Spectinomycin Resistance in *Neisseria gonorrhoeae*

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Declaration

This study represents the original work by the author and has not been submitted to any other university. Assistance rendered has been acknowledged. The research was carried out at the Department of Medical Microbiology, Nelson R. Mandela School of Medicine, University of KwaZulu Natal and was supervised by Professor Prashini Moodley.

Ethical approval was granted prior to the commencement of this study, reference number H233/05.

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List of Abbreviations

ABC	ATP Binding Cassette
ANT(3)	Adenyltransferase 3
ATP	Adenosine Triphosphate
$BaCl_2$	Barium Chloride
bp	Base Pair
CDC	Centers for Disease Control and Prevention
cfu	Colony Forming Unit
CMRNG	Chromosomally Mediated Resistant *Neisseria gonorrrhoeae*
CO_2	Carbon Dioxide
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
EF-G	Elongation Factor – G
GC	Gonococcal
H_2O	Water
H_2SO_4	Sulphuric Acid
H_3BO_3	Boric Acid
HA	Hydrophobic Agents
HIV	Human Immunodeficiency Virus
kb	Kilo Bases
KCl	Potassium Chloride
kDa	Kilo Daltons

KZN	KwaZulu Natal
LCAT	Lincomycin-Colistin-Amphoterricin-Trimethoprim
LOS	Lipooligosaccharide
MDa	Mega Dalton
MDR	Multiple Drug Resistant
MF	Major Facilitator
$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
mRNA	Messenger Ribonucleic Acid
mtr	Multiple Transferable Resistance
NaOH	Sodium Hydroxide
NCCLS	National Committee for Clinical and Laboratory Standards.
NYC	New York City
OM	Outer Membrane
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PEN-S	Penicillin Sensitivity
PPNG	Penicillinase producing *Neisseria gonorrhoeae*
RNA	Ribonucleic Acid
RND	Resistance-Nodulation-Cell Division
rpm	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
SMR	Small Multidrug Resistance
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection

TB	Tuberculosis
TBE	Tris-Borate-EDTA
TET-S	Tetracycline Susceptible
TM	Thayer-Martin
TNF	Tumor Necrosis Factor
tRNA	Transfer Ribonucleic Acid
TRNG	Tetracycline Resistant *Neisseria gonorrhoeae*
USA	United States of America
UV	Ultraviolet
WHO	World Health Organisation

Abstract

The potential molecular mechanism of resistance of *Neisseria gonorrhoeae* to spectinomycin was investigated using *N. gonorrhoeae* isolates collected between 2000 and 2004 from patients with male urethritis syndrome in Durban.

Stored isolates were grown on New York City agar plates. Minimum inhibitory concentrations (MIC) to spectinomycin were perfomed by means of agar dilution. PCR was performed using primers specific for the *aadA* gene which codes for a spectinomycin inactivating enzyme. A nested PCR was used to amplify the gene coding for the spectinomycin target site in the 16S rRNA. PCR was also done to amplify the *mtrR* gene of the *mtrRCDE* efflux pump since mutations in this gene are associated with increased antimicrobial resistance. The products were sequenced to detect mutations associated with increasing MICs.

MICs of the 53 isolates studied ranged from 4 to 64 mg/L. None of the isolates contained the *aadA* gene. The observed increase in MICs to spectinomycin in *N. gonorrhoeae* isolates from Durban could therefore not be attributed to the production of an antibiotic modifying enzyme. Sequencing of the 16S rRNA gene did not reveal any mutations that could be attributed to the increasing spectinomycin MICs observed. Sequencing of the *mtrR* gene revealed random mutations. However on phylogenetic analysis, no correlation could be found with the various MIC levels. The *mtrRCDE* efflux pump cannot be conclusively ruled out as a possibility for the increase in MICs but further investigation is needed.

The resistance mechanism causing the incremental increase in spectinomycin MICs amongst *N. gonorrhoeae* isolates in Durban currently remains unknown.

Chapter 1

Introduction

Gonorrhoea is a sexually transmitted infection caused by the organism *Neisseria gonorrhoeae*. It is a common cause of STIs (Alary, 1997), with a global estimate of 62 million new cases in 1999. The burden of infection varies among the different regions of the world. Western Europe and the USA reported a decline in the prevalence of gonococcal infections from 1980 to 1996 (WHO, 2001). These regions of the world are considered to be more developed and the decline in prevalence has been attributed to an increased awareness of STIs, including HIV (Alary, 1997).

The prevalence rates of *N. gonorrhoeae* in developing countries during the 1990's was reported to be 3.5% in Papua New Guinea, 3% in Cambodia, 1% in the Philippines and 0.5% in Vietnam (WHO, 2001). The African continent carries the heaviest burden of gonococcal infection with an estimated 17 million new cases in sub-Saharan Africa alone (WHO, 2001). Reports on the prevalence of gonorrhoea in Africa varies with rates of 0.02% in Gabon (1999), 5.7% in Benin (1998), 8.4% in Tanzania (1998), 7.8% in South Africa (1998), 17.1% in Malawi (1998) (WHO, 2001) and 0.6% in Ghana (2001) (Apea – Kubi *et al.*, 2004).

The increase in the prevalence of *N. gonorrhoeae* may be attributed to the high prevalence of asymptomatic infection with high infection rates, the lack of effective point of care diagnostic tests, as well as the development of resistance to many of the recommended antimicrobials for the treatment of this infection (Alary, 1997).

N. gonorrhoeae may be transmitted by sexual contact or vertically from mother to child. Patients infected with *N. gonorrhoeae* may be asymptomatic, or may present with genital discharge, urethritis, genital itchiness or conjunctivitis. The complications arising from this infection are numerous and include infertility, sepsis and sometimes death.

The development of resistance to the various antimicrobials used for the treatment of infection with *N. gonorrhoeae* is increasing (Shigemura *et al.*, 2004). Spectinomycin is an aminocyclitol antibiotic used to treat most forms of gonorrhoea. This antibiotic is administered intramuscularly to patients allergic to β-lactam agents or as an alternative to quinolones in pregnant women. (Medline, 1993).

Spectinomycin works by binding to the 30S ribosomal subunit and preventing the transfer of tRNA from the A site to the P site (Vakulenko *et al.*, 2003) thus halting protein synthesis, causing organism death.

Modification of the 16S rRNA of a particular organism can result in resistance to spectinomycin (Vakulenko *et al.,* 2003). Resistance can also be mediated by enzymatic modification of the antibiotic. The level of resistance depends on many factors including the type and strain of micro-organism. High level resistance is most commonly mediated by the production of transferases (Vakulenko *et al.,* 2003).

The *aadA* gene, which is present in Gram negative organisms codes for the adenyltransferase ANT(3) enzyme which mediates resistance to spectinomycin (Clark *et al.,* 1999). *aadA* genes are present on extrachromosomal elements and encode

adenyltransferases, which modify the position of the 9-OH group in the ring structure of spectinomycin (Read, 2000). This results in poor binding of the drug to the ribosome which impairs drug activity on the microbe (Vakulenko *et al.,* 2003). The *aadA* gene also mediates resistance to streptomycin and therefore a certain degree of cross resistance may occur between these two drugs. Streptomycin is commonly used to treat tuberculosis (TB) and this cross resistance can thus have an effect on the treatment of TB. Since STIs and TB are amongst the leading infections in South Africa, they may often occur together in an individual. The use of spectinomycin in dually infection individuals for the treatment of potential infection with *N. gonorrhoeae* may lead to the inadvertent treatment of tuberculosis with one agent only.

Although the *aadA* gene is responsible for resistance to spectinomycin in many species including *E. coli,* it is yet to be proven to be the reason for the increase in spectinomycin resistance in *N. gonorrhoeae*. Thus far the aforementioned increase in resistance has been attributed to a single step mutation in the 30S ribosomal subunit of *N. gonorrhoeae*, which alters the sensitivity of the ribosome to the drug, resulting in high level resistance to spectinomycin. This is essentially a chromosomally mediated resistance (Johnson and Morse, 1988; Tapsall, 2001). Studies carried out by Johnson and Morse (1988) and Maness *et al* (1974) concluded that the resistance to spectinomycin in *N. gonorrhoeae* was not related to antibiotic inactivating enzymes. Tapsall (2001), however, did suggest that it was possible for the gonococcus to acquire plasmid borne genes encoding resistance via inactivating enzymes.

Another possible resistance mechanism is the *mtrRCDE* efflux pump. This multidrug

efflux pump is known to be present in all *N. gonorrhoeae* isolates but is yet to be linked to spectinomycin resistance. This mtr (multiple transferable resistance) system consists of a transcriptional regulator (MtrR) and 3 linked genes viz. *mtrC*, *mtrD* and *mtrE* (Hagman *et al.,* 1995). This efflux pump, like most is located in the cytoplasmic membrane of organisms and is responsible for the energy dependent extrusion of a diverse range of hydrophobic agents (HA) including antibiotics (Veal *et al.,* 1998). The *mtrRCDE* efflux pump belongs to the resistance nodulation and cell division (RND) family of efflux pumps and therefore uses proton motive force to drive the active efflux of various agents (Grkovic *et al.,* 2002). According to Veal *et al* (1998) the MtrR protein is a repressor which regulates the transcription of the *mtrCDE* gene complex. Mutations in the *mtrR* gene which codes for the MtrR protein result in a decrease of the repressor function of this protein and therefore the expression of the *mtrC*, *mtrD* and *mtrE* genes is increased (Hagman *et al.,* 1995), causing enhanced activity of the *mtrCDE* complex which results in higher levels of resistance due to the increased rate of extrusion of HAs (Grkovic *et al.,* 2002).

Prior to the emergence of penicillinase producing *N. gonorrhoeae* (PPNG), penicillin was the drug of choice for the treatment of gonorrhoea, while spectinomycin was utilised as a second line agent (WHO, 2001). This high level resistance to penicillin is approximately 33% in KwaZulu Natal (KZN) (Moodley *et al*., 2001). The initial cases of spectinomycin resistance in *N. gonorrhoeae* were recorded in the Far East during the 1980's. Treatment failures with spectinomycin have also been noted and are most likely to be as a result of the antibiotic being poorly distributed from the injection site (Tapsall, 2001).

With the syndromic management approach being widely used to treat STIs in South Africa, another drug that is commonly used against *N. gonorrhoeae* is tetracycline. As with penicillin, however, high level resistance (MIC $\geq$ 16 mg/L) has also developed against tetracycline. The prevalence of high level tetracycline resistant *N. gonorrhoeae* in KZN is around 70%. Quinolones have also been used to treat gonorrhoea but the prevalence of resistance to ciprofloxacin in KZN has been reported to be around 42%. Isolates are still susceptible to the cephalosporins, ceftriaxone and cefixime which are used as second line agents against gonorrhoea during pregnancy, in South Africa (personal correspondence). Spectinomycin is thus an alternative treatment in our country but the MICs of this drug have been increasing.

Table 1.1 shows the trend of MICs to spectinomycin over a 9 year period in KZN (Moodley *et al.*, 2006). The minimum inhibitory concentration (MIC) of an organism is defined as the lowest concentration of a particular drug that will inhibit the growth of the organism. The breakpoint for susceptibility of spectinomycin in *N. gonorrhoeae* is 32 mg/L while the breakpoint for resistance is $\geq$ 128 mg/L. Table 1.1 shows that there is a definite shift to the right of MICs from 1997 to 2003. By 2003, 93% of isolates had an MIC of 16 mg/L as compared to 8% in 1997. The MICs for ciprofloxacin and ceftriaxone also show a shift to the right (Moodley *et al.*, 2006).

Table 1.1 : MIC trends of *N. gonorrhoeae* in South Africa from 1995 to 2003 (Moodley *et al.*, 2006)

Antimicrobial Agent	Year	Percentage of Isolates With Minimum Inhibitory Concentration (mg/L)													
		≤ 0.007	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64
Spectinomycin	95	Not Tested													
	97	9	3	4	4	5	1	2	7	13	10	17	20	8	
	98/99	3		2				7	2	6	16	10	48	2	2
	99/00												22	78	
	03												1	93	6
Ciprofloxacin	95	100													
	97	96	4												
	98/99	84	3	9	4										
	99/00	71	19	10											
	03	75	1	1		1				2	7	12	1		
Ceftriaxone	95	100													
	97	95	3	1	2										
	98/99	62	26	7	5										
	99/00	53	31	10	5										
	03	6	38	41	12	1	1		1						

Options for the treatment of gonorrhoea are declining while the prevalence of the infection appears to be on the increase. Infection with *N. gonorrhoeae*, is treatable but this pathogen is developing resistance to recommended antimicrobial agents at a rapid rate. This is the case with most first line agents and even with some alternative agents, like spectinomycin. It is therefore important to study and understand the mechanisms via which spectinomycin resistance is mediated in *N. gonorrhoeae* in order to develop more effective drugs for the treatment of this infection.

Hence, the aim of this investigation was to ascertain the mechanism via which spectinomycin resistance is mediated in *N. gonorrhoeae*.

Chapter 2

Review of Literature

2.1 Resistance Mechanisms to Antimicrobial Agents

Bacterial resistance to antimicrobial agents may occur through mutations or via plasmid and transposon specified genes (Bryan, 1988). Both these chromosomal and extra-chromosomal mechanisms may co-exist in one organism, thereby exponentially raising the level of resistance to an antibiotic (Tapsall, 2001). In order for an antimicrobial agent to carry out its function, it has to be able to enter the cell and then attach to and inactivate target sites (Conte Jr. and Barriere, 1992). It is usually at these target sites and / or entry sites that mechanisms of resistance develop.

According to Li *et al* (2000), the development of resistance and reduced susceptibility of *N. gonorrhoeae* to antimicrobial agents can be attributed to chromosomal mutations. Each mutant gene has the ability to increase resistance to a particular antibiotic until treatment failure occurs. The resistance genes can be transferred from resistant to susceptible strains of an organism (Hu *et al.*, 2005).

Resistance to bacteria can either be intrinsic or acquired. Intrinsic resistance refers to bacteria that are naturally insensitive to an antibiotic without acquiring resistance factors (Bryan, 1988). Acquired resistance would therefore refer to the acquisition of resistance factors in the form of chromosomal mutations, extra-chromosomal resistance genes via plasmids, transposons, integrons, gene cassettes and insertion

sequences (Powell, 2000). The most common mechanisms via which acquired resistance may occur include alteration of the target site which results in inhibition of the binding of the drug, inhibition of transport of the drug into the cell, destruction or inactivation of the antibiotic and metabolic bypass where the organism replaces the metabolic step inhibited by the antimicrobial agent (Conte Jr. and Barriere, 1992).

2.1.1 Target Alteration

This mechanism of resistance occurs when the target site in the cell is altered so that it is insensitive to the antimicrobial agent, yet the cell is still able to function normally (Gale *et al.,* 1981). According to Conte Jr. and Barriere (1992), this type of resistance is commonly associated with antibiotics that interfere with ribosomal function eg: streptomycin, spectinomycin and rifampicin. Resistance is thought to originate from the selection of pre-existing mutations in a sensitive population of bacteria (Conte Jr. and Barriere, 1992).

Often, the mutations that lead to resistance occur as a result of a single amino acid alteration at the target site thereby inhibiting binding of the antibiotic (Powell, 2000). In sensitive bacteria, streptomycin binds to protein S12 in the 30S ribosomal subunit of the organism but a single mutation in the polypeptide chain of this protein results in decreased affinity with subsequent resistance of the organism to the drug (Conte Jr. and Barriere, 1992). Resistance to spectinomycin in *N. gonorrhoeae* is mediated in a similar manner.

Mutations causing a decreased affinity in penicillin-binding protein (PBP) 2 to certain penicillins also results in resistance to these agents (Gale *et al.,* 1981).

2.1.2 Enzyme Inactivation of the Drug

Many bacteria produce enzymes that inactivate antimicrobial agents (Gale *et al.*, 1981). This can be done via enzyme cleavage or alteration of the drug so that it can no longer interact with the specified target (Powell, 2000). Aminoglycosides, cephalosporins and penicillins are among the antimicrobial agents that can be modified by enzymes, making this mechanism of resistance one of great clinical importance since these antimicrobial agents are widely used to treat a variety of infections (Conte Jr. and Barriere, 1992).

The genes that code for these modifying enzymes are located on plasmids and are therefore completely transferable. The enzymes produced include β - lactamases and cephalosporinases, which inhibit the action of β-lactam antibiotics. The acetyltransferases modify antibiotics via acetylation. Gram positive organisms encode a bifunctional aminoglycoside modifying enzyme that inactivates the antibiotic by the production of both acetyl- and phosphotransferases (Powell, 2000).

A number of organisms, including *E. coli* are resistant to spectinomycin via an antibiotic modifying enzyme. The *aadA* gene codes for an adenyltransferase that modifies the position of the 9-OH in the ring structure of spectinomycin resulting in the drug binding poorly to its target site (Read, 2000).

2.1.3 Synthesis of Resistant Metabolic Pathways

Certain antimicrobial agents such as trimethoprim work by inhibiting certain enzymes in the metabolic pathways of bacteria. Some bacteria, however, are capable of producing excess amounts of the enzyme in question so that the metabolic pathway

can still function in the presence of the antibiotic, despite its action. Some thymidine-dependent streptococci produce excess amounts of thymidine by an alternate pathway, so that trimethoprim and sulphonamides have a reduced or no effect (Powell, 2000).

2.1.4 Failure to Metabolise the Drug

Some drugs are only activated once they enter the bacterial cell. Failure to metabolise the drug means that it remains inactive and the organism becomes resistant, as is the case with *Bacteriodes fragilis* and metronidazole (Powell, 2000).

2.1.5 Movement of Resistance Genes

If genes encoding for resistance in bacteria do not occur as part of the bacterial chromosome, they may be found on extrachromosomal elements e.g.: plasmids (Gale *et al.*, 1981).

When exposed to adverse conditions bacteria may replicate and exchange genes that will confer a selective advantage. The most common genetic exchange mechanisms include exchange of plasmids via conjugation. Conjugation involves the direct contact of two cells via a sex pilus through which DNA passes unidirectionally from the donor cell to the recipient cell (Conte Jr. and Barriere, 1992).

Transposons or "jumping genes" commonly allow for the movement of genes between the chromosome and the plasmid and vice-versa, although genetic exchange via plasmids can occur in the absence of transposons. This exchange is even possible between organisms of different genera. Integrons are a subset of transposons often found on resistance (R) plasmids. Integrons consist of a "cassette" that codes for

various functions including antimicrobial resistance, flanked by two conserved segments (Powell, 2000).

Transformation, transduction and conjugation are the three most common methods for exchange of genetic information. Transformation involves the lysing of the bacterial cell so that the DNA is released into the surrounding medium to be taken up by other intact bacteria in the area. Transduction deals with the incorporation of bacterial DNA into a bacteriophage, which acts as a vector (Conte Jr. and Barriere, 1992).

2.1.6 Decrease of Concentration of the Drug

A decrease in the concentration of an antimicrobial agent at its target site can either be as a result of a decrease in the permeability of the cell envelope to the drug so that lower concentrations of the drug enter the cell or via an increase in the efflux pumps which pump that drug out of the cell (Conte Jr. and Barriere, 1992; Powell, 2000). Resistance seems to be attributed to the fact that critical concentrations of the antibiotic cannot be accumulated within the cell (Gale *et al.,* 1981). This seems to be the case with aminoglycosides and tetracyclines where interference with the transport of these antibiotics into the cell is a cause of resistance in certain organisms (Conte Jr. and Barriere, 1992).

Tetracycline is taken up, into normal cells via active transport and therefore accumulates within the cell (Conte Jr. and Barriere, 1992). The presence of tetracycline in a cell results in changes in the cell membrane that cause tetracycline to be excluded from inside the cell, thereby resulting in resistance to the antibiotic. A similar mechanism of resistance exists in Gram positive *Staphylococcus aureus* to

fusidic acid where changes in the cell envelope prevent the antibiotic from reaching its intended target (Gale *et al.,* 1981).

Another mechanism via which organisms decrease the concentration of the drug reaching its target site is via drug efflux pumps. These efflux systems are chromosomally encoded and are responsible for the extrusion of toxic substances from the cell. Because of their ability to cause drug resistance, these efflux pumps are present in many organisms and their gene sequences are conserved in some. Efflux pumps have been divided into four major families on the basis of assembly, mechanism and sequence homology (Powell, 2000).

In the ATP binding cassette (ABC) superfamily, each family works on a specific substrate including small molecules as well as proteins and carbohydrates produced in the cytoplasm. The major facilitator (MF) superfamily consists of more than three hundred families, each of which is specific for a different type of solute including sugars and organic and inorganic anions. Most drug resistance pumps belong to this family. The small multidrug resistance (SMR) family is comprised of only ten members, which extrude lipophilic cations. The sequences of these pumps are well conserved. Finally, the resistance-nodulation-cell division (RND) family is divided into three sub-families, the first is specific for divalent heavy metal ions, the second for lipooligosaccharides and the third for the efflux of a number of drugs (Powell, 2000).

2.1.6.1 The *mtrRCDE* Efflux Pump of *N. gonorrhoeae*

Efflux pumps can cause resistance to antibiotics as a result of their ability to extrude certain agents from the organisms in which they are present. These pumps may be

specific to a particular compound or may have the ability to extrude a diverse range of compounds, leading to multiple drug resistance (MDR). Efflux systems are usually energy dependent and are essentially transport proteins which facilitate the active pumping of toxic compounds out of the cell and into the surrounding environment. They exist in both Gram positive and Gram negative organisms (Webber and Piddock, 2003).

Gram negative bacteria are known to possess a cytoplasmic membrane as well as an outer membrane (OM) and sandwiched in between these 2 membranes is the periplasm. Efflux pumps are located in the cytoplasmic membranes of organisms. In the case of Gram negative organisms like *N. gonorrhoeae*, the presence of an accessory protein is required as part of the efflux system to bridge the gap between the cytoplasmic membrane and the OM so that the antibiotics are pumped out of the cell completely and not trapped in the periplasm once they have been extruded from the cytoplasm (Nikaido, 1994). It is for this reason that the RND family of efflux systems to which the *mtrRCDE* pump belongs consists of 3 proteins working synergistically to actively pump substances out of the organism and into the surrounding environment, similarly to the MexXY-OprM and MexJK-OprM efflux systems found in *Pseudomonas aeruginosa* (Schweizer, 2003).

Generally, efflux pumps in Gram negative organisms are thought to consist of a tripartite structure (Schweizer, 2003). Figure 2.1 illustrates this general structure. The three proteins which constitute this pump are a cytoplasmic membrane associated efflux transporter protein that is usually from the MF or RND families, this is attached

to an accessory protein which is in turn associated with an OM channel protein to ensure the extrusion of substances into the surrounding environment (Nikaido, 1994).

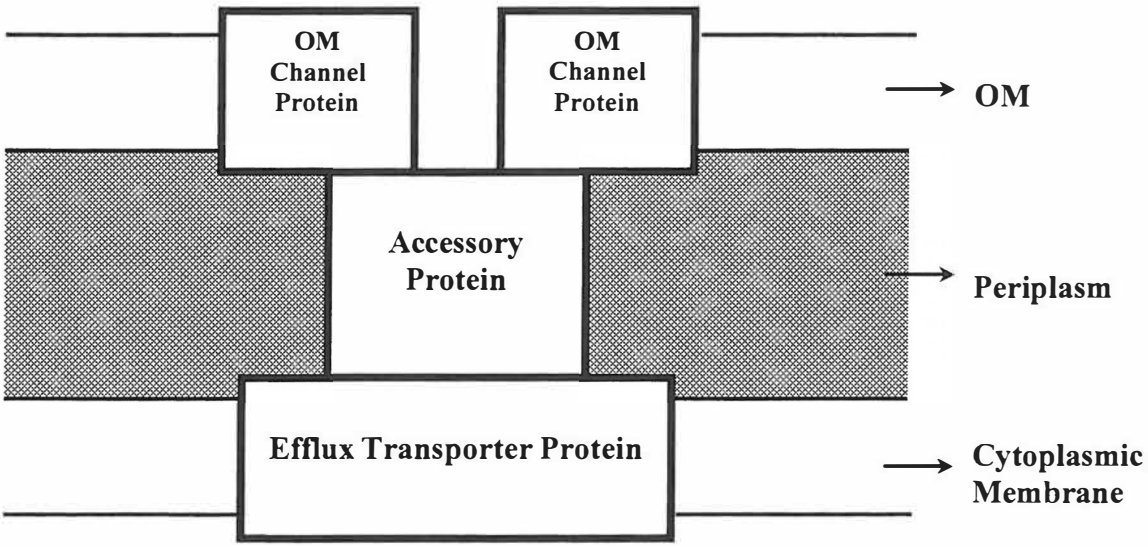


Figure 2.1 : General Structure of Efflux Systems in Gram Negative Organisms (Modified from Nikaido, 1994)

The *mtrRCDE* efflux pump of *N. gonorrhoeae* has a similar structure. The MtrD protein acts as the efflux transporter protein and belongs to the RND family, the MtrE protein is the OM channel protein and the MtrC protein is the accessory protein from the MF family (Veal *et al.,* 1998) that joins the MtrD and MtrE proteins (Rouquette-Loughlin *et al.,* 2002). It can therefore be assumed that the *mtrRCDE* efflux pump executes it function by the HA being captured by the MtrD protein in the cytoplasmic membrane, it is subsequently pumped through the MtrC protein and out of the organism via the MtrE protein (Nikaido, 1994).

The MtrR protein is also a significant component of the *mtrRCDE* efflux pump. It is encoded for by the *mtrR* gene which is situated 250 bp upstream and divergently

transcribed from the *mtrCDE* complex. MtrR is a transcriptional repressor that regulates the transcription of the *mtrCDE* efflux system (Veal *et al.,* 1998). This protein is similar to a variety of transcriptional repressors especially those of the *TetR* family (Hagman *et al.,* 1995). According to Grkovic *et al* (2002), MtrR is only a modulator and does not play a role in induction of *mtrCDE* expression. Although this is the case, mutations in the coding or promoter regions of the *mtrR* gene can result in a decrease in MtrR activity. Even a single base pair deletion in the promoter region of this gene can cause a reduction in repression of transcription of the *mtrCDE* complex (Veal *et al.,* 1998). This in turn results in increased expression of the *mtrCDE* genes, leading to increases in resistance to HAs as a consequence of higher efflux rates (Grkovic *et al.,* 2002). The level of resistance inferred by these pumps can differ depending on the type of mutation, for example, organisms which carry mutations in the *mtrR* transcriptional regulator display lower levels of resistance when compared to organisms with mutations in the *mtrR* promoter region (Hu *et al.*, 2005).

Mutations in the *mtrR* gene have been linked to increases in resistance in *N. gonorrhoeae* to azithromycin, erythromycin (Grkovic *et al.,* 2002), penicillin and tetracycline (Veal *et al.,* 2002). It is therefore possible that spectinomycin resistance may also be linked to this efflux pump.

2.2 General Characteristics of *Neisseria gonorrhoeae*

2.2.1 History of *N. gonorrhoeae*

Gonorrhoea is a very old infectious disease that was first identified and described by A. Neisser in 1879 (Matsuoka, 1989; Todar, 2004). Subsequently *Neisseria gonorrhoeae* was cultured by Bumm in 1885 fulfilling all of Koch's postulates for this organism (Matsuoka, 1989; Todar, 2004).

The infection was named gonorrhoea by Galen in the second century AD as a description of the urethral discharge that it caused which was thought to be seminal discharge (Oates and Csonka, 1990).

According to Cheng (1989), gonorrhoea is the most ubiquitous disease of mankind.

2.2.2 Description

Neisseria gonorrhoeae is one member of the genus *Neisseria* as well as the causative agent of gonorrhoea. The organism is a non-motile, Gram negative, aerobic diplococcus often referred to as the gonococcus. *N. gonorrhoeae*, most commonly occurs in pairs of kidney or bean shaped organisms (Cheng, 1989) with adjacent flattened sides (Todar, 2004).

2.2.3 Culture Characteristics of *N. gonorrhoeae*

As with most causes of sexually transmitted infections, *N. gonorrhoeae* is difficult to culture and requires specialised nutrients and specific conditions to grow (Conte Jr. and Barriere, 1992). Many variations and combinations of media have been developed

in an effort to optimise the growth of these organisms, for example Thayer-Martin (TM) Medium, Martin-Lewis Medium and New York City (NYC) Medium (Robertson *et al.,* 1989).

N. gonorrhoeae requires 3 – 10% CO_2 to grow at a temperature of 35 – 37°C, for 24 – 48 hours (Oates and Csonka, 1990; Todar, 2004) in a moist environment. These conditions also have to be maintained if *N. gonorrhoeae* specimens are to be transported, in order to avoid loss of viability of the organism. Media such as Stuarts Medium or Amies Medium are commercially available for the transport of *N. gonorrhoeae* and contain the appropriate nutrients to keep the organism viable (Robertson *et al.*, 1989) GC transport medium and Charcoal medium are also suitable for this purpose.

2.2.4 Identification Tests for *Neisseria gonorrhoeae*

A number of identification tests exist for the gonococcus but the most common include colony morphology, Gram staining, the oxidase test as well as carbohydrate utilisation tests.

2.2.4.1 Colony Morphology

The specimen/s should be streaked onto New York City agar plates and incubated at 37°C, in 3 – 10% CO_2 for 24 hours. After the incubation period colonies of *N. gonorrhoeae* should appear on the agar plates (except in the case of slow growing strains which may take up to 48 hours to grow). The colonies should be about 0.5 – 1.0 mm in diameter. They should appear glistening and raised as well as opaque and gray to white in colour (Morello and Bohnhoff, 1980).

2.2.4.2 Gram Stain

A smear of the specimen is made onto a glass slide, heat fixed using a Bunsen burner and subsequently stained using the standard Grams' staining technique. The slide should then be viewed under a microscope at 1000X magnification with 2 mm oil immersion (Oates and Csonka, 1990). *N. gonorrhoeae* is a Gram negative organism and therefore appears reddish-pink on a Gram stained smear (figure 2.2). The organism appears as kidney shaped diplococci (Whittington *et al.,* 1994).

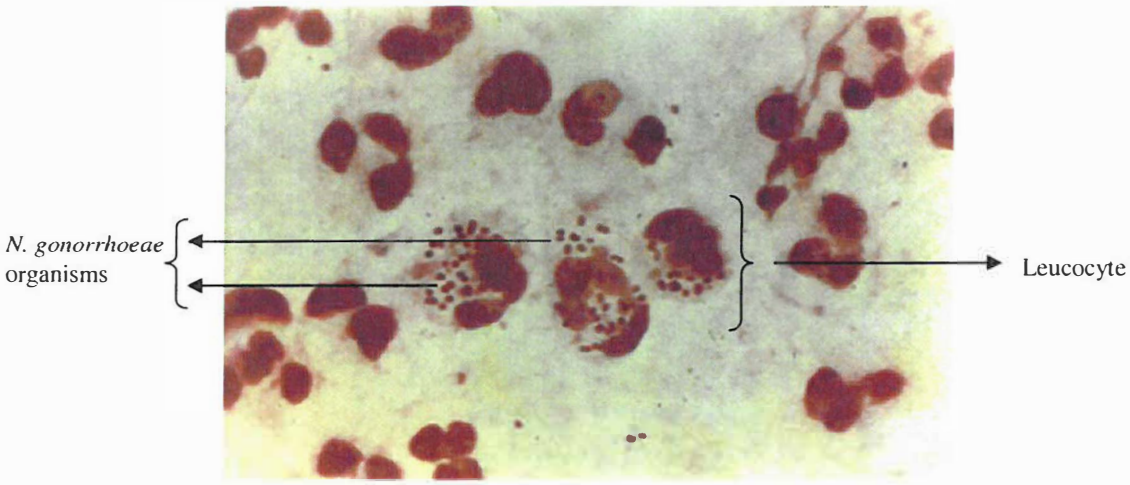


Figure 2.2. : *N. gonorrhoeae* organisms within polymorphonuclear leucocytes

2.2.4.3 Oxidase Test

This test is part of the battery of tests used in the identification of *N. gonorrhoeae*. It is positive in *N. gonorrhoeae*. The oxidase test is usually carried out on filter paper or on the agar plate on which the culture is growing. A drop of oxidase reagent, tetramethyl-p-phenylene-diamine dihydrochloride is added directly to the colony and the reaction is read after 15 to 20 seconds. A positive reaction causes a colour change in the reagent from colourless to purple (Morello and Bohnhoff, 1980).

2.2.4.4 Carbohydrate Utilisation Test

N. gonorrhoeae can also be identified by its ability to ferment glucose only. This is a characteristic unique to *N. gonorrhoeae* while most other *Neisseria* species ferment maltose with or without additional lactose and sucrose fermentation. (Whittington *et al.,* 1994) (table 2.1). According to Oates and Csonka (1990), this feature can be regarded as the most reliable for the identification of *N. gonorrhoeae*.

Table 2.1 : Fermentation reactions of *Neisseria* species (Oates and Csonka (1990))

	Glucose	**Maltose**	**Sucrose**	**Lactose**
N. gonorrhoeae	+	-	-	-
N. meningitides	+	+	-	-
N. pharyngis sicca	+	+	+	-
N. lactamica	+	+	-	+

2.3 Epidemiology of *Neisseria gonorrhoeae* Infection

Neisseria gonorrhoeae is one of the most prevalent sexually transmitted pathogens in the world. Gonococcal infection rates have fluctuated over the years. (Todar, 2004). Whilst the prevalence rates of gonococcal infections had declined in developed countries during the 1980s, the prevalence in Africa was estimated as being between 5 to 50 times higher than that of the USA. (Cheng 1989). Prevalence studies conducted in Africa, Latin America and Asia have also reported that the highest prevalence of gonorrhoea was found in Africa (Salam *et al.,* 2005).

As a result of inadequate diagnostic and treatment options in resource poor settings, the prevalence of the STI tends to be higher (Alary, 1997). Moodley *et al* (2002a)

attributed the high prevalence of STIs in KwaZulu Natal in South Africa to rapid re-infection following appropriate syndromic treatment (Moodley, 2002a).

In Africa, *N. gonorrhoeae* is responsible for 53 – 80% of male urethritis cases, 20 – 40% of female prostitutes are infected with gonorrhoea along with 3 – 10% of pregnant females (Alary, 1997). In KZN, the prevalence of *N. gonorrhoeae* was reported to be 52% amongst males (Sturm *et al.,* 2004) and 12% amongst females (Moodley *et al.,* 2002b).

2.4 Pathogenesis of *Neisseria gonorrhoeae*

Gonorrhoea is a highly infectious disease and requires a low organism load to cause infection (Freeman, 1985). Spread of the disease is primarily through sexual contact. Table 2.2 summarises the surface elements of gonococcal cells that play an important role in the initial interaction with host cells and which often determine whether infection will occur (Heckels, 1978).

The first step in the pathogenesis of *N. gonorrhoeae* is the attachment of the organism to the mucosal surface of non-ciliated epithelial cells which is mediated by pili (fimbriae) or by outer membrane proteins or both (Freeman, 1985). The main outer membrane components which are involved with adherence and invasion of the host cell are Opa, an opacity surface protein, Por, a major porin protein, Rmp, an outer membrane protein common to all strains of *N. gonorrhoeae* and lipooligosaccharide (LOS) a structural component of the outer membrane (Todar, 2004).

The Opa protein is important in establishing infection, since it plays a major role in adherence to epithelial cells (Ram and Rice, 2001). Once the organism has attached to the surface of the host cell, it undergoes endocytosis and is eventually released by exocytosis. Por, which is found in the outer membrane of *N. gonorrhoeae* is thought to mediate invasion of the host cell and promotes virulence in *N. gonorrhoeae*. LOS is released during infection and stimulates the production of tumor necrosis factor (TNF) which results in cell damage. Rmp blocks the bactericidal antibodies designed to eliminate LOS and Por. Transferrin and lactoferrin receptors scavenge transferrin- and lactoferrin-bound iron from the host (Todar, 2004).

Table 2.2 : Surface components of *N. gonorrhoeae* that play a role in virulence (Todar, 2004)

Component	Location	Role
PilE	Fimbrial protein	Initial binding to epithelial cells
Opa	Outer membrane protein	Contributes to invasion
Por	Outer membrane protein	Prevents phagolysosome formation in neutrophils
Rmp	Outer membrane protein	Responsible for the formation of ineffective antibodies that inhibit bactericidal activity against Por and LOS
LOS	Outer membrane lipooligosaccharide	Responsible for inflammatory response and release of TNF
Tbp1 and Tbp2	Outer membrane transferrin receptors	Iron acquisition
Lbp	Outer membrane lactoferrin receptor	Iron acquisition

2.5 Clinical Manifestations of *Neisseria gonorrhoeae*

Gonorrhoea commonly infects the mucous membranes and results in a discharge of pus that is more obvious in males than in females. *N. gonorrhoeae* may colonise and infect the urethra, pharynx, rectum and conjunctiva in males. Mucosal surfaces in females that are most commonly infected are the endocervix, urethra, rectum, pharynx and conjunctiva (Todar, 2004).

Asymptomatic infection with *N. gonorrhoeae* is commoner in females than males (Morello and Bohnhoff, 1980). Symptoms usually develop within 10 days of infection. The most common symptoms in females are increased vaginal discharge and dysuria (Ram and Rice, 2001) while urethral discharge, dysuria (Oates and Csonka, 1990), as well as swelling and pain in the urethral tissue are common in males (Todar, 2004).

Infection in women may spread from the endocervix to the uterus, fallopian tubes and ovaries. This may result in complications such as pelvic inflammatory disease, ectopic pregnancies and infertility (Todar, 2004). In addition, the organism may spread into the peritoneal cavity and cause a peri-hepatitis syndrome (Fitz-Hugh-Curtis Syndrome) (PeaceHealth, 2006). Gonorrhoea in males is also characterised by infection of the urethral mucosa, resulting in urethritis as well as infection of the testicles and the prostrate (Todar, 2004).

Often, gonorrhoea may be transmitted during birth. The most common manifestation in infants is a form of conjunctivitis called gonococcal ophthalmia neonatorum which

results in purulent discharge and if left untreated can lead to corneal scarring (Robertson *et al.,* 1989) and eventually to blindness.

2.6 Treatment of *Neisseria gonorrhoeae*

WHO guidelines recommend third generation cephalosporins, fluoroquinolones or spectinomycin for the treatment for gonococcal infection. (Lawung *et al.*, 2005). The United States Centers for Disease Control and Prevention currently recommends that uncomplicated infection with *N. gonorrhoeae* be treated with third generation cephalosporins. Fluoroquinolones are not recommended. Spectinomycin may be considered as an alternative to the third generation cephalosporins (Barclay, 2007).

According to Oates and Csonka (1990) and Zheng *et al* (2003) the purpose of treatment of infection with a pathogen is to eliminate the pathogen as quickly as possible from the body, thus limiting its transmission. However, many problems challenge the prompt treatment of gonorrhoea, including a lack of proper facilities to accurately diagnose and treat the infection especially in resource poor settings. This prompted WHO to adopt the syndromic approach for the management of sexually transmitted infections.

A treatment regimen in which the organism is susceptible to the drug will result in elimination of the pathogen (Robertson *et al.,* 1989). The antibiotics prescribed as part of the syndromic management package should therefore be based on the susceptibility pattern of the antimicrobial agent. This varies between different geographical areas

(Robertson *et al.,* 1989; Arreaza *et al.,* 2002; Salam *et al.,* 2005). Therefore treatment regimens should be guided by regular susceptibility pattern surveillance. WHO guidelines suggest that once resistance to a particular agent reaches a prevalence of 5%, it should no longer be used as empirical therapy (Tapsall, 2001).

South Africa adopted the syndromic approach for the management of STIs in 1995 and is now widely practiced (Moodley *et al.,* 2001). The syndromic management approach is based on algorithms which are formulated based on patients symptoms (Moodley *et al.,* 2001) as well as the causative organisms causing the symptoms and signs (Moodley *et al.,* 2006). Often individuals are infected with more than one STI causing organisms. Syndromic management allows treatment to be prescribed that can potentially eliminate the most prevalent microbial causes of infection presenting as a clinical syndrome. Furthermore it allows patients to begin treatment much sooner than if they had to wait for results from a laboratory test.

The drug currently used as part of syndromic management for the treatment of potential infection with *N. gonorrhoeae* in South Africa is ciprofloxacin. This recommendation from the National Department of Health has not changed despite widespread evidence that more than 5% of isolates of *N. gonorrhoeae* are resistant (personal correspondence: Prof P Moodley and Prof David Lewis). In KwaZulu Natal, this recommendation has recently been changed and ceftriaxone is the drug of choice. Spectinomycin is an older drug that has traditionally been used as part of syndromic management for the management of *N. gonorrhoeae* in pregnant women and in people who are allergic to β-lactam agents (Bala *et al*., 2005; Moodley *et al*., 2006).

With the increased resistance of *Neisseria gonorrhoeae* to ciprofloxacin, this drug has now became a potential candidate for use as a first line agent.

2.7 Antimicrobial Resistance of *Neisseria gonorrhoeae*

Treatment regimens have been developed for the treatment of infection with *N. gonorrheoae*. These regimens, however, regularly need to be altered as a result of the development of resistance to antimicrobial agents by *N. gonorrhoeae* and it is this resistance that makes it difficult to keep gonorrhoea under control. Resistance to antibiotics such as penicillin, tetracycline, spectinomycin, ciprofloxacin and ceftriaxone developed at varying intervals after these drugs were introduced as treatment agents against infection with *N. gonorrhoeae* (Li *et al*., 2000).

N. gonorrhoeae began to develop resistance to antibiotics in the late 1950's and this trend has continued to develop and spread worldwide (Arreaza *et al.,* 2003). For example, in investigations carried out in 1938, in the USA, it was found that the majority of *N. gonorrhoeae* strains were susceptible to sulphonamides, which was the treatment agent at the time but 10 years later, by 1948, 80% of *N. gonorrhoeae* isolates were resistant to the same drug making it an ineffective treatment modality for infection with *N. gonorrhoeae* (Conte Jr. and Barriere, 1992).

N. gonorrhoeae is capable of adjusting to adverse conditions in order to survive, The increased use of certain antimicrobial agents has allowed this bacterium to develop resistance mechanisms against many antimicrobials, rendering them ineffective.. Although certain drugs such as penicillin, are no longer prescribed for the treatment of

gonorrhoea, they can still be used to treat other unrelated infections thus exerting selective pressure on *N. gonorrhoeae* isolates (Lawung *et al.*, 2005). Acquired resistance may also exist in *N. gonorrhoeae*, where resistance genes are exchanged between organisms as well as organisms and the environment (Heritage, 2006).

2.7.1 Penicillin Resistant *N. gonorrhoeae*

It has been stated that the discovery of penicillin by Sir Alexander Fleming, around 1928, transformed the treatment of infectious diseases. This β - lactam antibiotic works by binding to an enzyme that produces peptidoglycan which is an essential part of the cell wall. Cell wall synthesis is thus prevented. Penicillin, which occurs naturally is active against both Gram negative and Gram positive organisms. There are also semi-synthetic penicillins which are produced by modification of the side chain of the β - lactam ring structure, which are active against a number of pathogens (Wilson, 2000).

For a number of years penicillin was used as a first line treatment agent against gonococcal infection (Wilson, 2000). According to Chenia *et al* (1997), penicillin has been used to treat gonorrhoea since 1943. However, in the mid 1970's, strains of *N. gonorrhoeae* capable of producing penicillinase emerged (Oates and Csonka, 1990; Salam *et al.,* 2005). This resulted in decreased efficacy of penicillin for the treatment of infection with *N. gonorrhoeae*. The mode of action of the enzyme penicillinase is to break open the β -lactam ring structure, thereby destroying the antibiotic (Wilson, 2000). Penicillinase-producing *Neisseria gonorrhoeae* (PPNG) was first isolated in 1976 in the Far East and West Africa (Chenia *et al.,* 1997; Cheng, 1989).

The TEM-1 type β -lactamase gene is carried on a plasmid (Tapsall, 2001) and codes for penicillinase, hence this type of resistance is plasmid mediated. It is thought that an R-plasmid carrying the β -lactamase gene was transferred to *N. gonorrhoeae* from *Enterobacteriaceae* (Conte Jr. and Barriere, 1992). Since then the plasmids carrying resistance genes have spread rapidly worldwide, rendering *N. gonorrhoeae* resistant to penicillin and in turn creating a major problem as far as treatment of gonorrhoea is concerned.

The resistance genes are carried on plasmids of varying sizes. The isolates from the Far East that were among the first PPNG isolates discovered were found to carry a 4.4 megadalton (MDa) plasmid named the "Asian" plasmid, which was associated with a 24.5 MDa plasmid. Similarly, the initial PPNG strains isolated in West Africa contained a 3.2 MDa plasmid coding for β -lactamase production named the "African" plasmid (Oates and Csonka, 1990; Cheng, 1989), which was discovered in 1981 (Tapsall, 2001). Altogether, six plasmids have been described (table 2.3) and it is believed that PPNG strains may contain a 2.6 MDa cryptic plasmid, a 24.5 MDa conjugative plasmid and a 25.2 MDa *TetM* conjugative plasmid (Chenia *et al.,* 1997).

Table 2.3 : Various plasmids carried by PPNG strains, responsible for β -lactamase production (Chenia *et al.,* 1997)

Name	Size (MDa)
African	3.2
Asian	4.4
New Zealand	6.0
Nimes	40
Rio	2.9
Toronto	3.05

As a result of its association with the large 24.5 MDa conjugative plasmid, the Asian plasmid is thought to disseminate more rapidly (Robertson *et al.,* 1989).

The first PPNG strains were isolated in Durban, South Africa in 1977 and have spread widely in the province and the country. Table 2.4 documents this increasing pattern. Currently, the breakpoint for penicillin sensitivity (PEN-S) is ≤ 0.06 mg/L, intermediate strains are at 0.5 mg/L while chromosomally mediated resistance is ≥ 2 mg/L and plasmid mediated resistance is ≥ 8 mg/L (NCCLS Guidelines, 1999; Moodley *et al.*, 2001).

Table 2.4 : Increase in PPNG strains in Durban, South Africa (Chenia *et al.,* 1997; Moodley *et al.,* 2001)

Year	Prevalence of PPNG (%)
1983	5
1985	29
1990	16
1994	19
2000	30

These statistics are relatively low when compared to countries like the Phillipines and Vietnam which had PPNG prevalence rates of 79% and 62.7% respectively by 1998 (Tapsall, 2001).

Plasmid mediated resistance is not the only type of resistance present in *N. gonorrhoeae*. Resistance to penicillin can also be chromosomally mediated and strains displaying this type of resistance are termed CMRNG (chromosomally mediated resistant *Neisseria gonorrrhoeae*). This type of resistance developed in a

stepwise manner, over many years as increases in penicillin doses to treat gonorrhoea resulted in treatment failures. Chromosomally mediated resistance is mainly as a result of mutations at various loci, namely *penA*, *penB* and *mtr* (Cheng, 1989).

According to Swanson and Meyer (1984), penicillin binding proteins (PBP's) are equivalent to penicillin sensitive enzymes and these are present in the cell envelope and play a role in cell wall metabolism in Gram negative organisms. The *penA* locus in *N. gonorrhoeae* codes for PBP1 and PBP2. Any mutations in either one of these PBP's results in their affinity for penicillin declining, thereby decreasing the susceptibility if the organism to the drug, resulting in resistance. Mutations to the *penB* locus cause a decline in the permeability of the cell wall to certain antibiotics while mutations to the *mtr* locus result in resistance to a number of antibiotics. Collectively these mutations result in penicillin resistance (Tapsall, 2001).

Neisseria gonorrhoeae has 4 PBP's, PBP1, 2, 3 and 4 (Ropp *et al.*, 2001). PBP's 1 and 2 are usually the target of penicillin activity as they have a high affinity for the drug especially PBP2. PBP1 is likely to be partially responsible for high level penicillin resistance and is known to cause a 1000-fold increase in penicillin MICs in combination with decreased cell wall permeability. The *ponA* gene codes for PBP1 (Ropp and Nicholas, 1997).

An investigation by Ropp *et al* (2001) concluded that chromosomally mediated resistance to penicillin in *N. gonorrhoeae* occurs as the result of the stepwise acquisition of resistance genes by susceptible *N. gonorrhoeae* organisms, causing an increase in penicillin resistance until treatment failure occurs. One such step is the

acquisition of an altered *ponA* gene which in turn encodes an altered PBP1 with a 3 to 4 fold lower penicillin acylation rate in comparison with the wildtype (Ropp *et al.*, 2001).

Since a decrease in acylation is insufficient to convert an intermediate resistant strain to a high level resistant strain the *penC* locus was also thought to play a role. Not much is known about this spontaneous mutation which occurs on an unidentified locus, except that it aids the *ponA* mutation in the mediation of high level penicillin resistance in *N. gonorrhoeae* (Ropp *et al.*, 2001).

It is possible for both plasmid and chromosomally mediated resistance to co-exist in a single isolate (Tapsall, 2001). Since the prevalence of penicillin resistance is so high, other antimicrobial agents have had to substitute penicillin as a treatment for gonorrhoea. Spectinomycin is most commonly used against PPNG strains together with the new generation cephalosporins (Salam *et al.,* 2005) while spectinomycin and ceftriaxone are used to counteract CMRNG strains (Robertson *et al.,* 1989).

2.7.2 Tetracycline Resistant *N. gonorrhoeae*

Tetracycline is a broad spectrum antibiotic that inhibits protein synthesis at the level of the 30S ribosomal subunit (Quesnel and Russell, 1983). This antibiotic binds reversibly to the ribosome and prevents the binding of tRNA to the acceptor site resulting in a bacteriostatic effect, in both Gram positive and Gram negative organisms (Wilson, 2000).

The gonococcus is however, resistant to tetracycline in many parts of the world hence the inability of the drug to be used as a first line treatment agent. Resistance to tetracycline can either be plasmid or chromosomally mediated. Plasmid mediated resistance is however more common and responsible for high level resistance (Conte Jr. and Barriere, 1992; Quesnel and Russell, 1983). High level resistance to tetracycline first emerged in the USA in 1983 followed by emergence in the Netherlands in 1985 (Chalkley *et al.,* 1997).

Plasmid mediated resistance occurs as a result of the presence of the *TetM* gene on a conjugative plasmid in *N. gonorrheoae* (Todar, 2004; Moodley *et al.,* 2006) while low level chromosomally mediated resistance is due to mutations in the *mtr* and *penB* genes (Tapsall, 2001). As previously described for penicillin, alterations in the *mtr* gene results in resistance to a number of antibiotics while alterations in the *penB* locus alters the permeability of the cell wall to certain drugs including tetracycline and penicillin (Tapsall, 2001). When these mutations occur in combination with mutations in the *tet* gene, the MIC is said to increase 16 fold. The presence of the *tet* gene mutation or the *mtr* mutation alone will result in a 4 fold increase and a 2 fold increase respectively while a combination of mutations in the *tet* and *mtr* genes or *mtr* and *penB* genes result in 4 fold increases in MICs when compared to the wildtype (Johnson and Morse, 1988).

Recently, another tetracycline resistance factor has been characterised by Hu *et al* (2005). The *rpsJ* resistance determinant has been described as the first gene that is specific for chromosomally mediated tetracycline resistance. This gene codes for the S10 ribosomal protein in *N. gonorrhoeae* and a G → A point mutation in this gene causes a val → met alteration (Hu *et al.*, 2005).

This mutation occurs at the binding site of tetracycline and decreases the affinity of the organism for the drug. The *rpsJ* gene mutation is the only tetracycline resistance determinant in *N. gonorrhoeae* to work independently of any other resistance determinant and can cause a 3 to 4 fold increase in tetracycline MICs whereas certain resistance factors require a synergistic relationship with other resistance factors to increase tetracycline MICs (e.g. *penB* and *mtr* only increase tetracycline MICs when they occur in combination in a single organism). A combination of *mtr*, *penB* and *rspJ* resistance determinants will result in high level tetracycline resistance (Hu *et al.*, 2005).

The breakpoints for tetracycline resistance are : tetracycline susceptible (TET-S) $\leq$0.25 mg/L, plasmid mediated tetracycline resistant *N. gonorrhoeae* (TRNG) $\geq$ 16 mg/L and chromosomally mediated tetracycline resistant *N. gonorrhoeae* (CMRNG) at between 1 – 8 mg/L (NCCLS Guidelines, 1999; Moodley *et al.,* 2001).

According to Moodley *et al* (2001), Zaire was the first African country to declare tetracycline resistant *N. gonorrhoeae* strains. High level resistance emerged in South Africa in 1994 from Bloemfontein and TRNG appeared in KwaZulu Natal in 1998.

High level tetracycline resistance in *N. gonorrhoeae* is mediated by via the plasmid borne *TetM* gene (Tapsall, 2001). The gene is carried on a 25.2 MDa conjugative plasmid and is responsible for inhibiting protein synthesis, leading to tetracycline resistance by coding for proteins that interact with the host ribosome (Johnson and Morse, 1988). The *TetM* determinant is said to be of *Streptococcal* origin (Todar, 2004). Two types of *TetM* gene are known to exist, namely the American and Dutch

types and according to Chalkley *et al* (1997), the Dutch type plasmid is 40.5 kb in size while the American type is 42.8 kb in size. The first TRNG isolates from Botswana were found to carry the American type plasmid which may have been transferred from Botswana or Namibia to South Africa.

Tetracycline can no longer be used effectively as a treatment against gonorrhoea as a result of the high levels of resistance that have developed (Tapsall, 2001), due mainly to the overuse of tetracycline as well as the ease with which resistance genes are transferred across borders. In KZN alone, within 18 to 24 months the prevalence of TRNG strains increased from 3% to more than 50% (Moodley *et al.,* 2001). TRNG prevalence rates are also high in countries like Singapore and the Solomon Islands, which showed rates of 84% and 74% of TRNG strains respectively by 1998 (Tapsall, 2001).

2.7.3 Quinolone Resistant *N. gonorrhoeae*

The quinolones are a group of antibiotics that inhibit protein synthesis (Johnson and Morse, 1988) by targeting the topoisomerases including DNA gyrase (Tapsall, 2001), which is responsible for the supercoiling of DNA. They have a bactericidal effect. Quinolones are usually derived from nalidixic acid and the addition of a fluorine molecule onto the structure has resulted in a new group of antibiotics called fluoroquinolones (Wilson, 2000).

As resistance of *N. gonorrhoeae* to penicillin, tetracycline, spectinomycin and other antimicrobial agents became more prevalent, fluoroquinolones were regarded as the most effective treatment against gonococcal infection. Resistance to these drugs is

becoming common (Knapp *et al.,* 1997) and this resistance seems to be exclusively chromosomally mediated (Johnson and Morse, 1988).

Tapsall (2001) describes resistance mechanisms that occur against quinolones in *N. gonorrhoeae*. These include : (i) the inability of the drug to reach its target site and (ii) the alteration of the DNA gyrase due to mutations in the *gyrA* gene, topoisomerase IV or the *parC* gene (Tapsall, 2001).

The mutations that occur in the *gyrA* and *parC* genes, which code for DNA gyrase subunit A and topoisomerase IV, respectively, result in multiple amino acid changes (Knapp *et al.,* 1997) resulting in an inability of the drug to bind to the target site. In addition mutations in *parC* seem to occur in the presence of mutations in *gyrA*. The mechanism of low level resistance is not well known (Tapsall, 2001).

Isolates of *N. gonorrhoeae* are considered resistant to ciprofloxacin at an MIC of ≥ 1 mg/L, susceptible at an MIC of ≤ 0.06 mg/L and intermediate at an MIC of 0.12 - 0.5 mg/L (NCCLS Guidelines, 1999).

Fluoroquinolone resistance is fast becoming a global phenomenon, occurring most frequently in the Far East, Philippines, Hong Kong and Japan (Knapp, 1997). The prevalence of fluoroquinolone resistance in Hong Kong increased from 7.7% in 1995 to 24% in 1996, while the prevalence in Japan increased from 6.6% in 1993 to 24.2% in 1998 (Tanaka *et al.,* 2000). Resistance to ciprofloxacin first appeared in 2000 in Spain and the prevalence of resistant strains rose dramatically from 2.3% in 2000 to 9.9% in 2001 (Arreaza *et al.,* 2003).

Quinolone resistance has also been on the increase in South Africa. In a study conducted by Moodley *et al* in Durban, KwaZulu Natal, it was recorded that 100% of *N. gonorrhoeae* isolates had MICs of ≤0.007 mg/L in 1995 but by 2000 only 71% of the isolates fell into this category. Furthermore, 4% of the isolates showed MICs of 0.125 mg/L to ciprofloxacin in 1999. Similar trends were observed for ofloxacin. These trends could be attributed to the fact that the use of quinolones rose dramatically in Durban from 1996 to 1997 (Moodley *et al.,* 2001). In another study conducted in Durban in 2006, 22% of isolates were resistant to ciprofloxacin at an MIC of ≥ 2 mg/L (Moodley *et al.,* 2006). Emergence of resistance to ciprofloxacin implies that the efficacy of quinolones against *N. gonorrheoae* has decreased.

2.7.4 Cephalosporin Resistant *N. gonorrhoeae*

Cephalosporins are structurally and functionally very similar to the penicillins. This means that they work by inhibiting cell wall synthesis. Cephalosporins also contain a β -lactam ring and modification of the side chains of this β - lactam ring results in the development of various drugs. It is in this way that the many different generations of cephalosporin drugs have been developed. The very first cephalosporin, cephalothin was developed in the 1960's and second generation cephalosporins were introduced in 1975 while the third generation cephalosporins were developed in the 1980's (Wilson, 2000). Cefepime, a fourth generation cephalosporin was approved for use by the FDA in 1996 (Mascaretti, 2003).

The third generation cephalosporins are currently recommended for the treatment of gonorrhoea. Ceftriaxone became a recommended first line agent against *N. gonorrhoeae* in 1989 in the USA. This was based on CDC recommendations after the

organism had developed resistance to penicillin, tetracycline and spectinomycin (Schwebke *et al.*, 1995). A recent study by Ison *et al* (2004) concluded that ceftriaxone (or cefixime) should be the treatment of choice, ahead of other cephalosporins, to replace ciprofloxacin against *N. gonorrhoeae* (Ison *et al.,* 2004). According to Schwebke *et al* (1995) decreased susceptibility to ceftriaxone is defined as ≥ 0.06 mg/L. Strains from various countries demonstrate decreased susceptibility to ceftriaxone and other cephalosporins while some countries display outright resistance (Schwebke *et al.,* 1995).

The mechanisms of resistance displayed by *N. gonorrhoeae* to cephalosporins are similar to those described for penicillin. Mutations at the *penA, penB* and *mtr* loci also cause resistance to cephalosporins (Tapsall, 2001). High level resistance of cephalosporins to *N. gonorrhoeae* has not been described. Resistance is therefore chromosomally mediated, by mutations which result in altered affinity to PBP's as well as decreased membrane permeability (Schwebke *et al.*, 1995). β - lactamases capable of inactivating cephalosporins, called cephalosporinases, are found in some Gram negative organisms but not yet in *N. gonorrhoeae* (Tapsall, 2001) and third generation cephalosporins like ceftriaxone are not hydrolysed by the TEM-type β - lactamase R plasmid in *N. gonorrhoeae* (Russo and Thompson, 1984). A certain degree of cross resistance occurs between cephalosporins and penicillin and according to Wilson (2000), about 10% of people who are hypersensitive to penicillins are also hypersensitive to cephalosporins.

In the USA, ceftriaxone was introduced against *N. gonorrhoeae* in 1985 and by 1991, 87.6% of gonorrhoea cases were treated with this drug. Despite this the MICs of

ceftriaxone began to decrease with more than 2.6% of isolates having an MIC that was ≥ 0.06 mg/L in 1991. Interestingly, the widespread use of ceftriaxone in the USA appeared not to promote resistance (Schwebke *et al.,* 1995).

In South Africa the use of ceftriaxone is also increasing (Moodley *et al.,* 2001). A study done in Durban in 2001, indicated that no resistance to ceftriaxone was present but MICs did increase significantly with 100% of isolates having an MIC ≤0.007 mg/L in 1995 while this figure decreased to 53% in 2000. Furthermore, 5% of isolates were at an MIC of 0.06 mg/L. These increasing trends are present on a global scale (Moodley *et al.,* 2006).

2.7.5 Macrolide Resistant *N. gonorrheoae*

Erythromycin and azithromycin are the most common macrolides used against *N. gonorrhoea.* These drugs work by binding to the 50S ribosomal subunit thereby inhibiting nucleic acid production and in turn preventing elongation of the peptide chain. Erythromycin, specifically, interferes with translocation (Russo and Thompson, 1984) and has a bactericidal effect but only at high concentrations (Wilson, 2000). Macrolides are usually used if the patient is allergic to cephalosporins (Ng *et al.,* 2002).

N. gonorrhoeae displays resistance to macrolides via efflux pumps, which mediate low level resistance or via modification of enzymes or mutations at the target site that result in a decreased affinity for the drug (Ng *et al.,* 2002). The *mtrRCDE* genes encode the *mtrC-mtrD-mtrE* efflux pump that is responsible for the export of azithromycin and erythromycin and is also one of the first efflux pumps described for

N. gonorrhoeae. The mutations occur due to the 23S rRNA methylases *erm(B), erm(C)* and *erm(F)* which alter the antibiotic target site (Ng *et al.,* 2002), by single base substitutions (Verster and Douthwaite, 2001). These methods of resistance are chromosomally mediated while plasmid mediated resistance is extremely rare (Russo and Thompson, 1984). Mutations that result in erythromycin resistance may also cause azithromycin resistance (Moodley *et al.,* 2001).

Erythromycin was first introduced in 1952 and became a first line treatment agent for pregnant women with gonorrhoea, who were allergic to the drug of choice, penicillin. This occurred in 1975 but by 1977 resistance was beginning to develop (Ehret *et al.,* 1995).

The breakpoints for erythromycin in *N. gonorrhoeae* are as follows : resistant $\geq$ 2 mg/L, intermediate 0.125 – 0.5 mg/L and susceptible $\leq$0.05 mg/L (NCCLS Guidelines, 1999). Azithromycin is a relatively new antibiotic, that was developed by introducing a nitrogen atom into the macrolide lactone ring (Ehret *et al.,* 1995) and susceptibility data is not readily available on the drug but it has been reported that treatment failure occurs with doses as low as 1g and high doses are not tolerated well, with numerous side effects (Tapsall, 2001). The high rates of treatment failure could be attributed to the fact that azithromycin is structurally very similar to erythromycin (Moodley *et al.,* 2001).

Azithromycin resistance was detected in Canada in 1997 with 95% of isolates in Quebec displaying resistance (Ng *et al.,* 2002). Moodley *et al* (2001) reported an increase in MICs of *N. gonorrhoeae* to azithromycin in Durban, South Africa. A

similar trend was reported for erythromycin with the MIC range falling between 0.007 mg/L to 0.5 mg/L. By 2000 the MIC range had increased to 0.03 mg/L to 4 mg/L. The trend of the erythromycin MICs leaned toward resistance with 26% of isolates displaying an MIC of 0.5 mg/L in 2000 while this figure was 14% in 1997 (Moodley *et al.*, 2001).

2.7.6 Spectinomycin Resistant *N. gonorrhoeae*

Spectinomycin is an aminocyclitol antibiotic which is related to the aminoglycoside antibiotics. It is closely related to streptomycin structurally and characteristically (Russo and Thompson, 1984). Spectinomycin does, however, possess qualities that distinguish it from true aminoglycosides. When compared to aminoglycosides, spectinomycin is known to display inferior bactericidal qualities, it also produces a bacteriostatic effect as opposed to the bactericidal effect achieved by the aminoglycosides (Conte Jr. and Barriere, 1992). Structural differences also exist between spectinomycin and the aminoglycosides, since spectinomycin lacks an amino sugar, present in aminoglycosides (Quesnel and Russell, 1983). According to Conte Jr and Barriere (1992) and Russo and Thompson (1984) the purpose of spectinomycin is for the treatment of uncomplicated gonococcal infection in individuals who are either allergic to penicillin (and / or other agents used to treat gonorrhoea) or who are infected with resistant strains.

The mode of action of spectinomycin is the inhibition of protein synthesis by binding to the 30S ribosomal subunit (Quesnel and Russell, 1983; Russo and Thompson, 1984). This drug does not however cause mis-reading of codons, hence its bacteriostatic effect (Quesnel and Russell, 1983).

The development of drug resistance and treatment failure with sulphonamides and penicillin led to new antimicrobial regimens being recommended for treatment of gonorrhoea. Spectinomycin was was recommended as a first line agent against *N. gonorrhoeae*, in the USA (Boslego *et al.,* 1986). It has also been recommended in patients who are allergic to cephalosporins. In some countries, where the prevalence of quinolone resistance is high, spectinomycin has also been recommended (Workowski *et al.,* 2002). In South Africa and other countries, spectinomycin is used as an alternative to ciprofloxacin during pregnancy due to the potential teratogenic effects of ciprofloxacin (Moodley *et al.,* 2006).

The inclusion of spectinomycin in treatment regimens against gonorrhoea is becoming more common worldwide although there is not much clinical evidence available to support the use of the drug against *N. gonorrhoeae.* MICs of this agent to *N. gonorrhoeae* has shown a trend to right. According to a World Health Organisation (WHO) report in 2001, penicillin and the quinolones have the highest resistance trends in *N. gonorrhoeae* followed closely by tetracycline.

A study carried out in Brazil showed that 46.7% of PPNG isolates were resistant to tetracycline while 100% of isolates were susceptible to spectinomycin (Belda Jr. *et al.,* 2002). Isolates from Korea, Bangladesh, USA, Finland and Dubai, tested during 1993 to 1998 demonstrated susceptibility to spectinomycin (Tapsall, 2001). In 1997, 33.4% of *N. gonorrhoeae* isolates in the USA were resistant to penicillin, tetracycline or both (WHO, 2001). In Bangladesh in 1999, 49% of isolates were resistant to ciprofloxacin (Rahman *et al.,* 2002). With gonococcal isolates showing such high levels of resistance to first line agents like penicillin, tetracycline and ciprofloxacin,

an alternative is needed. Spectinomycin would seem like the best option, considering that most isolates are susceptible.

The situation is identical on the African continent and in South Africa, where a large proportion of *N. gonorrhoeae* isolates demonstrate resistance to drugs recommended for syndromic management (Moodley *et al.,* 2001). Spectinomycin may prove to be an effective alternative.

Spectinomycin resistance can either be plasmid or chromosomally mediated. The *aadA* gene which is found on an R-plasmid codes for an adenyltransferase enzyme that results in spectinomycin resistance via drug inactivation. This gene is present in *E. coli* and many Gram negative bacteria but has not yet been transferred into *N. gonorrhoeae* (Russo and Thompson, 1984). There is, however, speculation that the genes coding for the antibiotic inactivating enzyme will one day be acquired by the gonococcus (Tapsall, 2001). Thus far, all spectinomycin resistance in *N. gonorrhoeae* has been chromosomally mediated via a single step mutation which results in high level resistance (Tapsall, 2001). The 30S ribosomal subunit is altered so that spectinomycin cannot bind to it and this results from mutations in the *spc* locus. The *spc* locus is also linked to the *str* and *rif* loci which mediate streptomycin resistance and rifampicin resistance, respectively (Johnson and Morse, 1988). Very little is known about the exact mechanism of resistance.

According to NCCLS guidelines, the breakpoints for spectinomycin are: susceptible $\leq$ 32 mg/L, intermediate = 64 mg/L and resistant $\geq$ 128 mg/L. In a study conducted by Moodley *et al* in Durban, KZN (2001) it was discovered that the MICs of

spectinomycin increased dramatically over a 3 year period. In 1997, 28% of *N. gonorrhoeae* isolates had MICs ≥ 16 mg/L, during 1998/1999 this increased to 52% of isolates while by 1999/2000, this figure was 100%. This clearly indicated the distinct movement toward decreased susceptibility (Moodley *et al.,* 2001).

A study conducted in Durban at the beginning of 2006 further supported this trend with 89% of isolates having an MIC of 16 mg/L and 3% of isolates having an MIC of 32 mg/L. Other antimicrobial agents tested against *N. gonorrhoeae* in this study included penicillin, tetracycline, ciprofloxacin and ceftriaxone. Twenty one percent of the isolates were resistant to penicillin some, at levels as high as 128 mg/L, of equal importance though, was the fact that only 2% of the isolates were susceptible to the drug. Seventy eight percent of the isolates showed tetracycline resistance with MICs also reaching levels of 128 mg/L while 43% of the isolates were resistant to ciprofloxacin and 99% showed a decreased susceptibility to ceftriaxone (personal correspondence). Spectinomycin therefore seems to be the most promising in terms of susceptibility as none of the MICs of the isolates have passed the resistance breakpoint.

During its use as a second line treatment agent for gonorrhoea (i.e. before the emergence of PPNG), spectinomycin resistance was rare. However, it is now becoming a common occurrence worldwide. In 2004, Bala *et al,* reported on the first *N. gonorrhoeae* isolate to show spectinomycin resistance in India. The MIC of the isolate was 128 mg/L and it was susceptible to penicillin, tetracycline, ciprofloxacin and ceftriaxone. At the same time, 11% of *N. gonorrhoeae* isolates in China were resistant to spectinomycin (Bala *et al.*, 2004).

2.7.6.1 Protein Synthesis and Spectinomycin

Spectinomycin is classified as an inhibitor of protein synthesis and works by binding to the 30S ribosomal subunit, halting its function (Quesnel and Russell, 1983). The first step in the process of protein synthesis is the formation of the initiation complex. Within this complex, the peptidyl donor site (P-site) on the large ribosomal subunit is occupied by the methionyl-transfer-RNA. The acceptor site is occupied by the aminoacyl tRNA appropriate to the next codon to be read. Peptidyl transferase is an enzyme which is responsible for attaching a methionine to the new amino acid via a peptide bond. During the next step, the partially formed peptide is translocated from the A-site to the P-site when the mRNA and the ribosome move, relative to one another. Simultaneously, the next mRNA codon moves into place, aligned with the A-site for the next aminoacyl tRNA. This process continues and a peptide chain is formed by the amino acids, following the order of the mRNA. Eventually the process is terminated when a "nonsense" codon is processed (Conte Jr. and Barriere, 1992).

Protein synthesis takes place in and around the ribosome. Ribosomes consist of 2 subunits and in bacteria like *N. gonorrhoeae*, the 2 subunits are the 30S subunit (small subunit) and the 50S subunit (large subunit) which combine to form the 70S subunit (Gale *et al.,* 1981).

Protein synthesis is inhibited by spectinomycin as a result of the drug affecting the interaction between the ribosome and elongation factor-G (EF-G) (Johanson and Hughes, 1995). Spectinomycin interferes with translation of the peptide from the A-site to the P-site by stabilizing helix 34, freezing the 30S subunit into an inactive conformation (Brink *et al.,* 1994).

Spectinomycin resistance has been found to be involved with the 30S ribosomal subunit. Mutations occurring in this subunit result in organisms like *N. gonorrhoeae* being resistant to spectinomycin. The mutation is a transversion that occurs in the 16S rRNA genes of the 30S ribosomal subunit at the binding site for spectinomycin, G_{1064} and C_{1192}. This base pair occurs in the upper stem of helix 34 (Brink *et al.,* 1994).

According to Johanson and Hughes (1995), the C_{1192} transition was the first mutation identified in the 16S rRNA resulting in spectinomycin resistance, the G_{1064} mutation was discovered later. It was also determined by these authors that any change of the G_{1064}-C_{1192} base pair will result in spectinomycin resistance. There was also speculation that mutations at other points of helix 34 may disrupt its conformation, preventing the formation of the G_{1064}-C_{1192} base pair, thereby not allowing spectinomycin to bind (Johanson and Hughes, 1995).

Although spectinomycin is widely used to treat gonorrhoea and there is a significant amount of resistance that has developed, there is very limited information available on resistance mechanisms for this drug.

Chapter 3

Materials and Methods

3.1 Bacterial Isolates

Fifty three stored isolates of *N. gonorrhoeae* were used in the experiments for this study. These isolates were selected based on their spectinomycin MICs so as to represent the spectrum from susceptible to decreased susceptibility. Of these, 28 isolates were collected in 2000 and 25 isolates were collected in 2004. All isolates were from male patients attending the Prince Cyril Zulu STD Clinic in Durban. The stored samples were inoculated onto New York City (NYC) agar plates. These plates were incubated in 5% CO_2 at 37°C for 24 to 48 hours.

DNA that was isolated from *N. gonorrhoeae* cultures grown on NYC agar plates was used in Polymerase Chain Reaction (PCR). The isolates were also inoculated into Mueller-Hinton II broth and used for Minimum Inhibitory Concentration (MIC) tests which were done via the agar dilution method.

3.2 Minimum Inhibitory Concentration (MIC)

The MIC of the *N. gonorrhoeae* isolates for spectinomycin was determined using the agar dilution method, according to NCCLS guidelines (NCCLS Guidelines, 1999). Table 3.1 shows the MICs of these isolates for spectinomycin and other antibiotics.

Table 3.1 : MICs of isolates used in this study

	MIC (mg/L)				
Sample	**Spectinomycin**	**Penicillin**	**Tetracycline**	**Ciprofloxacin**	**Ceftriaxone**
B1	32	1.0	128.0	8.0	0.031
B2	64	0.25	64.0	≤ 0.007	0.062
A9	32	0.5	2.0	≤ 0.007	0.031
B24	64	1.0	128.0	8.0	≤ 0.007
A25	64	0.25	64.0	≤ 0.007	≤ 0.007
A35	64	0.5	64.0	≤ 0.007	≤ 0.007
A42	64	0.125	1.0	≤ 0.007	≤ 0.007
B43	32	2.0	128.0	≤ 0.007	≤ 0.007
B58	64	2.0	2.0	≤ 0.007	0.031
A72	64	0.5	64.0	4.0	≤ 0.007
A80	32	0.25	64.0	4.0	≤ 0.007
A94	64	0.125	0.5	≤ 0.007	≤ 0.007
A102	64	0.5	32.0	≤ 0.007	≤ 0.007
A108	32	0.062	16.0	≤ 0.007	≤ 0.007
A120	64	0.25	64.0	8.0	≤ 0.007
B137	32	0.015	16.0	≤ 0.007	≤ 0.007
B153	64	1.0	64.0	≤ 0.007	≤ 0.007
A155	64	0.125	1.0	≤ 0.007	≤ 0.007
A164	32	0.25	128	8.0	≤ 0.007
A170	64	0.125	32.0	2.0	≤ 0.007
B186	32	0.125	1.0	≤ 0.007	≤ 0.007
B200	64	<128.0	2.0	≤ 0.007	≤ 0.007
T1124	32	Not Tested		≤ 0.007	≤ 0.007
T1189	16	Not Tested		≤ 0.007	≤ 0.007
T1192	32	Not Tested		≤ 0.007	≤ 0.007
127 065	32	Not Tested		≤ 0.007	0.015
127 072	64	Not Tested		≤ 0.007	≤ 0.007
127 222	16	Not Tested		≤ 0.007	≤ 0.007
USB 210	32	0.015	0.5	≤ 0.007	≤ 0.007
USB 213	16	0.5	128.0	≤ 0.007	≤ 0.007
USB 225	16	2.0	2.0	≤ 0.007	≤ 0.007
USB 235	32	0.125	0.5	≤ 0.007	≤ 0.007
USB 266	8	0.015	1.0	≤ 0.007	≤ 0.007
USB 312	32	2.0	128.0	≤ 0.007	≤ 0.007
USB 330	16	0.125	64.0	≤ 0.007	≤ 0.007
USB 393	16	0.062	0.5	≤ 0.007	≤ 0.007
USB 396	16	16.0	128.0	≤ 0.007	≤ 0.007
USB 397	16	16.0	64.0	≤ 0.007	≤ 0.007
USB 399	16	1.0	<128.0	≤ 0.007	≤ 0.007
USB 462	8	0.125	0.75	≤ 0.007	≤ 0.007
USB 491	16	32.0	64.0	≤ 0.007	≤ 0.007
USB 517	8	32.0	0.5	≤ 0.007	≤ 0.007
USB 553	4	0.25	0.25	≤ 0.007	≤ 0.007
USB 557	8	2.0	0.5	≤ 0.007	≤ 0.007
USB 563	8	1.0	64.0	≤ 0.007	≤ 0.007
USB 569	8	1.0	16.0	≤ 0.007	≤ 0.007

USB 594	8	0.062	16.0	≤ 0.007	≤ 0.007
USB 598	16	0.031	0.5	≤ 0.007	≤ 0.007
USB 599	64	2.0	64.0	0.015	0.015
USB 600	8	16.0	32.0	≤ 0.007	≤ 0.007
USB 646	8	0.0625	16.0	≤ 0.007	≤ 0.007
USB 648	8	0.25	0.5	≤ 0.007	≤ 0.007
USB 970	16	128.0	64.0	≤ 0.007	≤ 0.007

3.2.1 Stock Solution of Spectinomycin :

A 256 mg/L stock solution of spectinomycin was made by dissolving the spectinomycin powder in distilled water (dH_2O). The total volume of the stock solution was 10 ml. The solution was aliquoted into cryovials in 1 ml amounts and frozen at –40°C until use.

3.2.2 NYC Agar Plates for MIC Tests :

For the purpose of the MIC tests, the antibiotic was incorporated into NYC agar plates. Twofold serial dilutions of the antibiotic were made by adding 1 ml of the spectinomycin stock solution to 9 ml of distilled water and mixing well, 5 ml of the solution was then serially diluted out, fifteen times to give a concentration range of 128 mg/L to 0.007 mg/L. This was done in duplicate.

The final volume of each NYC agar plate was 25 ml. Seventy two grams of GC agar base (Oxoid) was dissolved in 1440 ml of distilled water and autoclaved at 120°C for 15 minutes. For the supplement, 1% Isovitalex was used. 5% saponin and 60 ml of yeast autolysate were added to 100 ml of horse blood, this was subsequently left at room temperature for 15 minutes in order for the yeast autolysate to lyse the horse blood, after which it was added to the autoclaved medium and mixed well.

Twenty millilitres of the NYC medium was mixed with 5 ml of spectinomycin from the dilution series and aliquoted into petri dishes giving a final volume of 25 ml per agar plate. The agar plates were left to solidify at room temperature and stored in the refrigerator until use. One plate from each batch was incubated at 37°C for 24 hours in order to ensure sterility of the agar plates. If after 24 hours of incubation, no contamination was observed on the agar plates then they were used for the MIC tests. These agar plates were made in duplicate.

3.2.3 Inoculation of NYC Agar Plates For MIC Tests :

3.2.3.1 Preparation of Broth For MIC Inoculum :

N. gonorrhoeae was cultured in GC broth medium for the purpose of inoculating the NYC agar plates for the MIC tests. Eleven grams of Mueller-Hinton II broth powder was dissolved in 500 ml of distilled water and autoclaved at 120°C for 15 minutes. The broth was allowed to cool and was dispensed into sterile tubes in 1 ml amounts.

3.2.3.2 Inoculation of Broth For MIC Inoculum :

A sterile inoculating loop was used to pick up a single colony of *N. gonorrhoeae* from 24 hour NYC agar plate cultures and inoculated into the Mueller-Hinton II broth for each sample and vortexed. The turbidity was adjusted to a 0.5 McFarland standard and a 1 in 10 dilution of each sample was made. The control strain, *Staphylococcus aureus* ATCC 25923 was subjected to the same procedure.

3.2.3.3 Inoculation of NYC Agar Plates for MIC Tests :

A multipoint inoculator was used to inoculate the NYC agar plates containing spectinomycin. The wells and the pins of the multipoint inoculator were autoclaved at

120°C for 15 minutes. The NYC agar plates were removed from the refrigerator and allowed to dry in an incubator at 37°C until no moisture was visible on the lids of the plates. The first well of the inoculator was filled with 450 µl of Nigrosin ink which was used as a marker while the rest of the wells were filled with 450 µl of the inoculum and the last well contained 450 µl of the control.

The pins of the multipoint inoculator were then dipped into the wells and subsequently touched onto the surface of each NYC agar plate containing spectinomycin. Each pin of the multipoint inoculator picks up 10 µl of sample and inoculates a spot onto the surface of the agar plates at a standardised concentration of 10^4 cfu/spot. Each agar plate containing spectinomycin was inoculated in this manner from the lowest concentration (i.e. 0.007 mg/L) to the highest concentration (i.e. 128 mg/L) in order to prevent carry over of antibiotic from plate to plate. One plate containing no antibiotic was also inoculated as a "growth" control plate, this plate was inoculated first.

These plates were incubated at 37°C, in 5% CO_2 for 24 hours. The plates were read after 24 hours, failure of a sample to grow at a particular concentration of spectinomycin, indicated the MIC for that sample.

According to NCCLS guidelines, the breakpoints for spectinomycin are defined as susceptible at $\leq$32 mg/L, intermediate at 64 mg/L and resistant at $\geq$128 mg/L.

3.3 Total DNA Isolation

The boiling method was used to isolate DNA from colonies of *N. gonorrhoeae*, taken off 24 - 48 hour NYC agar plate cultures that had been incubated at 37°C in 5% CO_2. This procedure was carried out in 1.5 ml Eppendorff tubes. A sterile inoculating loop was used to scrape a loopful of culture from the NYC agar plates and added to 250 µl of sterile distilled water in the Eppendorff tubes. The tubes were centrifuged until the culture had been homogenised. This suspension was then boiled for 15 minutes in a waterbath, which was preheated to 100°C. Once cooled, the tubes were centrifuged for 15 minutes at 12 000 rpm.

The cell debris formed a pellet at the bottom of the tube. The supernatent containing the gonococcal DNA was aliquoted into clean 1.5 ml Eppendorff tubes and used in PCR reactions.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 PCR for Detection of *aadA* Gene

PCR was performed on *N. gonorrhoeae* isolates so that the presence or absence of the *aadA* gene could be confirmed. This gene is meant to confer spectinomycin resistance in *N. gonorrhoeae*. The entire PCR procedure was done according to Clark *et al* (1999). The PCR reaction mixture had to be optimised in order to provide the best results. PCR for the detection of the *aadA* gene was only performed on the isolates from 2004 since these had higher MICs.

3.4.1.1 Optimisation of PCR for the Detection of the *aadA* Gene :

The dNTP and $MgCl_2$ concentrations were varied. The volumes of the PCR buffer, the primers and the Taq polymerase all stayed constant, while that of the distilled water was varied in order to keep the final volume of the PCR reaction mixture at 40 µl. The optimal PCR reaction mixture contained the components listed in Table 3.2.

3.4.1.2 PCR Reaction Mixture :

The total DNA obtained from the isolation was used as a template in polymerase chain reaction (PCR) amplification. Forty microlitres of a PCR mastermix was prepared, containing 10 mM Tris-2.0 mM $MgCl_2$-50 mM KCl (pH 8.3), 100 µM deoxynucleotide triphosphates (dNTPs), 0.5 µM primer and 2.5U taq DNA polymerase. Ten microlitres of the gonococcal DNA was added to the PCR mastermix, to bring the total volume of the reaction mixture to 50 µl.

3.4.1.3 PCR Cycling Conditions :

The PCR reaction mixture was amplified in a thermocycler (Gene Amp PCR system 9700). The cycling conditions were as follows : an initial denaturation for 10 minutes at 95°C, 30 cycles consisting of a 30 second denaturation step at 94°C, a 30 second annealing at 60°C and a 30 second extension step at 72°C, a 10 minute extension step at 72°C and incubation at 4°C until use.

3.4.1.4 PCR Primers and Controls :

The PCR positive control was an *E. coli* strain (C600, pHP 45Ω) which is known to contain the *aadA* gene (this strain was kindly provided by Nancye C. Clark from the Centres for Disease Control and Prevention in Atlanta, USA). In the negative control

tube, 10 µl of gonococcal DNA was replaced with 10 µl of distilled water.

The primers that were used to detect the *aadA* gene were as follows :

5'-TGA TTT GCT GGT TAC GGT GAC-3'

5'-CGC TAT GTT CTC TTG CTT TTG-3' (Clark *et al.*, 1999)

3.4.1.5 Electrophoresis of PCR Products :

Detection of the *aadA* gene was done via electrophoresis. Following PCR amplification, 10 µl aliquots of the amplified product were mixed with 3 µl of gel loading buffer and subsequently loaded into a 2% agarose gel submerged in a solution of 0.5X TBE buffer. A 100 bp ladder was used as a marker. The 2% agarose gel also contained ethidium bromide for visualisation of the DNA bands. The DNA was visualised by UV fluorescence in the Gene Genius Bioimaging System. A 284 bp product was expected.

Table 3.2 : Optimal volumes for *aadA* gene PCR reactions

Component	Volume (µl)
Distilled Water (dH_2O)	32
PCR Buffer	5
DNTP	0.5
Forward Primer	1
Reverse Primer	1
Taq Polymerase	0.5
Total	40

3.4.2 PCR for Detection of 16S rRNA

The *N. gonorrhoeae* isolates were subjected to further PCR amplification of the 16S rRNA region, which is known to carry a mutation that is responsible for spectinomycin resistance in *N. gonorrhoeae*, as well as other organisms, in various parts of the world. The PCR experiments were performed according to Galimand *et al* (2000). Optimisation of the PCR reaction mixture was done. Once amplified, the products were sequenced in order to determine the presence or absence of the mutation.

3.4.2.1 Optimisation of PCR for the Detection of the 16S rRNA :

The dNTP and $MgCl_2$ concentrations and volumes utilised in the PCR reaction mixture were varied. PCR buffer, primers and Taq polymerase volumes and concentrations remained the same. The final volume of the PCR reaction mixture was 90 µl. Optimal PCR conditions are listed in table 3.3.

3.4.2.2 PCR Reaction Mixture :

The PCR reaction mixture was prepared containing 10X PCR buffer, 250 µM deoxynucleotide triphosphates (dNTPs), 20 pmol of each primer, 2.5U taq DNA polymerase and water. 10 µl of total *N. gonorrhoeae* DNA (obtained by the boiling method) was used as a template and brought the total PCR reaction volume to 100 µl.

3.4.2.3 PCR Cycling Conditions :

Amplification was carried out in a thermocycler (Gene Amp PCR system 9700) under the following conditions : 2 minutes at 94°C, 30 cycles with 1 cycle consisting of 1 minute at 94°C, 45 seconds at 56°C and 1 minute at 72°C, finally 7 minutes at 72°C

and incubation at 4°C until use.

3.4.2.4 PCR Primers and Controls :

No positive control was used in this PCR as an organism containing this particular mutation was not easily accessible. Furthermore, the size of the product expected was known and the products obtained were sequenced thereby giving the exact sequence of the mutation, if it was present, this negated the need for a positive control to be included in the procedure. The primers and the experimental procedure were obtained from an article by Galimand *et al* (2000). The primers were designed to detect the 16S rRNA region in the *N. gonorrhoeae* genome that carry mutations G_{1064} and C_{1192} and gave a 373 bp product (Galimand *et al.*, 2000). 10 µl of distilled water was used to substitute gonococcal DNA as a negative control.

The primers used to detect the 16S rRNA were :

F980 : 5'-CTT ACC TGG TCT TGA CA-3'

R1353 : 5'- CGA TTA CTA GCG ATT CC-3' (Galimand *et al.*, 2000)

3.4.2.5 Electrophoresis of PCR Products :

Electrophoresis of the amplified PCR products was carried out in 2% agarose gel. Ten microlitres of the amplified PCR product was mixed with 3 µl of gel loading buffer and loaded into an agarose gel submerged in 0.5X TBE buffer. A 100 bp DNA ladder was used as a marker. Ethidium bromide staining allowed for visualisation of the DNA bands under UV light in the Gene Genius Bioimaging System, after electrophoresis. A 373 bp product was expected.

Table 3.3 : Optimal volumes for 16S rRNA PCR reactions

Component	Volume (µl)
Distilled Water (dH_2O)	73.75
PCR Buffer	10
$MgCl_2$	2
DNTP	1.25
Primer F980	1
Primer R1353	1
Taq Polymerase	1
Total	90

3.4.3 Nested PCR For the Detection of 16S rRNA

Even after optimisation, some of the bands appearing on the agarose gel subsequent to electrophoresis were still extremely faint. In light of this, a nested PCR had to be performed on the samples that were producing faint bands, in an attempt to intensify the DNA bands. The nested PCR method involves the amplification of DNA from the sample twice so as to increase the amount of DNA present, thereby increasing the intensity of the bands on the agarose gel.

The reaction mixture for the first amplification was identical to that originally used to detect the 16S rRNA region of the *N. gonorrhoeae* samples, after optimisation. 10 µl of template DNA was added to the 90 µl reaction mixture and amplified under the original conditions in a thermocycler (see section 3.4.2.3). Once the first round of amplification was complete, a second round was done by making a new reaction mixture with a total volume of 95 µl containing 10X PCR buffer, 10 mM deoxynucleotide triphosphates (dNTPs), 20 pmol of each primer, 2.5U taq DNA polymerase and water. The volumes of the components used in the reaction mixture are listed in table 3.4.

Table 3.4 : Volumes of components used in nested PCR reaction mixture

Component	Volume (µl)
Distilled Water (dH_2O)	78.75
PCR Buffer	10
$MgCl_2$	2
DNTP	1.25
Primer F980	1
Primer R1353	1
Taq Polymerase	1
Total	95

Instead of 10 µl of template DNA being added to the reaction mixture, 5 µl of amplified PCR product (from the first round of amplification) was used as a template and added to the reaction mixture, to bring the total volume to 100 µl. This was then amplified again in a thermocycler, under identical conditions to the first round of amplification. This results in a larger amount of DNA being amplified, it is therefore expected to increase the intensity of the DNA bands formed after electrophoresis and ethidium bromide staining.

3.4.4 PCR for the Detection of the *mtrR* Gene

The *mtrR* gene of the *mtrRCDE* efflux pump was amplified and subsequently sequenced to detect the presence or absence of mutations in this gene.

3.4.4.1 PCR Reaction Mixture :

The PCR reaction mixture contained 10X PCR buffer, 10 mM deoxynucleotide triphosphates (dNTPs), 5 pmol of each primer, 2.5U taq DNA polymerase and water. 45 µl of this PCR reaction mixture was prepared (table 3.5).

Table 3.5 : Volumes of components used in PCR for detection of *mtrR* gene

Component	Volume (µl)
Distilled Water (dH_2O)	36
PCR Buffer	5
$MgCl_2$	1
DNTP	0.5
Primer CEL # 1	1
Primer KH9 # 3	1
Taq Polymerase	0.5
Total	45

Five microlitres of template *N. gonorrhoeae* DNA was added to the PCR reaction mixture. The final volume of the reaction mixture was 50 µl.

3.4.4.2 PCR Cycling Conditions :

The reaction mixture was amplified in a Gene Amp PCR system 9700 thermocycler under the following conditions : initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 15 seconds and extension at 72°C for 5 minutes. Final extension was at 72°C for 5 minutes and then incubation at 4°C until use.

3.4.4.3 PCR Primers and Controls :

Gonococcal strain FA 19 was used as a positive control while distilled water was used as a negative control.

The following primers were used to detect the *mtrR* gene :

CEL # 1 : 5'-GAC AAT GTT CAT GCG ATG ATA GG-3'

KH9 # 3 : 5'-GAC GAC AGT GCC AAT GCA ACG-3' (Hagman *et al.*, 1995)

3.4.4.4 Electrophoresis of PCR Products :

Ten microlitres of the amplified PCR products were electrophoresed through a 2% agarose gel submerged in 0.5X TBE buffer. A 100 bp DNA marker was used. Visualisation of the DNA bands was carried out in the Gene Genius Bioimaging System after ethidium bromide staining. A 1087 bp product was expected.

3.5 Sequencing

The 16S rRNA PCR products were sequenced to determine the presence or absence of a mutation in the 16S rRNA which commonly causes spectinomycin resistance as well as in the *mtrR* gene of the *mtrRCDE* efflux pump.

Sequencing was performed by Inqaba Biotech using the Genetic Analysis System SCE2410 with 24 capillaries from SpectruMedix LLC in Pennsylvania, USA. For the reactions the BigDye version 3.1 Dye Terminator Cycle Sequencing kit from Applied Biosystems was used.

The software program used for the analysis of the sequences obtained was Chromas 2.31. BioEdit version 7.0.5.2 is a biological sequence alignment editor that was used to compile the sequences. The sequences were subsequently aligned using MAFFT which is a software program that uses a variety of methods to align multiple amino acid or nucleotide sequences (Katoh, 2006).

3.5.1 Sequencing of the 16S rRNA

Following the amplification of the 16S rRNA region of the *N. gonorrhoeae* isolates via PCR, the products were sequenced to determine if the G_{1064} - C_{1192} mutation was present in the isolates. Primers F980 (5'-CTT ACC TGG TCT TGA CA-3') and R1353 (5'- CGA TTA CTA GCG ATT CC-3') were used in the sequencing reactions.

3.5.2 Sequencing of the *mtrR* Gene

The *mtrR* gene was also sequenced to detect potential mutations in the gene. This was done using primers CEL # 1 (5'-GAC AAT GTT CAT GCG ATG ATA GG-3') and KH9 # 3 (5'-GAC GAC AGT GCC AAT GCA ACG-3').

Chapter 4

Results

4.1 MIC Results

Table 4.1 : Minimum Inhibitory Concentration (MIC) of Spectinomycin amongst *Neisseria gonorrhoeae* isolates obtained in 2000 and 2004

Year	MIC (mg/L)					
	4	8	16	32	64	128
2000 (n = 25)	1	10	10	3	1	-
2004 (n = 28)	-	-	2	11	15	-

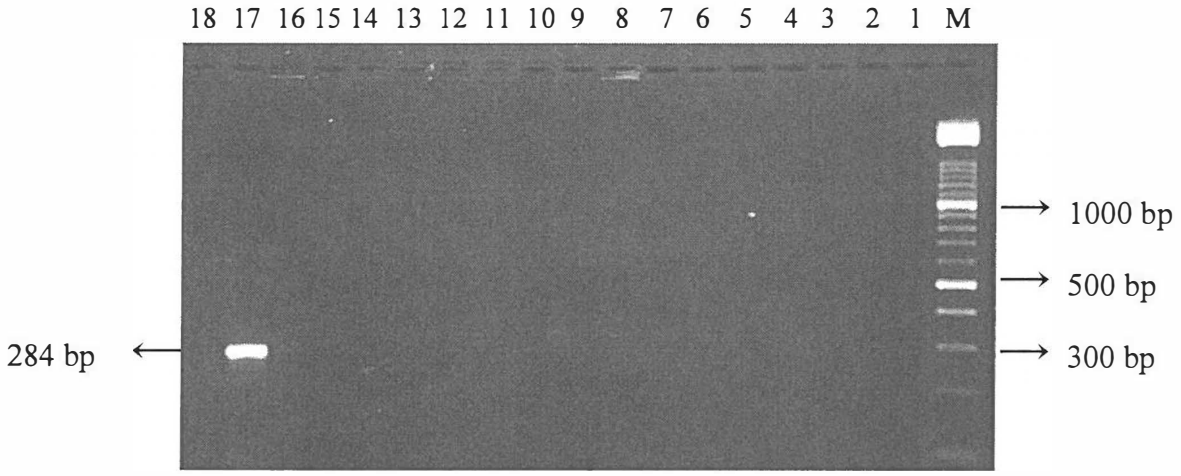
A comparison of the MICs from 2000 with that of 2004 shows a shift to the right. Amongst the 2004 set of isolates 60% show MIC values of 64 mg/L, which is above the breakpoint for susceptibility.

4.2 PCR Results

4.2.1 Detection of the *aadA* gene

Gels 1 and 2 represent results for all 28 *N. gonorrhoeae* samples collected in 2004.

Gel 1



Gel 2

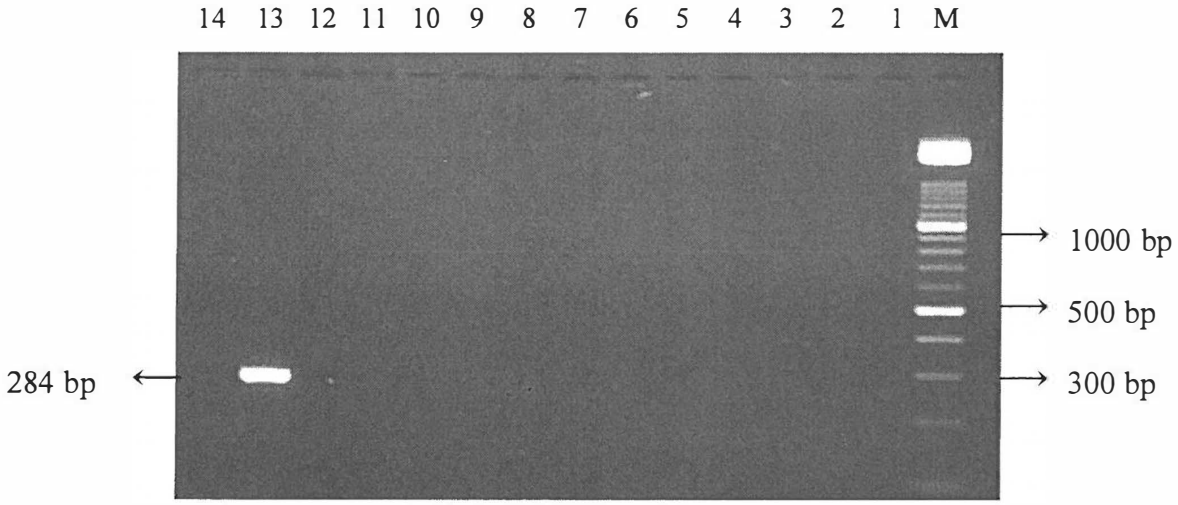


Figure 4.1 : Electrophoresis of PCR products for the detection of the *aadA* gene

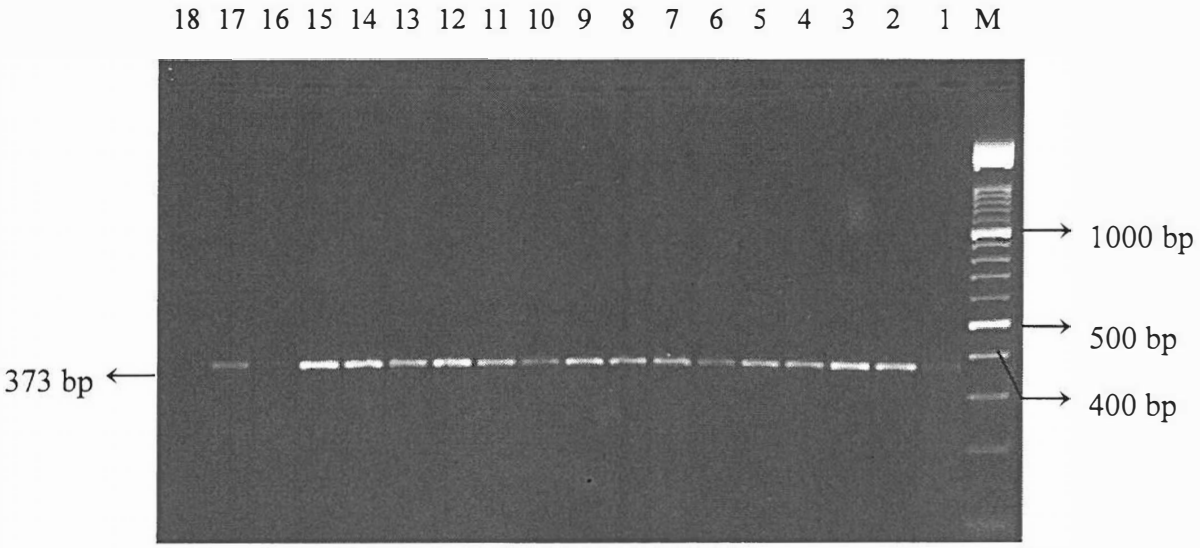
In gels 1 and 2, lane M contains a 100 bp DNA marker. With reference to gel 1, lanes 1 to 16 contain PCR amplified *N. gonorrhoeae* DNA. Lane 17 contains a positive control (*E. coli* (C600, pHP 45Ω), known to contain the *aadA* gene) and lane 18 contains a negative control (water). The only band to form was that of the positive control at 284 bp. The absence of bands on the gel indicates the absence of the *aadA* gene in the *N. gonorrhoeae* samples. The positive control band in lane 17 is intense. The negative control lane remained blank.

Lanes 1 to 12 in gel 2 contain PCR amplified *N. gonorrhoeae* DNA while lane 13 contains a positive control (*E. coli* (C600, pHP 45Ω), known to contain the *aadA* gene) and lane 14 contains a negative control (water). The results are identical to gel 1 with the only band to form being that of the intense positive control band at 284 bp, indicating the absence of the *aadA* gene in these samples as well as the absence of plasmid mediated resistance. Since the results indicate that the *aadA* gene is absent in these *N. gonorrhoeae* isolates, a region of the 16S rRNA was detected and sequenced to confirm the presence or absence of a mutation which is commonly responsible for spectinomycin resistance.

4.2.2 Detection of 16S rRNA

PCR for the detection of 16S rRNA was carried out on samples collected in 2000 and 2004.

Gel 1



Gel 2

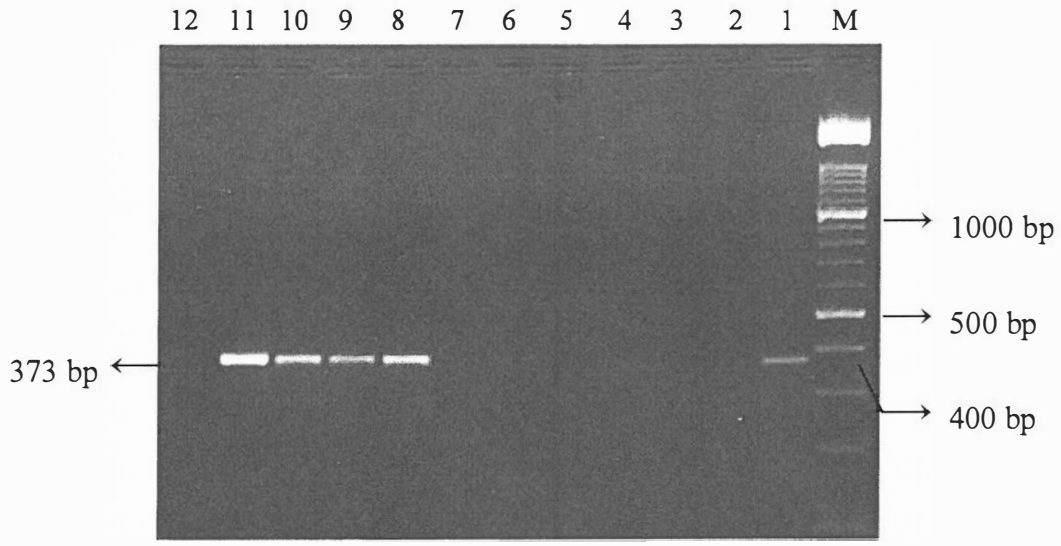


Figure 4.2 : Electrophoresis of PCR products for detection of 16S rRNA

Lanes M, in gels 1 and 2 contain a 100 bp DNA ladder as a marker. Gel 1 contains *N. gonorrhoeae* DNA from lanes 1 to 17, while lane 18 contains a negative control (water). Lanes 1 to 11 in gel 2 contain *N. gonorrhoeae* DNA and lane 12 contains a negative control (water). Both negative control lanes (i.e. lane 18 in gel 1 and lane 12 in gel 2) remained empty. Some bands that formed at the expected 373 bp were intense but not all the bands were clearly visible. As a result of this, a nested PCR was performed on the samples that gave faint bands.

4.2.3 Nested PCR for Detection of 16S rRNA

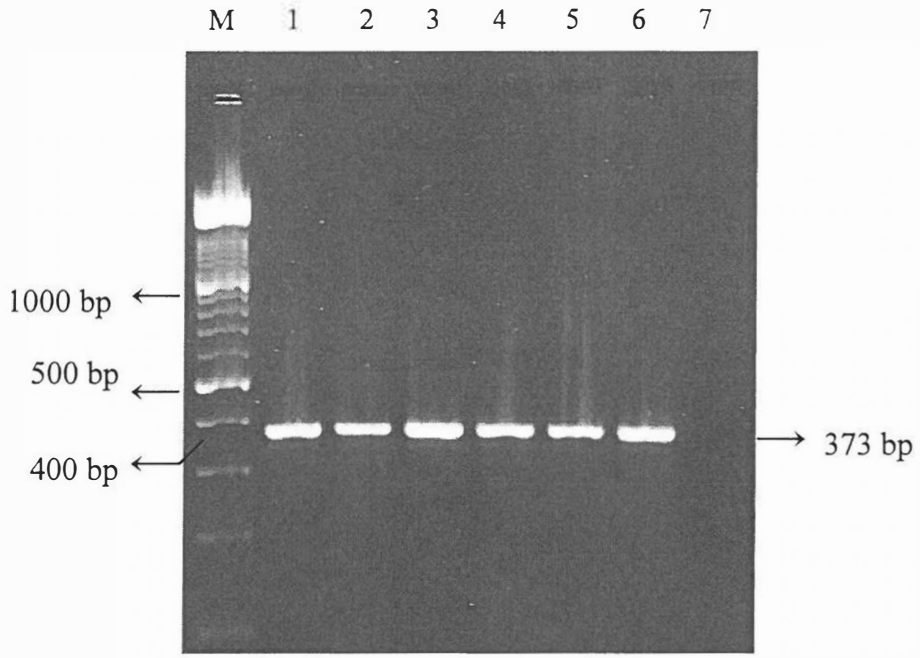


Figure 4.3 : Intense DNA bands present after nested PCR experiment

Lane M contains a 100 bp DNA marker. Lanes 1 to 6 contain *N. gonorrhoeae* DNA that has been amplified twice, while lane 7 contains a negative control (water). The

bands which formed at 373 bp are significantly more intense, this is due to the double amplification of the DNA, as opposed to the faint bands obtained with only a single amplification of the DNA (figure 4.2, gel 1, lane 16 and gel 2, lanes 2 to 6).

4.2.4 Detection of *mtrR* gene

PCR for the detection of the *mtrR* gene was carried out on samples collected in 2000 and 2004.

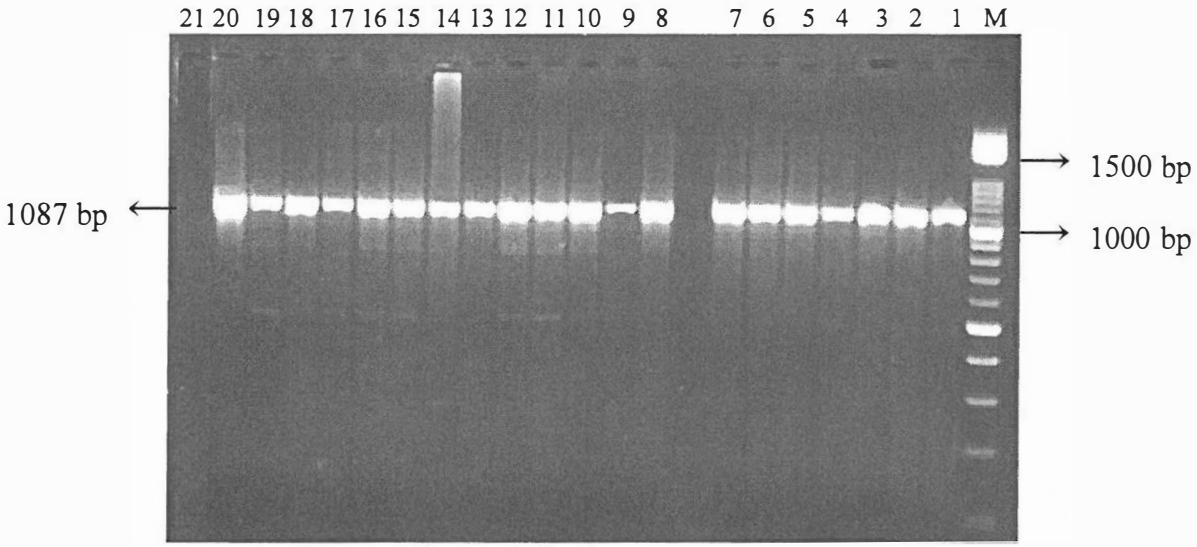


Figure 4.4 : Electrophoresis of PCR products for the detection of the *mtrR* gene

Lane M contains a 100 bp DNA marker while lanes 1 to 19 contain *N. gonorrhoeae* DNA. The positive control (FA 19) was run in lane 20 and a negative control (water) in lane 21. All the samples carried the *mtrR* gene as is indicated by the formation of bands at 1087 bp in all the lanes containing *N. gonorrhoeae* DNA. These PCR products were sequenced to determine whether mutations exist in the *mtrR* gene.

4.3 Sequencing Results

4.3.1 Sequencing of the 16S rRNA

Sequencing and analysis of all 53 *N. gonorrhoeae* isolates revealed that no mutation was present in any of the isolates. The region of the 16S rRNA of each isolate which usually contains the G_{1064}-C_{1192} mutation was compared to the same region of a fully susceptible *N. gonorrhoeae* strain from the Genbank database. No mutations were detected (see appendix 6 for sequences).

4.3.2 Sequencing of the *mtrR* Gene

Although some mutations were present in the *mtrR* gene, the phylogenetic analysis revealed that there were no distinct clusters which could group isolates according to the MIC values (Figure 4.5).

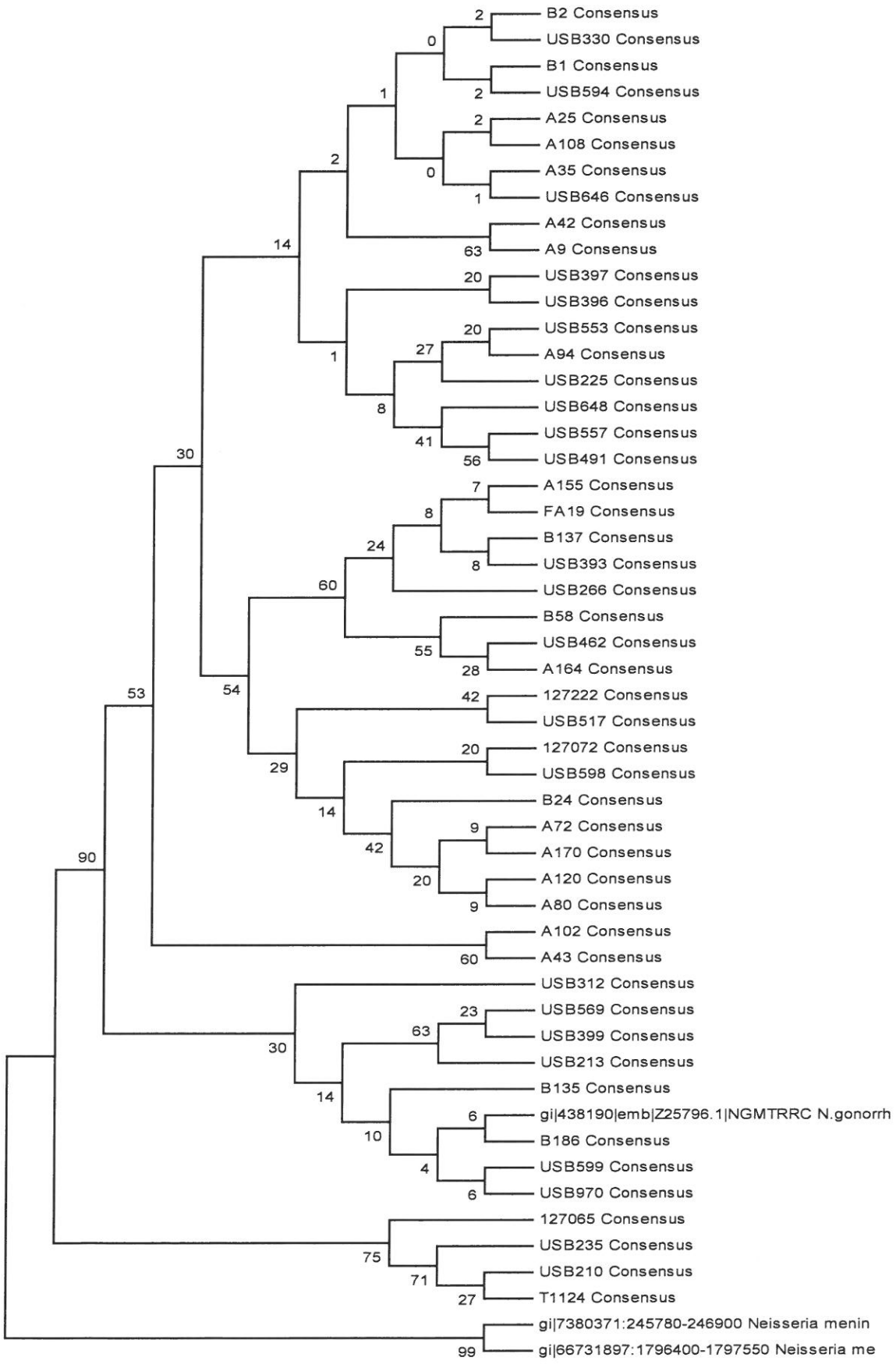


Figure 4.5 : Phylogenetic tree based on *mtrR* gene analysis

Chapter 5

Discussion

Sexually transmitted infections are a major health concern in KwaZulu Natal. The prevalence of discharge causing organisms has remained constant despite the introduction of syndrome management in 1995 (Moodley *et al.,* 2001). The lack of rapid and reliable point-of-care tests coupled with poor symptom recognition amongst patients and the increasing prevalence of organisms resistant to recommended antimicrobials, has made the treatment of sexually transmitted infections a challenge.

Neisseria gonorrhoeae has developed resistance mechanisms against all previously prescribed and most currently recommended antimicrobial agents. The development of resistance has been reported throughout the world (WHO, 2001) including KZN, South Africa (Chenia *et al.,* 1997; Moodley *et al.,* 2001).

Local reports have highlighted the increase in MIC values of first line antimicrobial agents like penicillin, tetracycline, ciprofloxacin and ceftriaxone against *N. gonorrhoeae* (Moodley *et al.,* 2001). This has led to scientists looking at alternative agents for the management of gonorrhoea. Spectinomycin which was classically used as part of syndromic management for vaginal discharge in pregnant women is one such agent. However there have been reports of increasing MICs to spectinomycin as well (Moodley *et al.,* 2001; Moodley *et al.,* 2006). This suggests that using spectinomycin as an alternative treatment agent may not be feasible.

One of two possible mechanisms is thought to be responsible for the increasing MICs of spectinomycin viz.: an antibiotic modifying enzyme encoded for by the *aadA* gene and a chromosomal mutation in the 16S rRNA. *N. gonorrhoeae* has developed a number of chromosomally and plasmid mediated mechanisms of resistance to various antimicrobial agents ranging from the alteration of the antibiotics target site to the acquisition of new DNA (Tapsall, 2001). The presence of the *aadA* gene on R-plasmids has been shown to be responsible for spectinomycin resistance in many Gram negative organisms (Clark *et al.,* 1999). This has not been shown in *N. gonorrhoeae* to date. Researchers have speculated that this may also occur with *N. gonorrhoeae* acquiring a similar mechanism of resistance (Johnson and Morse, 1987). The acquisition of this gene would adversely affect the treatment of infection with *N. gonorhoeae*, as plasmid mediated resistance has the potential to spread rapidly (Tapsall, 2001).

It has been shown that resistance due to the ribosomal mutation is a one step event (Tapsall, 2001). Since our MICs show an incremental rise, we postulated that the resistance is due to a modifying enzyme. PCR experiments were carried out to detect the presence or absence of the *aadA* gene amongst our *N. gonorrhoeae* isolates. Subsequent to optimisation, electrophoresis revealed that the *aadA* gene was completely absent in every isolate. The presence and intensity of the positive control band indicated that the PCR reaction mixture and conditions were all optimal. It can therefore be stated that the increase in MICs observed amongst *N. gonorrhoeae* isolates in Durban is not attributable to the antibiotic modifying enzyme which is encoded by the *aadA* gene.

A very common mechanism of spectinomycin resistance in *N. gonorrhoeae* is the 16S rRNA mutation which results in the alteration of the spectinomycin binding site (Johanson and Hughes, 1995). Although this has been linked to resistance as a result of a one step mutation, it is the only other reported mechanism and we therefore studied this. In order to establish whether the isolates in this study carried the mutation, a PCR was carried out to amplify the 16S rRNA of each isolate. Subsequently, the 373 bp product was sequenced to determine the presence or absence of the mutation.

A comparison of the sequence of the fully susceptible strain contained in Genbank with the local fully susceptible isolates as well as those with decreased susceptibility did not reveal any differences. The absence of the G_{1064}-C_{1192} mutation in our study isolates suggests that this mutation does not play a role in the increase in MICs to spectinomycin observed. It also suggests that another binding site for spectinomycin may exist, one which has not as yet been characterised.

In 1995, Johanson and Hughes showed the presence of another chromosomal mutation, C1066U, which also causes spectinomycin resistance in *E. coli* (Johanson and Hughes, 1995). It is, however, unlikely that this mutation is the cause for the increasing MICs seen locally. Whilst the mutation described by Johanson and Hughes, 1995 is located in the vicinity of G_{1064}-C_{1192}, the sequencing of our isolates did not reveal any changes in this region.

Another possibility for the increase in MICs to spectinomycin amongst our isolates includes a decrease in the concentration of the drug that reaches the target. This could

be the result of efflux pumps which extrude the drug (Powell, 2000). Although no efflux pumps have specifically been defined for spectinomycin in *N. gonorrhoeae*, there are a number of multidrug efflux systems present in other Gram negative bacteria which cause resistance to various antimicrobial agents (Vakulenko *et al.,* 2003).

The existence of the *mtrRCDE* efflux pump in *N. gonorrhoeae* organisms has contributed to the development of resistance to a range of antimicrobial agents. Previous studies have shown that mutations in the *mtrR* gene of this efflux system result in decreased repression of the *mtrCDE* genes and an over expression of the pump (Hagman *et al.,* 1995; Veal *et al.,* 1998; Grkovic *et al.,* 2002). Efflux pumps have been described locally in *N. gonorrhoeae* for ciprofloxacin (Abbai, 2005). It is for this reason that the *mtrR* gene was focused on for this investigation.

PCR was done to amplify the *mtrR* gene, which was detected in all samples. These PCR products were then sequenced to determine if mutations existed in the gene. Although mutations did exist in the *mtrR* gene, phylogenetic analysis and grouping of the samples revealed that no correlation existed between the mutations carried by the *mtrR* gene and the MICs of the study isolates.

This does not completely rule out the *mtrRCDE* efflux pump as a potential resistance mechanism as it is possible that the drug could be pumped out at varying efflux rates or in different amounts. Further investigation will be required to determine whether this is the case.

Other possibilities include the synthesis of resistant metabolic pathways in which pathways are altered to counteract the effect of the drug or the failure to metabolise the drug by the bacteria resulting in resistance (Powell, 2000). These mechanisms of resistance have been described for aminoglycosides, a drug class that is closely related to spectinomycin (Vakulenko *et al.*, 2003).

According to Powell (2000), there are five major categories of acquired antimicrobial resistance. Enzyme inactivation of a drug is one such mechanism (Powell, 2000). Although the *aadA* gene, which mediates spectinomycin resistance via the ANT(3) enzyme, was absent in the samples tested in this investigation, it does not completely rule out this mechanism. A study conducted in Denmark in 1999 revealed the sequence of a new gene in the *aadA* gene cassette. The *aadA5* gene is present in both Gram positive and Gram negative organisms and mediates resistance to spectinomycin and streptomycin (Sandvang, 1999). Another study carried out in Switzerland in 1981 investigated the presence of aminocyclitol modifying enzyme genes in the chromosomal DNA of *S. aureus* and no evidence for the presence of these genes on a plasmid could be found (Kayser *et al.*, 1981).

Chapter 6

Conclusion

In order to curb the growing epidemic of gonorrhoea in South Africa and around the world, effective antimicrobial treatment must complement the other components of syndromic management. Unfortunately, *N. gonorrhoeae* is notorious for constantly changing its genetic makeup and rapidly developing resistance mechanisms to drugs recommended for its treatment. The study of antimicrobial resistance mechanisms may help to detect early changes in the microbial makeup before clinical resistance occurs. It also plays a role in guiding the development of new antimicrobial agents.

The mechanism associated with the increase in MICs of *N. gonorrhoeae* to spectinomycin was shown not to be related to the antibiotic modifying enzyme encoded for by the *aadA* gene or the 16S rRNA mutation. Other undescribed antibiotic modifying enzymes may however play a role in the increase in MICs observed. Although sequencing of the *mtrR* gene did not reveal any MIC clusters, the *mtrRCDE* efflux pump is still a potential mechanism that could confer resistance to spectinomycin in *N. gonorrhoeae*. Further investigation looking at intracellular concentration of this drug to determine the extent of the role of the efflux pump is warranted. Other possible mechanisms that could infer resistance include the synthesis of resistant metabolic pathways or failure of the organism to metabolise the drug.

Increasing resistance of microbes to newer and more sophisticated antimicrobial agents have led policy makers to look at alternative agents for the treatment of infections. Unfortunately, development of antimicrobials with novel mechanisms of action have lagged behind the development of resistance mechanisms in microbes. As a result, older agents that have long been shelved are now making their appearance in the clinical arena. Spectinomycin is an old antimicrobial agent that is now re-emerging as an alternative for treatment of *N. gonorrhoeae*. However not much work has been done on this drug and its effect on *N. gonorrhoeae*.

The results of this study show that the resistance mechanisms classically related to spectinomycin and other microbes are not present in local *N. gonorrhoeae* isolates displaying increasing MICs to the drug. Alternative mechanisms of resistance need to be elucidated in future studies.

Chapter 7

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Chapter 8

Appendices

Appendix 1 : Preparation of Culture Media

1.1 New York City Agar Plates :

GC Agar Base	36 g
Water	850 ml
LCAT	20 ml
Yeast Autolysate	30 ml
0.5% Saponin	0.5 g
Horse Blood	100 ml

The GC agar base was dissolved in deionised water and autoclaved at 120°C for 15 minutes. The solution was then cooled to 55°C in a waterbath. The LCAT and yeast autolysate were subsequently added. Sapopnin was added to the horse blood in order to lyse it. Finally the lysed horse blood was added to the rest of the media solution, mixed well and aliquoted into Petri dishes.

1.2 GC Storage Broth :

Tryptic Soy Broth	12 g
Glycerol	100 ml

Water	400 ml

Tryptic soy broth and glycerol were mixed, added to deionised water and autoclaved at 120°C for 15 minutes. The mixture was then cooled, aliquoted into cryovials containing sterile glass beads and stored at 2 - 8°C until use.

Appendix 2 : Inoculation Standard for MIC

2.1 0.5 McFarland Standard :

2.1.1 1% H_2SO_4 Solution :

Concentrated H_2SO_4	0.5 ml
Water	49.5 ml

The concentrated H_2SO_4 and deionised water were mixed.

2.1.2 1% $BaCl_2$ Solution :

$BaCl_2.2H_2O$	0.175 g
Water	10 ml

The $BaCl_2.2H_2O$ and deionised water were mixed.

1 % H_2SO_4 Solution	4.950 ml
1% $BaCl_2$ Solution	50 µl

The 1% H_2SO_4 solution and 1% $BaCl_2$ solution were mixed well. The tubes were sealed and kept in a dark environment. The solution was vortexed thoroughly before use.

Appendix 3 : Solutions for Agarose Gel Electrophoresis

3.1 0.5M EDTA (pH 8.0) :

EDTA.$2H_2O$	186.1 g
Water	800 ml

EDTA was added to deionised water and stirred vigorously with a magnetic stirrer. The pH was adjusted to 8.0 using NaOH pellets. The volume of the solution was made up to 1000 ml and autoclaved at 120°C for 15 minutes.

3.2 5X TBE Buffer Stock Solution (Tris-Cl / Borate EDTA) (pH 8.5) :

Trisma Base	54 g
Boric Acid (H_3BO_3)	27.5 g
0.5M EDTA (pH8.0)	20 ml
Water	800 ml

Trisma base and boric acid were dissolved in deionised water. EDTA was added. pH was adjusted to 8.5 and the solution autoclaved at 120°C for 15 minutes.

3.3 0.5X TBE (Tris-Cl / Borate EDTA) Buffer :

5X TBE Buffer Stock Solution	100 ml
Water	900 ml

The 5X TBE buffer stock solution was added to deionised water.

3.4 Gel Loading Buffer :

Bromophenol Blue	0.25%
Sucrose	40%
Water	10 ml

Two point five grams of sucrose and 0.025 g of bromophenol blue were added to deionised water and mixed. The solution was stored at 4°C.

3.5 Ethidium Bromide :

Ethidium Bromide	0.5 mg
Water	1 ml

Ethidium bromide powder was dissolved in deionised water and stored at 4°C, in the dark.

3.5 2% Agarose Gel :

Agarose	2.0 g
0.5X TBE Buffer	100 ml
Ethidium Bromide	1µl / ml buffer

Agarose was added to 0.5X TBE buffer. The solution was boiled and then allowed to cool to 70°C at room temperature. Ethidium bromide was added. The solution was then thoroughly stirred and poured into the gel racks and a comb placed on one side of the gel to form the wells and allowed to set.

Appendix 4 : Optimisation of PCR

The optimisation experiments contained the combination of dNTP and $MgCl_2$ concentrations shown below.

Table 8.1 : dNTP and $MgCl_2$ concentrations and volumes for optimisation of PCR

		Concentration	Volume (μl)
Set A	**dNTP**	100 μM	0.5
	$MgCl_2$	1.5 mM	0
		2.0 mM	1
		2.5 mM	2
		3.0 mM	3
		3.5 mM	4
Set B	**dNTP**	200 μM	1.0
	$MgCl_2$	1.5 mM	0
		2.0 mM	1
		2.5 mM	2
		3.0 mM	3
		3.5 mM	4
Set C	**dNTP**	250 μM	1.25
	$MgCl_2$	1.5 mM	0
		2.0 mM	1
		2.5 mM	2
		3.0 mM	3
		3.5 mM	4

Table 8.2 : Volumes (µl) of PCR components used for optimisation of *aadA* gene PCR

	PCR Buffer	Forward Primer	Reverse Primer	Taq Polymerase	dH_2O	dNTP	$MgCl_2$
Set A	5	1	1	0.5	32	0.5	0
	5	1	1	0.5	31	0.5	1
	5	1	1	0.5	30	0.5	2
	5	1	1	0.5	29	0.5	3
	5	1	1	0.5	28	0.5	4
Set B	5	1	1	0.5	31.5	1	0
	5	1	1	0.5	30.5	1	1
	5	1	1	0.5	29.5	1	2
	5	1	1	0.5	28.5	1	3
	5	1	1	0.5	27.5	1	4
Set C	5	1	1	0.5	31.25	1.25	0
	5	1	1	0.5	30.25	1.25	1
	5	1	1	0.5	29.25	1.25	2
	5	1	1	0.5	28.25	1.25	3
	5	1	1	0.5	27.25	1.25	4

Table 8.3 : Volumes (µl) of PCR components used for optimisation of 16S rRNA PCR

	PCR Buffer	Forward Primer	Reverse Primer	Taq Polymerase	dH_2O	DNTP	$MgCl_2$
Set A	10	1	1	1	76.5	0.5	0
	10	1	1	1	75.5	0.5	1
	10	1	1	1	74.5	0.5	2
	10	1	1	1	73.5	0.5	3
	10	1	1	1	72.5	0.5	4
Set B	10	1	1	1	76	1	0
	10	1	1	1	75	1	1
	10	1	1	1	74	1	2
	10	1	1	1	73	1	3
	10	1	1	1	72	1	4
Set C	10	1	1	1	75.75	1.25	0
	10	1	1	1	74.75	1.25	1
	10	1	1	1	73.75	1.25	2
	10	1	1	1	72.75	1.25	3
	10	1	1	1	71.75	1.25	4

Appendix 5 : PCR Results

5.1 Initial Detection of the *aadA* gene

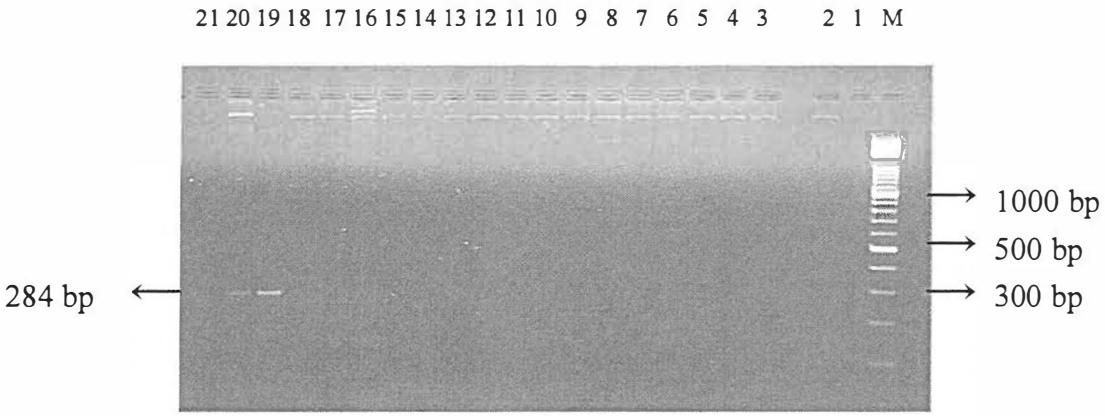


Figure 8.1 : Electrophoresis gel for the detection of the *aadA* gene

Lane M contains a 100 bp DNA marker, while lanes 1 to 18 contain PCR amplified *N. gonorrhoeae* DNA. Lanes 19 and 20 contain a positive control (*E. coli* (C600, pHP 45Ω), known to contain the *aadA* gene) and lane 21 contains a negative control (water). As is evident, the only bands to form were those of the positive control and were 284 bp in size. Although present, these bands were extremely faint, indicating that optimisation was required. The remaining lanes, including the negative control lane remained empty.

5.2 Optimisation of *aadA* gene PCR

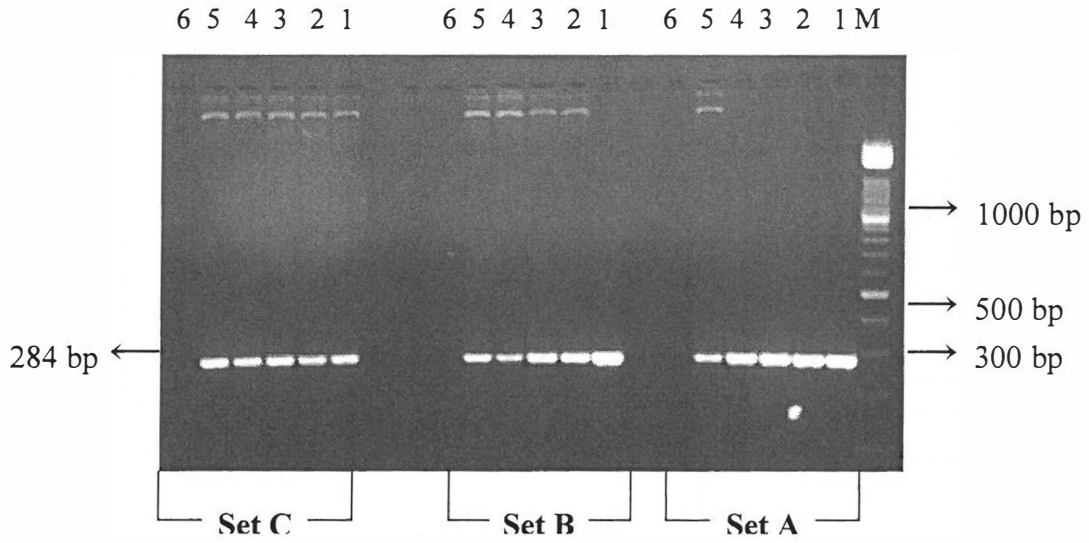
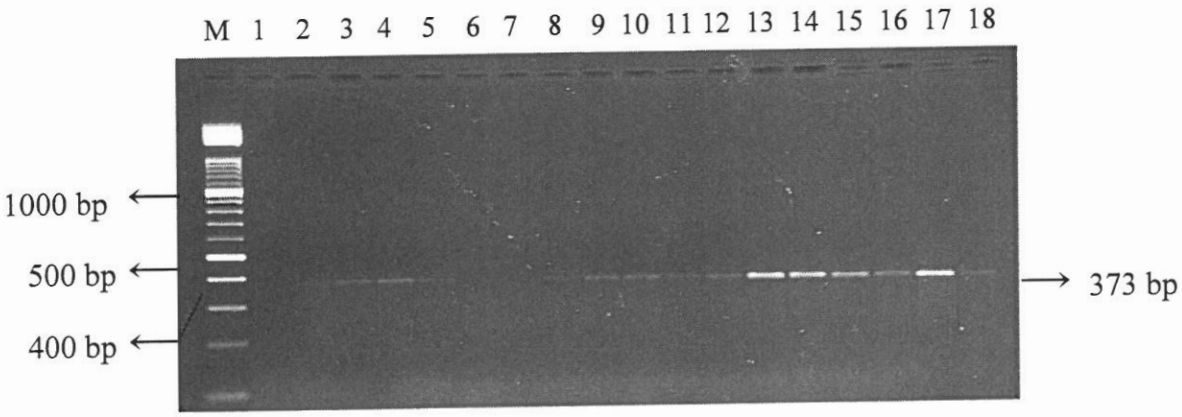


Figure 8.2 : Optimisation of the *aadA* gene PCR

Lane M contains a 100 bp DNA marker. Lanes 1 to 5 in sets A, B and C all contain the PCR amplified positive control, *E. coli* (C600, pHP 45Ω) while lane 6 contains a negative control (water). All bands formed were 284 bp in size. The brightest band formed in lane 1 of set A.

5.3 Initial Detection of 16S rRNA – Gel 1



Gel 2

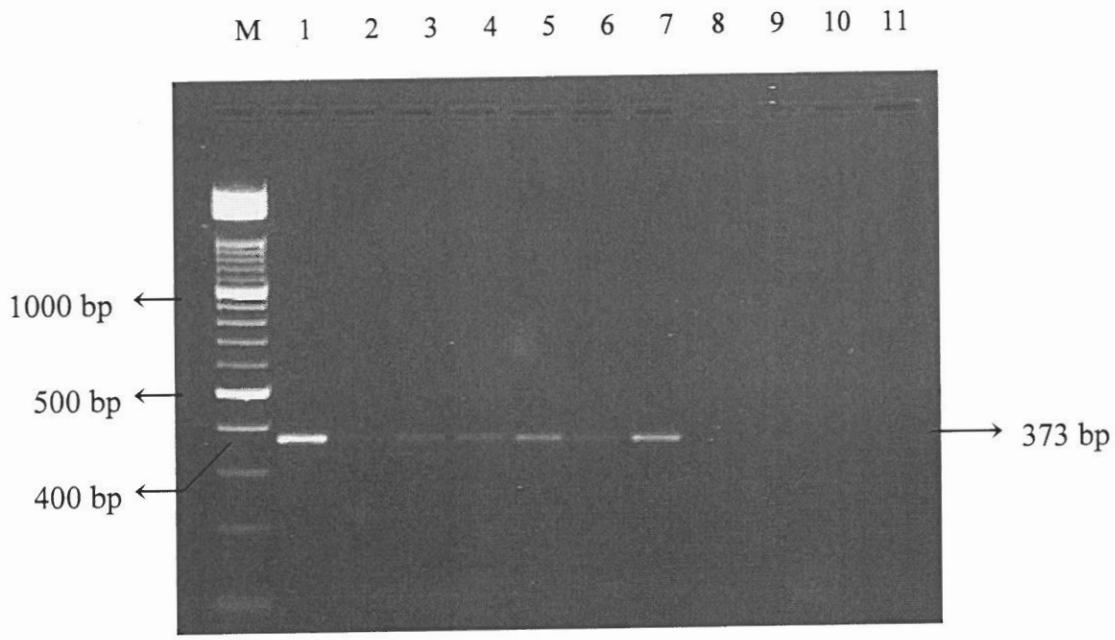


Figure 8.3 : Electrophoresis gel for the detection of the 16S rRNA of *N. gonorrhoeae* isolates

Lanes M in both gel 1 and 2 contain a 100 bp DNA ladder as a marker. Lanes 1 to 18 in gel 1 as well as lanes 1 to 10 in gel 2 contain *N. gonorrhoeae* DNA, lane 11 of gel 2 contains a negative control (water). A positive control was not used in these PCR experiments as the size of the expected product was known and because the samples

were sequenced. As can be seen, bands formed on the gel at the 373 bp mark, as expected. The bands were however of varying intensity, this meant that certain bands may have been present and not visible to the naked eye, therefore optimisation of the PCR reaction mixture was done in order to make sure that all bands that were present were visible. The negative control lane remained empty.

5.4 Optimisation of 16S rRNA PCR

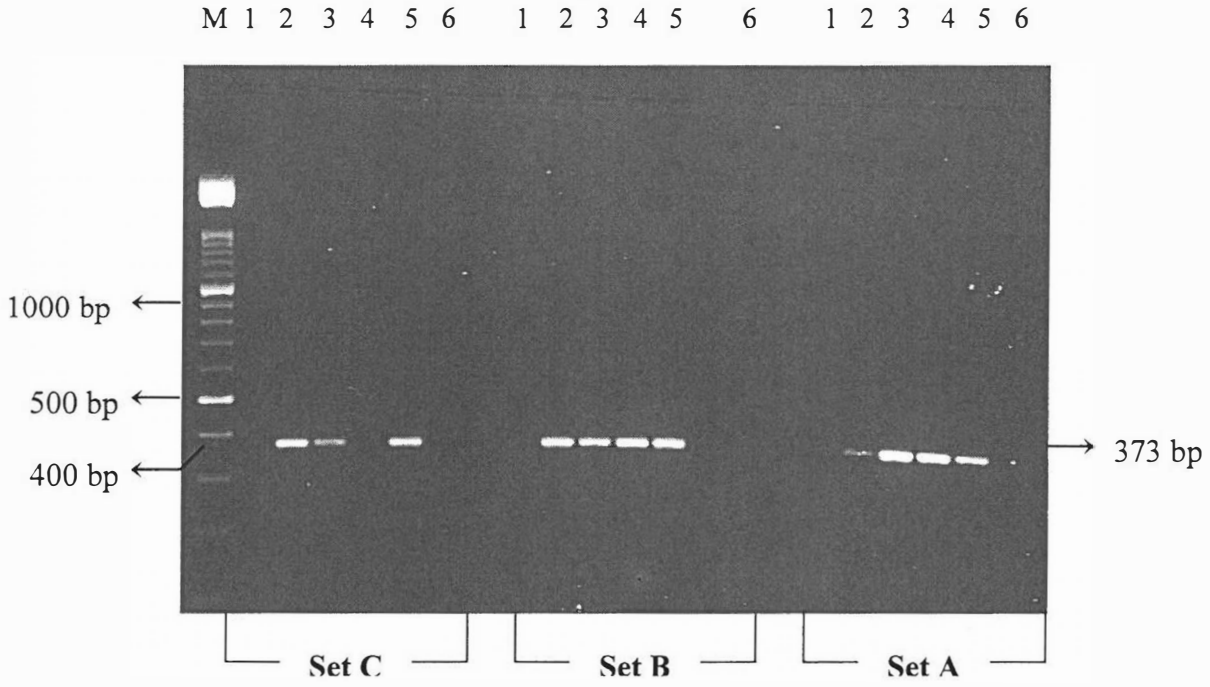


Figure 8.4 : Optimisation of the 16S rRNA PCR

Lane M contains a 100 bp DNA marker. Lane 6 in sets A, B and C all contain a negative control (water) and as can be seen, no bands have formed in any of the negative control lanes. Lanes 1 to 5 in each set contain the *N. gonorrhoeae* DNA of sample B2, which formed a very light band in the initial PCR that was run (figure 4.5, gel 1, lane 2). This experiment was done to determine the PCR reaction mixture that

would result in bands of a high intensity. All the bands formed at 373 bp, as expected and the brightest band formed in lane 3 of set C.

Appendix 6 : Sequencing Results

6.1 Mutations in *mtrR* Gene

Table 8.4 : *mtrR* gene mutations an MICs for each isolate

Sample	Nucleotide Differences	Position/s	MIC (mg/L)
B1	A → G	37, 856	32
	C → G	565	
	C → T	568	
	G → A	594, 841, 1226	
	C → A	800	
	G → T	811	
	T → A	812	
	T → C	850	
	A → T	1227	
	T → G	1229	
B2	A → G	27, 856	64
	G → T	28, 811	
	C → G	565, 1231	
	C → T	568	
	G → A	594, 628, 841	
	C → A	800	
	T → A	812	
	T → C	850	
A9	G → C	21, 22, 31, 41	32
	C → T	24, 568, 1231	
	T → A	26, 34, 812	
	A → G	27, 30, 40, 856	
	G → A	28, 594, 841, 1029	
	C → A	32, 800	
	A → T	33	
	C → G	565	
	G → T	811	
	T → C	850, 1229	
	A → C	1234	
B24	G → C	21, 22	64
	C → T	23, 24, 568	
	T → A	25, 812, 1236	
	T → G	26	
	A → T	27, 29	
	G → A	28, 594, 614	
	T → C	433	

	C → A	434, 480, 800	
	A → G	476, 481, 1208	
	C → G	565	
	G → T	811	
A25	T → A	47, 812, 1221, 1236	64
	A → C	48	
	G → A	391, 594, 841	
	C → G	565	
	C → T	568, 1217, 1220, 1231	
	C → A	800	
	G → T	811, 1226	
	T → C	850, 1216, 1219, 1229, 1235	
	A → G	856	
	A → T	1234	
A35	C → A	24, 32, 800	64
	T → C	25, 34, 69, 850, 1233	
	G → T	28, 31, 811	
	A → G	30, 856, 1189, 1211, 1224	
	A → T	33, 1222	
	T → G	59, 1221, 1229	
	G → A	395, 594, 841, 1226	
	C → G	565	
	C → T	568, 1231	
	T → A	812, 1236	
A42	A → C	30	64
	G → T	31, 811	
	C → T	32, 568	
	A → T	33	
	T → C	34, 49, 850	
	C → G	42, 64, 565	
	A → G	43, 856, 1225	
	T → G	46	
	G → A	594, 841, 1029, 1226	
	C → A	800	
	T → A	812	
A43	G→ C	22	32
	G → T	811	
	C → T	568, 808, 1217, 1220	
	C → G	565	
	A → G	856	
	G → A	594, 841	
	C → A	800	
	T → A	812, 1221	

	T → C	850,1216, 1219	
B58	T → C	54, 1185	64
	T → G	63	
	G → A	615	
	C → A	800, 1186, 1213	
	G → T	811	
	T → A	812	
	A → G	971, 1173	
	A → C	981, 1195, 1209, 1214	
	G → C	985, 1192	
	C → T	995, 1182	
	C → G	1194	
A72	C → G	565	64
	C → T	568, 1217, 1220	
	G → A	594, 614	
	C → A	800	
	G → T	811	
	T → A	812, 1221	
	A → G	821	
	T → C	1216, 1219	
A80	G → C	21, 28	32
	C → T	24, 568	
	T → A	26, 812	
	A → G	27, 1225, 1227	
	G → A	448, 594, 614, 1226	
	C → G	565	
	C → A	800	
	G → T	811	
A94	G → C	21	64
	C → T	23, 24, 568, 1217, 1220	
	T → A	25, 812, 1221	
	T → G	26	
	A → C	27, 29, 43	
	G → A	28, 328, 594, 841	
	T → C	57, 850, 1216, 1219	
	C → G	565	
	C → A	800	
	G → T	811	
	A → G	856, 930	
A102	G → C	22, 28	64
	C → T	24, 568, 808	
	T → A	26, 812	

	A → G	27, 856, 1173, 1225	
	C → G	565	
	G → A	594, 841, 1226	
	C → A	800	
	G → T	811	
	T → C	850	
A108	G → T	21, 811	32
	G → C	22, 28	
	A → T	29, 53	
	T → G	46, 69	
	T → C	63, 47, 850	
	C → T	64, 568	
	T → A	76, 812, 1215	
	C → G	89, 565, 1213	
	G → A	594, 841, 1212, 1226	
	C → A	800, 1231	
	A → G	856, 1211, 1230	
	A → C	1214, 1232	
A120	A → C	30	64
	T → C	51	
	A → G	437	
	C → G	565	
	C → T	568	
	G → A	594, 614, 1226	
	C → A	800	
	G → T	811	
	T → A	812, 1216	
	T → Y	1039	
B137	G → C	21	32
	T → G	433, 1229	
	C → T	434, 1217, 1220	
	G → A	506, 1226	
	C → A	800	
	G → T	811	
	T → A	812, 1221	
	T → C	1216, 1219	
	A → T	1225, 1227	
B153	G → A	31, 841	64
	C → T	32, 808, 1217, 1220	
	A → G	40, 856	
	G → C	41	
	C → A	800	
	G → T	811, 1226	

	T → A	812, 1221	
	T → C	850, 1216, 1219	
	T → G	1229	
	A → T	1234	
A155	C → G	23, 24	64
	A → C	29	
	C → S	486	
	C → A	800	
	G → T	811	
	T → A	812, 1221	
	T → C	1216, 1219	
	C → T	1217, 1220	
A 164	G → A	615, 1226	32
	C → A	800	
	G → T	811	
	T → A	812	
	A→ G	1173, 1227	
A170	G → C	22, 41	64
	C → T	24, 568, 1217, 1220	
	T → A	26, 812, 1221	
	A → G	27, 40	
	G → A	28, 594, 614	
	A → C	29	
	C → G	565	
	C → A	800	
	G → T	811	
	T → C	1216, 1219	
B186	G → C	21, 22, 28	32
	C → T	23, 24, 808, 1217, 1220	
	T → A	26, 812, 1221	
	A → G	27, 856	
	A → T	29, 1234	
	T → G	433, 1229	
	C → A	800	
	G → T	811, 1226	
	G → A	841	
	T → C	850, 1216, 1219	
B200*	A → G	535, 549, 555, 556, 631, 690, 700, 803, 809, 856, 1208, 1211	64
	G → A	552, 558, 563, 594, 614, 728, 841, 1212	

* Sample B200 was sequenced in one direction only using primer CEL#1.

	C → A	553, 570, 685, 800	
	G → T	559, 697, 811	
	T → G	562	
	A → C	569, 1214	
	C → G	603, 1213	
	T → C	626, 850	
	A → T	701	
	T → A	739, 812, 1215	
	C → T	808	
T1124	G → C	21, 22	32
	C → A	23, 800, 1220, 1231	
	C → T	24, 622, 808, 1217	
	T → A	25, 26, 812, 1221	
	A → T	27, 29, 1214, 1230, 1232	
	G → A	28, 841, 1226	
	A → C	30, 1227	
	G → T	31, 811	
	T → C	63, 657, 665, 850, 1216, 1218, 1233	
	T → G	739	
	A → G	747, 856, 1141, 1173, 1177, 1224	
T1192	G → A	21, 28, 471, 534, 1083, 1226	16
	G → T	22, 31, 588, 602, 811	
	C → A	23, 480, 800	
	C → T	24, 462, 521, 590, 608, 1231	
	T → A	25, 517, 812, 1236	
	A → C	27, 458, 484, 1214, 1232	
	A → T	29, 463, 593, 607, 1225	
	A → G	43, 356, 466, 516, 1227	
	C → G	325, 564, 565, 587, 603	
	T → C	358, 375, 514, 1215	
	T → G	374, 433, 523, 562	
	G → C	432, 563, 594, 598, 601	
T1189**	G → C	21, 22, 731	32
	C → G	23, 42, 798	
	C → A	24, 766, 800	
	T → A	26, 669, 777	
	A → T	27, 29, 33, 48, 700, 701, 724, 774	
	G → A	28, 41, 725, 784, 797	
	A → C	30, 43	
	G → T	31, 327, 648	
	A → G	36, 37, 483, 505, 707	
	T → G	46, 562	
	T → C	51, 63, 666, 739	
	C → T	581	

** Sample T1189 was sequenced in one direction only using primer KH9#3.

127 065	G → C	21, 22	32
	C → T	24, 622, 808	
	T → A	26, 812	
	A → C	27	
	G → T	28, 811	
	T → C	409, 657, 665, 850	
	G → A	448, 725, 841, 1226	
	T → G	739	
	A→ G	747, 856, 1227	
	C → A	800	
127 072	C → G	565, 1213, 1231	64
	C → T	568	
	G → A	594, 1212	
	C → A	800	
	G → T	811, 1226	
	T → A	812, 1229	
	A → G	1208, 1211	
	A → C	1214, 1230	
	T → C	1215	
127 222	G → C	21, 22, 28, 31	16
	C → T	23, 32, 568	
	C → A	24, 800	
	T → A	26, 812	
	A → G	30, 971, 1209	
	C → G	565	
	G → T	811	
USB210	G → C	21	32
	T → G	84, 739	
	C → T	622, 808	
	T → C	657, 665, 850	
	A → G	747, 856, 1141, 1173	
	C → A	800	
	G → T	811	
	T → A	812	
	G → A	841	
USB213	A → C	40	16
	T → C	54, 57, 61, 433, 850, 1236	
	C → T	55, 60, 808	
	A → T	58, 407	
	T → A	59, 812	
	G → T	62, 306, 811	
	T → G	63, 307, 374	

	G → C	371, 985	
	C → A	434, 800	
	C → G	461	
	G → A	841, 1226	
	A → G	856, 979, 1225	
USB225	C → T	23, 24, 568, 995	16
	T → A	25, 812	
	T → G	26	
	A → C	27, 29, 981	
	G → A	28, 328, 594, 841	
	T → C	57, 850	
	C → G	565	
	C → A	800	
	G → T	811, 969	
	A → G	856	
	G → C	985	
	A → T	993	
USB235	G → C	21	32
	C → T	622, 808	
	T → C	657, 665, 850	
	T → G	739	
	A → G	747, 856, 1141, 1173	
	C → A	800	
	G → T	811	
	T → A	812	
	G → A	841	
USB266	G → A	31, 1226	8
	C → A	800	
	G → T	811	
	T → A	812	
	A → G	1225, 1227	
USB312	G → C	21	32
	G → A	22, 31, 448, 841, 1226	
	C → G	23	
	T → C	25, 26, 49, 63, 850, 1236	
	A → T	27, 36, 463, 466, 1222	
	G → T	28, 811	
	A → C	30, 33	
	C → T	32, 64, 808	
	T → A	34, 812	
	T → G	69, 467, 1221	
	C → A	800	
	A → G	856, 1227	

USB330	A → T	29	16
	G → A	31, 594, 841, 1226	
	G → T	408, 811	
	T → G	409	
	C → G	565	
	C → T	568	
	C → A	800	
	T → A	812	
	T → C	850	
	A → G	856	
USB393	A → G	40	16
	G → C	41	
	T → C	86	
	C → A	800	
	G → T	811	
	T → A	812	
USB396	G → C	31	16
	C → T	32, 493, 568, 995	
	G → A	408, 594, 841	
	T → G	409, 467	
	A → T	463, 466	
	T → A	465, 812	
	C → A	480, 800	
	A → C	484, 500	
	C → G	527, 565	
	G → T	811	
	T → C	850, 1236	
	A → G	856, 971	
USB397	G → C	31	16
	C → T	32, 388, 462, 568	
	T → C	84, 850	
	A → T	463, 473	
	C → A	479, 800	
	C → G	565	
	G → A	594, 841	
	G → T	811	
	T → A	812	
	A → G	856, 1175, 1225	
	T → G	1236	
USB399	C → A	32, 800	16
	A → T	33, 403, 1222	
	T → A	34, 402, 812,1215	

	A → C	40, 1230	
	G → C	62, 399	
	T → C	63, 850, 1218, 1221	
	C → T	285, 808, 1217, 1220, 1231	
	G → T	306, 395, 811	
	T → G	307, 409	
	C → G	396	
	A → G	401, 856	
	G → A	408, 841	
USB462	G → C	21	8
	G → T	22, 811	
	C → T	24	
	G → A	615	
	C → A	800	
	T → A	812	
	A → G	1173	
USB491	T → G	69, 374	16
	G → A	72, 75, 328, 594, 841	
	A → T	73	
	T → A	268, 812	
	C → T	269, 440, 568	
	G → T	302, 487, 811, 1226	
	G → C	371, 425, 728	
	A → G	407, 856, 1225	
	C → G	427, 565, 729	
	C → A	800	
	T → C	850, 1236	
USB517	G → C	22	8
	C → A	23, 800	
	C → T	24, 64, 568	
	T → C	25, 57, 63	
	T → G	26, 69	
	A → C	27	
	G → T	28, 811, 933	
	A → T	29, 33, 36	
	G → A	31, 594, 978, 1226	
	T → A	34, 76, 812	
	A → G	43, 971, 1225, 1227	
	C → G	89, 565	
USB553	G → C	21	4
	G → A	328, 594, 841, 1037	
	C → G	565	
	C → T	568	

	C → A	800	
	G → T	811	
	T → A	812	
	T → C	850	
	A → G	856	
USB557	T → C	57, 850, 1216, 1219	8
	G → A	328, 594, 841, 1226	
	A → G	407, 856, 1224, 1225	
	G → C	425	
	C → G	427, 565	
	C → T	568, 1217, 1220	
	C → A	800	
	G → T	811	
	T → A	812, 1221	
	A → T	1232	
USB569	T → A	61, 812	8
	G → T	62, 306, 811	
	T → G	63, 69, 76, 307	
	T → C	66, 850	
	C → G	89	
	A → G	407, 856	
	C → A	800	
	C → T	808	
	G → A	841	
USB594	C → T	24, 32, 568	8
	T → A	25, 812	
	G → A	28, 594, 841	
	A → C	29	
	T → C	51, 850	
	C → G	565	
	C → A	800	
	G → T	811	
	A → G	856	
USB598	G → A	21, 81, 594	16
	G → T	22, 28, 75, 811	
	C → G	24, 31, 89, 565	
	T → C	25, 26, 52, 57, 63, 433	
	A → T	27, 53	
	C → T	32, 55, 568	
	T → G	46, 47, 74, 409	
	A → G	48, 979	
	T → A	49, 54, 812	
	G → C	408	

	C → A	434, 800	
USB599	G → C	21	64
	C → A	800, 1231	
	C → T	808	
	G → T	811	
	T → A	812	
	G → A	841, 1226	
	T → C	850	
	A → G	856, 1225, 1227	
	T → G	1229	
	A → T	1230	
	A → C	1232	
USB600	A → G	98, 856, 971	8
	G → A	99, 841	
	G → T	306, 327, 346, 811	
	T → G	307, 347	
	A → T	407, 993	
	C → T	429, 431, 808, 995	
	T → C	430, 433, 850, 991, 994, 1229	
	C → A	434, 800, 992	
	A → C	437	
	C → G	461, 758, 766, 770	
	G → C	757, 765, 769	
	T → A	812	
USB646	G → A	21, 594, 841	8
	G → C	22, 28, 31	
	C → A	23, 800	
	C → T	24, 32, 440, 568	
	T → A	25, 812	
	T → G	26, 409	
	A → G	30, 856	
	C → G	565	
	G → T	811	
	T → C	850, 1236	
USB648	A → T	27, 463	8
	G → A	28, 31, 448, 594, 841	
	A → G	29, 407, 856	
	G → C	425	
	C → G	427, 446, 565	
	C → A	439, 452, 800	
	A → C	451	
	C → T	568	
	G → T	811	

	T → A	812	
	T → C	850	
	T → G	1236	
USB970	G → T	21, 811	16
	G → C	22, 31	
	C → G	23	
	T → C	25, 850	
	A → T	27	
	G → A	28, 841	
	A → G	29, 856	
	C → A	800	
	C → T	808	
	T → A	812	

6.2 16S rRNA Sequences :

Neisseria gonorrhoeae 16S ribosomal RNA gene, partial sequence (Genbank)

-CTTACCTGGTTTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGC

Sample B2

TCTTACCTGGTCTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGA

Sample A108

TCTTACCTGGTCTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGA

Sample T1192

TCTTACCTGGTCTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGA

Sample USB 213

TCTTACCTGGTCTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGA

Sample USB 312

TCTTACCTGGTCTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGA

Sample USB 462

TCTTACCTGGTCTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGA

Sample USB 563

TCTTACCTGGTCTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGA

Sample USB 599

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TCTTACCTGGTCTTGACATGTGCGGAATCCTCCGGAGACGGAGGAGTGCCTTCGGGAGCCGTAACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTT
GTCATTAGTTGCCATCATTCGGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATGACCAGGGCTTCACACGTCATACAATGGTCGGTACAGAGGG
TAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGCAACTCGA
GTGCATGAAGTCGGAATCGCTAGTAATCGA
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6.3 *mtrR* Gene Sequences :

Neisseria gonorrheae mtrR gene, partial sequence (Genbank)

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GCCAACGCGGCCGCACGCATCGCCTTAGAAGCATAAAAAGCCA--TTAT-TTATCCTATC
TGTC-TGGTTTGATGTA-AAGGGTTTTGCCAATCAACAGGCATTCT--------------
------------------------------------------------------------
------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAA-CCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGGTTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGAT-AAAAGTCTTTTTTATAA
T-CCG-CCCTCGTCAAA-CCG-ACCC-GAAACG-AAAACGCCATTAT-GAGAAAAA--CC
AAAACCG-AAGCCTTGAAAACCAAAGAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCCCGCACCTCGCTC-AACGAAATCGCCCAAG-CCGCC
GGCGTAAC-GCGCGGCGCGCTCTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTTGAC
GCG-TTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGTTC-TTGGACGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCAACGACATCTACTACAAATTCCACAACAT-CCTGTTTTTAAA
ATGCGAACACACGGAGCAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
ACGA-TTTGGA-CAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGGTTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
ATCAAGCCTTGGTAACAATGCCGTCTG-AAACAAACAAACCCTTTCAAAC-GGCATCAAA
ATGACACAA-AGCATTCTTCTAAAAA--TACATATTCACTAAATTGCATTTTTAATTTCC
CCT
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Positive Control - FA19

```
--------------------GGCCTTAGAAGCAT-AAAAAGCA--TTAT-TTATCCTATC
TGTC-T-GTTTGATGTA-AAGGGTTTTGCCAATCAACAGGCATTCT--------------
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------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAA-CCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGGTTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGATAAAAAGTCTTTTTTATAA
T-CCG-CCCTCGTCAAA-CCG-ACCC-GAAACG-AAAACGCCATTAT-GAGAAAAA--CC
AAAACCG-AAGCCTTGAAAACCAAAGAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCCCGCACCTCGCTC-AACGAAATCGCCCAAG-CCGCC
GGCGTAAC-GCGCGGCGCGCTCTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTTGAC
GCG-TTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGTTC-TTGGACGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCACCGACATCCACGTCAAATTCCACAACAT-CCTGTTTTTAAA
GTGCGAACATACGGAACAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
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ACGA-TTTGGA-CAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGGTTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
ATCAAGCCTTGGTAACAATGCCGTCTG-AAACAAACAAA-CCTTTCAAAC-GGCATCAAA
ATGACACAA-AGCATTCTTCTAAAAGA-TACATATT----------------------
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Sample B137

```
--------------------CGCCTTAGAAGCAT-AAAAAGCA--TTAT-TTATCCTATC
TGTC-TGGTTTGATGTA-AAGGGTTTTGCCAATCAACAGGCATTCT-------------
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------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAA-CCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGGTTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGATAAAAAGTCTTTTTTATAA
T-CCG-CCCTCGGTAAA-CCG-ACCC-GAAACG-AAAACGCCATTAT-GAGAAAAA--CC
AAAACCG-AAGCCTTGAAAACCAAAAAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCCCGCACCTCGCTC-AACGAAATCGCCCAAG-CCGCC
GGCGTAAC-GCGCGGCGCGCTCTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTTGAC
GCG-TTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGTTC-TTGGACGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCAACGACATCCACTACAAATTCCACAACAT-CCTGTTTTTAAA
GTGCGAACATACGGAACAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
ACGA-TTTGGA-CAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGGTTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
ATCAAGCCTTGGTAACAATGCCGTCTG-AAACAAACAAACCCTTTCAAAC-GGCATCAAA
ATGACACAA-AGCATCTTCTAAAATAT-GACA---------------------------
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Sample A155

```
----------------------GGTTAGCAGCAT-AAAAAGCA--TTAT-TTATCCTATC
TGTC-TGGTTTGATGTA-AAGGGTTTTGCCAATCAACAGGCATTCT-------------
------------------------------------------------------------
------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAA-CCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGGTTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGATAAAAAGTCTTTTTTATAA
T-CCG-CCCTCGTCAAA-CCG-ACCC-GAAACG-AAAACGCCATTAT-GAGAAAAA--CC
AAAACSG-AAGCCTTGAAAACCAAAGAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCCCGCACCTCGCTC-AACGAAATCGCCCAAG-CCGCC
GGCGTAAC-GCGCGGCGCGCTCTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTTGAC
GCG-TTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGTTC-TTGGACGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCAACGACATCCACTACAAATTCCACAACAT-CCTGTTTTTAAA
GTGCGAACATACGGAACAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
ACGA-TTTGGA-CAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGGTTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
ATCAAGCCTTGGTAACAATGCCGTCTG-AAACAAACAAACCCTTTCAAAC-GGCATCAAA
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ATGACACAA-AGCATCTTCTAAA-------------------------------
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Sample 127 222

```
-----------------GG-CCTATAACAGCTAT-AAAAAGCA--TTAT-TTATCCTATC
TGTC-T-GTTTGATGTA-AA-GGTTTTGCCAATCAACAGGCATTCT-------------
------------------------------------------------------------
------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAA-CCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGGTTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGATAAAAAGTCTTTTTTATAA
T-CCG-CCCTCGTCAAA-CCG-ACCC-GAAACG-AAAACGCCATTAT-GAGAAAAA--CC
AAAACCG-AAGCCTTGAAAACCAAAGAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCGCGTACCTCGCTC-AACGAAATCGCCCAAG-CCGCC
GGCGTAAC-GCGCGGCGCGCTCTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTTGAC
GCG-TTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGTTC-TTGGACGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCAACGACATCCACTACAAATTCCACAACAT-CCTGTTTTTAAA
GTGCGAACATACGGAACAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
ACGA-TTTGGGCCAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGGTTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
ATCAAGCCTTGGTAACAATGCCGTCTG-AAACAAACAAACCCTTTCAAAC-GGCATCAAA
ATGACACAGAAGCATTCTTCTAAAAGA-TACATATTCACTAAATCTGCATC---------
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Sample USB 210

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---------GCCGCACGCATCGCCTTAGAAGCAT-AAAAAGCA--TTAT-TTATCCTATC
TG-C-TGGTTTGATGTA-AA-GGGTTTGCCAATCAACAGGCATTCT-------------
------------------------------------------------------------
------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAA-CCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGGTTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGATAAAAAGTCTTTTTTATAA
T-CCG-CCCTCGTCAAA-CCG-ACCC-GAAACG-AAAACGCCATTAT-GAGAAAAA--CC
AAAACCG-AAGCCTTGAAAACCAAAGAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCCCGCACCTCGCTC-AACGAAATCGCCCAAG-CCGCC
GGCGTAAC-GCGCGGCGCGCTTTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTCGAC
GCG-CTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGGTC-TTGGGCGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCAACGACATCTACTACAAATTCCACAACAT-CCTGTTTTTAAA
ATGCGAACACACGGAGCAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
ACGA-TTTGGA-CAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGGTTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
GTCAAGCCTTGGTAACAATGCCGTCTG-AAACGAACAAA-CCTTTCAAAC-GGCATCAAA
ATGACACAA-AGCATTCTTCTAAAAGA-TACATATTCACTA------------------
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Sample USB 266

```
----------------------------AACAT-AAAAAGCA--TTAT-TTATCCTATC
TG-C-TAGTTTGATGTA-AA-GGTTTTGCCAATCAACAGGCATTCT-------------
------------------------------------------------------------
------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAACCCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGGTTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGATAAAAAG-CTTTTTTATAA
T-CCG-CCCTCGTCAAACCCG-ACCC-GAAACG-AAAACGCCATTAT-GAGAAAAA--CC
AAAACCG-AAGCCTTGAAAACCAAAGAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCCCGCACCTCGCTC-AACGAAATCGCCCAAG-CCGCC
GGCGTAAC-GCGCGGCGCGCTCTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTTGAC
GCG-TTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGTTC-TTGGACGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCAACGACATCCACTACAAATTCCACAACAT-CCTGTTTTTAAA
GTGCGAACATACGGAACAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
ACGA-TTTGGA-CAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGGTTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
ATCAAGCCTTGGTAACAATGCCGTCTG-AAACAAACAAA-CCTTTCAAAC-GGCATCAAA
ATGACACAA-AGCATTCTTCTAAAGAGATACATATTCACTAA-----------------
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Sample USB 553

```
--------------------CGCCTTAGAAGCAT-AAAAAGCA--TTAT-TTATCCCATC
TGTC-TGGTTTGATGTA-AA-GGTTTTGCCAATCAACAGGCATTCT-------------
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------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAA-CCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGATTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGAT-AAAAGTCTTTTTTATAA
T-CCG-CCCTCGTCAAA-CCG-ACCC-GAAACG-AAAACGCCATTAT-GAGAAAAA--CC
AAAACCG-AAGCCTTGAAAACCAAAGAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCGCGTACCTCGCTC-AACGAAATCGCCCAAA-CCGCC
GGCGTAAC-GCGCGGCGCGCTCTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTTGAC
GCG-TTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGTTC-TTGGACGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCAACGACATCCACTACAAATTCCACAACAT-CCTGTTTTTAAA
ATGCGAACACACGGAGCAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
ACGATTTTGGA-CAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGATTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
ATCAAGCCTTGGTAACAATGCCGTCTG-AAACAAACAAACCCTTTCAAAC-GGCATCAAA
ATGACACAA-AGCATTCTTCTAAAAGA-TACATATTCACTAAATTGCTA-----------
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Sample USB 970

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GCCAACGCGGCCGCACGCA-TCGCCTTAGACCAT-AAAAAGCA--TTAT-TTATCCTATC
TGTC-TGGTTTGATGTA-AAGGGTTTTGCCAATCAACAGGCATTCT--------------
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------------------------------------------------------------
----------------------TATTTCAGGATAT-AAAAA-CCGCCTGCTTTGATACC
CGAATGTTCGAACGGGTTGCAAAGCAGGTTATACCTGTTTTCAAAGTTGAGATGCAGTCT
CAATTTTAT-GGGTTTCATTATACATACACGATTGCACGGAT-AAAAGTCTTTTTTATAA
T-CCG-CCCTCGTCAAA-CCG-ACCCGGAAACG-AAAACGCCATTATGGAGAAAAA--CC
AAAACCG-AAGCCTTGAAAACCAAAGAACACC-TGATG--C-TTGCCGCCTTGGAAACC-
TTTT----ACCGCAAAGGGATTGCCCGCACCTCGCTC-AACGAAATCGCCCAAG-CCGCC
GGCGTAAC-GCGCGGCGCGCTCTA-TTGGCATTTCAAAAATAAGGAAGACTTG-TTTGAC
GCG-TTGTT-CCAACGTAT-CT-GCGACGACATC--G-AAAACTGCATCGCGCAAGATGC
CGCAGATGCCGAAGGAGGTTC-TTGGACGGTATTCCGCCA-CACGC-TGCTGCAC-TTTT
TC-GAGCGGCTGCAAAGCAACGACATCTACTACAAATTCCACAACAT-CCTGTTTTTAAA
ATGCGAACACACGGAGCAAAACGCCGCCGTTATCGCCATTGCCCGCAAGCATCAGGCAAT
C-TGGCGCGAGAAAATTACCGCCGTTTTGACCGAAGCGGTGGAAAATCAGGATTTGGCTG
ACGA-TTTGGA-CAA-GGAAA-CGGCGG--TCATC-TTCATCAAATCGACGTTGGACGGG
C-TGATTTGGCGT-TGGTTCTCTTCCGGCGAAAGTTTCGATTTGGGCAAAACCGCCCCGC
--GCATCATCGGGATAATGATGGACAACTTGGAAAACCATCCCTGCCTGCGCCGGAAATA
ATCAAGCCTTGGTAACAATGCCGTCTG-AAACAAACAAACCCTTTCAAAC-GGCATCAAA
ATGACACAA-AGCATTCTTCTAAAAGA-TACATATTC----------------------
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