gonorrhoeae*

Numerous microorganisms have evolved and developed resistance over time leading to diminished treatment options. This subsequently leads to use of multi drug therapy, which is a combination of antimicrobials with different mechanisms of action that decreases the propensity of resistance development. The proposed treatment combinations for gonococcal infections are one of the cephalosporins and azithromycin (Bignell & Fitzgerald 2011). Single doses of ceftriaxone (250mg IM) plus azithromycin (1g orally)are currently recommended in the 2015 treatment guidelines for STIs (National Department of Health South Africa 2015). This dual antimicrobial therapy has been implemented in USA and Europe (CDC 2012) (Bignell & Unemo 2013). Where there is documented azithromycin allergy, doxycycline can be used in dual therapy with either cefixime or ceftriaxone (Workowski KA 2015). However, the cost of implementing dual therapy has serious implications for control programs in poorly resourced settings (Unemo & Shafer 2014).

1.7 Mechanisms of antimicrobial resistance

Prolonged antimicrobial exposure supports adaptation and survival mechanisms in bacteria. *N.gonorrhoeae* has developed or acquired various mechanisms of resistance to the antimicrobials that have been used to treat gonorrhoea over the years (Patel *et al*. 2011). These mechanisms allow *N.gonorrhoeae* to adapt to and survive in the human host (Unemo & Shafer 2014). *N.gonorrhoeae* can undergo transformation by altering its genetic material (Unemo & Shafer 2014).

N.gonorrhoeae exhibits two types of resistance chromosomally mediated, genetic transformation that is responsible for acquiring drug resistance and plasmid mediated resistance. The latter is restricted to penicillin and tetracycline, transmitted by conjugation (Patel *et al*. 2011). Plasmid mediated resistance is linked to the bla_{TEM} and *tetM* genes (Morse *et al*. 1986; Unemo & Shafer 2014).

1.7.1 Sulfonamide resistance

The mode of action of this antimicrobial is through competition with p-aminobenzoic acid (PABA) for the enzyme dihydropteroate synthase (DHPS) that prevents the formation of tetrahydrofolate, essential for DNA synthesis (Dillon *et al*. 2015). Mutations linked to sulphonamide resistance were reported in the early 1940's. A decade later, most *N.gonorrhoeae* isolates were resistant to various sulphonamides (Dillon *et al*. 2015). Excessive synthesis of PABA dilutes the antimicrobial concentration and may induce alteration in the *folP* gene leading to sulfonamide resistance (Unemo & Shafer 2014). The *folP* gene encodes the drug target DHPS. Alterations in the DHPS target results in lower affinity for sulfonamide agents (Unemo & Shafer 2014).

1.7.2 Penicillin resistance

Resistance to penicillin is mediated by genetic mutations and the acquisition of plasmids. Penicillin acts by inhibiting the formation of peptidoglycan cross-links in the bacterial cell wall through binding of the β-lactam ring to transpeptidase enzymes (penicillin-binding proteins [PBPs]) (Unemo & Shafer 2014). High-level penicillin resistance in *N.gonorrhoeae* is mediated by bla_{TEM-1} plasmids which encode TEM-1 βlactamase (Patel *et al*. 2011; Unemo & Shafer 2014). There have been reports of the global dissemination of *N.gonorrhoeae* isolates that bear the Asian (4.4 MDa) and African (3.2 MDa) plasmids (Tapsall *et al*. 2014; Speer *et al*. 1992). The African plasmid was more prominent in South Africa (Dangor *et al*. 2010). There are other types of β-lactamase producing plasmids which are Toronto, Rio, Nîmes, New Zealand and Johannesburg (Brett 1989; Dillon & Yeung 1989; Gouby *et al*. 1986; Müller *et al*. 2011; Pagotto *et al*. 2000; Palmer *et al*. 2000). Strains of PPNG was first reported in 1977 in South Africa in Johannesburg and Durban clinics (Hallett *et al*. 1977; Robins-Browne *et al*. 1977). KwaZulu-Natal (KZN) had the highest rates of PPNG during 1999/2000.

Increase in penicillin resistance was associated with an additive effect of multiple chromosomal mutations resulting in altered penicillin binding proteins, increase in the antibiotic efflux system and a probable decrease in the antibiotic uptake from the membrane (Patel *et al*. 2011). Mutations in the *ponA* gene which encodes PBP 1 is involved in high level of penicillin resistance (Unemo *et al*. 2012). PBP 2 is encoded by the *penA* gene which is the lethal target for β-lactam antibiotics (Powell *et al*. 2009). There has been 5 to 9 mutations in the *penA* gene of penicillin resistant *N.gonorrhoeae* strains (Ropp *et al*. 2002). Insertion of an aspartic codon (Asp345a) in the *penA* gene along with downstream mutations causes a reduced affinity of penicillin binding protein 2 (Lindberg *et al*. 2007). Alterations in PBP1 and PBP2 are

associated with a decreased affinity for penicillin (Patel *et al*. 2011).

1.7.3 Tetracycline resistance

Tetracycline acts by binding to bacterial ribosome thereby inhibiting protein synthesis (Speer *et al*. 1992). Tetracycline resistance is mediated by genetic mutations and the acquisition of plasmids. Chromosomal resistance is linked to mutations in the *mtrR*, *penB* and *rpsJ* genes that modify the ribosomal protein that is the target of tetracycline (Unemo & Shafer 2014). Bacteria have the ability to develop tetracycline resistance using 3 strategies: (i) by restricting access of tetracycline to the ribosome, (ii) modifying the ribosome to prevent effective binding of tetracycline and (iii) by producing tetracycline inactivating enzymes. (Speer *et al*. 1992). The first strategy of limiting access of tetracycline is via efflux pump mechanisms. Bacteria that carry genes encoding efflux pumps produce a cytoplasmic membrane protein which pump tetracycline out of the cell at the same rate as it is pumped in (Speer *et al*. 1992). The second strategy occurs by bacteria carrying a resistant gene that protects the ribosome and allows the ribosome to proceed with protein synthesis. The third strategy is, bacteria carrying a tetracycline modification resistant gene that produces an enzyme which chemically modifies tetracycline to an inactive form which diffuses out of the cell (Speer *et al*. 1992). This allows protein synthesis to continue.

Adjunct to these target modifications, an increased and decreased influx of tetracycline due to the *mtrR* and *penB* resistance determinants result in enhanced tetracycline resistance (Folster *et al*. 2009; Ohneck *et al*. 2011; Olesky *et al*. 2002; Olesky *et al*. 2006; Zhao *et al*. 2009).

The high level of tetracycline resistance in *Neisseria gonorrhoeae* is mediated by the acquisition of the *tetM* gene carried on a conjugative plasmid (Hakenbeck & Coyette 1998). Acquistion of the *tetM* gene offers protection to the ribosome and confers resistance to

tetracycline (Hakenbeck & Coyette 1998). In 1998/1999, 51% of gonococcal isolates in KZN were reported to be tetracycline resistant *N.gonorrhoeae* (TRNG) (Dangor *et al*. 2010). In *N.gonorrhoeae,* the *tetM* gene has 2 variants viz: the American and Dutch types. Molecular epidemiology studies report that the American type may have originated on the African continent while the Dutch type may have originated in the Far East. (Turner *et al*. 1999).

A study in KZN in 2001, reported the prevalence of the American variant of the gene and no occurrences of the Dutch variant of the gene in any of the isolates screened in the study. (Moodley *et al*. 2001). The Dutch variant was detected in Johannesburg amongst clinical isolates collected during 2008. However, this variant was not as prevalent as the American variant (Fayemiwo *et al*. 2011).

1.7.4 Spectinomycin resistance

Spectinomycin acts by inhibiting protein translation by binding to the 30S ribosomal subunit of 16S rRNA gene. (Unemo & Shafer 2014). During polypeptide elongation, the interaction of spectinomycin with 16S rRNA blocks EF-G catalysed translocation of the peptidyl-tRNA from the A site to the P site (Ramakrishnan & White 1992; Sigmund *et al*. 1984). High level spectinomycin resistance in *N.gonorrhoeae* is linked to Val25 and K26E alteration in the 30S ribosomal protein 5S encoded by the *rpsE* gene (Unemo *et al*. 2013). Low level resistance is associated with T24P mutation in 5S. This results in a disruption of binding to 16S rRNA gene (Magnus Unemo *et al*. 2013).

1.7.5 Quinolone resistance

Fluoroquinolones act by binding to the DNA gyrase and topoisomerase IV enzymes, inhibiting DNA replication (Unemo & Shafer 2014). The DNA gyrase and topoisomerase IV and type II

topoisomerases are significant for DNA metabolism (Unemo & Shafer 2014). GyrA and GyrB subunits make up DNA gyrase, encoded by the *gyrA* and *gyrB* genes respectively. Topoisomerase IV consists of the ParC and ParE subunits, encoded by the *parC* and *parE* genes respectively (Unemo & Shafer 2014). High-level, clinically relevant resistance is mediated by alteration of the target sites. Fluoroquinolone resistance is a result of alterations in *gyrA* and *parC* genes as well as efflux activation and decreased antibiotic cell permeation (Patel *et al*. 2011). Variations in *ParC* seem to occur in conjunction with presence of mutations affecting *gyrA* (Patel et al. 2011).

1.7.6 Macrolide resistance

Macrolides act by inhibiting protein synthesis. Resistance to erythromycin and azithromycin is mediated by single nucleotide polymorphism (SNP) in 23S rRNA, the binding site of macrolides. This leads to either low or high level resistance (Dillon *et al*. 2015). Efflux pump mediated macrolides resistance is mediated by the *mef* gene expression (Dillon *et al*. 2015). Mutations in the *mtr* gene result in an overexpression of an enhanced efflux pump of the antibiotic resulting in resistance (Dillon *et al*. 2015). Low level resistance to azithromycin has been reported to occur by the methylation of 23S rRNA by RNA methylases encoded by *ermB*, *ermC* and *ermF* (Dillon *et al*. 2015). Resistance to rifampicin, azithromycin and erythromycin is conferred by mutations in the *mtrR* gene and increased expression of the MtrCDE efflux pump system (Tanaka *et al*. 2006). Azithromycin is not recommended as monotherapy for a single dose treatment of gonorrhoea infections due to the concern of rapid emergence and spread of resistance and is therefore recommended for use in combination with other drugs (Dillon *et al*. 2015).

1.7.7 Cephalosporin resistance

Cephalosprins are β-lactam antimicrobials and with the same mode of action as penicillin antibiotics. Mutations in the target PBPs are responsible for resistance to cephalosporins in *N.gonorrhoeae* (Unemo & Shafer 2014). The most common mechanism of resistance to ESCs is the acquisition of the *penA* mosaic allele by horizontal gene transfer (Dillon *et al*. 2015). Mutations/alterations in the *penA* encoded penicillin binding protein 2 (PBP2) is responsible for cefixime resistance. Minor contributions of the *mtrR* and *ponB* genes may mediate cefixime resistance. Ceftriaxone resistance is linked to all three of these genes (Dillon *et al*. 2015; Ohnishi *et al*. 2010). The *penA* gene contains 60 to 70 amino acid alterations. However, it lacks the Asp345A insertion that is found in penicillin resistant *N.gonorrhoeae* (Lindberg *et al*. 2007; Ohnishi *et al*. 2011). The acquisition of a *penA* mosaic allele and alterations to the A501, G545 and P551 in the transpeptidase/β-lactam binding domain of PBP2 is associated with the decreased susceptibility to ceftriaxone (Unemo *et al*. 2012). The HO41 XDR strain displayed high level resistance to cephalosporins (cefixime and ceftriaxone). The strain displayed novel *penA* mosaic allele with 12 additional amino acid alterations (Ohnishi *et al*. 2011; Unemo & Shafer 2014). *N.gonorrhoeae* F89 is an XDR strain that has emerged from Europe (Unemo *et al*. 2012). These are the first 2 XDR strains that have been reported thus far (Goire *et al*. 2014). The first 2 cases of cefixime resistant *N.gonorrhoeae* reported in SA showed that they had the mosaic *penA* gene detected by PCR and the full-length sequencing showed that these 2 isolates had the identical *penA* mosaic allele XXXIV (Lewis *et al*. 2013). This allele has been correlated with decreased susceptibility to 3rd generation cephalosporins and cefixime treatment failure in many countries (Unemo & Nicholas 2012; Hess *et al*. 2012; Pandori *et al*. 2009; Ohnishi *et al*. 2011).

1.8. Problem Statement

Recent literature highlights the major concern regarding effective treatment of *N.gonorrhoeae* in many parts of the world including KZN amid the ever increasing burden of antimicrobial resistance. *N.gonorrhoeae*, a once curable disease has evolved into extensively drug resistant forms. Extended spectrum cephalosporins have been advised for treatment of *N.gonorrhoeae* infection. However, reports of resistance to these agents have started to emerge. Due to the limitations in treatment options available for the eradication of the disease, we screened isolates currently circulating in the KZN province to determine the resistance profiles of the isolates. We also explore tetracycline that was previously used to treat *N.gonorrhoeae* infections and is currently part of syndromic management protocols to determine if this drug may be repurposed in our setting.

Aims of this study:

1. To determine the antimicrobial resistance profiles of *N.gonorrhoeae* isolates circulating in KwaZulu-Natal, South Africa.
2. To investigate if the *tetM* gene is responsible for high-level tetracycline in *Neisseria gonorrhoeae*

Objectives

1. To determine the MIC of the *N.gonorrhoeae* isolates to azithromycin, cefixime, ceftriaxone, ciprofloxacin, ofloxacin, penicillin and tetracycline.
2. To detect the presence of the *tetM* gene in tetracycline resistant *N.gonorrhoeae* isolates by PCR.

CHAPTER 2: MATERIALS AND METHODS

2.1. Ethics Approval and Informed Consent

The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal UKZN (BE220/13) as well as the Research Committee of the KwaZulu-Natal Department of Health (Appendix 6).

Patients

Male and female patients 18 years and older were recruited after signed informed consent was obtained. A clinical history was taken and the patients were examined.

2.2. Study Site

KwaZulu-Natal is one of the most populous Provinces in South Africa with approximately 10.3 million people. The Province is divided into 11 health districts. The eThekwini and uMgungundlovu Districts are home to the 2 largest cities in the Province viz: Durban and Pietermaritzburg respectively. The population of these cities account for approximately half of the provincial population.

Patients were recruited from two clinics within these cities:

The first site, East Boom Clinic is situated in the uMgungundlovu district in Pietermaritzburg. It is an urban Community Health Centre (CHC) that services patients from the surrounding urban and peri-urban areas. This CHC provides a comprehensive package of services and operates 24 hours per day. Patient recruitment occurred from 16 September 2013 to 1 October 2014 at this site.

The second site, Umlazi Section D Clinic is situated in the eThekwini district in Umlazi, Durban. This clinic also primarily serves an urban and peri-urban community. Patient recruitment occurred from 6 October 2014 to 19 December 2014 at this site.

2.3. Specimen Collection

Specimens were collected from patients presenting with male urethritis syndrome and vaginal discharge syndrome.

2.3.1. Male patients:

A sterile Dacron® urethral swab was inserted 2 to 3 cm into the urethra and rotated before removal.

2.3.2. Female patients:

A speculum was passed into the vagina to visualise the cervix. An endocervical swab was then inserted and rotated in the endocervix before removal.

The swabs were inoculated onto New York City (NYC) agar plates [prepared with GC agar base supplemented with Lincomycin, Colistin, Amphotericin and Trimethoprim (LCAT), yeast autolysate (Oxoid, England) and horse blood lysed with saponin (Oxoid, England)]. The inoculated plates were placed in a candle extinction jar and transported to the laboratory in the Department of Infection, Prevention and Control (IPC) situated at the Nelson R Mandela School of Medicine, University of KwaZulu-Natal (UKZN) within 4 hours.

2.4. Bacterial isolation of *N.gonorrhoeae*

In the laboratory, the inoculated plates were streaked using a four-way streak method to obtain single colonies. The plates were incubated for 48h at 37^{o}C in 5% CO_2.

Following the 48h incubation, plates were examined and a phenotypic assessment was made. Presumptive colonies of *N.gonorrhoeae* were identified based on the colony morphology, Gram stain characteristics, a positive oxidase reaction and acid production from glucose.

2.5. Phenotypic Characterization

2.5.1. Presumptive identification of *N.gonorrhoeae*

2.5.1.1. Colony morphology

N.gonorrhoeae colonies appear as grey, smooth, round shaped colonies on selective NYC agar plates.

2.5.1.2. Gram Stain

A Gram stain was performed to confirm the presence of Gram negative diplococci. A presumptive colony was picked off using a straight wire and smeared onto a glass slide. The smear was heat fixed and stained with crystal violet solution for 20 seconds. The slide was gently washed with tap water, followed by staining with iodine for 20 seconds. The slide was flooded with acetone for a few seconds and washed off with water. Carbol fucshin solution was added onto the slide for 20-50 seconds, the slide was rinsed, blot and air dried. The slide was examined microscopically for the presence of pink diplococci. *Staphylococcus aureus* (ATCC 25923) (Gram positive) and *Escherichia coli* (ATCC 25922) (Gram negative) were used as controls for the staining process.

2.5.1.3 Oxidase test

An oxidase test was performed to detect oxidase production. A commercial oxidase test strip (Merck, SA) was used to determine cytochrome c oxidase activity which is dependent on the presence of an intracellular cytochrome oxidase system. The intracellular cytochrome oxidase system catalyses the oxidation of cytochrome c by molecular oxygen, which serves as the terminal electron acceptor in the organism's electron transport system. A wooden orange stick was used to transfer a few colonies to the pink coated end of the strip. A positive reaction was recorded by observing a colour change to a dark blue or purple. *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used as negative and positive controls respectively.

2.6 Confirmation of *N.gonorrhoeae* isolates

Confirmatory tests were performed to differentiate *N.gonorrhoeae* from other *Neisseria* species.

2.6.1. Carbohydrate Utilization Test

The carbohydrate utilization test differentiates between *Neisseria* species based on their patterns of acid production from glucose, maltose, lactose and sucrose. These tests were conducted on agar plates prepared with GC agar base (Oxoid, England) incorporated with a phenol red indicator. The bacterial suspension was prepared by picking off colonies from the culture plate and transferring it into Brain Heart Infusion (BHI) broth (Oxoid, England) and incubating for approximately 30minutes at 37^{o}C in 5% CO_2 until the broth was turbid. 100µl of the bacterial suspension was spread evenly covering the entire surface of the agar plate.

Carbohydrate discs were prepared by saturating sterile 9mm antibiotic discs (Lasec, SA) with 50µl of 20% solutions of glucose, maltose, lactose and sucrose respectively (prepared as described in Appendix 1). Individual carbohydrate containing discs were placed onto the agar plate in respectively marked quadrants. The plate was incubated for 24h at 37°C in 5% CO_2.

Differentiation of fastidious organisms such as *N.gonorrhoeae* is based on fermentation reactions. Utilization of carbohydrates produces organic acids resulting in a colour change of the media from red to yellow. *N.gonorrhoeae* was identified by its fermentation of glucose only.

Colonies that were confirmed as *N.gonorrhoeae* were picked off, and sub-cultured onto chocolate agar plates to obtain a pure culture for further work up and storage.

2.7 Storage of Isolates

All isolates were stored for further testing. Colonies were picked off from the purity plate and added to cryovials containing glass beads and BHI broth + 20% glycerol and mixed well by inverting the cryovial (preparation as per Appendix 1). The cryovials containing the isolates were stored at -80°C in duplicates.

2.8. Determination of Minimum Inhibitory Concentration (MIC)

2.8.1 Preparation of antimicrobial solutions

The antimicrobial drugs were purchased from Sigma-Aldrich, USA and stored at temperatures recommended by the manufacturer, until use. The minimum inhibitory concentration (MIC) of each of the following antimicrobial drugs were obtained for all *N.gonorrhoeae* isolates: Azithromycin (AZITHRO), Cefixime (CEFIX), Ceftriaxone (CEFTRIAX), Ciprofloxacin (CIPRO), Ofloxacin (OFLOX), Penicillin (PEN) and Tetracycline (TET).

The range of antimicrobial drug concentrations used in the experiments is shown in Table 1. The antimicrobial drug stock solutions were prepared in triplicate and the tests were performed in triplicate. For each antimicrobial drug, the appropriate amount was weighed out using the calculation shown in appendix 2. The antibiotic stocks were prepared as follows: 0.034g of azithromycin dissolved in 1ml methanol and topped up to 10ml with distilled water (128mg/L), 0.032g of cefixime dissolved in 1ml methanol and topped up to 10ml with distilled water (128mg/L), 0.032g of ceftriaxone dissolved in distilled water (128mg/L), 0.033g of ciprofloxacin dissolved in 1ml acetic acid and topped up to 10ml with distilled water (128mg/L), 0.032g of ofloxacin dissolved in 1ml acetic acid and topped up to 10ml with distilled water (128mg/L), 0.128g of penicillin dissolved in distilled water (512mg/L) and 0.033g of tetracycline in 10ml distilled water (128mg/L). All antibiotic solutions were filter sterilized using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA) and aliquoted into cryovials with a volume of 1.1ml in each and stored at -20^{o}C until use.

2.8.2 Preparation of bacterial suspensions

N.gonorrhoeae isolates were retrieved from -80^{o}C storage and cultured onto chocolate agar plates and incubated for 24h at 37◦C in 5% CO_2. Following the incubation, colonies were picked off and a standardised bacterial suspension equivalent to a 0.5 McFarland standard was prepared for each isolate as well as the controls. Controls were selected from WHO reference strains, characteristics can be seen in table 1. The WHO reference strains used were WHO F, K, L, O and P. A 0.5 McFarland standard was prepared as described in Appendix 2.

Table 1: Antimicrobial susceptibility phenotypic and genetic (*tetM*) characteristics of the 2008 WHO *N.gonorrhoeae* reference strains

	WHO reference strain						
Characteristics	**F**	**G**	**K**	**L**	**O**	**P**	**N**
Azithromycin	S[1] (0.125)	S[1] (0.25)	S[1] (0.25)	I[2] (0.5)	S[1] (0.25)	R[3] (2)	S[1] (0.125)
Cefixime	S[1] (<0.016)	S[1] (<0.016)	NS[4] (0.5)	NS[4] (0.25)	S[1] (0.016)	S[1] (<0.016)	S[1] (<0.016)
Ceftriaxone	S[1] (<0.002)	S[1] (0.008)	NS[4] (0.064)	NS[4] (0.125)	S[1] (0.032)	S[1] (0.004)	S[1] (0.004)
Ciprofloxacin	S[1] (0.004)	LLR[5] (0.25)	HLR[6] (>32)	HLR[6] (>32)	S[1] (0.008)	S[1] (0.004)	R[3] (4)
Penicillin	S[1] (0.032)	I[2] (0.5)	CMRNG[7] (2)	CMRNG[7] (2)	PPNG[8] (>32)	I[2] (0.25)	PPNG[8] (8)
Tetracycline	I[2] (0.25)	TRNG[9] (32)	R[3] (2)	R[3] (4)	I[2] (1)	I[2] (0.5)	TRNG[9] (16)
tetM plasmid type	----------	Dutch	----------	----------	--------	----------	Dutch

[1] S - Susceptible
[2] I – Intermediate
[3] R - Resistant
[4] NS - non susceptible, contains genetic resistance markers but clinical/laboratory correlates are insufficient to allow resistance phenotype designation at present
[5] LLR – Low-Level Resistance
[6] HLR – High-level Resistance
[7] CMRNG - chromosomally mediated resistant *N.gonorrhoeae*
[8] PPNG - penicillinase producing *N.gonorrhoeae*
[9] TRNG - plasmid mediated tetracycline resistant *N.gonorrhoeae*

2.8.3 Minimum Inhibitory Concentration (MIC) using agar dilution method

The agar dilution method is the "gold standard" recommended for MIC determination of *N.gonorrhoeae* isolates. MICs were performed in 90mm petri dishes containing different concentrations of antibiotic solution incorporated with GC agar base supplemented with yeast autolysate and lysed horse blood (Modified New York City).

2.8.3.1 Preparation of antimicrobial drug dilutions and plates for agar dilution method

All antimicrobial drug dilutions were done using distilled water. One millilitre of the prepared antibiotic stock was added to 9ml of distilled water, mixed well and 5ml was transferred into the next tube containing 5ml of distilled water. Twofold serial dilutions were made by transferring 5ml from one tube to the next, consecutively and the final 5ml was discarded.

All antimicrobial dilutions were made in triplicate. Twenty millilitres (20ml) of the modified New York City media was added to each dilution tube (5ml) that gave a final volume of 25ml. The media and antibiotic dilution was mixed in the tube by inverting 3 times and poured into 90mm Petri dishes. The plates were left overnight to set and inoculated the next morning.

2.8.4 Inoculation of plates

Four hundred microliters (400µl) of each bacterial suspension was transferred into the wells of the multipoint inoculator. The 1st well was loaded with a crystal violet dye to serve as a marker for orientation of the wells. Wells 2 and 3 were loaded with controls and the remaining wells were loaded with the test isolates. The multipoint inoculator transfers approximately 1-2µl of each suspension onto the surface of the agar. A drug-free control plate was included as a growth control. Plates were inoculated starting with a drug-free plate followed by the lowest to the

highest drug concentrations and ending with a drug-free plate. This is done to avoid carry-over of the antibiotic between plates. Plates were incubated for 24h at 37^{o}C with 5% CO_2.

2.8.5 Interpretation of MIC results

Following 24 hours incubation, MIC plates were placed on a multipoint plate reader and the MIC results were recorded as the lowest concentration of the antimicrobial drug that inhibited growth of the organism on the respective agar plates. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints (2015) for all antimicrobial drugs (Table 2).

2.9 β-lactamase detection

This test is done to detect β-lactamase production as *N.gonorrhoeae* may carry plasmids that produce the β-lactamase enzyme. This is a chromogenic cephalosporin method that is widely used to detected β-lactamase in *N.gonorrhoeae*. β-lactamase hydrolyses the β-lactam ring of the chromogenic cephalosporin which is nitrocefin and produces a colour change from yellow to red. This test is performed by adding one drop of nitrocefin solution (Oxoid, England) onto filter paper. Colonies from a pure overnight *N.gonorrhoeae* culture are picked off and placed onto the nitrocefin drop. A colour change from yellow to red indicates a positive result.

Table 2: Antimicrobial drug concentrations used for MIC determination

Antibiotic	Concentrations (mg/L)												
Azithromycin	0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8		
Cefixime	0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8		
Ceftriaxone	0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8		
Ciprofloxacin	0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	
Ofloxacin	0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	
Penicillin	0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32
Tetracycline	0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32

Table 3: MIC interpretive standards for *Neisseria gonorrhoeae* according to EUCAST Criteria

Antimicrobial drug	MIC breakpoint (mg/L)	
	Susceptible ≤	Resistant >
Azithromycin	0.25	0.5
Cefixime	0.125	0.125
Ceftriaxone	0.125	0.125
Ciprofloxacin	0.03	0.06
Ofloxacin	0.125	0.25
Penicillin	0.06	1
Tetracycline	0.5	1

2.10 Polymerase Chain Reaction (PCR) Amplification

2.10.1 DNA Extraction

All isolates resistant to tetracycline were used and these were grown on modified New York City agar and incubated for 24h at 37ºC in 5% CO_2. Crude DNA extraction was done using a boiling method. This was done by picking off colonies from the culture plate. The colonies were inoculated into 1ml BHI broth and vortexed until the suspension was homogenous. This suspension was then standardised to a McFarland 1 standard solution (prepared as in Appendix 2). This was done by aligning the bacterial suspension tube next to the prepared McFarland 1 standard solution and eyeballing the turbidity. The suspension was centrifuged at 6500rpm for 5minutes. The supernatant was discarded and the pellet was re-suspended in 500µl of 1 X TE buffer. The sample was boiled for 30minutes at 100ºC using a heating block. The DNA was stored at -20ºC until further use.

2.10.2 Amplification of the *tetM* gene

PCR amplification was conducted to determine the presence of 2 variants of the *tetM* gene. The assay was performed in final volume of 50µl containing 5µl of the template DNA, 5µl of AmpliTaq GOLD LD buffer (10X), 3µl 25mM $MgCl_2$, 1µl deoxy-nucleotides triphosphates (dNTPs), 0.45µl of Taq DNA Polymerase (5U/µl) (Life Technologies), 1µl (10pmol) of each primer (Universal forward, American reverse and Dutch reverse), and 32.55µl of PCR grade water (Roche Diagnostics) refer to Appendix 3 table 11. Primer sequences are shown in table 3. The cycling protocol utilised was an initial denaturation at 94ºC for 10mins followed by 35 cycles of denaturation at 94ºC, annealing at 55ºC; extension at 72ºC for 45secs each and a final extension of 5mins at 72ºC. WHO strain N was used as positive control for the Dutch variant

and distilled water was used as a negative control. The expected size of the PCR amplicon for the WHO Dutch strain was 443bp. An internal control was designed in our laboratory to serve as positive control for the detection of the American variant. A clinical isolate containing the American variant, matched to 778bp on an agarose gel was purified and submitted for Sanger sequencing. The PCR was conducted in triplicate in 3 separate runs and all amplicons were submitted for sequencing. The final sequences of the internal control were aligned to the *N.gonorrhoeae* tetracycline resistant protein (*tetM*) gene reference sequence. (GenBank accession number: AF116348.1) Analysis was done using the Geneious (Biomatters Limited) version 8.1 software.

Table 4: Oligonucleotide primer sequences used for the amplification of the *tetM* gene

Gene	Primer	Nucleotide Sequence (5'→3')	Amplicon Size	Reference
tetM American Variant	Universal FWD	[CTCGAACAAGAGGAAAGC]	778bp	Turner A *et al*. 1999
	American REV	[GCATTCCACTTCCCAAC]		
tetM Dutch Variant	Universal FWD	[CTCGAACAAGAGGAAAGC]	443bp	Turner A *et al*. 1999
	Dutch REV	[TGCAGCAGAGGGAGG]		

2.10.3 Detection of PCR products by gel electrophoresis

The presence of the *tetM* gene variants were detected using gel electrophoresis. A 2% agarose gel was prepared by mixing agarose with 1x Tris-borate-EDTA buffer (TBE buffer) (Appendix 3). The agarose was dissolved by heating in a microwave. The solution was cooled to 40-45^{o}C and thereafter poured into a gel casting tray secured in a gel casting holder that had a 20 well plastic comb attached. The gel was left to solidify at room temperature for 45minutes after which the combs and the gel casting tray were removed. The gel was placed in an electrophoresis tank filled with 1x TBE buffer. A template was made and placed under a sheet of parafilm on which 5ul of the PCR product was mixed with 3µl of gel loading dye containing gel red dye (Anatech, SA). A 100bp molecular weight marker XIV (Roche) was included to estimate size of PCR products. The gel was run at 100 volts for 2 hours. The gel was viewed under UV illumination using the gel dock and images of the bands were captured using the Syngene gel imaging system and GeneSnap Software (InGeniusBio Imaging, Syngene).

CHAPTER 3: RESULTS

3.1 Patient demographic data and Prevalence of Gonorrhoea

A total of 1220 patients were recruited during the study period from September 2013 to December 2014: Boom Street Clinic (n=875) and Umlazi Section D Clinic (n=345). Five hundred and six (506) male patients and 714 female patients were recruited. *N.gonorrhoeae* was isolated from 264 (30%) patients at the Boom Street Clinic and 55 (16%) patients from the Umlazi Section D Clinic. Overall, there were 319 patients from both clinics from whom *N.gonorrhoeae* was isolated. Two hundred and forty eight (78%) of the positive *N.gonorrhoeae* isolates were from male patients and 71 (22%) isolates were from female patients. The dominant age group in male and female patients was the 25-39 year age category. Seventy three (29%) of the male patients were between 18-24 years, 164 (66%) were between 25-39 years and the remaining 11 patients were over 40 years. In female patients, 30 (42%) were between the ages of 18-24 years, 40 (56%) were between 25-39 years and 1 patient (2%) was over the age of 40. Figure 1 shows the occurrence of *N.gonorrhoeae* isolates stratified by age and gender.

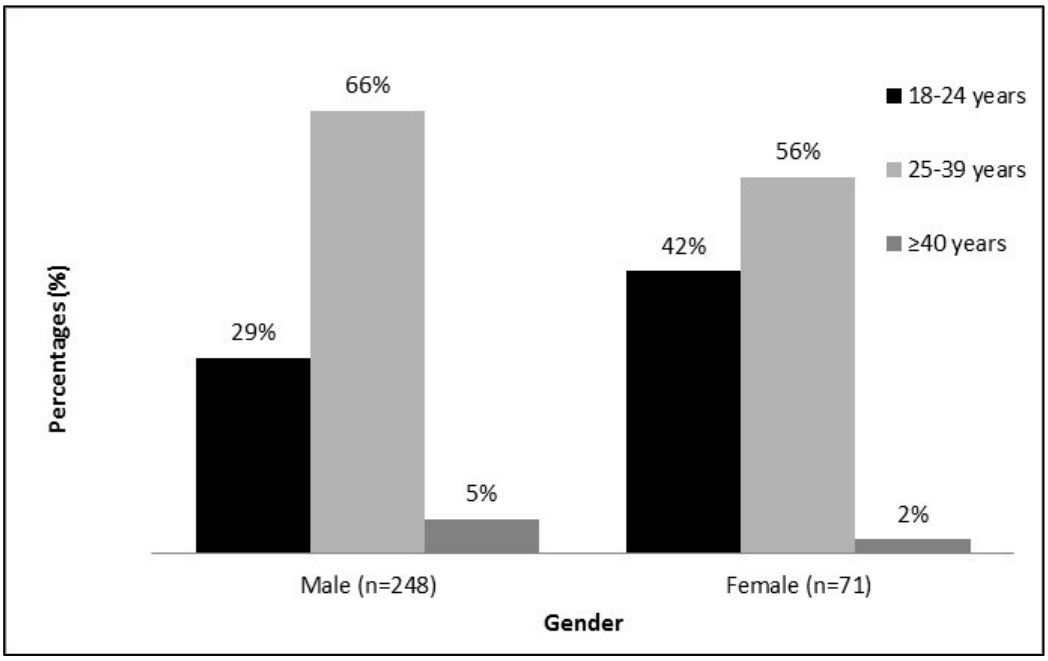


Figure 1: Patients with gonorrhoea stratified by Age and Gender

3.2 MIC Results

The MIC distribution of the various antimicrobials for *N.gonorrhoeae* is shown in tables 5, 6, 7, 8, 9, 10 and 11.

3.2.1 Cephalosporins

3.2.1.1 Cefixime

Three hundred and seventeen (99%) of the tested isolates were susceptible to cefixime with MIC values ranging from ≤0.007mg/L to 0.125mg/L. The remaining 2 (1%) isolates were resistant with an MIC value of 0.25mg/L. The distribution of cefixime MIC values stratified by year of collection and study site are shown in table 5. In 2014, there was a shift of MIC values to the right implying decreasing susceptibility.

Table 5: MIC (mg/L) distribution of *N.gonorrhoeae* isolates (n=319) for Cefixime

Year of Collection	Study site	MIC (mg/L) ≤0.007	0.015	0.03	0.06	0.125	0.25
2013 (n=107)	PMB[x] n=107	107	0	0	0	0	0
	Umlazi n=0	Specimens not collected at this site in 2013					
2014 (n=212)	PMB[x] n=157	49	39	39	21	7	2
	Umlazi n=55	11	15	16	13	0	0

[x] PMB - Pietermaritzburg

3.2.1.2 Ceftriaxone

All 319 (100%) tested isolates were susceptible to ceftriaxone with MIC values ranging from ≤0.007mg/L to 0.125mg/L. The distribution of ceftriaxone MIC stratified by year of collection and study site are shown in table 6. In 2014, there was a shift of MIC values to the right implying decreasing susceptibility.

Table 6: MIC (mg/L) distribution of *N.gonorrhoeae* isolates (n=319) for Ceftriaxone

Year of collection	Study site	MIC (mg/L)					
		≤0.007	**0.015**	**0.03**	**0.06**	**0.125**	**0.25**
2013 (n=107)	PMB[x] n=107	107	0	0	0	0	0
	Umlazi n=0	Specimens not collected at this site in 2013					
2014 (n=212)	PMB[x] n=157	106	33	14	3	1	0
	Umlazi n=55	32	16	7	0	0	0

[x] PMB - Pietermaritzburg

3.2.2 Fluoroquinolones

3.2.2.1 Ciprofloxacin

Ninety two (29%) tested *N.gonorrhoeae* isolates were susceptible to ciprofloxacin with MIC values ranging from ≤0.007mg/L to 0.03mg/L. Four (1%) isolates displayed intermediate resistance with an MIC value of 0.06mg/L. The remaining 223 (70%) isolates were resistant with MIC values ranging from 0.125mg/L to 8mg/L. The distribution of ciprofloxacin MIC values stratified by year of collection and study site are shown in table 7.

Table 7: MIC (mg/L) distribution of *N.gonorrhoeae* isolates (n=319) for Ciprofloxacin

Year of collection	MIC (mg/L)											
	Study site	**≤0.007**	**0.015**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**
2013 (n=107)	PMB[x] n=107	17	8	11	4	1	3	5	13	2	42	1
	Umlazi n=0	Specimens not collected at this site in 2013										
2014 (n=212)	PMB[x] n=157	27	13	3	0	2	5	6	25	18	58	0
	Umlazi n=55	6	7	0	0	0	0	0	10	6	23	3

[x] PMB - Pietermaritzburg

3.2.2.2 Ofloxacin

Ninety six of the 319 (30%) tested *N.gonorrhoeae* isolates were susceptible with MIC values ranging from ≤0.007 mg/L to 0.125mg/L. Two isolates (1%) displayed intermediate resistance with an MIC value of 0.25 mg/L. The remaining 221 isolates (69%) were resistant with MIC values ranging from 0.5mg/L to 8mg/L. The distribution of ofloxacin MIC values stratified by year of collection and study site are shown in table 8.

Table 8: MIC (mg/L) distribution of *N.gonorrhoeae* isolates (n=319) for Ofloxacin

Year of collection		MIC (mg/L)										
	Study site	**≤0.007**	**0.015**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**
2013 (n=107)	PMB[x] n=107	14	18	6	1	1	1	3	5	11	40	7
	Umlazi n=0	Specimens not collected at this site in 2013										
2014 (n=212)	PMB[x] n=157	25	9	6	3	0	1	5	9	25	66	8
	Umlazi n=55	3	10	0	0	0	0	0	0	10	26	6

[x] PMB - Pietermaritzburg

3.2.3 Macrolides

3.2.3.1 Azithromycin

Fourteen isolates (4%) were susceptible, with MIC values below the susceptibility breakpoint of 0.25 mg/L. Eighty eight (28%) displayed intermediate resistance with an MIC value of 0.5. The remaining 217 isolates (68%) were resistant with MIC values ranging from 1mg/L to 4mg/L. The distribution of azithromycin MIC values stratified by year of collection and study site are shown in table 9.

Table 9: MIC (mg/L) distribution of *N.gonorrhoeae* isolates (n=319) for Azithromycin

Year of collection		MIC (mg/L)					
	Study site	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**
2013 (n=107)	PMB[x] n=107	2	4	31	43	25	2
	Umlazi n=0	Specimens not collected at this site in 2013					
2014 (n=212)	PMB[x] n=157	0	6	47	61	40	3
	Umlazi n=55	0	2	10	28	15	0

[x] PMB - Pietermaritzburg

3.2.4 Penicillin

Five of the 319 (2%) tested *N.gonorrhoeae* isolates were susceptible with an MIC value of ≤0.06mg/L. One hundred and five isolates (33%) displayed intermediate resistance with MIC values ranging from 0.125 mg/L to 1 mg/L. The remaining 209 isolates (65%) were resistant with MIC values ranging from 2mg/L to 8mg/L. The distribution of penicillin MIC values stratified by year of collection and study site are shown in table 10.

One hundred and ninety one isolates (60%) were β-lacamase positive.

Table 10: MIC (mg/L) distribution *N.gonorrhoeae* isolates (n=319) for Penicillin

Isolates Year of collection (n)		MIC (mg/L)							
	Study site	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**
2013 (n=107)	PMB[x] n=107	5	11	7	10	11	6	14	43
	Umlazi n=0	Specimens not collected at this site in 2013							
2014 (n=212)	PMB[x] n=157	0	17	25	17	4	21	23	50
	Umlazi n=55	0	4	8	6	1	8	18	10

[x] PMB - Pietermaritzburg

3.2.5 Tetracycline

All 319 (100%) *N.gonorrhoeae* isolates were resistant to tetracycline with MIC values > 16mg/L. There were no susceptible isolates. The distribution of tetracycline MIC values stratified by year of collection and study site are shown in table 11.

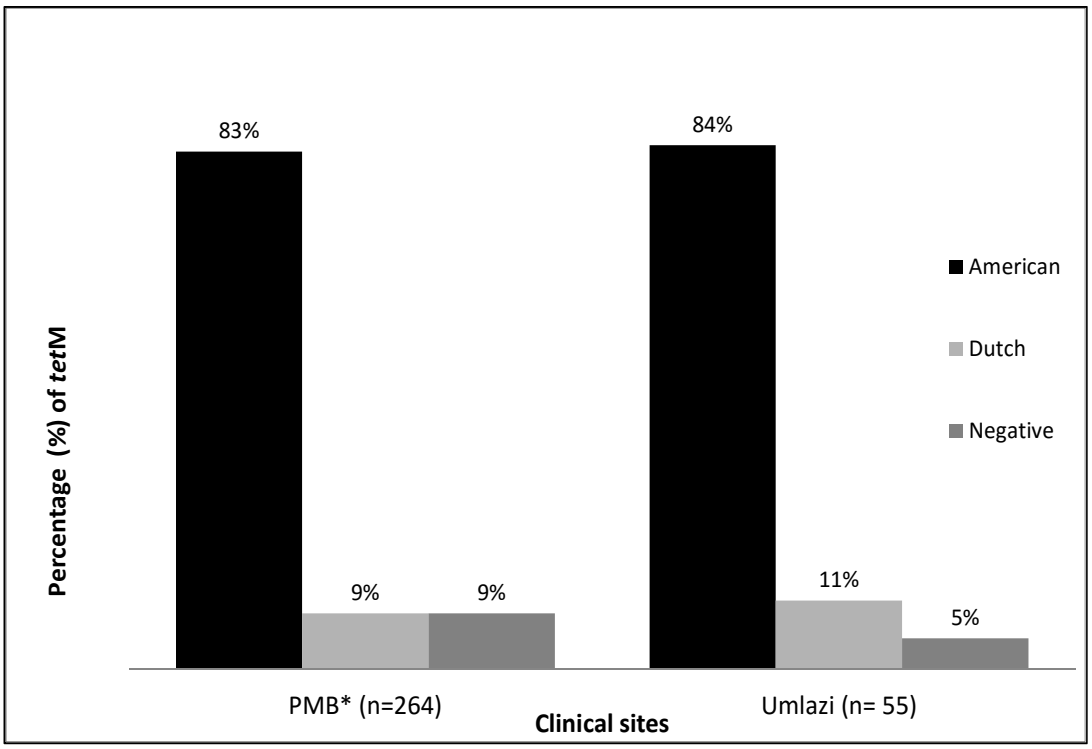
Table 11: MIC (mg/L) distribution of *N.gonorrhoeae* isolates (n=319) for Tetracycline

Isolates **Year of collection (n)**	**Study site**	MIC (mg/L)			
		4	**8**	**16**	**≥32**
2013 (n=107)	PMB[x] n=107	0	0	5	102
	Umlazi n=0	Specimens not collected at this site in 2013			
2014 (n=212)	PMB[x] n=157	0	0	6	151
	Umlazi n=55			1	54

[x] PMB - Pietermaritzburg

3.3 Molecular characterization of tetracycline resistance (PCR results)

The *tetM* gene was detected in 293 (92%) of the 319 isolates that were resistant to tetracycline. Two hundred and sixty four of the 293 (90%) isolates had the American variant of *tetM* gene and 29 (10%) had the Dutch variant of *tetM* gene. Twenty six (8%) of the 319 isolates did not harbour any *tetM* gene. The distribution of the 2 variants of the gene between the 2 study sites was similar (figure 2). Figure 3 shows a picture of the gel electrophoresis run with both types of the *tetM* gene. The sequencing alignment of the internal control for the American variant of the *tetM* gene and detailed patient demographic data, complete MIC data and PCR results are attached in the appendices.



* PMB - Pietermaritzburg

Figure 2: Distribution of *tetM* variants amongst tetracycline resistant *N.gonorrhoeae* isolates at the two clinical sites

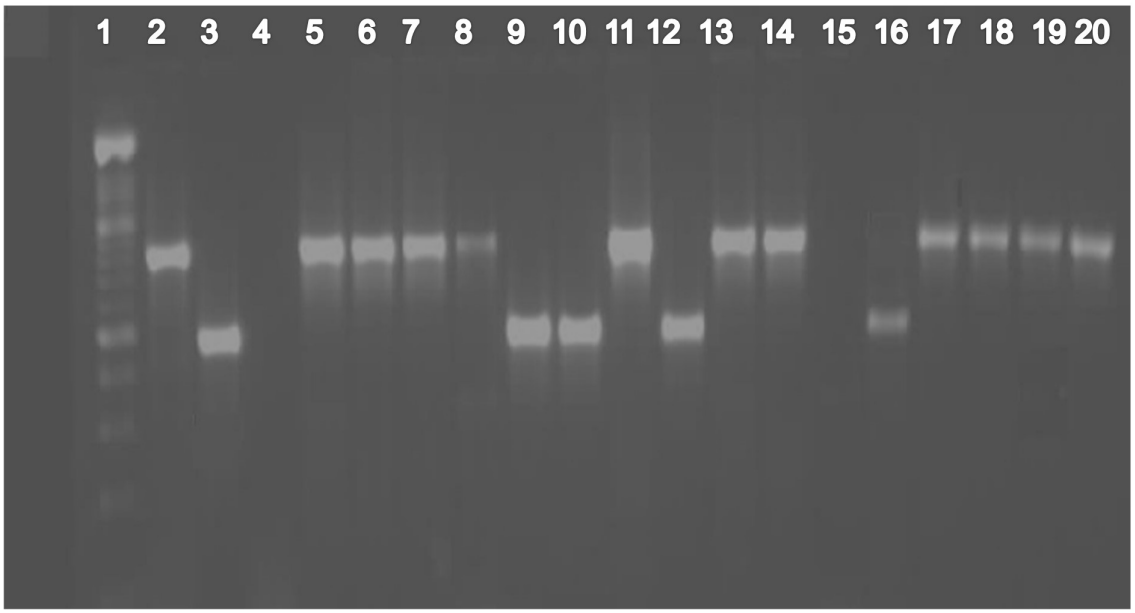


Figure 3: Detection of the *tetM* gene in clinical isolates of *N.gonorrhoeae*

Lane 1: Molecular weight marker XIV 100bp (Roche)

Lane 2: American Control (778bp)

Lane 3: Dutch Control WHO N (443bp)

Lane 4: Negative Control

Lanes 5-8, 11, 13, 14 and 17-20: Clinical isolates of *N.gonorrhoeae* (American)

Lanes 9, 10, 12 and 16: Clinical isolates of *N.gonorrhoeae* (Dutch)

Lane 15: Clinical isolate of *N.gonorrhoeae* (Negative)

CHAPTER 4: DISCUSSION

The emergence of drug resistant strains of *N.gonorrhoeae* is a growing public health concern. Maintaining effective treatment of gonococcal infections remains a challenge given the ability of *N.gonorrhoeae* to rapidly acquire resistance mechanisms (Bignell & Garley 2010). Routine surveillance and drug susceptibility testing of *N.gonorrhoeae* does not feature on STI control and management programs in the country. Such surveillance is limited to research settings and STI data is mainly acquired from patients visiting ante-natal clinics. In an attempt to understand the dynamics of antimicrobial resistance amongst *N.gonorrhoeae* isolates currently circulating in our setting, we collected clinical specimens from 2 study sites in the two largest cities in KZN over a 2 year period and screened isolates for resistance to various antimicrobials. We included antimicrobials previously used for treatment, as well as the currently recommended for the management of infections with *N.gonorrhoeae*.

The results of this study confirm that majority of the isolates are still resistant to antibiotics that were historically used to treat gonorrhoea viz: penicillin, tetracycline and the fluoroquinolones. Penicillin has been extensively used in the treatment of gonorrhoea infections. The evolution of penicillin resistance and its resistant determinants has been well established globally (Elwell *et al.* 1977). Our data supports the trends that have been reported over the past two decades which have demonstrated an increase in number of gonococcal strains resistant to penicillin (Amies 1967). In this study, 98% of the isolates displayed MIC values in the intermediate or resistant range for penicillin. Sixty percent (60%) of the *N.gonorrhoeae* isolates in this study carry plasmids which produce the β-lactamase enzyme and are resistant to penicillin. The remaining 40% of the isolates that were resistant to penicillin but did not carry the β-lactamase

plasmid could be using other mechanisms of resistance. These isolates could possibly have the *penA* or *ponA* genes present that are responsible for penicillin resistance or mutations in these genes. Penicillin resistance is also due to the acquisition of plasmids which could be present in these isolates. However PCR was not done to confirm the presence of these genes or plasmids.

Ciprofloxacin resistance was reported in 60% of patients with gonorrhoea in a Durban clinic in 2006 compared to 42% in 2005 (Moodley & Sturm 2005). Similar trends were reported for a Johannesburg clinic (Lewis 2007). Ciprofloxacin was introduced into the treatment guidelines in KZN in 1995 and was discontinued following reports of decreased susceptibility to the drug (Lewis & Marumo 2009). This was in keeping with Asian and European countries that had removed ciprofloxacin as part of first line treatment protocols for gonorrhoea due to the high levels of resistance (Unemo & Shafer 2011). Our data supports the study by Moodley *et al*, 2001, that demonstrated increased MICs to these antimicrobial drugs. In the current study, 70% of the isolates displayed resistance to the fluoroquinolones.

Azithromycin has been used to treat gonococcal infections in many countries. A previous study by Moodley *et al*, 2001 showed that even though azithromycin was not in the recommended treatment guidelines in South Africa at the time, there was an alarming increase in resistance to the drug (Moodley *et al*. 2001). This was most likely due to antibiotic pressure from the widespread use of this drug for other infections. Our study supports this finding as our MIC results for azithromycin show that 68% of the isolates are resistant to this antimicrobial drug. High-level resistance (MIC ≥256mg/L) to azithromycin was described in Scotland (Palmer *et al*. 2008), England (Chisholm *et al*. 2010), Argentina (Galarza *et al*. 2010), Italy (Starnino & Stefanelli 2009), United States (Katz *et al*. 2012) and Sweden (Unemo *et al*. 2013). However from our data we have not seen MIC values greater than 4mg/L.

The isolates in the current study displayed high-level tetracycline resistance (MIC ≥16 mg/L), 4 to 5 fold higher than the current recommended breakpoint for the drug. Three hundred and seven of the 319 isolates displayed an MIC value of ≥32 mg/L and the remaining 12 isolates had an MIC value of 16 mg/L. In South Africa, high-level tetracycline resistance was first reported in Bloemfontein in 1994 (Chalkley *et al*. 1997). A previous study conducted in 1999 in KZN reported that 67% isolates had a MIC to tetracycline ≥16mg/L (Moodley *et al*. 2001). The results of the current study indicate that *N.gonorrhoeae* isolates in our setting have evolved and are fully resistant to tetracycline. Genetic mutations, acquisition of plasmids and modifications of drug targets have allowed for *N.gonorrhoeae* to become resistant to tetracycline. Our study also supports the findings of many other studies that have shown an increase in tetracycline MIC over the years (Moodley *et al*. 2001; Dangor *et al*. 2010).

High-level tetracycline resistance attributed to the acquisition of the *tetM* gene which protects the ribosome from tetracycline and therefore renders the drug ineffective (Moodley *et al*. 2001). In an attempt to characterize the molecular basis for high level tetracycline resistance, we screened isolates for the presence of either the American or Dutch variant of the *tetM* gene. In keeping with previous findings, the *tetM* gene was detected in 92% of the isolates (Van Dyck *et al*. 1992; Moodley *et al*. 2001). Furthermore, our data shows the predominance of the American variant of the *tetM* gene carried by 90% of the isolates. This aligns to the premise that the American variant of the *tetM* originated on the African continent (Turner *et al*. 1999). This is further supported by reports from Botswana and Namibia in Southern Africa which attribute high level tetracycline resistance to the American variant of the *tetM* gene (Chalkley *et al*. 1995). Previous reports suggest that the American variant is widely distributed in South Africa (Chalkley *et al*. 1997). Previous reports have shown high numbers of the Dutch variant in Indonesia, UK, Caribbean and Europe (Turner *et al*. 1999).

We report the presence of the Dutch variant in 10% of isolates. Although the Dutch variant is not commonly seen in the country, it is slowly emerging and circulating (Fayemiwo *et al*. 2011; Chalkley *et al*. 1997). To the best of our knowledge, this is the first report of the Dutch variant of the *tetM* gene in KZN. Both variants displayed similar MIC values for tetracycline and were present in isolates from both study sites.

Twenty six isolates representing 8% of the tetracycline resistant isolates displayed high level tetracycline resistance in the absence of any of the *tetM* genes. This indicates the possibility of an alternate mechanism of tetracycline resistance such as an efflux pump. Interestingly, this is the first report of isolates displaying high level tetracycline resistance in the absence of the *tetM* gene. Identification of alternate mechanisms of resistance or alternate targets will provide invaluable information regarding tetracycline resistance. Concerns have been raised regarding the transfer of the *tetM* gene between various organisms as the gene is carried on a plasmid. Due to the possible interaction between chlamydial and gonococcal infections it is questionable whether tetracycline can still be used for the treatment chlamydial of infections.

The cephalosporins are the only remaining class of antibiotics that are available for gonorrhoea monotherapy. Ceftriaxone and cefixime are recommended for treatment of this infection. Our results showed that 2 isolates were resistant to cefixime, whilst none displayed resistance to ceftriaxone. However, we did note a gradual increase in the MIC values of gonococcal isolates over the 2 year collection period. In 2013, all isolates that were collected were susceptible to both cephalosporins with MIC values of ≤ 0.007mg/L. In 2014, the isolates displayed a gradual increase in MIC values. Whether the increase in MIC values indicates *in vivo* clinical resistance requires further elucidation of the mechanisms mediating resistance to these drugs. To date, we have not had any reports of ceftriaxone treatment failures in South Africa. However, cefixime

treatment failures have been reported in 2 homosexual men in Johannesburg (Lewis *et al*. 2013).

A significant factor contributing to the emergence and spread of resistant forms of gonococcal infections is the pressure created by syndromic management of STIs. South Africa and many resource-limited settings apply syndromic management for the treatment of STIs. Syndromic management involves treating patients based on signs and symptoms in the absence of a confirmed laboratory diagnosis. Reports show that only 13% of symptomatic STIs are cured with the current STI services in KZN (White *et al*. 2008; Mlisana *et al*. 2012). Drug pressure from these empiric regimens has resulted in widespread resistance (Moodley, Pillay, *et al*. 2001). There have been conflicting reports regarding the utility of syndromic management. Whilst it aims to ensure that the patient receives quality treatment at the first visit to the healthcare facility without the delay of a laboratory diagnosis this approach underestimates the prevalence of STIs, especially in the cases of asymptomatic infection (Grosskurth *et al*. 2000; Kamali *et al*. 2003; Gregson *et al*. 2007).

A study by White *et al.* 2008 estimated the effectiveness of treatment of STIs in rural KZN before and after the implementation of syndromic management in South Africa in 1995. This study showed that after the introduction of syndromic management the prevalence of STIs especially chlamydia and gonorrhoea did not decrease but increased (White *et al*. 2008). Syndromic management of STIs is seen as a cost-saving HIV prevention strategy however due to the emergence of high-levels of drug resistance in *N.gonorrhoeae* and the interaction of the HIV epidemic, the utility of syndromic management requires urgent re-consideration in our setting that has a high HIV incidence (White *et al.* 2008). Reports of increasing STI resistance to antimicrobials and increasing prevalence of STIs demonstrate that syndromic management

might not be the most effective solution for the management of STIs. The most recent new STI guidelines (CDC 2015) advocate inclusion of diagnostic testing algorithms to improve patient outcomes. Whilst the individualized approach may offer a great improvement in the management of STIs, it might not be widely implemented due to financial constraints especially in developing countries.

The emergence of *N.gonorrhoeae* displaying resistance to most antimicrobials previously and currently available for the treatment of gonococcal infections is a major concern. Such strains have been defined as XDR strains displaying resistance to 2 or more of the classes that are generally recommended for treatment gonorrhoea or 3 or more of the classes that are less frequently recommended for treatment (Goire *et al*. 2014). The first 2 XDR strains displaying high level antimicrobial resistance to most agents including the cephalosporins were described in Japan (Ohnishi *et al*. 2011) and Europe (Magnus Unemo *et al*. 2012). These were identified in 2 high risk populations which included a commercial sex worker and homosexual male. Detection of such strains has naturally led to the implementation of dual-therapy regimens. To date, dual-therapy has been implemented in the United States (CDC 2012), United Kingdom (Bignell & Fitzgerald 2011) and throughout Europe (Bignell & Unemo 2013). Dual-therapy regimens mainly includes the combined use of azithromycin and ceftriaxone. However, due to the decreased susceptibility of *N.gonorrhoeae* to the cephalosporins and previously reported azithromycin resistance, dual-therapy is not an effective long term solution.

We caution against the application of this combination in our setting for 2 main reasons. Firstly, 68% of the isolates in the current study display resistance to azithromycin. Secondly, our isolates show an increase in MIC for the cephalosporins over the 2 year collection period. In addition to these, reports of cefixime resistance in Johannesburg raise the concern of the spread of cephalosporin resistance throughout South Africa (Lewis *et al*. 2013). Taken together, these

provide evidence that empiric use of this combination in our setting will only drive further resistance in *N.gonorrhoeae* and will be disastrous from a public health perspective. Drug susceptibility, where possible should be conducted prior to the initiation of treatment.

The increase in resistance to tetracycline and increasing MIC to cephalosporins highlights the urgent need for routine surveillance and drug susceptibility monitoring systems in the country. Data from research settings does not provide a universal representation of the drug resistance problem for the country. Furthermore, discrepancies in reporting guidelines from EUCAST and CLSI are potentially underestimating or overestimating resistance. In both cases, inadequate treatment contributes to the burden of drug resistance. In the current study, we have utilized EUCAST breakpoints for all drugs. CLSI guidelines are widely used however; their breakpoints are higher than EUCAST guidelines. This results in misclassification of a proportion of resistant isolates. Forty five (45) resistant isolates would have been misclassified as susceptible if CLSI breakpoints were used. Ideally genotypic characterization should be conducted to confirm and correctly identify resistant isolates from those that are susceptible. However this would only apply to known mutations associated with resistance. Correlations between phenotypic and genotypic data should be applied in order to produce uniform guidelines that should be implemented globally.

New rapid diagnostic assays offer a ray of hope. The Cepheid GeneXpert® *Chlamydia trachomatis* (CT)/*Neisseria gonorrhoea* (NG) test is capable of producing test results in 90 minutes. This technology can provide testing whilst the patient is still present at the clinic, eliminating treatment delays and inappropriate results. If this is implemented in a clinical setting it will reduce over prescribing of antibiotics when and if not necessary and assist with syndromic management. Furthermore, the cartridge based test can be applied to the same GeneXpert machines already in use for tuberculosis testing (Cepheid®, US). This will be a

major advantage in South Africa as the testing platform has been implemented throughout the country as part of the national tuberculosis control programme. The ThermoFisher Open-Array real-time PCR based test has the ability to detect multiple infections simultaneously in just 2 hours. This test however, will require expertise and training which may not be ideal to be used as a rapid diagnostic but rather in the research setting for surveillance studies. This system could be a better option than the real-time multiplex PCR because not only does it have high specificity but it also uses less reagents that minimizes costs.

In conclusion, the results of this study provide compelling evidence of increased antimicrobial drug resistance in clinical isolates of *N.gonorrhoeae* currently circulating in KZN. This study has important implications for the local STI control programme as it highlights the looming crisis of multidrug resistant *N.gonorrhoeae* strains and the urgent need for routine surveillance of these organisms. We have shown that empirical dual-therapy with azithromycin and a 3rd generation cephalosporin might not be an option in our setting. Furthermore, in a resource limited setting like South Africa, the cost of new drugs and dual-therapy will pose an additional financial burden, whilst driving antibiotic resistance. We support the judicious use of cephalosporins in our setting for the treatment of infection with *N.gonorrhoeae*.

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APPENDICES

APPENDIX 1

Primary isolation agar plates

1.1 New York City Agar

Weigh out 36g GC agar base (Oxoid, England) and dissolve in 850ml of distilled water, autoclave for 15mins at 121ºC. Dissolve supplements 2 X yeast autolysate (Oxoid, England) by adding 15ml of distilled autoclaved water into each vial. Dissolve 2x LCAT supplement (Oxoid, England) by adding 5ml of distilled autoclaved water to each vial and filter sterilising through a 0.2µm Millipore filter. Lyse horse blood using saponin (Sigma-Aldrich, USA). Pour 100ml of horse blood into autoclaved bottle. Weigh out 0.5g of saponin and dissolve in 10ml of distilled autoclaved water, filter through Millipore and add to blood for 30mins. Supplements and blood are added when media is cooled to 55 ºC.

1.2 Candle Extinction Jar

A jar with a lid that seals tightly is used to create a humid atmosphere for the transportation of the inoculated agar plates. Paper towel is moistened with water and placed at the bottom of the jar. Once plates are placed inside the jar, a candle is lit inside and closed with the lid of the jar.

1.3 Chocolate agar plates (subculture for storage of isolates)

Weigh out 39g of Columbia Blood Agar Base (Oxoid, England) and dissolve in 1000ml of distilled water. Autoclave for 15 minutes at 121 ºC. Once autoclave complete, transfer flask to waterbath and pour 50ml of horse blood immediately around 80 ºC. Mix well, allow to cool and pour.

1.4 Preparation of 0.5% phenol red solution

Weigh out 0.5g of phenol red powder and dissolve in 100ml of distilled water. Autoclave for 15 minutes at 121 ºC.

1.4.1 GC sugar plates for acid production

Weigh out 36g of GC agar base (Oxoid, England) and dissolve in 1000ml of distilled water, add 5ml of a 0.5% phenol red solution mix well. Adjust pH to 7.6 and autoclave for 15 minutes at 121 ºC.

1.4.2 Preparation of 20% sugar solutions for glucose, maltose, lactose and sucrose

Weigh out 20g of each sugar and dissolve in 100ml of distilled water. Filter each sugar through Millipore filter size 0.2µm (Merck, SA) into autoclaved bottles.

Autoclave 9mm antibiotic discs (Lasec, SA) and separate into 4 Petri dishes for each sugar. Saturate discs with 50µl of each sugar solution and allow to dry at 37 ºC overnight.

1.5 Storage media (BHI + 20% glycerol medium) for isolates

Weigh 3.7g of BHI powder (Oxoid, England), add 20ml glycerol and add 80ml distilled water. Autoclave for 15 minutes at 121 ºC. Allow to cool and keep at 4 ºC.

Dispense glass beads into bottles and autoclave for 15 minutes at 121 ºC.

1.6 Preparation of storage vials

Dispense autoclaved glass beads into cryovials and add 1ml of storage media (BHI + 20% glycerol).

APPENDIX 2

MIC media and antimicrobial stock solutions preparation

2.1 Preparation of modified New York City agar for antimicrobial susceptibility testing of MICs

Weigh out 36g GC agar base (Oxoid, England) and dissolve in 860ml of distilled water, autoclave for 15mins at 121^{o}C. Dissolve supplements 2 X yeast autolysate (Oxoid, England) by adding 15ml of distilled autoclaved water into each vial. Pour 100ml of horse blood into autoclaved bottle. Weigh out 0.5g of saponin and dissolve in 10ml of distilled autoclaved water, filter through Millipore and add to blood for 30mins. Supplements and blood are added when media is cooled to 55 ^{o}C.

2.2 Preparation of 0.5 McFarland Standard

0.5 McFarland standard is prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4).

Confirm with spectrophotometer at 625nm the reading should range from 0.08 to 0.10

2.2.1 Preparation of McFarland No. 1 Standard

0.5 ml of concentrated H_2SO_4 (Merck, SA) was added to 49.5 ml of triple distilled water, to make a 1 % H_2SO_4 solution. 0.175g of $BaCl_2.2H_2O$ (Sigma-Aldrich, USA) was added to 10 ml of triple distilled water to make a 1 % $BaCl_2.2H_2O$ solution.

Thereafter 4.95 ml of 1 % H_2SO_4 solution was combined with 50 µl of 1 % $BaCl_2.2H_2O$ solution. The tube containing the McFarland 1 suspension was wrapped in foil and stored away from direct sunlight.

2.3 Antibiotic stock solutions

$$\textbf{Weight of powder} = \frac{[\text{highest concentration } (\mu g/ml)] \times [\text{volume (ml)}]}{(\text{potency } \mu g/mg)}$$

$$= \text{powder weight } (\mu g/ml)$$

Diluent required = [weight of powder (µg/ml)] x [volume (ml)]

2.4 BHI broth for culture suspensions

Weigh out 3.7g of BHI powder (OXOID, England), add 100ml of distilled water and mix well. Autoclave for 15 minutes at 121^{o}C.

APPENDIX 3

PCR master mix reagents and cycling conditions

All three primers used were from publication Turner *et al.* 1999 and synthesized for this study by Applied Biosystems.

Add 100µl PCR grade water to lyophilized primers to reconstitute. Vortex until dissolved and quick spin.

3.1 Working solution of primers (10pmol)

Add 10µl of primer solution to 90µl of PCR water

Table 12: PCR MasterMix for *tetM* gene variants

Gene	MasterMix (1X Reaction)	
tetM	1. AmpliTaq GOLD LD Buffer (10X	5µl
	2. $MgCl_2$ (25mM)	3µl
	3. dNTPs Mix	1µl
	4. Primer Universal Forward (10pmol)	1µl
	5. Primer American Reverse (10pmol)	1µl
	6. Primer Dutch Reverse (10pmol)	1µl
	7. Enzyme (5U)	0.45µl
	8. PCR Grade Water	32.55µl
	Total Volume for 1X reaction	**45µl**

3.2 PCR Cycling Conditions

35 cycles of Denaturation, Annealing and Extension

Cycling condition	Time and temperature
Initial Denaturation	10 minutes at 94°C//
Denaturation	45 seconds at 94°C
Annealing	45 seconds at 50°C
Extension	45 seconds at 72°C
Final Extension	5 minutes at 94 °C
Hold	4 °C

3.3 Agarose gel electrophoresis

3.3.1 10X TBE Buffer

Trizma base (Sigma-Aldrich, USA) 108g

Boric acid (Sigma-Aldrich, USA) 55g

EDTA (Sigma-Aldrich, USA) 9.3g

Weigh out the required amounts of the reagents and dissolve in 1000ml of distilled water.

3.3.2 1X TBE Buffer

Add 100ml of 10X TBE buffer to 900ml of distilled water.

3.3.3 2% agarose gel (150ml)

Agarose powder (LONZA, USA) 3g

1X TBE buffer 150ml

The agarose powder was weighed and added to a flask containing 1X TBE buffer. The mixture was boiled in a microwave until the powder was dissolved.

The agarose and TBE solution was allowed to cool to about 45^{o}C and poured into a casting gel tray secured in a tray holder that had a 20 well comb attached. The gel was allowed to set at room temperature for 40-55minutes before removing the combs.

3.3.4 Gel loading dye

Glycerol 50g

1M Tris/HCl, pH 7.5 5ml

100mM EDTA 5ml

Bromophenol blue (Sigma-Aldrich, USA) 0.05g

10mg/ml RNase 300µl

Weigh out the reagent powders and their required amounts. Measure the required volumes of the solutions. Add distilled water to make a final volume of 100ml. Heat for 15 minutes in a

100ºC water bath to dissolve. Store at 4 ºC for no longer than one year.

980ul was dispensed into cryovials and stored. 20ul of Gel Red (Biotium) was added to the 980ul of gel loading dye.

APPENDIX 4

Raw data

ISOLATE NUMBER		PEN	CIPRO	OFLOX	TET	CEFIX	CEFTR	AZITHRO	AGE	GENDER	*tetM*
1		0.25	4	4	≥32	≤0.007	≤0.007	2	19	M	A
2		0.25	≤0.007	0.015	≥32	≤0.007	≤0.007	2	28	M	A
3		0.25	4	4	≥32	≤0.007	≤0.007	0.5	21	M	A
4		8	4	4	≥32	≤0.007	≤0.007	4	24	M	A
5		8	0.015	0.015	≥32	≤0.007	≤0.007	1	28	M	A
6		0.125	0.03	0.015	≥32	≤0.007	≤0.007	2	29	M	A
7		0.5	0.5	0.5	≥32	≤0.007	≤0.007	0.5	33	M	A
8		4	4	4	≥32	≤0.007	≤0.007	1	27	M	A
9		0.125	0.25	≤0.007	≥32	≤0.007	≤0.007	1	30	M	A
10		0.5	0.03	0.015	16	≤0.007	≤0.007	1	25	M	A
11		4	8	8	≥32	≤0.007	≤0.007	4	30	M	N
12		8	4	4	≥32	≤0.007	≤0.007	1	21	M	D
13		8	4	4	≥32	≤0.007	≤0.007	2	40	M	A
14		8	≤0.007	0.015	≥32	≤0.007	≤0.007	0.5	18	M	A
15		4	4	4	≥32	≤0.007	≤0.007	1	24	M	A
16		8	0.03	0.015	≥32	≤0.007	≤0.007	0.5	44	M	N
17		2	4	4	≥32	≤0.007	≤0.007	0.25	21	M	A
18		0.125	0.015	0.015	≥32	≤0.007	≤0.007	1	23	M	A
19		8	4	4	≥32	≤0.007	≤0.007	2	33	M	D
20		1	4	8	≥32	≤0.007	≤0.007	1	19	M	A

21		8	2	4	≥32	≤0.007	≤0.007	2	37	M	A
22		0.5	4	8	≥32	≤0.007	≤0.007	2	35	M	A
23		8	1	2	≥32	≤0.007	≤0.007	1	30	M	A
24		4	1	2	≥32	≤0.007	≤0.007	1	33	M	A
25		8	0.06	0.015	≥32	≤0.007	≤0.007	0.5	42	M	A
26		1	1	2	≥32	≤0.007	≤0.007	2	25	M	N
27		4	0.03	0.015	≥32	≤0.007	≤0.007	0.5	29	M	A
28		8	4	4	≥32	≤0.007	≤0.007	1	27	M	A
29		2	1	1	≥32	≤0.007	≤0.007	1	22	M	A
30		0.5	4	4	≥32	≤0.007	≤0.007	1	28	M	A
31		2	4	8	≥32	≤0.007	≤0.007	0.5	38	M	A
32		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	1	31	M	A
33		4	4	8	≥32	≤0.007	≤0.007	2	30	M	D
34		8	0.06	0.015	≥32	≤0.007	≤0.007	0.5	26	M	D
35		4	0.03	0.015	16	≤0.007	≤0.007	0.25	21	M	N
36		8	0.03	0.015	≥32	≤0.007	≤0.007	0.5	25	M	A
37		0.5	1	2	≥32	≤0.007	≤0.007	1	32	M	A
38		1	0.03	4	≥32	≤0.007	≤0.007	2	30	M	A
39		0.5	4	8	≥32	≤0.007	≤0.007	2	22	M	A
40		0.25	4	8	≥32	≤0.007	≤0.007	1	30	M	A
41		0.125	0.25	2	≥32	≤0.007	≤0.007	2	24	M	A
42		8	4	4	≥32	≤0.007	≤0.007	1	30	M	A
43		8	0.5	0.5	≥32	≤0.007	≤0.007	0.5	23	M	N
44		8	0.03	0.015	≥32	≤0.007	≤0.007	0.5	29	M	A
45		1	4	4	≥32	≤0.007	≤0.007	2	28	M	D
46		0.125	4	4	≥32	≤0.007	≤0.007	2	21	M	A
47		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.25	36	M	A
48		0.25	4	4	≥32	≤0.007	≤0.007	0.5	18	M	N

49		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	28	M	A
50		1	4	4	≥32	≤0.007	≤0.007	2	31	M	A
51		8	≤0.007	0.03	≥32	≤0.007	≤0.007	0.5	33	M	A
52		0.25	0.125	0.25	≥32	≤0.007	≤0.007	2	26	M	A
53		0.06	0.06	0.03	≥32	≤0.007	≤0.007	0.5	30	M	A
54		8	1	2	≥32	≤0.007	≤0.007	1	28	M	A
55		0.125	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.125	21	M	A
56		0.125	0.03	0.015	≥32	≤0.007	≤0.007	1	27	M	N
57		8	4	4	≥32	≤0.007	≤0.007	2	31	M	D
58		8	0.015	0.015	≥32	≤0.007	≤0.007	0.5	29	M	A
59		0.5	4	4	≥32	≤0.007	≤0.007	1	37	M	A
60		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	1	26	M	A
61		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	1	27	M	A
62		1	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	29	M	A
63		4	4	4	≥32	≤0.007	≤0.007	1	30	M	A
64		8	1	4	≥32	≤0.007	≤0.007	0.5	35	M	A
65		1	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	35	M	A
66		8	4	4	≥32	≤0.007	≤0.007	1	21	F	D
67		0.06	≤0.007	0.015	≥32	≤0.007	≤0.007	1	22	M	A
68		0.125	4	4	≥32	≤0.007	≤0.007	2	23	M	A
69		0.06	4	4	≥32	≤0.007	≤0.007	2	23	F	N
70		0.25	4	4	≥32	≤0.007	≤0.007	2	29	M	A
71		8	4	4	≥32	≤0.007	≤0.007	1	26	M	D
72		0.06	0.06	0.03	≥32	≤0.007	≤0.007	0.5	18	M	A
73		1	0.015	0.06	16	≤0.007	≤0.007	2	30	F	N
74		4	4	4	≥32	≤0.007	≤0.007	1	27	M	D
75		8	0.5	1	≥32	≤0.007	≤0.007	1	35	M	A
76		8	4	4	≥32	≤0.007	≤0.007	1	25	F	A

77		4	4	4	≥32	≤0.007	≤0.007	1	30	F	D
78		0.5	0.03	0.03	≥32	≤0.007	≤0.007	1	29	M	A
79		2	0.25	0.5	16	≤0.007	≤0.007	0.25	31	F	A
80		8	1	2	≥32	≤0.007	≤0.007	0.5	27	M	A
81		0.06	≤0.007	≤0.007	≥32	≤0.007	≤0.007	1	30	M	N
82		2	1	1	16	≤0.007	≤0.007	1	38	M	A
83		0.125	0.5	1	≥32	≤0.007	≤0.007	0.5	30	F	A
84		8	1	2	≥32	≤0.007	≤0.007	1	19	M	A
85		4	4	4	≥32	≤0.007	≤0.007	1	28	F	D
86		1	4	4	≥32	≤0.007	≤0.007	2	28	M	A
87		0.125	0.015	0.015	≥32	≤0.007	≤0.007	0.5	49	M	A
88		8	0.5	1	≥32	≤0.007	≤0.007	0.5	21	M	A
89		4	4	4	≥32	≤0.007	≤0.007	0.5	32	M	D
90		0.5	2	4	≥32	≤0.007	≤0.007	1	31	M	A
91		8	1	2	≥32	≤0.007	≤0.007	0.125	25	M	A
92		8	4	4	≥32	≤0.007	≤0.007	1	33	M	A
93		4	4	4	≥32	≤0.007	≤0.007	0.5	29	M	A
94		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	20	M	A
95		4	4	4	≥32	≤0.007	≤0.007	0.5	28	M	D
96		8	0.015	0.03	≥32	≤0.007	≤0.007	2	24	M	D
97		8	4	4	≥32	≤0.007	≤0.007	1	22	F	A
98		2	1	2	≥32	≤0.007	≤0.007	1	18	M	A
99		8	0.015	0.015	≥32	≤0.007	≤0.007	0.5	25	M	A
100		1	0.03	0.03	≥32	≤0.007	≤0.007	0.5	37	M	N
101		1	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	35	M	A
102		8	1	2	≥32	≤0.007	≤0.007	2	32	M	A
103		0.5	4	4	≥32	≤0.007	≤0.007	1	27	M	A
104		8	4	4	≥32	≤0.007	≤0.007	1	35	M	D

105		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	2	20	M	A
106		8	0.015	0.125	≥32	≤0.007	≤0.007	1	32	M	A
107		0.125	≤0.007	≤0.007	≥32	≤0.007	≤0.007	1	32	M	N
108		8	1	1	≥32	0.06	0.03	0.5	28	M	A
109		4	1	2	≥32	0.06	0.03	1	40	M	A
110		0.5	≤0.007	≤0.007	≥32	≤0.007	≤0.007	2	25	M	A
111		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	28	M	A
112		2	≤0.007	≤0.007	≥32	0.03	≤0.007	0.5	29	M	A
113		8	0.25	0.5	≥32	≤0.007	≤0.007	1	26	M	A
114		2	2	4	≥32	0.06	0.015	0.5	32	F	A
115		0.25	4	4	≥32	0.015	0.03	1	28	M	A
116		0.5	4	4	≥32	0.03	0.015	1	27	F	A
117		8	≤0.007	≤0.007	≥32	0.015	0.015	1	23	M	A
118		8	2	4	≥32	≤0.007	≤0.007	1	33	M	A
119		0.25	4	4	≥32	≤0.007	≤0.007	0.5	29	F	A
120		0.5	4	4	≥32	≤0.007	≤0.007	2	37	M	A
121		0.125	4	4	≥32	≤0.007	≤0.007	1	76	F	N
122		2	≤0.007	≤0.007	≥32	≤0.007	0.015	0.5	29	M	A
123		0.125	≤0.007	≤0.007	≥32	0.06	0.015	1	32	F	A
124		0.25	0.015	0.015	≥32	≤0.007	≤0.007	0.5	24	F	A
125		8	0.015	0.015	≥32	0.015	≤0.007	2	18	M	N
126		4	4	8	≥32	≤0.007	0.015	0.5	21	M	A
127		2	4	4	≥32	≤0.007	≤0.007	2	35	M	A
128		8	1	1	≥32	≤0.007	≤0.007	2	21	F	A
129		0.125	4	4	≥32	≤0.007	≤0.007	4	20	F	A
130		8	4	4	≥32	≤0.007	0.015	2	27	F	A
131		0.125	0.25	4	≥32	≤0.007	≤0.007	2	27	F	N
132		4	4	4	≥32	≤0.007	≤0.007	2	30	F	A

133		0.5	4	4	≥32	0.015	≤0.007	1	24	M	A
134		0.125	4	4	≥32	≤0.007	≤0.007	0.5	29	F	A
135		8	4	4	≥32	0.06	0.015	1	31	M	A
136		0.5	4	4	≥32	≤0.007	≤0.007	2	24	M	A
137		1	4	4	≥32	0.03	0.015	1	25	M	A
138		8	≤0.007	≤0.007	≥32	0.015	0.015	2	35	M	A
139		2	0.25	0.5	≥32	≤0.007	≤0.007	0.5	44	M	A
140		0.25	4	4	≥32	0.015	≤0.007	0.5	20	M	A
141		8	1	2	≥32	≤0.007	≤0.007	0.5	31	M	A
142		2	4	4	≥32	0.125	0.06	1	27	M	A
143		0.5	4	4	≥32	≤0.007	≤0.007	0.5	34	M	A
144		8	1	2	≥32	0.03	0.015	0.5	35	M	A
145		0.5	1	2	≥32	0.06	0.015	1	23	M	A
146		8	0.015	0.015	≥32	≤0.007	≤0.007	0.5	34	F	A
147		0.125	4	4	≥32	≤0.007	≤0.007	2	36	M	A
148		4	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	32	F	A
149		2	≤0.007	≤0.007	≥32	0.06	0.03	2	52	M	N
150		8	4	4	≥32	0.06	0.03	2	22	M	D
151		4	1	2	≥32	0.06	0.03	1	39	M	A
152		0.125	≤0.007	≤0.007	16	0.03	≤0.007	1	26	M	A
153		4	≤0.007	≤0.007	≥32	0.03	0.015	0.5	25	F	A
154		8	0.25	0.5	≥32	0.015	0.015	0.5	31	M	A
155		2	1	2	≥32	0.06	0.015	1	26	M	A
156		0.25	4	4	≥32	0.03	≤0.007	1	24	M	A
157		0.25	4	4	≥32	0.015	≤0.007	1	23	M	A
158		0.5	4	4	≥32	0.015	≤0.007	2	23	M	A
159		8	4	4	≥32	0.03	≤0.007	1	23	M	A
160		0.125	≤0.007	≤0.007	≥32	0.03	≤0.007	2	26	M	A

161		0.125	≤0.007	≤0.007	≥32	0.015	≤0.007	1	27	F	A
162		0.25	4	4	≥32	0.015	≤0.007	1	33	M	A
163		0.25	4	4	≥32	0.015	≤0.007	2	26	M	A
164		0.125	≤0.007	≤0.007	≥32	0.015	≤0.007	0.5	30	M	A
165		0.5	≤0.007	≤0.007	16	0.06	≤0.007	1	30	M	A
166		8	1	2	≥32	0.06	0.03	1	26	F	A
167		8	2	4	≥32	0.015	≤0.007	2	38	M	A
168		0.125	≤0.007	≤0.007	16	0.015	≤0.007	0.5	30	M	N
169		4	4	4	≥32	≤0.007	≤0.007	0.5	32	M	A
170		8	4	4	≥32	0.125	0.06	2	28	M	D
171		4	1	2	≥32	0.125	0.03	1	35	M	A
172		1	2	4	≥32	≤0.007	≤0.007	2	26	M	A
173		4	0.015	0.03	≥32	0.125	0.03	2	21	F	N
174		0.25	4	4	≥32	0.015	≤0.007	1	26	M	A
175		8	4	4	≥32	0.125	0.015	2	24	M	D
176		4	1	2	≥32	0.03	0.015	0.5	23	M	A
177		2	0.015	0.03	≥32	0.06	≤0.007	1	22	M	A
178		8	2	4	16	0.06	0.015	1	32	M	D
179		8	0.015	0.03	≥32	0.015	≤0.007	0.5	23	M	A
180		2	4	4	≥32	0.25	0.03	1	25	M	A
181		8	0.5	1	≥32	0.015	≤0.007	1	33	M	A
182		0.5	2	4	≥32	0.03	≤0.007	2	32	M	A
183		4	4	4	≥32	0.03	0.015	2	24	M	A
184		2	4	4	≥32	≤0.007	≤0.007	4	18	F	N
185		8	≤0.007	≤0.007	≥32	0.03	≤0.007	0.25	26	F	A
186		0.25	0.015	0.03	≥32	0.03	≤0.007	2	22	F	N
187		0.5	1	2	≥32	0.25	0.125	1	26	M	A
188		0.5	1	4	≥32	0.06	0.015	2	66	M	N

189		0.5	2	4	≥32	0.03	≤0.007	2	27	M	A
190		8	2	4	≥32	≤0.007	≤0.007	0.25	32	M	A
191		0.25	2	4	≥32	≤0.007	≤0.007	2	25	F	A
192		0.125	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	23	F	A
193		8	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	26	M	A
194		0.5	4	4	≥32	≤0.007	≤0.007	1	20	M	A
195		8	1	2	≥32	0.06	0.015	2	44	M	A
196		4	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	26	M	A
197		2	0.5	1	≥32	0.03	0.015	1	36	M	A
198		8	0.5	2	≥32	0.03	0.015	1	19	F	A
199		4	1	2	≥32	0.03	0.03	1	27	F	A
200		8	0.5	2	≥32	0.06	0.03	1	20	M	A
201		0.25	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	31	M	A
202		8	0.5	1	≥32	0.03	≤0.007	0.5	25	M	A
203		0.25	1	2	≥32	0.03	≤0.007	1	24	M	A
204		8	2	4	≥32	0.015	≤0.007	2	29	F	A
205		8	2	4	≥32	0.03	≤0.007	1	28	F	A
206		0.25	0.125	0.5	≥32	≤0.007	≤0.007	1	28	M	A
207		8	1	2	≥32	0.06	0.03	1	25	M	A
208		0.25	0.125	0.25	≥32	≤0.007	≤0.007	4	31	M	A
209		8	0.03	0.06	≥32	≤0.007	≤0.007	0.5	34	M	A
210		2	1	1	≥32	0.015	≤0.007	1	22	M	A
211		8	4	4	≥32	0.06	0.015	2	27	F	A
212		0.25	4	2	≥32	0.015	≤0.007	2	23	F	A
213		8	2	2	≥32	0.03	≤0.007	0.5	19	M	D
214		4	0.03	0.06	≥32	≤0.007	≤0.007	1	26	M	A
215		0.125	0.25	0.5	≥32	≤0.007	≤0.007	0.25	28	M	A
216		1	0.015	0.015	≥32	≤0.007	≤0.007	1	38	F	A

217		8	≤0.007	≤0.007	≥32	0.015	≤0.007	0.5	25	M	A
218		4	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	29	F	A
219		2	0.5	1	≥32	0.03	≤0.007	1	25	F	A
220		0.25	≤0.007	0.015	≥32	≤0.007	≤0.007	2	31	M	N
221		0.5	2	4	≥32	0.015	≤0.007	1	38	M	A
222		0.125	4	8	≥32	≤0.007	≤0.007	0.25	30	F	A
223		8	≤0.007	0.03	≥32	≤0.007	≤0.007	2	20	M	D
224		2	4	4	≥32	0.03	≤0.007	0.5	22	F	A
225		0.25	4	8	≥32	≤0.007	≤0.007	2	24	F	A
226		8	≤0.007	≤0.007	≥32	0.015	≤0.007	1	22	M	A
227		4	4	4	≥32	0.015	≤0.007	0.25	29	M	A
228		8	4	4	≥32	0.015	0.015	0.5	22	F	A
229		4	4	8	≥32	0.015	≤0.007	1	29	F	A
230		2	4	4	≥32	0.015	≤0.007	0.5	19	F	A
231		8	4	4	≥32	0.015	≤0.007	0.5	32	M	A
232		1	1	2	≥32	0.03	0.015	0.5	34	M	A
233		8	4	4	≥32	0.03	≤0.007	0.5	32	M	A
234		8	4	4	≥32	0.03	≤0.007	2	24	F	A
235		2	2	4	≥32	0.03	≤0.007	1	27	M	A
236		2	2	2	≥32	0.03	≤0.007	0.25	29	M	A
237		8	0.015	0.015	≥32	0.015	0.015	2	29	M	N
238		8	1	1	≥32	0.03	0.015	0.5	23	M	A
239		0.5	4	4	≥32	0.03	≤0.007	0.5	29	M	A
240		0.25	2	4	≥32	0.015	≤0.007	0.5	18	F	A
241		0.25	0.015	0.03	≥32	0.015	≤0.007	1	28	M	A
242		0.25	4	8	≥32	0.03	≤0.007	1	27	M	A
243		8	4	4	≥32	0.06	0.015	0.5	29	M	A
244		4	1	1	≥32	0.03	0.015	1	24	F	A

245		2	4	4	≥32	0.03	≤0.007	0.5	19	M	D
246		2	4	4	≥32	0.06	0.015	2	25	M	D
247		0.125	2	2	≥32	0.015	≤0.007	2	24	M	A
248		0.25	4	8	16	0.015	≤0.007	2	26	F	A
249		8	4	4	≥32	0.015	≤0.007	1	31	M	A
250		2	0.015	0.015	≥32	≤0.007	≤0.007	0.5	20	F	A
251		4	1	2	≥32	0.125	0.06	1	35	M	A
252		4	0.015	0.015	≥32	0.03	≤0.007	0.5	32	F	A
253		0.25	4	4	≥32	0.125	0.03	1	32	M	A
254		8	2	4	≥32	0.015	≤0.007	1	29	M	A
255		0.125	0.015	0.015	16	0.015	≤0.007	1	26	M	A
256		0.25	≤0.007	≤0.007	≥32	0.015	≤0.007	2	26	M	A
257		0.5	4	4	≥32	0.03	≤0.007	1	25	M	A
258		4	1	2	≥32	0.03	0.015	1	24	M	A
259		8	4	8	≥32	≤0.007	≤0.007	1	26	M	A
260		4	0.03	0.06	≥32	≤0.007	≤0.007	1	39	M	A
261		0.25	4	8	≥32	0.03	≤0.007	1	23	M	A
262		0.125	4	4	≥32	≤0.007	≤0.007	0.5	34	F	A
263		8	1	2	≥32	0.03	0.015	1	21	M	A
264		4	1	2	≥32	0.015	≤0.007	0.5	37	M	A
265		4	1	2	≥32	0.03	0.03	2	31	M	A
266		4	1	2	≥32	0.03	0.015	1	26	F	A
267		0.25	0.015	0.015	≥32	0.015	≤0.007	1	25	M	N
268		0.5	4	8	≥32	≤0.007	≤0.007	1	25	F	A
269		0.25	2	4	≥32	0.015	≤0.007	2	54	M	A
270		4	≤0.007	≤0.007	≥32	≤0.007	≤0.007	0.5	32	F	A
271		0.5	2	4	≥32	0.06	0.015	1	24	M	A
272		1	4	4	≥32	0.015	≤0.007	1	26	F	A

273		4	1	2	≥32	0.06	0.03	2	31	M	A
274		8	4	4	≥32	0.03	0.015	1	25	M	A
275		8	≤0.007	≤0.007	≥32	≤0.007	0.015	1	23	M	A
276		4	4	4	≥32	0.06	0.03	2	19	M	D
277		4	1	2	≥32	0.03	≤0.007	1	21	F	A
278		0.25	0.015	0.015	≥32	0.015	≤0.007	2	27	M	A
279		0.25	4	4	≥32	0.03	0.015	1	22	M	A
280		4	1	2	≥32	0.03	≤0.007	1	42	M	A
281		4	≤0.007	≤0.007	≥32	≤0.007	≤0.007	1	27	M	A
282		2	8	4	≥32	0.03	≤0.007	1	37	M	A
283		8	≤0.007	0.015	≥32	0.015	≤0.007	1	28	F	A
284		0.25	4	8	≥32	0.015	≤0.007	2	20	M	A
285		0.25	≤0.007	0.015	16	0.03	0.015	2	24	M	N
286		0.5	2	4	≥32	0.03	0.015	0.5	23	M	A
287		4	≤0.007	0.015	≥32	≤0.007	≤0.007	0.5	22	M	A
288		4	4	4	≥32	0.03	0.03	2	30	M	D
289		8	2	4	≥32	0.015	≤0.007	1	23	M	A
290		4	4	4	≥32	0.03	≤0.007	1	20	M	A
291		8	4	4	≥32	≤0.007	≤0.007	1	24	F	A
292		2	4	4	≥32	0.06	≤0.007	1	21	M	A
293		0.25	4	8	≥32	0.015	≤0.007	2	29	M	A
294		8	4	4	≥32	≤0.007	≤0.007	2	27	F	A
295		0.125	0.015	0.015	≥32	0.015	≤0.007	1	25	M	A
296		0.5	0.015	0.015	≥32	0.015	≤0.007	0.25	23	F	A
297		2	4	4	≥32	0.06	0.03	1	20	F	D
298		2	8	8	≥32	0.06	0.015	2	18	F	A
299		8	4	4	≥32	0.06	0.015	2	19	M	A
300		8	1	2	≥32	0.06	0.015	0.5	28	M	A

301		4	4	4	≥32	0.06	0.015	1	21	M	D
302		2	4	4	≥32	≤0.007	≤0.007	1	26	M	A
303		4	4	8	≥32	≤0.007	≤0.007	2	24	F	A
304		4	2	4	≥32	0.03	0.015	1	23	M	A
305		0.125	0.015	0.015	≥32	0.015	≤0.007	0.5	22	F	N
306		8	4	4	≥32	0.06	0.03	1	24	M	D
307		2	0.015	0.015	≥32	0.015	≤0.007	0.5	34	M	A
308		2	4	4	≥32	0.015	≤0.007	1	22	M	A
309		4	4	4	≥32	0.06	0.015	0.5	27	M	D
310		4	1	2	≥32	0.03	0.015	0.5	26	M	A
311		8	2	2	≥32	0.03	0.015	1	18	F	A
312		0.5	4	4	≥32	≤0.007	≤0.007	2	22	F	A
313		0.25	0.015	0.015	≥32	0.015	≤0.007	2	25	F	A
314		2	1	2	≥32	0.06	0.015	1	26	M	A
315		0.125	1	2	≥32	0.03	≤0.007	0.5	21	F	A
316		4	8	8	≥32	0.015	≤0.007	1	25	M	A
317		0.5	1	4	≥32	0.03	≤0.007	1	23	M	A
318		0.125	4	4	≥32	≤0.007	≤0.007	0.25	25	M	A
319		4	4	4	≥32	0.06	0.03	0.5	29	M	A

APPENDIX 5

Sequencing results of internal *tetM* American control

Figure 4: Alignment of sequences of internal American control to reference sequence

APPENDIX 6

Biomedical Research Ethical Approval and Recertification for the duration of the study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

16 September 2013

Prof P Moodley
719 Umbilo Road
Congella
moodleyp@ukzn.ac.za

Dear Prof Moodley

PROTOCOL: Surveillance for Sexually Transmitted Infections in KZN. REF: BE220/13.

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 03 May 2013.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 14 August 2013 to queries raised on 19 July 2013 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 16 September 2013.

This approval is valid for one year from **16 September 2013.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **08 October 2013.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

Professor D.R Wassenaar
Chair: Biomedical Research Ethics Committee

Professor D Wassenaar (Chair)
Biomedical Research Ethics Committee
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx
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24 August 2015

Prof P Moodley
719 Umbilo Road
Congella
moodleyp@ukzn.ac.za

Dear Prof Moodley

PROTOCOL: Surveillance for Sexually Transmitted Infections in KZN. REF: BE220/13.

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 16 September 2015
Expiration of Ethical Approval: 15 September 2016

I wish to advise you that your application for Recertification received on 12 August 2015 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

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

The approval will be ratified by a full Committee at a meeting to be held on 08 September 2015.

Yours sincerely

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics