PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF *NEISSERIA GONORRHOEAE* ISOLATES FROM NEW ZEALAND WITH REDUCED SUSCEPTIBILITY TO CEFTRIAXONE

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ABSTRACT

Objectives

Currently, ceftriaxone is the last remaining drug recommended for empirical treatment of gonorrhoea. *Neisseria gonorrhoeae* with reduced susceptibility to ceftriaxone have been isolated worldwide in countries such as Japan, France, Spain, Slovenia, Australia and Sweden. These have led to treatment failures and the emergence of ceftriaxone-resistant *N. gonorrhoeae*. Various mutations in *penA* (mosaic and nonmosaic), which encodes the penicillin-binding protein 2 (PBP2), have been reported to be the primary reason for reduced ceftriaxone susceptibility, but it can be reduced further by mutations in *mtrR*, $porB_{IB}$ and *ponA*. In this study, we aimed to determine the antimicrobial resistance patterns of New Zealand isolates of *N. gonorrhoeae* with reduced susceptibility to ceftriaxone and to characterise the *penA*, *mtrR,* $porB_{IB}$ and *ponA* in the isolates.

Methods

A total of 28 *N. gonorrhoeae* isolates with elevated ceftriaxone MIC (0.03 to 0.12 mg/L), collected from 2012 to 2015 and obtained from the Institute of Environmental Science and Research (ESR), were examined in this study. Samples came from laboratories in Auckland (26), Wellington (1) and Taranaki (1). The antimicrobial resistance of penicillin G, tetracycline, ciprofloxacin, azithromycin and ceftriaxone were determined through antimicrobial susceptibility test, using minimum inhibitory concentration (MIC) test strips. Polymerase chain reactions (PCRs) and sequencing to identify specific mutations in *penA*, *mtrR*, $porB_{IB}$ and *ponA,* that are associated with elevated minimum inhibitory concentrations (MICs) to ceftriaxone, were undertaken. The association between the phenotypic and genotypic results was investigated by comparing the presence of the number of mutated genes and the MIC level of ceftriaxone.

Results

Based on the AST results using MIC test strips and interpreted using The European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria, 23 out of 28 isolates (82%) showed reduced susceptibility to ceftriaxone, with MICs of 0.03 to 0.06 mg/L. All of the isolates were resistant to ciprofloxacin, while 36%, 25% and 7% were resistant to penicillin G, tetracycline and azithromycin, respectively. Two azithromycin-resistant *N. gonorrhoeae* isolates were observed, and isolate 264 (azithromycin MIC: 4mg/L) also exhibited reduced susceptibility to ceftriaxone (MIC: 0.03 mg/L). A total of 21% (6/28) of the isolates produced β-lactamase. The 23 isolates that conveyed reduced ceftriaxone susceptibility were found to harbour three or four mutated genes (*penA, mtrR and/or porB$_{IB}$ and ponA*). Reduced susceptibility to ceftriaxone among *N. gonorrhoeae* isolates in this study was associated with mosaic PBP2 (encoded by *penA*) with G545S/A501V mutations, with nonmosaic PBP2 with an A501V mutation, plus the presence of mutation in *mtrR* promoter with G120 and A121 alterations in PorBIB. A total of 65% (15/23) of the *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone harboured mosaic PBP2 XXXIV, a pattern found in *N. gonorrhoeae* associated with ceftriaxone treatment failures in Europe and Australia. The current study also revealed that the partial sequences of four mosaic PBP2 (M-2, M-3, M-4, M-5) were different from the common mosaic PBP2 sequences reported in various studies.

Conclusion

There is an association between the phenotypic and genotypic character of *N. gonorrhoeae* isolates expressing reduced susceptibility to ceftriaxone in this study population. Furthermore, the presence of important mosaic PBP2 that link to ceftriaxone treatment failure might be circulating among *N. gonorrhoeae* isolates in New Zealand .

Keywords: *Neisseria gonorrhoeae,* ceftriaxone, reduced susceptibility, New Zealand

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Abbreviations

AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
ATCC	American type culture collection
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CLSI	Clinical Laboratory Standards Institute
CMRNG	Chromosomally-mediated resistant *Neisseria gonorrhoeae*
CO_2	Carbon dioxide
CRO	Ceftriaxone
DDBJ	DNA Data Bank of Japan
DHB	District health board
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ESCs	Extended-Spectrum Cephalosporins
ESR	Institute of Environmental Science and Research
EtBr	Ethidium Bromide
EUCAST	The European Committee on Antimicrobial Testing
FDA	Food and Drug Administration
GISP	Gonococcal Isolate Surveillance Project
HIV	Human Immunodeficiency Virus
HLR	High-level resistance
HTH	Helix-turn-helix
IM	Intramuscular injection
MDR	Multidrug-resistant
$MgCl_2$	Magnesium Chloride
MGS	Massey Genome Service
MIC	Minimum Inhibitory Concentration
MLST	Multi-locus sequence typing

MSM	Men who have sex with men
mtr	Multiple transferable system
MUHEC	Massey University Human Ethics Committee
NAAT	Nucleic Acid Amplification Test
NCBI	National Center for Biotechnology Information
NETs	Neutrophil Extracellular Traps
NG-MAST	*Neisseria gonorrhoeae* Multi-antigen Sequence Typing
NPV	Negative predictive value
NZSHS	The New Zealand Sexual Health Society Incorporation
PBP	Penicillin Binding Protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEN	Penicillin
PHE	Public Health England
PID	Pelvic Inflammatory Disease
PMNs	Polymorphonuclear leukocytes
PPNG	Penicillinase-producing *Neisseria gonorrhoeae*
PPV	Positive predictive value
qPCR	Quantitative Polymerase Chain Reaction
STIs	Sexually transmitted infections
TMP-SMX	Trimethoprim/sulfamethoxazole
TAE	Tris-Acetate-EDTA
TrisHCL	Tris hydrochloride
TRNG	Tetracycline-resistant *Neisseria gonorrhoeae*
WHO	World Health Organization
XDR	Extensively drug resistant

Nucleotides Abbreviations

A	Adenine
G	Guanine
C	Cytosine
U	Uracil
T	Thymine

Amino Acids Abbreviations

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic Acid
C	Cysteine
E	Glutamic Acid
Q	Glutamine
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
T	Threonine
V	Valine
Y	Tyrosine
S	Serine
W	Tryptophan

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

Gonorrhoea is a sexually transmitted disease that remains a public health problem globally. Gonorrhoea was identified by Centers for Disease Control and Prevention (CDC) as the second most commonly reported notifiable infection in the United States in 2014, with 110.7 cases per 100,000 people reported as infected (CDC, 2014b). In New Zealand, the Institute of Environmental Science and Research (ESR) reported that the gonorrhoea rate for 2014 was 70 cases per 100,000 people.

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Gonorrhoea is caused by *Neisseria gonorrhoeae*, an aerobic Gram-negative bacterium that is found intracellularly in white blood cells (Frobisher and Fuerst, 1978). Symptoms include abnormal discharge from vagina or penis, burning when urinating, pelvic or abdominal pain and bleeding between periods (CDC, 2014a). Gonorrhoea can cause a substantial impact on the wellbeing of those who have been infected. Long-term effects range from pelvic inflammatory disease (PID), ectopic pregnancy, infertility (in both men and women) to blindness in babies (Tapsall *et al.*, 2009a; Goire *et al.*, 2014). The inflammation caused by gonorrhoea also increases the probability of the patient contracting HIV (Cohen *et al.*, 1997; Tapsall *et al.*, 2009a).

Providing effective treatment for gonorrhoea is a challenge due to the rapid emergence of multi-drug resistant *N. gonorrhoeae*. In the past seven decades, *N. gonorrhoeae* has acquired resistance to almost all groups of antimicrobials agents used to treat it (Unemo & Shafer, 2014). Nowadays, ceftriaxone (a cephalosporin) is the only antibiotic recommended to treat gonorrhoea infection empirically (Tapsall *et al.*, 2009a; Unemo and Shafer, 2014). Many countries practise the current treatment recommendation by CDC where a single intramuscular injection (IM) dose of 250mg of ceftriaxone is administered in combination with 1g azithromycin or 100 mg of doxycycline (orally twice daily for seven days (CDC, 2012c). Dual treatment is advised because of the likelihood of

concurrent *Chlamydia trachomatis* infection and to slow down the emergence of ceftriaxone-resistant *N. gonorrhoeae* (CDC, 2012c).

Alarmingly, therapeutic failure with ceftriaxone has been reported in several countries such as Australia, Sweden, Slovenia and Japan (Unemo *et al.*, 2011a; Unemo *et al.*, 2011b; Read *et al.*, 2013; Golparian *et al.*, 2014). *N. gonorrhoeae* strains expressing full resistance to ceftriaxone have been isolated from Japan, France and Spain (Ohnishi *et al.*, 2011; Camara *et al.*, 2012; Unemo *et al.*, 2012), and the number of *N. gonorrhoeae* strains with reduced susceptibility to cefixime and ceftriaxone has been increasing globally (CDC, 2013b). These findings suggest that *N. gonorrhoeae* will probably become untreatable in the near future.

The aim of this literature review is to provide insights into *N. gonorrhoeae* and its epidemiology, its history of antimicrobial resistance and the current issues related to its reduced susceptibility to ceftriaxone.

1.1. Gonorrhoea: Epidemiology and Health Impact

Gonorrhoea, caused by *N. gonorrhoeae*, is a sexually transmitted disease found worldwide. 106 million new cases of gonorrhoea are reported globally every year and this represents 25% of the estimated total of new cases of curable sexually transmitted infections (STIs) that occur globally every year (WHO, 2012). In 2013, the Centers for Disease and Prevention (CDC) stated that *N. gonorrhoeae* is “an immediate public health threat that requires urgent and aggressive action” (CDC, 2013b). CDC acknowledges gonorrhoea as the second most commonly reported notifiable disease in the United States, with a total of 333,004 cases reported in 2013. However, it is estimated that more than 800,000 cases of gonorrhoea occur annually in the United States (PHE, 2013).The report also states that there are differences in infection rates and severity among different sexes, races and ethnic minorities. The differences might be influenced by the access to health services, how these services are utilised, geographical distribution, socioeconomic factors

and choice of partner (Toomey *et al.*, 1993). Nevertheless, changes in gonorrhoea rates have been observed over the years due to changes in treatment regime, the use of improved screening procedures and changes in reporting practices (Tapsall *et al.*, 2009a).

In 2014, gonorrhoea infection in New Zealand represented an estimated 10% of the total curable sexually transmitted infections (STIs) which included chlamydia and syphilis (ESR, 2014). In the latest *Sexually Transmitted Infections (STIs) Annual Surveillance Report 2014* report released by the Institute of Environmental Science and Research Limited (ESR), New Zealand, the reported gonorrhoea rate was 70 cases per 100,000 people (ESR, 2014). The reported gonorrhoea rate in this country has been steady over the past five years (ESR, 2009; ESR, 2010; ESR, 2011). The highest rate in 2014 was reported from the Tairawhiti District Health Board (Gisborne), with 316 cases per 100,000 people (ESR, 2014). The number of anorectal and pharyngeal infections in men has more than doubled between 2009 and 2014 (ESR, 2014). The increase in number of laboratory diagnoses could have been due to the introduction of nucleic acid amplification testing (NAAT) in 2009, which has improved the detection of *N. gonorrhoeae* (ESR, 2014).

N. gonorrhoeae causes asymptomatic urogenital infections in the majority of women (≥50%) and some men (10%) (Unemo and Shafer, 2014), with rectal and pharyngeal infections most frequently observed in men who have sex with men (MSM). The finding is supported by Morris *et al*. (2006), whose study revealed that the pharynx is a primary site of gonorrhoea infection among sexually active MSM. The prevalence of pharyngeal gonorrhoea among MSM determined through both ligase chain reaction and strand displacement amplification testing was 5.5%. Ninety-two percent of the cases were asymptomatic (Morris *et al.*, 2006). Regardless, rectal and pharyngeal infections can also occur in women, depending on their sexual practice.

If left untreated, or inadequately treated, the infection of the lower urogenital tract will move upwards, infecting the reproductive tract. In the male, *N. gonorrhoeae* infection usually causes urethritis, which leads to acute scrotal pain (epididymo-orchitis) (Goire *et al.*, 2014), while in the female sequelae such as pelvic inflammatory disease (PID) can result in outcomes such as ectopic pregnancy. Gonorrhoea can also cause infertility in both men and women (Goire *et al.*, 2014). Where a baby is born through vaginal delivery to an infected mother, the infection can cause blindness (gonococcal ophthalmia) (Tapsall *et al.*, 2009a). Failure to treat gonorrhoea will also enhance the transmission of the human immunodeficiency virus (HIV) infection (Cohen *et al.*, 1997; Tapsall *et al.*, 2009a). Fleming and Wasserheit (1999) state that gonorrhoea increases the risk of HIV by recruiting HIV target cells (CD4 lymphocytes) to the endocervix (Fleming and Wasserheit, 1999). Active intervention plays a crucial role in containing the infection (preventing transmission) and avoiding complications. However, this is a challenge because the disease is asymptomatic most of the time and *N. gonorrhoeae* is resistant to many antibiotic classes. Both these factors contribute to delays in receiving treatment, ineffective treatment and further dissemination

1.2. *Neisseria gonorrhoeae* morphology and traits

N. gonorrhoeae are diplococci that resemble two coffee beans pressed together at their flat surfaces (Frobisher and Fuerst, 1978). It is an aerobic bacterium, which is incapable of anaerobic growth (James-Holmquest *et al.*, 1973). *N. gonorrhoeae* might have evolved from a nasopharyngeal ancestor such as *N. meningitidis,* since its 16S rRNA sequence has 98% similarity with that of *N. meningitidis* (Vázquez *et al.*, 1995), indicating that the bacterium is closely related genetically to the other pathogenic *Neisseria* species.

N. gonorrhoeae also has the intrinsic ability to exchange its chromosomal DNA actively via horizontal transfer among *Neisseria* species (Biswas *et al.*, 1989; O'Rourke and Stevens, 1993). The exchange of chromosomal DNA encoding resistance genes between *Neisseria* species causes resistance mechanisms to

be disseminated freely among the species, thus resulting in the spread of antimicrobial resistant among *Neisseria* species (Tapsall *et al.*, 2009b). The best example of this phenomenon is the contribution of the *penA* gene fragment, which contains various point mutations, from *Neisseria* commensals to susceptible *N. gonorrhoeae* strains. Most *N. gonorrhoeae* strains that show reduced susceptibility to extended-spectrum cephalosporins (ESCs) harbour mosaic *penA* sequences that resemble those of *Neisseria* commensals such as *N. cinerea*, *N. perflava/sicca, N. flavescens and N.meningitidis* (Ameyama *et al.*, 2002). Also, the tendency of *N. gonorrhoeae* to gain and lose its genes can also cause strain misidentification, either through molecular or phenotypic methods (Tapsall *et al.*, 2009b; Upton *et al.*, 2013).

N. gonorrhoeae is one of the fastest-evolving bacteria. Since sulphonamides were released, the bacterium has successfully developed resistance to all available antimicrobial agents for gonorrhoea treatment (Tapsall *et al.*, 2009b; Unemo and Shafer, 2014). The development of AMR *N. gonorrhoeae* is either plasmid-mediated or chromosomally-mediated (Unemo and Shafer, 2014). Antimicrobial resistant *N. gonorrhoeae* has caused significant problems for physicians in providing effective treatment for their patients. To make the situation worse, *N. gonorrhoeae* is also reported to maintain resistance genes against these antimicrobial agents, such as penicillin and tetracycline, even though these antimicrobial agents are no longer used to treat gonorrhoea (CDC, 2005; Unemo and Shafer, 2014).

Another trait of *N. gonorrhoeae* is the ability to adapt to the host defence system and maintain itself in the host, particularly in the genital tract, in the presence of a vigorous inflammatory response (Gunderson and Seifert, 2015). Gunderson and Seifert (2015) have recently shown that the gonococci have developed a way to control polymorphonuclear leukocyte (PMNs) responses. Normally, PMNs that are infected by the gonococcus will undergo oxidative burst and release neutrophil extracellular traps (NETs) as the last line of defence. NETs can kill various fungi and bacteria directly. However, *N. gonorrhoeae* stimulates PMNs to

produce NETs even without the process of oxidative burst (Gunderson and Seifert, 2015). As a result, other commensals and normal resident flora are affected by the released NETs, while the gonococci remain safe and protected inside polymorphonuclear leukocytes (PMNs). Consequently, *N. gonorrhoeae* can persist in the host for an extended period and the situation encourages further dissemination of the bacteria to another host (Gunderson and Seifert, 2015).

Other survival mechanisms of *N. gonorrhoeae* involve changing the composition of its antigens, particularly its pili and opacity protein, to avoid being eradicated by the immune system (Hombach *et al.*, 2013; Wolfensberger *et al.*, 2013). It can also delay phagosomal maturation (Johnson & Criss, 2011) and inhibit the apoptosis PMNs (Boom *et al.*, 1990).

1.3. Methods to diagnose gonorrhoea

To collect accurate information regarding the status of the infection, the application of a robust diagnostic test is essential. A reliable diagnostic test should have a high positive predictive value (PPV) and negative predictive value (NPV), plus be able to provide rapid results to enable appropriate treatment for patients. Additionally, the method should also help enhance the monitoring of AMR of *N. gonorrhoeae* (Tapsall *et al.*, 2009b). Some authors have discussed the different methods available for laboratory diagnosis of *N. gonorrhoeae* (Ng and Martin, 2005; Whiley *et al.*, 2006). Three methods are used to diagnose *N. gonorrhoeae*: microscopy, conventional culture and nucleic acid amplification tests (NAATs).

Gram stain and bacterial culture were the earliest methods used to identify the organism (Whiley *et al.*, 2006). Ng and Martin (2005) describe the use of Gram stain, which can act as a presumptive test for *N. gonorrhoeae* in urethral specimens from males. Gonococci in smear samples can be seen as a Gram-negative diplococsi associated with or within polymorphonuclear leukocytes

(Jorgensen *et al.*, 2015). Compared to the bacterial culture method, a Gram stain is simple and provides rapid outcomes. However, the microscopy method is not applicable to certain types of samples, such as an endocervical smear from females and a rectal smear (Weyant *et al.*, 1996). The presence of other Gram-negative commensals, such as *Moraxella osloensis* and *Moraxella phenylpyruvica,* are said to interfere with the interpretation of the smear. The Gram stain was considered adequate for a urethral smear from males, but not suitable for cervical smears from females due to the presence of other commensal *Neisseria* species (Whiley *et al.*, 2006). The sensitivity of endocervical smears is much lower (50% to 70%) than the sensitivity of urethral smears, which is 90% (Gaydos *et al.*, 2003). Due to this limitation, the direct Gram stain is not recommended for specimens other than urethral smears from men.

Bacterial culture is the gold standard method used for identification of *N. gonorrhoeae* (Whiley *et al.*, 2006; Goire *et al.*, 2014). The method has a specificity of 100% and was used in most clinical health laboratories to confirm the organism before the introduction of NAATs (Van Dyck *et al.*, 2001). The culture-based method is valuable since it means the isolate's AMR profile can be determined phenotypically, and this can be done through antimicrobial susceptibility testing (AST).

Currently, the agar dilution method and the use of a predefined gradient of antibiotic concentrations on a plastic strip (MIC test strips) are the two AST methods that are most commonly used. Although the agar dilution method is the gold standard method suggested by CLSI to determine the MIC level of antimicrobial agents (Jorgensen *et al.*, 2015), the use of the MIC test strips is widely opted because of its ease of use, rapid and reproducibility (Sanchez *et al.*, 1992; Van Dyck *et al.*, 1994). There are plenty of MIC test kits available in the market with large price difference and varied performances, however there is limited data regarding MIC test kits comparisons for *N. gonorrhoeae*.

Regardless, the MIC results is interpreted by either Clinical Laboratory Standard Institute (CLSI) or European Committee on Antimicrobial Testing (EUCAST) AST breakpoints guidelines indirectly benefits physicians in providing more effective treatment to their patients by providing a clear picture of the current level of resistance of *N. gonorrhoeae* strains. It helps the monitoring program to identify potential new strains that show previously unrecognised resistance to drugs (Hook *et al.*, 2013).

However, the classic bacteria culture suffers from several limitations (Ng and Martin, 2005; Goire *et al.*, 2014). First, *N. gonorrhoeae* can be difficult to manage in a laboratory and health setting. The gonococcus is a fastidious organism and requires specially enriched media to enhance its growth, such as chocolate-supplemented agar that contains 1% IsoVitaleX growth supplement, or selective media such as Thayer-Martin or New York City Media that contain antimicrobial agents that will inhibit the growth of other commensals (Ng and Martin, 2005). The gonococcus grows well at 35°C to 37°C in a humid environment enriched with 3% to 5% carbon dioxide (CO_2) (Ng and Martin, 2005; Brown *et al.*, 2016). When trying to culture and grow the bacteria in the laboratory, it is important to meet all of the stated criteria to maintain its viability.

Secondly, the culture-based method can be tedious, particularly the collection and maintenance of the control samples in storage. Collecting fresh samples such as urethral swabs is a complicated process and it can cause discomfort for patients. At the laboratory level, experienced technicians are required to carry out the test and a high level of resources is needed. The application of this method is not suitable for some anatomical sites such as rectum and endocervix, due to the presence of the normal flora that might overgrow *N. gonorrhoeae* in culture. Lack of clear guidelines to carry out the test, plus the political and financial constraints, are some of other factors that have led to a decrease in the use of the culture-based method (Goire *et al.*, 2014). Due to these limitations, there has been a significant shift towards the use of NAATs in many clinical laboratories. The preference for NAATs over culture has decreased the number of viable

cultures available for determining the resistance profile of the strains. This change poses a significant challenge in monitoring the spread and the emergence of AMR *N. gonorrhoeae* in many countries including New Zealand due to the lack of AST data (Heffernan *et al.*, 2004) .

Although a part of determining the profile of *N. gonorrhoeae* isolates from bacterial culture, it is worth noting that the issue regarding the AST interpretation using CLSI and EUCAST guidelines. Both AST guidelines are the most commonly used AST breakpoints in many countries. The issue between the two guidelines are due to the different breakpoints set up for antimicrobial agents, with EUCAST guidelines tending to have lower resistant breakpoints to many of the antimicrobial agents used in this study, particularly from gram negative bacteria, compared to the CLSI guidelines (Hombach *et al.*, 2013; Wolfensberger *et al.*, 2013). As a result of the different breakpoints, higher number of isolates have been classified as resistant when the EUCAST guidelines were used, compared to when the CLSI guidelines were used. The unsynchronized breakpoints of the CLSI and EUCAST might affect the international surveillance of antimicrobial resistance, which will lead to different reports of susceptibility and cause a false comparison of resistant rates in different countries (Kahlmeter and Brown, 2002). The difference between the breakpoints set by both guidelines are due to the different objectives that the guidelines want to achieve, different approaches used in setting the breakpoints and establishment of their own susceptibility methods (Kahlmeter and Brown, 2002; Brown *et al.*, 2016).

Nucleic acis tests for *N. gonorrhoeae* were introduced in the 1990s, and since then have significantly improved the diagnosis of gonorrhoea infection (CDC, 2012a; ESR, 2013; Goire *et al.*, 2014). Nowadays, many diagnostic laboratories have opted for commercial NAATs assay that have largely replaced the hybridization assay (Jorgensen *et al.*, 2015). The NAATs test were designed to target *both N. gonorrhoeae* and *Chlamydia trachomatis.* Due to their robustness, highly sensitive and the ability to provide a rapid result, the test is favoured over the bacterial culture method. Additionally, a viable sample is not needed, which

makes it a favourable method in most laboratories. The method can also be used to diagnose both symptomatic and asymptomatic infections (Whiley *et al.*, 2006). In 2012, the reported gonorrhoea rate in New Zealand increased by a third after NAATs for gonorrhoeae were introduced (ESR, 2013).

Furthermore, the NAATs can also be used to identify the AMR *N. gonorrhoeae* strain that might pose a public health threat. For this purpose, Goire *et al*. (2014) have divided the NAATs into two approaches: typing-based, where gonococcal resistance is predicted by association with genotype, and through detecting specific genes or mutations of AMR *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) is the validated typing method that requires the sequence of a *porB* gene and *tbp* gene, and uses an open-access NG-MAST database. According to Goire and colleagues, isolates that belong to similar or almost identical NG-MAST groups usually will have similar AMR profiles. Although the method is useful, the test cannot be fully relied on especially in the long term due to the loss and acquisition of chromosomal DNA by *N. gonorrhoeae* (Goire *et al.*, 2013). Nevertheless, NG-MAST has been intensively used in Europe to identify the presence of NG-MAST type 1407 (*N. gonorrhoeae* F89 strain), a ceftriaxone-resistant *N. gonorrhoeae* strain (Chisholm *et al.*, 2013). Another important clone is the NG-MAST 4220 which is a ceftriaxone-resistant *N. gonorrhoeae* strain, H041, isolated in Japan (Ohnishi *et al.*, 2011).

On the other hand, numerous studies have attempted to develop PCR assays that are able to rapidly identify AMR in *N. gonorrhoeae* based on its genotype (Vernel-Pauillac and Merien, 2006; Goire *et al.*, 2011; Goire *et al.*, 2012a; Goire *et al.*, 2012b; Balashov *et al.*, 2013; Goire *et al.*, 2013). Some of these PCR assays have been validated to have 100% sensitivity and specificity and have proven to correlate well with the phenotypic character of the AMR strains. For example, Vernel-Pauillac *et al*. (2006) and Goire *et al*. (2011) have developed assays to detect directly the penicillin-resistant *N. gonorrhoeae* based on chromosomal mutations in *penA* and *ponA*, and plasmids encoding β-lactamase. While a qPCR assay to detect ciprofloxacin resistance in *N. gonorrhoeae* based

on single nucleotide polymorphisms (SNPs) associated with the resistance show high sensitivity and specificity value (Peterson *et al.*, 2015).

In 2013, a more advanced molecular technology was developed by Balashov and colleagues, using multiplex bead suspension array assay, which can detect 29 mutations and two plasmid genes that cause resistance to six antibiotics (Balashov *et al.*, 2013). However, this assay has not yet been adopted by diagnostic laboratories.

With the current emergence of *N. gonorrhoeae* with reduced susceptibility to ceftriaxone, most scientists now focus on developing a molecular assay to detect this important strain. Ochiai *et al*. (2008) developed a Quantitative PCR (qPCR) assay that directly identifies the mosaic *penA,* said to be the primary factor associated with reduced susceptibility to ceftriaxone. In the same vein, Goire and colleagues (2012, 2013) more specificlydeveloped qPCR assays that target novel mutations in ceftriaxone-resistant strains, F89 and H041. Recently, a molecular assay was developed by Peterson *et al*. (2015b) to detect *N. gonorrhoeae* with reduced susceptibility to ceftriaxone based on four genetic markers, *penA, mtrR*, $porB_{IB}$*, ponA* and the *N. gonorrhoeae* specific marker (*porA*). Peterson and colleagues reported that the sensitivity of the test to detect at least two single nucleotide polymorphisms (SNPs) is more than 98% (Weyant *et al.*, 1996). Peterson and colleagues also added that the technology can reduce the high cost of molecular testing since the method can detect many resistance markers at the same time.

Despite these benefits, the molecular approaches still suffer from some disadvantages. One of the limitations includes the need to revise and validate the assays from time-to-time due to the ever-evolving nature of *N. gonorrhoeae* and its resistance mechanisms (Whiley *et al.*, 2006). Over time, these assays will lose their sensitivity and specificity due to the gonococcus undergoing constant genetic changes (Biswas *et al.*, 1989; Goire *et al.*, 2014). Furthermore, carrying

out molecular testing to determine a complete resistance profile of the strains will involve a high cost and resources (Whiley *et al.*, 2006; Goire *et al.*, 2014). Last, but not least, only a few of these molecular tests show a good correlation between the MIC and the genes present. When Balashov *et al*. (2013) developed their multiplex bead suspension array assay, they did not correlate back with the phenotypic character of the strains used in the study. While for a qPCR assay developed to detect ciprofloxacin-resistant strains, only six *N. gonorrhoeae* isolates were used in the study (Peterson *et al.*, 2015).

NAAT testing also suffers from low specificity for two other reasons. First, detection of other types of *Neisseria* commensals might occur since *Neisseria* species frequently exchange their DNA within the species (Whiley *et al.*, 2006). Secondly, the low specificity of the test is also due to some of the *Neisseria* species having a high similarity of sequence homology with *N. gonorrhoeae* (Goire *et al.*, 2013). For example, Ochiai and colleagues (2008) have developed a RT-PCR assay that can detect the presence of mosaic *penA* genes that have been linked with reduced susceptibility to ceftriaxone (Lahra, 2014). However, the assay was not sensitive enough to distinguish between *N. gonorrhoeae* and *N. meningitidis* due to the high level of similarity of the mosaic *penA* sequence (Lahra, 2014).

Some studies have also reported a false-positive result through using a molecular method, signalling the low specificity of NAAT (Upton *et al.*, 2013; Chow *et al.*, 2015). This issue has highlighted the need to review the guideline on the usage of NAAT testing to diagnose *N. gonorrhoeae* infection, particularly for low-risk groups. A positive NAAT result should be confirmed with a second NAAT test or a supplementary ‘confirmatory’ test to validate the result (Spratt *et al.*, 1992; Tapsall *et al.*, 2010; Sethi *et al.*, 2013; Upton *et al.*, 2013). It is crucial to confirm the result since reporting false-positive results in gonorrhoea reporting will lead to unnecessary treatment, and importantly, affect the personal relationship of the patients (Chow *et al.*, 2015).

Perhaps, the most important limitation of the NAATs is that the method is unable to provide data on new forms of antibiotic resistance which is essential for the development of an efficient AMR surveillance programme for *N. gonorrhoeae* (Whiley *et al.*, 2006). The method is unable to provide data on the resistance level that is essential for both treatment and a monitoring programme for AMR *N. gonorrhoea*. Hence, laboratories such as public health laboratories still need to rely on conventional culture to obtain data for the AMR *N. gonorrhoeae.*

In summary, there are advantages and disadvantages of both conventional and NAAT methods for detection of *N. gonorrhoeae* and antibiotic resistance in this pathogen. Both methods are equally important in providing complete information about the genotypic and phenotypic character of *N. gonorrhoeae* and are best used together. However, a suitable method will depend on the scope and the objective that needs to be achieved by the clinical laboratories. Private laboratories in New Zealand might benefit more from the use of both methods to screen and confirm the infection, providing that the laboratories are financially capable and have enough staff.

1.4. Chronology of multidrug-resistant (MDR) *Neisseria gonorrhoeae*

The emergence of multidrug-resistant (MDR) *N. gonorrhoeae* and extensively drug resistant (XDR) *N. gonorrhoeae* that have developed resistance to older and recently introduced antibiotics have complicated gonorrhoea treatment (Tapsall *et al.*, 2009b). MDR strains are resistant to one of the antibiotics currently used for gonorrhoea treatment (Class I), plus two or more of the antibiotics in that are less frequently administered for gonorrhoea treatment, or else proposed for extensive use (Class II). On the other hand, XDR *N. gonorrhoeae* are described as strains that are resistant to two or more of antibiotic in class I, or three or more antibiotics in class II **(Table 1-1).**

According to a CDC (1987) report "Antibiotic-resistant strains of *Neisseria gonorrhoeae*: policy guidelines for detection, management, and control", an antibiotic used to treat gonorrhoea must cure 95% or more gonorrhoea infections. In other words, for an antibiotic to remain in the empirical gonorrhoea treatment regime, the resistance rate must not be more than 5%. This guideline has been used since then to decide whether the current treatment options are still effective for gonorrhoea treatment.

The timeline of the development of *N. gonorrhoeae* antimicrobial resistance can be seen in **Table 1-2**. Gonorrhoea treatment began as early as the 1930s when antiseptics such as Merbromin were used to treat the infection (Unemo and Shafer, 2014). However, due to their toxicity and side effects, the use of the antiseptics was terminated (CDC, 2012b). Sulfonamides were introduced in 1935 and were used worldwide as the first line antimicrobial agent for gonorrhoea treatment (Hook *et al.*, 2013). However, the sulfonamides started to show signs of losing their effectiveness in the 1940s when 35% of the patients receiving a high dose of sulfonamides suffered a failure of treatment and more than 90% of gonococcal isolates were resistant to sulfonamides *in vitro* (Osaka *et al.*, 2008; Unemo and Shafer, 2014). Nevertheless, sulfonamides were still used for several more decades to treat gonorrhoea in combination with trimethoprim (TMP-SMX) (Tapsall *et al.*, 2009b).

Table 1-1 Classification of antibiotics used to treat *N. gonorrhoeae.* The original concept was from Tapsall *et al.* (2009b)

Class	Antibiotic
Class I **Antibiotics recommended for gonorrhoea treatment**	• Extended-Spectrum Cephalosporins (ESCs) • Azithromycin • Doxycycline (Tetracycline)

Class II	
Antibiotics that are less frequently administered for gonorrhoea treatment, or else proposed for extensive use	• Penicillins • Fluoroquinolones • Aminoglycosides • Carbapenems
Class III **Antibiotics that are inappropriate for gonorrhoea treatment**	• Chloramphenicol • Rifampicin • Co-trimoxazole • Erythromycin

Note:

1. MDR: Strains that are resistant to one of the antibiotics currently used for gonorrhoea treatment (Class I), plus two or more of the antibiotics in that are less frequently administered for gonorrhoea treatment, or else proposed for extensive use (Class II).
2. XDR: Strains that are resistant to two or more of antibiotic in class I, or three or more antibiotics in class II.

Table 1-2 The development antimicrobial resistance in *N. gonorrhoeae*

Antimicrobial agents	Year it was introduced for gonorrhoea treatment	Year of the antimicrobial-resistant strain first reported (globally)	Year of the antimicrobial - resistant strain first reported in New Zealand
Merbromin	1930s	-	-
Sulfonamides	1935	1944	-
Penicillin	1943	1976	1976 (Green, 1979)
Tetracycline	1949	1986 [a]	1978 [b] (Green, 1978)
Spectinomycin	1965	1967	Predicted later than 2002 [c]
Ciprofloxacin	1985	1990	2001 (ESR, 2008b)

Azithromycin	1990	1997	-

(-): No information was found

[a] Data is based on the first reported plasmid-mediated tetracycline resistance causing high-level resistance (TRNG) (Unemo and Shafer, 2014). No information regarding the first chromosomal-mediated tetracycline resistance which caused low-level of resistant.

[b] The first *N. gonorrhoeae* with low-level, chromosomal resistance (2-4 mg/L) in New Zealand (Green, 1978).

[c] No information on when the first spectinomycin-resistant *N. gonorrhoeae* was found in New Zealand. The latest update was in 2002, where *N. gonorrhoeae* strains were still susceptible to spectinomycin.

Penicillin was introduced in 1943 for the treatment of gonococcal urethritis and it was used for nearly 40 years. As reported by Van Slyke and colleagues (1941) (cited in Unemo & Shafer, 2014), penicillin initially successfully cured 95% cases of gonorrhoea. Nonetheless, several years after it was introduced, the penicillin dose needed to be increased due to the increasing prevalence of chromosomally-mediated resistant *Neisseria gonorrhoeae* (CMRNG) (Vetlab, 2014). By the 1960s, treatment failure with penicillin was reported, due to the gradual increase of resistance of *N. gonorrhoeae* to penicillin, even at the higher dose (Unemo and Shafer, 2014).

Multiple mutations in the chromosome involving *penA, mtrR, penB (porB$_{IB}$)* and *ponA* led to alterations of the gonococcus cell wall causing the strain to become resistant to penicillin (Ropp and Nicholas, 1997). However, the primary alteration that led to this condition is the insertion of aspartic acid (D) at position 345 of the penicillin-binding protein 2 (PBP2) (Brannigan *et al.*, 1990). Other point mutations in PBP2 *(penA)* such as F504L, A510V, A5016G and P55IS have also been verified to cause increase in penicillin MIC (Powell *et al.*, 2009).

In 1976, as a result of the dissemination of penicillinase-producing *N. gonorrhoeae* (PPNG), along with the increasing prevalence of CMRNG, penicillin gradually lost its effectiveness in countries such as the United Stated (CDC, 2013a) and the United Kingdom (Bignell and Unemo, 2013). The spread of CMRNG across vast areas of the United States, including a significant outbreak of penicillin-resistant *N. gonorrhoeae* in North Carolina in 1986, caused penicillin to be finally deleted from the recommended treatment regime of gonorrhoea in the United States and some other countries (Unemo and Shafer, 2014).

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In New Zealand, PPNG was first reported in October 1976 from a patient whose treatment with penicillin failed (Green, 1979). Amoxicillin was still widely used until 2002, along with ciprofloxacin as the recommended treatment for gonorrhoea in this country. Nevertheless, the prevalence of penicillin-resistant *N. gonorrhoeae* in New Zealand had increased from 2.5% to 9% between 1988 and 2002 as a result of a gradual rise in the incidence of CMRNG (Heffernan *et al.*, 2004). Despite this increase in prevalence, it was still relatively low compared to countries from the Asian region, Africa and non-Pacific Island nations, including Australia (Brett *et al.*, 1992; Heffernan *et al.*, 2004). The low prevalence of the penicillin-resistant *N. gonorrhoeae* in New Zealand might have been due to the relatively isolated geographical location of the country (Heffernan *et al.*, 2004).

During the period when penicillin was the drug of choice for treatment of gonorrhoea, tetracycline was also administered, especially for patients who had an allergy to penicillin (Unemo and Shafer, 2014). However, not long after the antibiotic was introduced, Reyn, Korner and Bentzon (1958) reported an increase in the MIC of tetracycline due to the emergence of CMRNG. In 1986, Morse *et al*. reported the emergence of strains resistant to tetracyclines that contained the plasmid-encoded *tetM* determinant, causing high-level tetracycline resistance (Morse *et al.*, 1986). As a result, tetracycline was abandoned as a treatment regimen in the United States and many other countries (Morse *et al.*, 1986; Unemo and Shafer, 2014). The plasmid-encoded *tetM* determinants is associated

with tetracycline-resistant *N. gonorrhoeae* (TRNG), with MICs ≥ 16 mg/L (Reyn *et al.*, 1958; Heffernan *et al.*, 2004; Unemo and Shafer, 2014).

In New Zealand, *N. gonorrhoeae* with low-level resistance to tetracycline (MIC ≥ 2mg/L) was first reported in 1978 (Green, 1978), and the rate had increased by 1988 (Brett *et al.*, 1992). However in 2002, Heffernan and colleagues reported that the rate of gonococcus with low-level of resistant has decreased, but high-level resistance (TRNG) with MIC ≥ 16mg/L of had emerged and reached a rate of 6.5% (Heffernan *et al.,* 2004). By 2008, the tetracycline resistant rate reported in New Zealand was 31.4% (ESR, 2008a). Regardless, 1g of doxycycline was suggested as a second antibiotic besides azithromycin to be administered alongside 250 mg of ceftriaxone for the current treatment option of uncomplicated gonorrhoea by CDC (CDC, 2012c). In New Zealand, for complicated gonorrhoea infection such as PID, 500 mg IM ceftriaxone was given with 100 mg doxycycline (twice daily for 14 days), plus 400 mg metronidazole (twice daily for 14 days) (NZSHS, 2015).

In 1965, spectinomycin was also used to treat gonorrhoea, but by 1967 the drug started to show a sign of losing its effectiveness when the first spectinomycin-resistant *N. gonorrhoeae* was isolated in The Netherlands (Downey, 2003). Almost two decades after spectinomycin was introduced, Ashford and colleagues (as cited in Unemo & Shafer, 2014) reported an isolate of spectinomycin-resistant gonococcus in the Philippines, which was also found to be a penicillinase-producer (Unemo and Shafer, 2014). Spectinomycin was abandoned as an empirical monotherapy treatment for the infection internationally in the 1980s. The decision was due to the high rate of treatment failure with spectinomycin (8.2%) and the many spectinomycin-resistant gonococcal strains circulating in the United Kingdom (Boslego *et al.*, 1987; ESR, 2012). In New Zealand, no spectinomycin-resistant *N. gonorrhoeae* isolates were reported until 2002 (Heffernan *et al.*, 2004).

In 1986, CDC introduced the Gonococcal Isolate Surveillance System (GISP) to suggest treatment options based on scientific findings from regional laboratories and sentinel sites before therapy failures become a widespread problem (Unemo and Shafer, 2014). Fluoroquinolones such as ciprofloxacin, ofloxacin and levofloxacin were originally suggested for gonorrhoea treatment in the 1980s since these antibiotics are inexpensive, and can be administered orally in a single dose therapy (CDC, 2002; Goire *et al.*, 2014; Unemo and Shafer, 2014). GISP started to play its part in 2004 when ciprofloxacin and ofloxacin were removed from the empirical gonorrhoea treatment regimen for MSM due to the prevalence of >5% resistance among samples isolated in the United States (Lindbäck *et al.*, 2006).

In New Zealand, ciprofloxacin was widely used after the emergence of penicillin-resistant *N. gonorrhoeae* in the 1980s (Heffernan *et al.*, 2004). However, there was a significant 4-fold increase in ciprofloxacin resistance in Auckland, with a rate of 10.1% in 2001 (Heffernan *et al.*, 2004). By 2007, the prevalence of ciprofloxacin-resistant *N. gonorrhoeae* was higher than penicillin-resistant *N. gonorrhoeae* (ESR, 2008b). The primary determinant of ciprofloxacin resistance in *N. gonorrhoeae* is the alteration of the quinolone target sites in *gyrA* and *parC* (Belland *et al.*, 1994). Although ceftriaxone is the main treatment option for gonorrhoea treatment in the country, another drug (ciprofloxacin) is still recommended alongside 1 g azithromycin if the *N. gonorrhoeae* isolate is ciprofloxacin susceptible (NZSHS, 2015).

Regardless, until the current time, many countries including New Zealand have followed the CDC guideline, where azithromycin or doxycycline are prescribed together with ceftriaxone as a dual antimicrobial therapy regime to treat gonorrhoea infection (CDC, 2012c; NZSHS, 2015). However, azithromycin has an advantage over doxycycline because of the convenience of single-dose therapy. In addition, the prevalence of azithromycin resistance among *N. gonorrhoeae* is much lower than tetracycline resistance, particularly in *N. gonorrhoeae* isolates with reduced susceptibility to cephalosporins (CDC,

2012c). Additionally, azithromycin is effective against *Chlamydia trachomatis* which can commonly cause infection along with *N. gonorrhoeae* (CDC, 2012c).

1.5. Extended-spectrum cephalosporins (ESCs), the current treatment for gonorrhoea, and the emergence of *N. gonorrhoeae* with reduced susceptibility to ESCs

After the fluoroquinolones lost their effectiveness, the third generation extended-spectrum cephalosporins (ESCs) were recommended as an empirical treatment for gonorrhoea, in oral (cefixime) and injectable (ceftriaxone) form (Unemo and Shafer, 2014). Not long after ESCs were introduced, the prevalence of *N. gonorrhoeae* strains with high MIC to ESCs increased in Japan from 1995 to 2002 (Ito *et al.*, 2004). The reason behind the emergence of *N. gonorrhoeae* strains with high MIC to ESCs in Japan might have been due to the administration of low-dose regimens of cefixime (300 mg) instead of the recommended 400 mg dose per patient. The continuous regimen of low-dose cefixime may have selected for ESC resistance (Ito *et al.*, 2004).

By 2006, due to four treatment failures with cefixime being reported in Japan (Yokoi *et al.*, 2007), and the increased prevalence of cefixime resistance around the country, Japan removed cefixime from its recommended gonorrhoea treatment (Unemo and Shafer, 2014). A similar trend has also been seen in the United States between 2006 and 2011 (Bolan *et al.*, 2012). More treatment failures with cefixime were reported in countries such as Norway, France, the United Kingdom, Austria, Canada and South Africa (Unemo *et al.*, 2010; Allen *et al.*, 2011; Ison *et al.*, 2011; Unemo *et al.*, 2011b; Unemo *et al.*, 2012; Lewis *et al.*, 2013). These incidents signalled the broad spread of cefixime-resistant *N. gonorrhoeae* strains globally. By 2011, as a result of a prevalence of >5% resistance among samples isolated in the United States, CDC no longer recommended cefixime in the treatment regimen of gonorrhoea in that country (CDC, 2012c).

Currently, ceftriaxone is the only treatment option left used for gonorrhoea treatment, at least until a new drug is introduced (CDC, 2012c). Now, the treatment recommendation by CDC consists of 250 mg of a single IM dose of ceftriaxone administered along with 1g of azithromycin, or 100 mg of doxycycline to treat uncomplicated urogenital, anorectal and pharyngeal gonorrhoea. Some countries in Europe, and countries such as Australia and New Zealand, recommend the administration of a 500 mg dose of ceftriaxone to overcome the emerging resistance of *N. gonorrhoeae* to ESCs (Bignell and FitzGerald, 2011; Bignell and Unemo, 2012; BPAC, 2012; NZSHS, 2015). The administration of two antimicrobials with different mechanisms of action should delay the emergence of *N. gonorrhoeae* resistant to cephalosporins (CDC, 2012c).

However, treatment failures with ceftriaxone have already been reported from countries such as Slovenia, Japan, Australia and Sweden (Ohnishi *et al.*, 2011; Unemo *et al.*, 2011b; Read *et al.*, 2013; Golparian *et al.*, 2014). The treatment failures require physicians to increase the dose of ceftriaxone from the routine 250 mg to 500 mg. Most of the *N. gonorrhoeae* isolates exhibit an MIC from 0.03 to 0.125 mg/L, which is susceptible based on European Committee on Antimicrobial Testing (EUCAST) (R>0.125 mg/L) or the Clinical Laboratory Standard Institute (CLSI) guideline (R>0.25 mg/L). However, these strains exhibit higher MICs of ceftriaxone compared to the wild-type *N. gonorrhoeae*. Alarmingly, in 2011 and 2012, ceftriaxone-resistant *N. gonorrhoeae* that are also resistant to nearly all suitable drugs including ceftriaxone were isolated in Japan, France and Spain (Ohnishi *et al.*, 2011; Camara *et al.*, 2012; Unemo *et al.*, 2012). The emergence of gonococcal strains showing decreased susceptibility to ceftriaxone leaves few therapeutic options to treat this severe disease.

In New Zealand, the first *N. gonorrhoeae* strains with reduced susceptibility to ceftriaxone were isolated from Auckland in 2010, later in Waikato in 2013, and the most recent report is from the Canterbury District (ESR, 2011; ESR, 2013; ESR, 2014). All of these strains showed an MIC of ceftriaxone of 0.06 mg/L. Although no treatment failures with ceftriaxone have been reported in this

country, this emergence of *N. gonorrhoeae* strains that show reduced susceptibility to ceftriaxone is a serious concern.

It is important to highlight that, based on several studies on *N. gonorrhoeae* with elevated ceftriaxone MIC, the definite MIC cut-off point to define reduced susceptibility level of ceftriaxone among *N. gonorrhoeae* is varied and vague. Different studies have its interpretation of the MIC level for reduced susceptibility of ceftriaxone. For an example, Tapsall *et al.* (2009a) stated that the MIC of 0.03 mg/L and 0.016 mg/L for ceftriaxone, although considered as 'susceptible', is still considered high compared to the wild-type gonococcus (MIC ≤ 0.0005 mg/L) reported by Tapsall & Philips (1995). In a different study, Chen *et al*. (2013) defined MICs of ceftriaxone of 0.03 mg/L to 0.06 mg/L as reduced susceptibility to ceftriaxone among *N. gonorrhoeae*, while Whiley *et al.* (2009) defined MIC of 0.125 mg/L as reduced susceptibility level of ceftriaxone. In New Zealand, ESR has reported that all *N. gonorrhoeae* isolates that show reduced susceptibility to ceftriaxone (between 2010 to 2014) exhibit an MIC of 0.06 mg/L. Based on the majority of these studies, it is safe to say that an isolate with an MIC of ceftriaxone between 0.03 mg/L to 0.12 mg/L exhibits reduced susceptibility to ceftriaxone. Importantly, based on the EUCAST guideline that has recently started to be used in many laboratories in New Zealand, the cut-off point for ceftriaxone-resistance is >0.125 mg/L. Hence, any MIC values that are shifted towards the resistant cut-off point can be considered as showing reduced susceptibility to ceftriaxone.

With the global emergence of *N. gonorrhoeae* with reduced susceptibility to ESCs, many researchers are focused on elucidating the responsible genes and their mechanisms of action, with the hope that the new findings would provide in-depth information about the character of the strains and alternatives for gonorrhoea treatment in the future. Additionally, research regarding this resistance mechanism is necessary to help control the burden of antimicrobial-resistant *N. gonorrhoeae* and enhance the surveillance programme to control this infection. The mutations that have been reported to contribute to reduced

susceptibility to ceftriaxone are in *penA*, *mtrR*, *porB$_{IB}$* and *ponA*(Unemo and Shafer, 2014).

1.6. Chromosomal gene mutations in *penA, mtrR, porB$_{IB}$* and *ponA* cause reduced susceptibility to ESCs

The mutated *penA*, *mtrR*, *porB$_{IB}$* and *ponA*, need to be acquired in a stepwise manner to cause elevated MIC to β-lactam drugs such as penicillin and cephalosporins (Sparling *et al.*, 1975; CDC, 2005; Zhao *et al.*, 2009). A transformation experiment carried out by Sparling and colleagues (1975) showed that penicillin-resistant *N. gonorrhoeae* can transfer its mutated genes to susceptible *N. gonorrhoeae* in a stepwise manner, starting with mutated *penA, mtrR* and followed by *porB$_{IB}$*. Ropp *et al*. (2002) later found a fourth mutated allele, (*ponA*), increases the MIC of penicillin G to a higher level if it is present together with another mutated allele *penC* (now called *pilQ*). However, the impact of *pilQ* mutation on the increased MIC of penicillin was not fully characterised and verified in the study.

The primary gene that contributes to the increase in MIC to β-lactam drugs is *penA* (mosaic or nonmosaic type) which encodes the penicillin-binding protein 2 (PBP2) which crosslinks the peptidoglycan cell wall of the gonococcus. The mosaic-like structure of *penA* (mosaic *penA*) occurs due to multiple alterations in the gene which includes the isoleucine substitution with methionine at position 312 (I312M), valine with threonine at position 316 (V316T) and glycine with serine at 545 (G545S).The sequences resemble the *penA* of *Neisseria perflava (Neisseria sicca), Neisseria cinerea, Neisseria flavescens* and *Neisseria meningitidis* (Ameyama *et al.*, 2002). On the other hand, the nonmosaic *penA* sequence do not resemble any *penA* sequence of *Neisseria* commensals, but possess significant mutations that can increase MIC of to β-lactam drugs .

Ameyama and colleagues (2002) found that the insertion of *penA* from *N. gonorrhoeae* NG-3 strain (*N. gonorrhoeae* with reduced susceptibility to cefixime) into *N. gonorrhoeae* ATCC19424 (wild-type strain) causes a substantial increase in ceftriaxone MIC. In another comprehensive study carried out by Zhao *et al.* (2009), insertion of *penA35* (mosaic type) into the wild-type *N. gonorrhoeae* FA19 strain increased the ceftriaxone MIC by up to 20-fold. The *penA35* belongs to *N. gonorrhoeae* 35/02, which conveyed reduced susceptibility to ceftriaxone (MIC:0.095 mg/L) (Warner *et al.*, 2008). On the other hand, in a different study carried out by Whiley *et al.* (2007), 41% of *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone in Australia were observed to harbour nonmosaic *penA* (Whiley *et al.*, 2007b). These findings indicate that mutated *penA* is the primary resistance gene determinant due to its significant impact on ceftriaxone MIC in *N. gonorrhoeae*.

However, despite the significant impact of the mutated *penA,* the ceftriaxone MIC of the transformants in both Ameyama *et al.* (2002) and Zhao *et al.* (2009) studies was still lower than ceftriaxone MIC of the donor strains, which indicates the requirement of additional resistance gene determinant(s) to further increase the ceftriaxone MIC.

Table 1-3 Stepwise transformation of *N. gonorrhoeae* FA19 strains with mosaic *penA, mtrR, porB$_{IB}$* and *ponA* of *N. gonorrhoeae* 35/02 (adapted from Zhao *et al.* (2009))

Strains/ transformants	Ceftriaxone MIC (mg/L)	Fold increase of ceftriaxone MIC (mg/L)
***N. gonorrhoeae* FA19** [a]	0.0006 mg/L	N/A [c]
***N. gonorrhoeae* 35/02** [b]	0.094 mg/L	N/A
FA19 *penA35*	0.012 mg/L	20-fold
FA19 *penA35 mtrR*	0.015 mg/L	Little to no effect at all [d]
FA19 *penA35 mtrR penB35*	0.04 mg/L	2.5-fold increase
FA19 penA35 *mtrR penB35 ponA1*	0.03 mg/L	No effect [e]

[a] *N. gonorrhoeae* FA19 strain is a penicillin and cephalosporin susceptible strain (Zhao *et al.*, 2009).

[b] *N. gonorrhoeae* 35/02 strain showed reduced susceptibility to cephalosporin (Zhao *et al.*, 2009).

[c] NA: Not applicable

[d] Ceftriaxone MIC was not stated in the study. The estimate ceftriaxone MIC in this table is based on the Fig. 2 (page 3747) from Zhao et al. (2009) study.

[e] Relative to FA19 *penA35 mtrR penB35*. The fold increase is less than 1 fold (0.75 fold increase)

In the study carried out by Zhao *et al.* (2009), further insertion of mutated *mtrR* and *penB* (contain $porB_{IB}$ alterations) of *N. gonorrhoeae* strain 35/02 into FA19 *penA35* transformant, further increased the ceftriaxone MIC by 2.5-fold, indicating the importance of additional mutated genes to the increased ceftriaxone MIC (as shown in **Table 1-3**). Interestingly, *ponA* insertion only was associated with an increase of penicillin MIC, but not ceftriaxone MIC (Zhao *et al.*, 2009). However, the overall impact all four resistance determinants led to an estimated 50-fold increase of ceftriaxone MIC in the *N. gonorrhoeae* FA19 strain (Zhao *et al.*, 2009).

The association of mosaic *penA, mtrR,* $porB_{IB}$ *and ponA* mutations in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone has been widely observed (Unemo *et al.*, 2002; Takahata *et al.*, 2006; Tanaka *et al.*, 2006; Warner *et al.*, 2008; Allen *et al.*, 2013). Lindberg *et al*. (2006) reported the presence of mosaic *penA, mtrR*, $porB_{IB}$ and *ponA* mutations in 61% of *N. gonorrhoeae* isolates that conveyed reduced susceptibility to ceftriaxone, with MIC of 0.064 to 0.125 mg/L. Similarly, Allen *et al.* (2013), Lee *et al*. (2010) and Tanaka *et al*. (2006) observed the presence of the four mutated genes in *N. gonorrhoeae* with a ceftriaxone MIC range between 0.062 mg/L to 0.126 mg/L. The findings are listed in **Table 1-4**. Similarly, the impact of nonmosaic PBP2 alongside *mtrR* and $porB_{IB}$ has also been verified (Whiley *et al.*, 2010a) and observed in several studies (as listed in **Table 1-4**).

In conclusion, the combination and synergism of mutated *penA, mtrR, porB$_{IB}$* and *ponA* mechanisms may lead to high levels of resistance to ESCs. It is important to understand the background of these mutations and how the resistance mechanisms work in causing reduced susceptibility to ESCs. The next section will focus on the genes above in detail.

Table 1-4 Susceptibility to ceftriaxone and mutated *penA, mtrR, porB$_{IB}$ and ponA* observed in *N. gonorrhoeae* in published journals

Countries	Authors	Mutated genes observed in *N. gonorrhoeae* [a]	Ceftriaxone MIC
Korea	Lee *et al.* (2010)	Mosaic *penA* + *porB$_{IB}$* + *mtrR* + *ponA*	0.25 mg/L
		Nonmosaic *penA* + *porB$_{IB}$* + *mtrR* + *ponA*	0.03 to 0.25 mg/L
Japan	Takahata *et al*. (2006)	Mosaic *penA* + *porB$_{IB}$* + *mtrR* + *ponA*	0.12 to 0.5 mg/L
		Nonmosaic *penA* + *porB$_{IB}$* + *ponA*	0.008 to 0.03 mg/L
	Tanaka *et al.* (2006)	Mosaic *penA* + *porB$_{IB}$* + *mtrR* + *ponA*	0.5 mg/L
Canada	Allen *et al*. (2011)	Mosaic *penA* + *porB$_{IB}$* + *mtrR* + *ponA*	0.063 to 0.125 mg/L
		Mosaic PBP2 + *mtrR* + *ponA*	0.125 mg/L
Europe	Lindberg *et al*. (2007) [b]	Mosaic *penA* + *porB$_{IB}$* + *mtrR* + *ponA*	0.094 to 0.125 mg/L

[a] The PorBIB *(porB$_{IB}$)* alterations involved amino acid substitution at codon 120 and 121; *mtrR* alteration involved adenine (A) deletion in *mtrR* promoter; PBP1 *(ponA)* alteration involved amino acid substitution at codon 421.

[b] One of the *N. gonorrhoeae* isolates found by Lindberg *et al*. (2007) was the 35/02 strain, where the mosaic *penA*, *mtrR*, *porB$_{IB}$* and *ponA* gene of the strain was used to transform a wild-type *N. gonorrhoeae* FA19 strain in Zhao *et al*. (2009) study. The ceftriaxone MIC of by *N. gonorrhoeae* 35/02 is 0.094 mg/L as it was for seven other isolates with a mosaic *penA* allele.

1.6.1. Penicillin-binding protein 2 (*penA*) gene

The *penA* encodes the penicillin-binding protein 2 (PBP2), which is essential for linking the peptidoglycan strands to build the inner layer of the cell wall of the gonococcus. Plus, PBP2 is a lethal target of β-lactam antibiotics. β-Lactam antibiotics act by covalently binding to the serine residue at the PBP2 (transpeptidase) active site, disrupting the ability of the enzyme to link the peptidoglycan molecules and causing the dysregulation of cell wall formation. The accumulation of peptidoglycan precursors activates bacterial autolysins, causing the gonococcus to autolyse (Dougherty, 1985). Dougherty (1985) also state that the mutated *penA* encodes a new form of PBP2 with low-affinity binding of the β-lactam drugs. As a result, the β-lactam drugs have lost their effectiveness against *N. gonorrhoeae* causing a rapid increase in penicillin-resistant *N. gonorrhoeae*.

In studies of gonococcal isolates conveying high MICs to ESCs two types of *penA* alterations were observed: the mosaic *penA* and the nonmosaic *penA* (Ameyama *et al.*, 2002; Takahata *et al.*, 2006; Whiley *et al.*, 2007b; Tomberg *et al.*, 2010; Ohnishi *et al.*, 2011). The emergence of various amino acid changes in the PBP2 region contributes to the divergence of the PBP2 sequence into different patterns. Currently, 42 PBP2 patterns consisting of various mosaic and various nonmosaic sequences have been observed in *N. gonorrhoeae*. The PBP2 nomenclature consists of patterns I to X (Ito *et al.*, 2005), patterns XI to XXIII (Whiley *et al.*, 2007b), pattern XXIV (Unemo *et al.*, 2002), patterns XXV to XXX (Ohnishi *et al.*, 2011), patterns XXX to XXXII (Ohnishi *et al.*, 2010), patterns XXXIII to XXXVI (Ohnishi *et al.*, 2011), patterns XXXIV to XXXVIII (Pandori *et al.*, 2009; Allen *et al.*, 2013), pattern XXXVII (Allen *et al.*, 2013), mosaic-2 to mosaic-4 (Takahata *et al.*, 2006), and patterns IIIg and IIIh (Takahashi *et al.*, 2013).

1.6.2. The role of mosaic *penA* (PBP2) in causing reduced susceptibility to ESCs

Ameyama and colleagues (2002) have carried out a thorough study on the character of mosaic *penA*, and its association with decreased susceptibility to ESCs. They reported that when the *penA* of the cefixime-resistant *N. gonorrhoeae* strain (NG-3) was inserted into an ESC-susceptible strain of gonococcus, ATCC 19424, the strain showed an elevated MIC to cefixime of 62.5-fold. Even though the MICs of cefixime and ceftriaxone for the transformed ATCC 19424 were still lower than the donor NG-3 strain, the experiment indicates the significant role of *penA* in elevating the MIC of ESCs.

In the same study, when *penA* NG-3 strain was sequenced and compared to that of the *penA* sequence of the cefixime-susceptible strain NG-12, for which the sequence is similar to the wild-type strain (*N. gonorrhoeae* LM306, GenBank accession no. M320921), Ameyama and colleagues found 59 amino acid alterations in the PBP2 sequence. The finding indicates the PBP2 of the NG-3 strain has gone through significant changes that might affect the susceptibility of the strain to ESCs. The multiple changes of *penA* led to the formation of the mosaic-like structure of the gene. The study done by Ameyama *et al*. (2002) was also supported by Ito *et al*. (2005), where they found one PBP2 sequence similar to that described by Ameyama *et al*. (2002). The mosaic PBP2 pattern X exhibits a higher MIC to ceftriaxone compared to nine other PBP2 patterns that were found in their study.

Another important finding from Ameyama and colleagues' study (2002) is that the mosaic *penA* region of NG-3, which is estimated between 600 bp to 1550 bp (167 to 482 amino acid residues), resembles the sequences of other *Neisseria* commensals such as *N. perflava/sicca*, *N. cinerea, N. flavescens* and *N. meningitidis.* Hence, the assumption has been made that *Neisseria* commensals have contributed a part of their DNA via horizontal genetic exchange to the wild-

type *N. gonorrhoeae* (Spratt *et al.*, 1992). The *Neisseria* commensals that inhabit the pharynx are usually intrinsically resistant and may harbour resistance genes in their DNA (Tapsall *et al*., 2009b, p. 824). Ameyama and colleagues speculated that ESCs-susceptible *N. gonorrhoeae* might have obtained the DNA containing multiple alterations in *penA* from these commensals during the colonisation of the pharynx (Ameyama *et al.*, 2002).

The study carried out by Ameyama and colleagues (2002) is the earliest comprehensive study done to characterise mosaic *penA*. The sequence of the mosaic *penA* of the NG-3 strain (mosaic *penA* pattern X) (deposited in the DNA Data of Bank Japan (DDBJ) and the National Center for Biotechnology Information (NCBI) under accession number AB071984) has been used in many subsequent studies as a basis for mosaic *penA* (PBP2) comparison. Later studies have observed other patterns of mosaic PBP2 in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone, such as mosaic PBP2 pattern XXXV and XXXVIII (Allen *et al.*, 2011), XXXIV (Pandori *et al.*, 2009), and XXXII (Ohnishi *et al.*, 2010). Mosaic PBP2 pattern XXXIV is another significant mosaic PBP2 observed in the past few years. Two ceftriaxone-resistant *N. gonorrhoeae* strains from France (Unemo *et al.*, 2012) and Spain (Camara *et al.*, 2012) were found to harbour this PBP2 pattern. Additionally, mosaic PBP2 pattern XXXIV was also observed in *N. gonorrhoeae* isolates associate with ceftriaxone treatment failure reported in Sweden (Golparian *et al.*, 2014), Australia (Chen *et al.*, 2013) and Slovenia (Unemo *et al.*, 2011b). Examples of various mosaic PBP2 sequences are depicted in **Figure 1-1**.

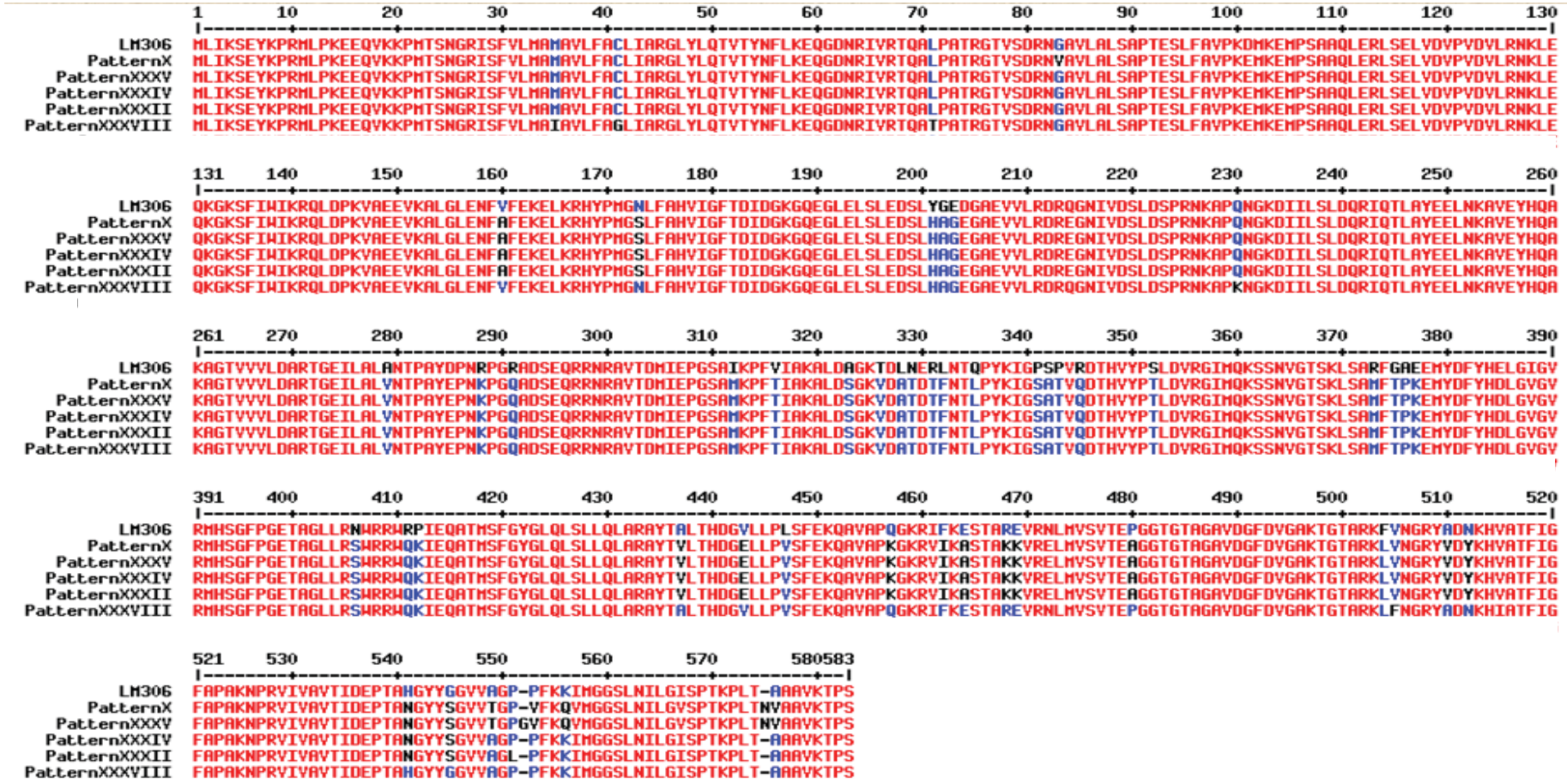

Figure 1-1 Alignment of published mosaic PBP2 patterns X (Ameyama *et al.*, 2002), XXXV and XXXVIII (Allen *et al.*, 2011), XXXIV (Pandori *et al.*, 2009) and XXXII (Ohnishi *et al.*, 2010) with the wild-type sequence of *N. gonorrhoeae* LM306.

1.6.3. Significant involvement of mosaic PBP2 (I312M, V316T and G545S alterations)

In the detailed study conducted by Takahata *et al*. (2006) on the character of mosaic PBP2, the transformation of full and partial recombinants of *penA* into the cefixime-susceptible FA1090 strain revealed a significant involvement of the I312M, and V316T (Takahata *et al.*, 2006). I312M and V316T were reported to increase resistance to cefixime, ceftibuten and cefpodoxime by at least 4-fold (Takahata *et al.*, 2006). I312M and V316T might have originated from *N. perflava* and *N. flavescens* since similar alterations have been found in these commensals strains, hence strengthening the belief that *Neisseria* commensals have contributed to the mosaic PBP2.

Takahata *et al*. also noted another significant alteration, glycine to serine at position 545 (G545S). This change might occur through antibiotic selective pressure in *N. gonorrhoeae* since no *Neisseria* commensal has been found to harbour this mutation. However, the G545S alteration is commonly observed alongside I312M and V316T alterations in mosaic PBP2 (Takahata *et al.*, 2006; Whiley *et al.*, 2007b; Pandori *et al.*, 2009; Tomberg *et al.*, 2010; Allen *et al.*, 2013). Together, the three mutations cause an increase in ceftriaxone MIC. Several studies (Ito *et al.*, 2005; Takahata *et al.*, 2006; Whiley *et al.*, 2007b; Tomberg *et al.*, 2010) refer to mosaic PBP2 as the combination of I312M, V316T and G545S alterations due to its importance in increasing the MIC of ESCs. The impact of G545S on the increased MIC of ceftriaxone is more significant than the combination of both I312M and V316T, and this is discussed by Takahata *et al*. (2006) and Tomberg *et al*. (2010). According to Takahata *et al*. (2006), the insertion of G545S together with either I312M or V316T into the *N. gonorrhoeae* FA1090 strain increased the MIC of ceftriaxone by 4-fold (Takahata *et al.*, 2006).

However, there has been an issue with the results from Takahata *et al*. (2006). In a different transformation study carried out by Tomberg *et al.* (2010), only a

1.5-fold increase of ceftriaxone MIC was observed when the *penA* containing all these three primary alterations (I312M, V316T and G545S) was inserted into the penicillin and cephalosporin-susceptible FA19 strain. Tomberg and colleagues concluded that the cefixime-susceptible FA1090 strains utilised in the study by Takahata and colleagues contain a penicillin-resistant mutation, the Asp-345a, which might have slightly contributed to the increase of the MIC of ESCs. Also, the reversion of G545S to wild-type decreased the MIC of ceftriaxone by 6-fold, whereas the reversion of the double mutation I312M and V316T to wild-type decreased the MIC of ceftriaxone by 4-fold (Tomberg *et al.*, 2010). As a conclusion, both studies by Takahata *et al.* (2006) and Tomberg *et al*. (2010) reveal the significant involvement of G545S alterations alongside the mosaic PBP2 I312M and V316T mutations.

G545S, I312M and V316T are located in the active sites of PBP2 (transpeptidase), where the enzyme and the ESCs drugs bind (Takahata *et al.*, 2006; Tomberg *et al.*, 2010). The mutation G545S is located downstream of the $K^{497}TG$ (KTG) motif while the mutations I312M and V316T are situated in the $S^{310}AIK$ (SXXK) motif (Takahata *et al.*, 2006). According to Tomberg *et al*., the location of these mutations near the active site of the enzyme causes a reduction in the acylation rate of the transpeptidase, hence causing an increase in the MIC level. On the other hand, Osaka *et al.* (2006) has confirmed the mosaic PBP2 as the main contributor of reduced susceptibility to ceftriaxone, and stated that based on the modelling study, the alterations cause conformational alterations of the β-lactam-binding pocket which lead to reduced susceptibility to ceftriaxone in *N. gonorrhoeae.*

1.6.1. Other PBP2 alterations and their role in reduced susceptibility to ESCs

Even though it is well known that mosaic PBP2 (I312M, V316T and G545S) is the primary cause of decreased susceptibility to ESCs, several studies have observed the presence of other alterations in PBP2 (nonmosaic PBP2) among *N. gonorrhoeae* with reduced susceptibility to ceftriaxone (Takahata *et al.*, 2006; Whiley *et al.*, 2007b). In a study carried out Whiley *et al*. (2007), more isolates exhibited a nonmosaic PBP2 pattern than the mosaic PBP2. Studies have verified the significance of A501V/T, A501P, N512Y (Tomberg *et al.*, 2010), G542S and P551S/L (Whiley *et al.*, 2010a) alterations, which were commonly observed in a nonmosaic PBP2 sequence. This change might arise through antibiotic selective pressure in *N. gonorrhoeae* (Tomberg *et al.*, 2010; Whiley *et al.*, 2010a)

The amino acid substitution of alanine (A) with valine (V) at position 501 of the amino acid sequence (A501V) is usually found in a nonmosaic PBP2 (Takahata *et al.*, 2006). Although very rare, A501V mutations, alongside mosaic PBP2 alterations have been reported in mosaic pattern-4 (pattern XXVI) (Takahata *et al.*, 2006; Ohnishi *et al.*, 2010) and pattern XXX (Ohnishi *et al.*, 2010). Unlike pattern XXVI, mosaic PBP2 XXX showed the presence of a rare combination of G545S and A501V. Based on the experiment carried out by Tomberg *et al*. (2010), the combination of A501V with the mosaic PBP2 (I312M, V316T and G545S) combination might lead to resistance to the ESCs. One case of cefixime-resistant *N. gonorrhoeae* found in Japan supports this finding. The strain harboured mosaic PBP2 pattern XXX, with an MIC of cefixime 1.0 mg/L (Ohnishi *et al.*, 2010). Regardless, the mosaic PBP2 pattern XXX has never been reported in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone. Unlike A501V, A501T has never been reported to appear together with mosaic PBP2.

The impact of A501V on the increase of ceftriaxone MIC has been verified by Tomberg *et al*. (2010). The insertion of A501V into the FA19 wild-type strain leads

to a 2-fold increase in the MIC of ceftriaxone while together with mosaic PBP2 *penA35*, a 4-fold increase of ceftriaxone MIC was observed. According to Tomberg *et al.* (2010), the A501T mutation also causes a similar effect to the A501V. On the other hand, Osaka *et al.* (2008) confirmed that, similar to mosaic PBP2, the A501V mutation is one of the major contributors to reduced susceptibility to ceftriaxone in *N. gonorrhoeae*. The mutation also causes conformation change of the β-lactam-binding pocket since it is situated near the β-lactam binding site, hence lowering the binding affinity between PBP2 and β-lactam drugs (Osaka *et al.*, 2008).

Unlike the A501V mutation, the substitution of alanine (A) with proline (P) (A501P) is mostly found alongside the mosaic PBP2 sequence, and the reason behind it remains unclear (Unemo *et al.*, 2012). Two *N. gonorrhoeae* strains that are highly resistant to ceftriaxone reported from France and Spain showed the presence of the A501P mutation together with mosaic *penA* allele XXXIV (Camara *et al.*, 2012; Unemo *et al.*, 2012). These findings reinforce the theory of Tomberg *et al*. (2010) that the alteration at position 501 alongside mosaic PBP2 can cause *N. gonorrhoeae* to become more resistant to ESCs. According to Unemo *et al*. (2012), the replacement of the methyl side chain of alanine 501, which is located close to the KTG active-site motif of PBP2, with the more bulky side chains of either valine (V) or threonine (T) inhibits the binding between ESCs and PBP2. Additionally, insertion of a proline (P) introduces secondary structure alterations that cause more dramatic changes (Unemo *et al.*, 2012).

Other mutations in nonmosaic PBP2 that have been found in *N. gonorrhoeae* with increased MIC of ceftriaxone are the substitution of glycine (G) with serine (S) at position 542 (G542S), and the substitution of proline (P) with serine (S) or leucine (L) at position 551 of PBP2 (P551S/L). According to Whiley *et al*. (2010), G542S and P551S/ P551L involvement in the increase in MIC of ceftriaxone in their study was statistically significant (P=0.0186 and 0.001, respectively). However, a transformation experiment needs to be carried out to confirm the effect of both mutations. Osaka *et al*. (2008) have clarified the significant impact of P551S on

the acylation rate of penicillin G, but not for ESCs. Another *penA* mutation that was reported to contribute to the decreased susceptibility to ESCs is the substitution of asparagine (N) with tyrosine (Y) at position 512 of PBP2 (N512Y). This reversion of the mutation to wild-type leads to a decrease of MICs of cefixime and ceftriaxone by 2-fold (Tomberg *et al.*, 2010).

In conclusion, there is an intricate involvement of various mutations in *penA* that contribute to the increased MICs of ESCs. With the ever-changing state of *N. gonorrhoeae* DNA, plus the exposure to ESCs drugs, it is not surprising that new mutations arise and enhance resistance. A summary of the verified mutations within the *penA* gene that contributes to elevate the MIC of ceftriaxone can be seen in **Table 1-5** and **Figure 1-2**.

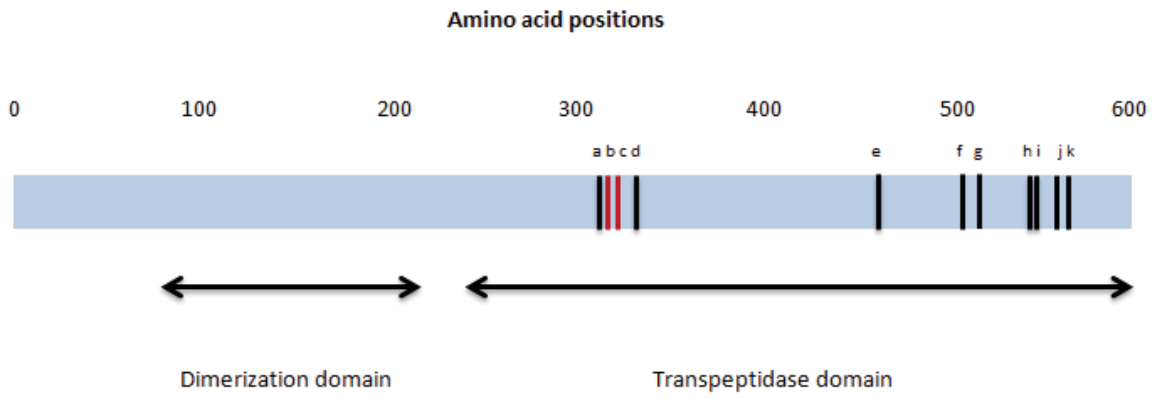

Figure 1-2 PBP2 structure consists of a dimerisation domain (residues 71 to 221) and a transpeptidase domain (residues 263 to 557) [adapted from Ameyama *et al*. (2002), Ito *et al*. (2004), Osaka *et al*. (2008)]. Shaded boxes represent verified amino acid changes published for *N. gonorrhoeae* with reduced susceptibility to ESCs (as stated in **Table 1-5**). Shaded red boxes represent the amino acid changes that occur in mosaic PBP2 while the black shaded boxes represent other amino acid changes that might occur alongside either mosaic or nonmosaic PBP2

Table 1-5 Verified mutations within PBP2 that contribute the decreased susceptibility to ceftriaxone as seen in Figure 1-2

Mutations in PBP2 [a]	Impact on MIC of ceftriaxone	Note	Transformants	References
a) A311V	~2.5-fold increase	When present together with T316P, it causes an additional 2-fold increase	FA19 *penA35* [b]	Tomberg *et al*. (2013)
	~2-fold increase	Estimated from the 4-fold increase for G545S and I312M and 2-fold increase for G545S	FA1090 [c]	Takahata *et al*. (2006)
b) I312M	<1.5-fold increase	Combination of all three G545S, I312M & V316T, increased the MIC of ceftriaxone by 1.5-fold	FA19 [d]	Tomberg *et al*. (2010)
		Reversion of I312M & V316T to wild-type decreased the MIC of ceftriaxone by 4-fold	FA19 *penA35*	
	~2-fold increase	Estimated from the 4-fold increase of G545S and I312M and 2-fold increase of G545S	FA1090	Takahata *et al*. (2006)
c) V316T	<1.5-fold increase	Combination of all three G545S, I312M and V316T increase of MIC by 1.5-fold	FA19	Tomberg *et al*. (2010)
		Reversion of I312M & V316T to wild-type decreased the MIC of ceftriaxone by 4-fold	Fa19 *penA35*	

Mutations in PBP2	Impact on MIC of ceftriaxone	Note	Transformants	References
d) T316P	~2.5-fold increase	When present together with A311V, either mutation causes 2-fold increase	FA19 *penA35*	Tomberg *et al*. (2013)
e) T484S/T483S [e]	~2-fold increase		FA19 *penA35*	Tomberg *et al*. (2013)
f) A501P		No transformation has been reported. However, it might cause a greater increase compared to the A501V mutation since the proline (P) insertion caused a more dramatic impact on the binding of PBP2 by β-lactam drugs		Unemo *et al*. (2012)
g) A501V	~2-fold increase in FA19 strain	The impact of this mutation is similar to that caused by the G545S mutation	FA19	Tomberg *et al*. (2010)
	~4-fold increase in FA19 *penA35* strain		FA19 *penA35*	

Mutations in PBP2	Impact on MIC of ceftriaxone	Note	Transformants	References
h) N512Y	Reversion of N512Y to wild-type decreased the MIC of ceftriaxone by 2-fold		FA19 *penA35* N512Y [f]	Tomberg *et al*. (2010)
i) G542S	No transformation experiment was carried out	However, the alteration has been widely reported to be found in *N. gonorrhoeae,* which showed reduced susceptibility of ceftriaxone (with MIC between 0.03 mg/L to 0.125 mg/L)		Whiley *et al*. (2010)
j) G545S	~2-fold increase		FA1090	Takahata *et al*. (2006)
	<1.5-fold increase	Combination of all three G545S, I312M and V316T, increased the MIC by 1.5-fold	FA19	Tomberg *et al*. (2010)
	Reversion of G545S to wild-type decreased the MIC of ceftriaxone by 6-fold	Reversion of G545S, I312M and V316T to wild-type decreased the MIC of ceftriaxone by 16-fold	FA19 *penA35*	Tomberg *et al*. (2010)
k) P551S/ P551L	~2-fold increase	Increase when the mutation is present together with *penA* XXXIV	NG9807 [g]	Shimuta *et al*. (2013)

[a] The letter labelled for each mutations can be seen in **Figure 1-2**.

[b] FA19 *penA35*: A penicillin and ESCs susceptible strain FA19 (wild-type strain), which has been transformed with mosaic *penA* from the 35/02 strain. The MIC of ceftriaxone showed by this transformant is between 0.012 mg/L to 0.05 mg/L (Zhao *et al.*, 2009; Tomberg *et al.*, 2010).

[c] FA1090: Penicillin-resistant strains that harbour Asp345a mutation in PBP2.

[d] FA19*:* A penicillin and ESCs susceptible strain FA19 (wild-type strain).

[e] In the original report by Ohnishi *et al*. (2011), the alteration was stated as T484S. However, based on the Tomberg *et al*. (2010) study, the alteration is listed as T483S because the PBP2 alteration was determined based on the comparison with the 35/02 strain.

[f] FA19 *penA35* N512Y: A penicillin and ESCs susceptible strain FA19 (wild-type strain), which has been transformed with mosaic *penA* from the 35/02 and N512Y mutations.

[g] NG9807: ESCs susceptible *N. gonorrhoeae* strain (MIC of ceftriaxone: 0.016 mg/L) (Shimuta *et al.*, 2013).

1.7. Introduction to the multiple transferable resistance (*mtr*) locus

Research into the function of the multiple transferable resistance (*mtr*) system has a long history. In the early years, a large body of literature focused on understanding the character of the *mtr* system and its involvement with gonococcal susceptibilities to hydrophobic agents, such as detergent-like fatty acids, dyes, bile salts and erythromycin. Basically alterations of the *mtrR* caused a change in the cell envelope structure (Guymon *et al.*, 1978). The increased amounts of 52,000-molecular-weight outer membrane protein and cross-linking of peptidoglycan reduced the permeability of the cell envelope, causing the gonococci to become resistant to hydrophobic agents (Guymon *et al.*, 1978)

In 1994, Pan and Spratt described several aspects of the *mtr* system. According to them, the role of the *mtrR* was to encode MtrR repressor protein. The protein suppresses the activity of the nearest gene *mtrC* that encodes the lipoprotein that is essential for the development of the gonococcal cell envelope (Hagman *et al.*, 1995). When the activity of the *mtrC* is suppresssed, lipoprotein production decreases, increasing the permeability of the gonococcal cell envelope. As a result, the gonococci become hyper-susceptible to hydrophobic agents such as drugs (tetracycline and erythromycin), dye (crystal violet) and detergents (Triton-X) (Morse *et al.*, 1982). Pan and Spratt added that, by inactivating the *mtrR*, repressor protein fails to be produced, leading to *mtrC* expression of the lipoprotein and resulting in decreased permeability of the cell envelope (Pan and Spratt, 1994). Therefore, *N. gonorrhoeae* becomes resistant to hydrophobic agents. The study carried out by Pan and Spratt clearly showed the important role the *mtrR* played in regulating the expression of the *mtrC* and the permeability of the gonococcal cell envelope. Additionally, both Morse *et al*. (1982) and Pan and Spratt (1994) agree that the *mtr* system evolved to protect *N. gonorrhoeae* in certain environments rather than encoding resistance to antibiotics.

The structure and functions of the *mtr* system were further characterised by Hagman *et al*. (1995). The *mtr* system consists of a 13-bp inverted sequence in

the promoter gene, which is located between the *mtrR* coding sequence and *mtrC*. Hagman and colleagues also identified the *mtrD* and the *mtrE* that are located after the *mtrC*. Together with the *mtrC*, a single transcriptional unit (operon) *mtrCDE* is formed **(Figure 1-3).** Altogether, the *mtrCDE* region of the gonococcus encodes an envelope lipoprotein, which forms channels in the cell envelope, and an energy-dependent efflux pump system. The efflux pump system transfers substrates through the channel using proton motive forces (Veal *et al.*, 1998). Since each gene in the *mtrCDE* region is dependent on the others for the activity of the efflux pump, inactivation of any of the genes will affect the overall efflux pump activity, causing the gonococci to become hyper-susceptible to hydrophobic agents (Hagman *et al.*, 1995; Veal *et al.*, 1998).

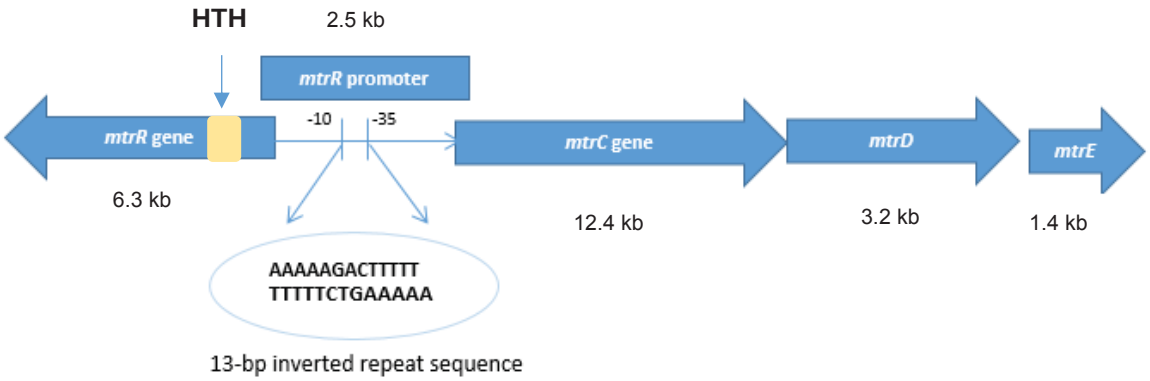

Figure 1-3 Organisation of the *mtrCDE* region in gonococci, adapted from Kayla *et al*. (1995, 1997), Veal *et al*. (1998) and Warner *et al*. (2008). The diagram is not drawn to scale. The arrows represent the direction of transcription of the *mtrR* coding sequence, *mtrR* promoter and *mtrCDE* region. Helix-turn-helix (HTH) is situated in the MtrR coding region between residues 32 to 53.

1.7.1. Mutations in the *mtrR* cause *N. gonorrhoeae* to become resistant to hydrophobic agents, penicillin and azithromycin

Mutation in *mtrR* increases the level of MtrC and causes the strain to become resistant to hydrophobic agents (Hagman *et al.*, 1995). A comparison of the *mtrR* sequences of wild-type FA19 strain with *mtrR* sequences of hydrophobic agents-resistant strains, FA171 and BR87, confirmed two essential mutations (Hagman *et al.*, 1995). First, a single point mutation in the MtrR coding region at codon 45 caused glycine (G) to be changed to aspartic acid (D) (G45D). The second mutation is the single-base-pair thymine (T) or adenine (A) deletion in the 13-bp inverted sequence of the *mtrR* promoter region.

The transformed wild-type FA19 strain (KH 15 strain), harbouring the mutated T/A deletion in the 13-bp promoter, showed higher MIC value for hydrophobic agents compared to the FA19 strain transformed by the G45D MtrR mutation (KH16 strain). This finding led to the conclusion that the impact of *mtrR* 13-bp promoter on resistance to hydrophobic agents is more significant than the G45D mutation in the MtrR coding region. This result also signalled that the *mtrR* promoter is a part of a transcriptional regulatory region that regulates the efflux pump activity of the *mtrCDE* region. Hagman *et al*. (1995) added that the single deletion might shorten the length of the promoter region, thereby reducing expression of *mtrR* gene, and intensify the action of the efflux pump. On the other hand, the G45D mutation in the MtrR coding region only causes an intermediate level of resistance in the absence of the promoter mutation. The location of the G45D mutation within the second helical domain of helix-turn-helix (HTH) DNA-binding motif (between residues 32 to 53) might increase the helical characteristics of the region. As a result, the binding of MtrR repressor protein to the target DNA upstream of *mtrCDE* will be reduced (Hagman *et al.*, 1995).

The previous body of literature mainly discussed the effect of *mtrR* alterations in causing resistance to hydrophobic agents among *N. gonorrhoeae*. However, similar alterations in *mtrR* also cause the level of susceptibility in gonococci to hydrophilic antibiotics such as penicillin, azithromycin and ESCs to increase (Faruki and Sparling, 1986; Zarantonelli *et al.*, 1999; Warner *et al.*, 2008; Zhao *et al.*, 2009; Shigemura *et al.*, 2015).

1.7.2. Mutations in the *mtrR* region enhanced the reduced susceptibility to ceftriaxone in *N. gonorrhoeae*

Several studies of *N. gonorrhoeae* with elevated MICs to ESCs have reported the presence of an *mtrR* mutation (Unemo *et al.*, 2002; Takahata *et al.*, 2006; Tanaka *et al.*, 2006; Warner *et al.*, 2008; Allen *et al.*, 2011). Lindberg *et al*. (2006) found that 39% of *N. gonorrhoeae* with elevated MICs to ESCs have a single A deletion in the *mtrR* promoter; 33% of isolates harboured both an A deletion in the *mtrR* promoter and a G45D mutation in the MtrR coding region, while 28% display only a G45D mutation. Novel MtrR mutations, A39T and L47P, were also found in a study done by Lee *et al*. (2010). Similar to G45D, the location of the two alterations in the helix-turn-helix (HTH) DNA-binding motif can enhance gonococcal resistance to hydrophobic antimicrobials (Hagman *et al.*, 1995). However, the significance of these variations was not elucidated.

On the other hand, the substitution of histidine (H) with tyrosine (Y) at position 105 (H105Y) of MtrR amino acid was first observed by Pan and Spratt (1994) during a comparison study of FA19 wildtype strain and two penicillin-resistant clinical isolates. H105Y is situated outside the HTH DNA-binding site region and it could affect the tertiary structure or sub-unit interactions that are important in DNA-binding (Shafer *et al.*, 1995). Another mutation which is also situated outside the HTH binding site is the T86A which was observed by Liao *et al.* (2011). Both H105Y and T86A mutation have also been observed in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone (Liao *et al.*, 2011). The list of amino acid changes in *mtrR* region that have been reported can be seen in **Table 1-6**.

Table 1-6 Mutations within the *mtrR* region that contribute to the decreased susceptibility of *N. gonorrhoeae* to hydrophobic agents, penicillin and ceftriaxone

Mutations in *mtrR* (MtrR) region	**Location**	**Reference**
H105Y	MtrR coding region	Pan & Spratt (1994)
G45D	MtrR coding region	Hagman *et al*. (1995)
T/ A deletion	*mtrR* promoter	Hagman *et al*. (1995)
Dinucleotide insertion (TT)	*mtrR* promoter	Zarantonella *et al*. (1999)
A39T	MtrR coding region	Lee *et al*. (2010)
L47P	MtrR coding region	Lee *et al*. (2010)
T86A	MtrR coding region	Liao *et al*. (2011)

Interestingly, Zhao *et al*. (2009) found that the presence of only the mutated *mtrR* (containing both the A deletion in the promoter and G45D MtrR repressor protein mutation), as well as the presence of mosaic *penA,* has little to no effect on the MIC to ceftriaxone. Nevertheless, when both mutated *mtrR* and *penB* (containing $porB_{IB}$ alteration) were transferred into an *FA19penA35 strain* (which harbours mosaic *penA* from cefixime-resistant strain 35/02) via stepwise acquisition, the MIC of ceftriaxone increased about 2.5-fold. Interestingly, neither the involvement of the *mtrR* alone, nor the presence of both *mtrR* and $porB_{IB}$, has an effect on the MIC of cefixime. Zhao and colleagues came to the conclusion that, besides increasing the efflux pump activity, the *mtrR* mutation also might play a part in

activation of the *porB$_{IB}$* mutation. This finding indicates the synergism between the *mtrR* and *porB$_{IB}$* required to increase MIC to ceftriaxone, i.e. a combination of increased efflux and reduced entry (Zhao *et al.*, 2009).

1.8. Introduction to *penB* and *porB$_{IB}$*

Much of the earlier research on mutated *penB* and *porB$_{IB}$* was focused on the contribution of these genes to increased resistance to antibiotics, plus the mechanisms and mutations involved in causing antibiotic resistance, particularly for β-lactam drugs and tetracycline.

Guymon and colleagues (1978) and Cannon *et al*. (1980) found a significant increase in the molecular weight of the principal outer membrane protein (now protein I) of mutant *N. gonorrhoeae* (transformed with mutated *penA* and *mtrR*), after a mutated *penB* was introduced. The molecular weight of protein I increased from 36.5K to 39K (Cannon *et al.*, 1980). Both Guymon *et al*. and Cannon *et al*. hypothesised that the mutation in *penB* causes an alteration in cell protein I, changing the membrane permeability, and making the strains resistant to penicillin and tetracycline. However, besides *penB,* Cannon *et al*. also noticed another gene involved in the changes. This locus, called “new membrane protein” gene, or nowadays, *por* gene, was also involved in the changes. According to Cannon *et al*., *penB* and *por* are closely linked to each other, based on the 98% co-transformation frequency of the two loci.

Protein I can be classified into two classes, protein I class A (PIA) and protein I class B (PIB), encoded by *porA* and *porB,* respectively (Carbonetti *et al.*, 1988). As a result of selective pressure, the *por* undergoes various alterations, resulting in the diversity of sequence (Smith *et al.*, 1995). The classification is based on a considerable diversity of amino acid sequence in protein I (Carbonetti *et al.*, 1988). The PIA and PIB are encoded by most gonococci, with strains showing chromosomally-mediated resistance to penicillin and tetracycline belonging to

PIB serovars, suggesting the role of *porB* in *penB* phenotype strains (Woodford *et al.*, 1989).

Another investigation of gonococci with the *penB* phenotype was carried out by Gill *et al*. (1998). The transformation of susceptible gonococcus H1 with chromosomal DNA from penicillin-intermediate strain FA140 (*penA mtrR, penB;* porin serovar IB1) showed a decrease in equilibrium penicillin concentration in the cell periplasm. This decrease is associated with changes in *penB*, followed by *mtrR*. Gill *et al*. suggest that the fall in equilibrium penicillin concentration in the cell periplasm is due to changes in the structure of cell porin, although no porin structural data was presented in the study. Based on alignments of *por* with other porin super-family members, proposed by Jeanteur *et al*. (1991), *penB* is actually a chromosomal mutation located within the loop 3 area of *porB (porB$_{IB}$)*, the area that is responsible for forming the pore constriction area of cell porin.

1.8.1. The alterations of loop 3 area of PorB (*porB$_{IB}$*)

Glycine-101 and Alanine-102 to aspartic acid (D) (G101D and A102D) alterations, located within the loop 3 area of PorB (*porB$_{IB}$*), cause an increase in negative charge in the loop area (Gill *et al.*, 1998). The increase in the negative charge causes reduced porin permeability for antibiotics via anionic repulsion, hence the decrease in equilibrium penicillin concentration in the cell periplasm (Gill *et al.*, 1998). However, this hypothesis was not proven by laboratory testing. In fact, it was based on a sequence comparison of Por protein of *penB*-phenotype gonococcus and other Por protein super-families, such as the OmpF protein of *E.coli* (Olesky *et al.*, 2002).

To determine the specific amino acid alterations in the PorBIB that are responsible for an increase resistance to penicillin and tetracycline, Olesky *et al*. (2002) carried out a laboratory investigation. In this study, the PorBIB sequence of one of the tetracycline-resistant clones revealed the alteration of glycine (G) to

aspartic acid (D) at position 120 (G120D), and alanine (A) to aspartic acid (D) at position 121 (A121D) (Olesky *et al.*, 2002). Presumably, the G120D and A121D alterations in PIB equate to the G101D and A102D alterations in the loop 3 area of PorBIB described by Gill *et al*. (1998) since the locations of the mutations are so similar.

Besides the G120D A121D double mutation, the presence of G120R A121H, G120P A121P, or a single mutation, G120K, are also sufficient to cause a significant increase in resistance to penicillin and tetracycline (Olesky *et al.*, 2002). This increase is similar to the level of resistance caused by both G120D and A121D (Olesky *et al.*, 2002). The G120K and A121D changes in the PorBIB protein are the most frequent changes reported, based on 47% of clinical isolates in the GenBank database harbouring these mutations. The high frequency of the changes indicates that the mutations occur naturally (Olesky *et al.*, 2002).

However, in contrast to the hypothesis by Gill and colleagues (1998), Olesky *et al*. suggested that the modifications of residues 120 and 121 in loop 3 do not cause anionic repulsion of penicillin and tetracycline. Instead, they cause structural changes in pore channels that lead to a decrease in the flux of antibiotics into the periplasmic space (Olesky *et al.*, 2002). However, a more recent study by Olesky *et al*. (2006) showed that neither the pore size nor the permeation rate of β-lactam drugs and tetracycline is significantly changed by the alterations above.

The presence PorBIB alterations alone are not sufficient to cause increased resistance to penicillin and tetracycline. Further study on the influence of other mutated genes showed that changes in PorBIB act synergistically with the efflux pump activity of MtrCDE system. Consistent with the earlier findings by Sparling *et al*. (1975) and Gill *et al*. (1998), mutations in *mtrR* are required along with those in $porB_{IB}$, to cause a significant increase in resistance to both antibiotics. One hypothesis of Olesky and colleagues (2006) is that PorBIB variants form a

complex with MtrC-MtrD-MtrE, and work together through porin regulation and efflux pump activity to decrease the periplasmic concentrations of antibiotics.

1.8.2. Mutations in the *porB$_{IB}$* cause reduced susceptibility to ESCs in *N. gonorrhoeae*

Many studies on *N. gonorrhoeae* with elevated MICs to ESCs, have encountered *porB$_{IB}$* mutations alongside other resistance determinants (Unemo *et al.*, 2002; Takahata *et al.*, 2006; Tanaka *et al.*, 2006; Warner *et al.*, 2008; Allen *et al.*, 2011). As previously mentioned, Zhao *et al*. highlighted the requirement for both mutated *penB* (containing PorBIB alterations) and *mtrR* to increase the MIC of ceftriaxone to a higher level (Zhao *et al.*, 2009). Together with mosaic *penA35*, the MIC of ceftriaxone increased by 2.5-fold (Zhao *et al.*, 2009). The reversion of *penB* allele back to the wild type did not cause any changes to the MIC of ceftriaxone, indicating the insufficient impact of *porB$_{IB}$* mutations when it is present alone (Zhao *et al.*, 2009).

Regardless, when PorBIB alterations were observed, the G120K and A121D changes in PorBIB protein are the most common alterations observed in *N. gonorrhoeae* strains with reduced susceptibility to ESCs (Unemo *et al.*, 2002; Takahata *et al.*, 2006; Warner *et al.*, 2008). Interestingly, two ceftriaxone-resistant *N. gonorrhoeae* strains, found in France (Unemo *et al.*, 2012) and Spain (Camara *et al.*, 2012), plus one strain that was found to cause treatment failure with 500 mg ceftriaxone in Australia (Chen *et al.*, 2013), harboured the double mutations, G120K and A121N. This combination of changes has not been reported elsewhere in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone. Nevertheless, these findings revealed that the G120K A121D and G120K A121N are the most common PorBIB mutations that occur among *N. gonorrhoeae* with reduced susceptibility to ESCs. **Table 1-7** and **Figure 1-4** shows the most common published alterations in residues 120 and 121 of PorBIB amino acid sequence of *N. gonorrhoeae* with reduced susceptibility to ceftriaxone.

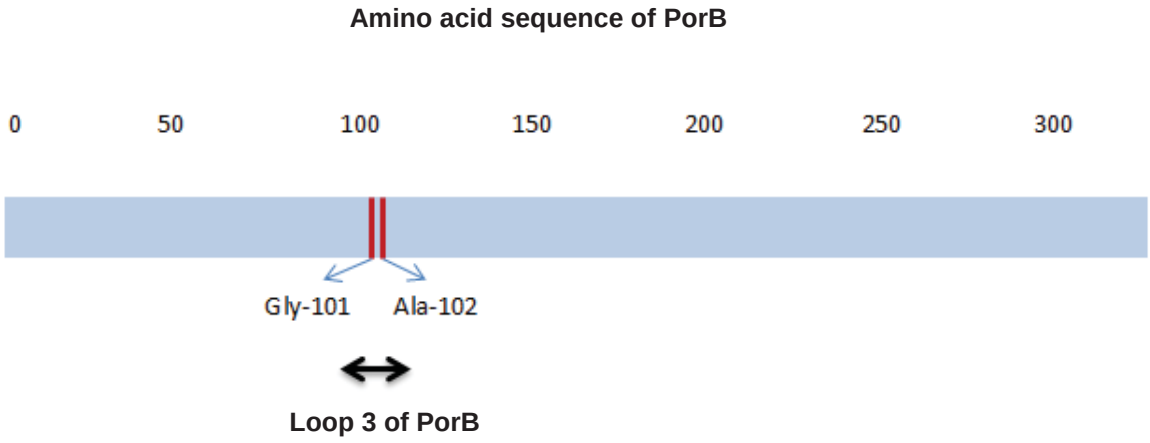

Figure 1-4 Amino acid sequences from PorBIB of *N. gonorrhoeae* strains adapted from Gill *et al*. (1998) and Olesky *et al*. (2002) (not drawn to scale). The arrows represent the area of loop three region of PorBIB that is responsible for changes in the porin structure of *N. gonorrhoeae*. Shaded red boxes represent the location of amino acids G120 and A121, usually involved in *N. gonorrhoeae* with reduced susceptibility to ESCs (as stated in **Table 1-7**)

Table 1-7 Published PorBIB mutations that contribute to reduced susceptibility to ESCs (e.g. ceftibuten, cefpodoxime, cefuroxime, cefotaxime, and ceftriaxone)

PorBIB mutations	Reference [a]
G120K A121N	(Unemo *et al.*, 2012) [b] (Camara *et al.*, 2012) [c] (Chen *et al.*, 2013) [d]

G120K A121D	(Lee *et al.*, 2015) (Warner *et al.*, 2008) (Takahata *et al.*, 2006)

[a] All *N. gonorrhoeae* strains published in these journals, except for Chen *et al.* (2012) showed the presence of the *mtrR* mutation.

[b] Ceftriaxone-resistant *N. gonorrhoeae* strain (F89) from France.

[c] Ceftriaxone-resistant *N. gonorrhoeae* strain (F89) from Spain.

[d] *N. gonorrhoeae* strain that caused treatment failure to 500 mg ceftriaxone in Australia.

1.9. Introduction to *ponA*

Besides penicillin-binding protein 2 (PBP2), *N. gonorrhoeae* harbours three other PBPs: PBP1, PBP3 and PBP4 (Ropp and Nicholas, 1997). PBP3 and PBP4 are not highly significant for the survival of the cell, but PBP1 and PBP2 are the lethal targets of β-lactam antibiotics. However, compared to PBP1, PBP2 has a higher affinity for β-lactam drugs (Ropp and Nicholas, 1997)

In 1986, Dougherty briefly discussed the association of PBP1 with penicillin-resistant gonococcal strains. The penicillin-resistant strains showed changes in the PBP1 structure and also had reduced affinity for penicillin (Dougherty, 1986). On the other hand, Ropp and Nicholas (1997) found that PBP1 consists of three major regions, the highly hydrophobic region, the transglycosylase region and the

transpeptidase region. The highly hydrophobic region located at the first 25-amino-acid region acts as the transmembrane anchor to the cell. The transglycosylase domain consists of residues 88 to 97; 117 to 127 and 286 to 293 is involved in the polymerisation of the glycan chains of peptidoglycan, a process that is essential for the construction of bacteria cell wall. The transpeptidase area contains three active sites that form an active-site pocket that connects with the β-lactam antibiotics, the SXXK, SXN and KTG (Ropp and Nicholas, 1997) (as depicted in **Figure 5**). Additionally, Ropp and Nicholas have verified the role of *ponA* in the production of PBP1, since inactivation of *ponA* in their study disrupted the production of PBP1 and influenced cell morphogenesis.

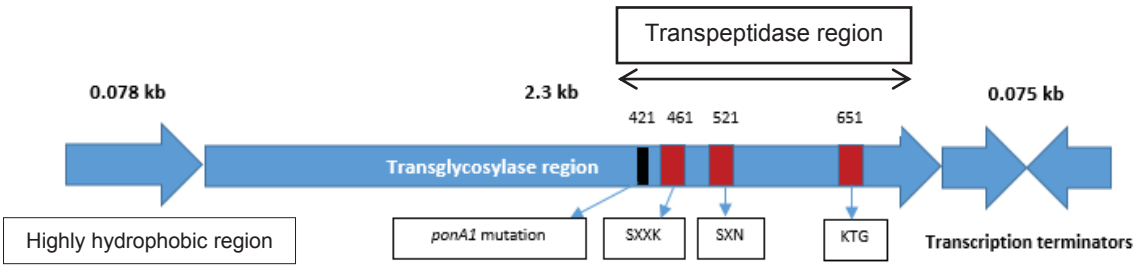

Figure 1-5 Organisation of *ponA* in gonococci, adapted from Ropp and Nicholas (1997), and Ropp *et al*. (2001). The *ponA* region includes the estimated location of the active sites (SXXK motif, SXN motif and KTG motif) in the transpeptidase region or domain and the *ponA1* mutation (Leu-421 → Pro) (not drawn to scale). The arrows represent the direction of transcription of the *ponA* sequence.

1.9.1. Mutation in *ponA* which causes *N. gonorrhoeae* to become resistant to ESCs.

In a different study, Ropp and colleagues (2002) identified a single point mutation at nucleotide position 1261 (T-to-C transition), while the rest of the amino acid sequence was similar to the wild-type *ponA.* The mutation correlates with the alteration of leucine (L) to proline (P) at position 421 of the amino acid sequence (L421P) **(Figure 1-5).** Ropp and colleagues reported L421P causes a 3-fold to 4-fold lower rate of acylation by β-lactam drugs, such as penicillin and ceftriaxone, compared to *ponA* from the wild-type strain. Ropp and colleagues added that the mutation near the active-serine residue SXXK changes the PBP1 structure, leading to functional disruption of PBP1. Interestingly, for the *ponA* gene to cause a high level of penicillin resistance, a novel mutation in *penC* is required (Ropp *et al.*, 2002; CDC, 2005; Shigemura *et al.*, 2005).

Surprsingly, several studies have observed the presence of the *ponA* mutation (L421P) in both *N. gonorrhoeae* isolates that were fully susceptible to ceftriaxone and showed reduced susceptibility to ceftriaxone (Unemo *et al.*, 2002; Takahata *et al.*, 2006; Tanaka *et al.*, 2006; Warner *et al.*, 2008; Allen *et al.*, 2011). Unlike penicillin G, a stepwise transformation of FA19 strain carried out by Zhao *et al*. (2009) revealed that the L421P mutation did not cause any significant increase in both cefixime and ceftriaxone MICs. Zhao *et al.* (2009) hypothesised that, besides PBP2, PBP1 could also be a lethal target of penicillin G. However, this does not apply for cephalosporins where only PBP2 is the lethal target of the antibiotic.

Regardless, after the last resistance determinant (*ponA)* was transferred into the FA19 *penA35 mtrR penB35* transformant, the ceftriaxone MIC of the transformant was still low (MIC: 0.03 mg/L) compared to the ceftriaxone MIC of the donor strain *N. gonorrhoeae* 35/02 (MIC: 0.094 mg/L). Zhao *et al*. hypothesised an additional resistance gene determinant(s) is required to increase the ceftriaxone MIC to the same level as the donor strain. Similarly, Ropp *et al.* (2002) found that another

novel resistance gene determinant *pilQ* (previously named *penC*) enhanced the effect of the *ponA* mutation, leading to an increase of penicillin MIC to a higher level. In a separate study, Whiley *et al*. (2010) found that one D526A alteration in the *pilQ* is associated with the increase in ceftriaxone MIC, and the association is statistically significant ($P<0.01$). However, overall, the contribution of *pilQ* to the increase of cephalosporins MIC is still unlikely (Whiley *et al.*, 2010b).

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1.10. Treatment failure of infections due to *N. gonorrhoeae* with reduced susceptibility to ceftriaxone

The emergence of *N. gonorrhoeae* displaying reduced susceptibility to ceftriaxone is an alarming issue that needs to be tackled and continually monitored in every country. *N. gonorrhoeae* that harbour the mutations in the resistance gene, *penA, mtrR,* $porB_{IB}$ and *ponA*, have been found to complicate patients' treatment with ceftriaxone.

Up until December 2015, six treatment failures of pharyngeal gonorrhoea with ceftriaxone had been reported: in Australia (n=1), Slovenia (n=1), Sweden (n=3) and Japan (n=1) (Tapsall *et al.*, 2009a; Ohnishi *et al.*, 2011; Unemo *et al.*, 2011a; Unemo *et al.*, 2011b). Excluding the case from Japan where the gonococcus strain is a ceftriaxone-resistant strain, five other treatment failures were caused by *N. gonorrhoeae* showing reduced susceptibility to ceftriaxone, with MIC ranging from 0.03 mg/L to 0.25 mg/L. A genotypic study of *N. gonorrhoeae* strains that were associated with treatment failures from Australia, Slovenia and Sweden revealed the presence of mosaic *penA* pattern XXXIV, with significant PBP2 alterations I312M, V316T, N512Y and G545S. Alongside mosaic PBP2, *mtrR* and $porB_{IB}$ mutations were also observed.

Although not as often reported as the mosaic PBP2, ceftriaxone treatment failures associated with *N. gonorrhoeae* and the nonmosaic PBP2 sequence have been reported in Australia in 2009 (Tapsall *et al.*, 2009a). Two treatment failures of

gonococcal pharyngitis with 250 mg ceftriaxone were reported, causing the physician to increase the dose to 500 mg and 1g. *N. gonorrhoeae* isolates with a nonmosaic PBP2 (pattern VII and V) were observed in this study. According to PBP2 nomenclature set by Ito *et al*. (2005), PBP2 pattern VII possess the A501V mutation, while pattern V possesses G542S. However, since the MIC of *N. gonorrhoeae* isolates that harbour nonmosaic PBP2, patterns VII and V are too low to cause treatment failure (0.03 mg/L and 0.016 mg/L, respectively), Tapsall's group hypothesised that the presence of other mutations might also partially be associated with the treatment failure. The presence of *mtrR* and $porB_{IB}$ mutations were not specified in the study.

In 2011, the first *N. gonorrhoeae* strain (H041) that is resistant to ceftriaxone was isolated from the pharynx of a sex worker in Japan (Ohnishi *et al.*, 2011). Analysis by Ohnishi and colleagues showed that the gonococcal strain exhibited an MIC of 2 to 4mg/L for ceftriaxone and was resistant to nearly all antibiotics. The mosaic PBP2 pattern X plus four additional novel mutations, A331V, T316P, A328T and T484S, that had never before been described were found in this strain. This finding signalled that mosaic PBP2 is evolving to cause an even higher MIC of ceftriaxone (Shimuta *et al.*, 2013) Three out of four of these new mutations, A311V, T316P and T484S, have been verified by Tomberg and colleagues (2013) to cause a substantial increase in the MICs level of ESCs. The *N. gonorrhoeae* H041 strain also harboured mutations in *mtrR,* $porB_{IB}$ and *ponA*. The occurrence of these additional PBP2 mutations plus *mtrR,* $porB_{IB}$ and *ponA* mutations, might have caused the *N. gonorrhoeae* strain to become fully resistant to ceftriaxone and other antibiotics.

The second ceftriaxone-resistant *N. gonorrhoeae* was isolated from the urethra of a 50-year-old man in France, with an MIC of 1 to 2mg/L (Unemo *et al.*, 2012). Similarly, Unemo *et al*. found that this ceftriaxone-resistant *N. gonorrhoeae* strain (F89) harboured mosaic PBP2 pattern XXXIV, with an additional A501P alteration. When Unemo and colleagues demonstrated the effect of the F89 mosaic *penA* by transforming it into a recipient strain, WHO F, the MIC of cefixime

increased between 8-fold and 500-fold and of ceftriaxone from 31-fold to 500-fold. Like XDR *N. gonorrhoeae* H041, *N. gonorrhoeae* F89 also contains an A deletion in the inverted *mtrR* promoter sequence, plus G120K and A121N alterations in $porB_{IB}$ and L421P mutation in *ponA*. The F89 strain belongs to the sequence type ST1407 (Camara *et al.*, 2012; Unemo *et al.*, 2012)

A third ceftriaxone-resistant *N. gonorrhoeae* was reported from Spain, with MIC of 1.5 mg/L to ceftriaxone (Camara *et al.*, 2012). This group revealed that the strain also harbours a mosaic PBP2 XXXIV with A501P mutation, very similar to that described by Unemo *et al*. (2012). The study showed that the ceftriaxone-resistant *N. gonorrhoeae* (F89) has probably spread around Europe. The gonococcal strains were isolated from two male patients who reported sexual activity with each other (MSM). This marks the first inter-patient spread of a ceftriaxone-resistant of *N. gonorrhoeae* (Camara *et al.*, 2012). Like the previously reported ceftriaxone-resistant gonococcus strains, the third strain also harboured an A deletion in the *mtrR* promoter region, the G120K and A121N mutation in $porB_{IB}$ and the L421P alteration in *ponA*.

In conclusion, between 2011 and 2012, ceftriaxone-resistant *N. gonorrhoeae* strains have emerged, even though they are still rare and sporadic. The spread of ceftriaxone-resistant *N. gonorrhoeae* shows that the strains can circulate within high-risk groups, such as sex workers and MSM. Importantly, four mutated genes *penA, mtrR,* $porB_{IB}$ *and ponA,* were found in all of these resistant strains, showing the significant role of these mutations in causing elevated MIC of ceftriaxone in *N. gonorrhoeae*. The same mutated genes have also been reported in other studies on *N. gonorrhoeae* with reduced susceptibility to ceftriaxone, and also in cases of treatment failures **(Table 1-8).**

Table 1-8 Published ceftriaxone-resistant *N. gonorrhoeae* strains, and *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone associate with ceftriaxone treatment failures

Country	MIC (mg/L)	Mutated genes	Note	Reference
Sweden (2014)	0.064 - 0.125	• mosaic PBP2 XXXIV • PorBIB alteration (not specified) • *mtrR* alteration (not specified)	• Three cases of treatment failures reported • Treatment failure to 500 mg ceftriaxone	(Golparian *et al.*, 2014)
Australia (2013)	0.03 - 0.06	• mosaic PBP2 XXXIV • PorBIB (G120K/A121N)	• Two cases of treatment failures reported • Treatment failure to 500 mg ceftriaxone	(Chen *et al.*, 2013)
Spain (2012)	1.5	• mosaic PBP2 XXXIV + A501P • *mtrR* (A deletion) • PorBIB (G120K/A121N) • *ponA* mutation	• Ceftriaxone resistant strain	(Camara *et al.*, 2012)
France (2012)	1.0 - 2.0	• mosaic PBP2 XXXIV + A501P • *mtrR* (A deletion) • PorBIB (G120K/A121N) • *ponA* mutation	• Ceftriaxone-resistant strain	(Unemo *et al.*, 2012)

Table 1-8 (Continue) Published ceftriaxone-resistant *N. gonorrhoeae* strains, and *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone associate with ceftriaxone treatment failures

Japan (2011)	2.0 – 4.0 mg/L	• mosaic X with A311V, T316P, A328T and T484S • *mtrR* alteration (not specified) • PorBIB alteration (not specified) • *ponA* mutation	• Ceftriaxone-resistant strain	(Ohnishi *et al.*, 2011)
Sweden (2011)	0.125/ 0.25	• mosaic PBP2 X • *mtrR* alteration (not specified) • PorBIB alteration (not specified)	• One treatment failure reported • Treatment failure to 250 mg ceftriaxone	(Unemo *et al.*, 2011a)
Slovenia (2011)	0.12	• mosaic PBP2 XXXIV • *mtrR* (Not specified) • PorBIB alteration (not specified)	• One treatment failure reported • Treatment failure to 250 mg ceftriaxone	(Unemo *et al.*, 2011b)
Australia	0.03	• nonmosaic PBP2 XIII (with A501V alteration), and type V (with G542S) were found • the presence of other mutated genes were not specified	• Two treatment failures reported • Treatment failure to 250 mg ceftriaxone	(Tapsall *et al.*, 2009a)

1.11. Susceptibility of New Zealand isolates of *N. gonorrhoeae* to ceftriaxone

In New Zealand, neither treatment failures nor ceftriaxone resistance have been detected among *N. gonorrhoeae* isolates from patients so far. However, gonococcal strains with reduced susceptibility to ceftriaxone were first identified in the Auckland region in 2010 (ESR, 2011), followed by in 2011(ESR, 2012) and 2013 . The isolates was also found in Hamilton in 2013 (ESR, 2013). In 2014, one *N. gonorrhoeae* isolate with reduced susceptibility to ceftriaxone was reported in the Canterbury region (ESR, 2014) **(Table 1-9).** An investigation into the presence of mosaic *penA* was done in 2013, where 5% of the residuals DNA from *N. gonorrhoeae* strains was found to be positive for the presence of the mosaic *penA* gene (Nicol *et al.*, 2014). However, no phenotypic data was obtained for these isolates, since viable cultures were not available. It is important to carry out phenotypic and genotypic studies on *N. gonorrhoeae* showing reduced susceptibility to ceftriaxone at an early stage, so that action can be taken to curb the emergence of ceftriaxone-resistant *N. gonorrhoeae,* to avoid treatment failures and assist in the successful eradication of the bacteria

Table 1-9 *N. gonorrhoeae* with reduced susceptibility to ceftriaxone in New Zealand and the presence of mosaic *penA*

Year	Location	MIC of ceftriaxone	Resistance determinants
2010	Auckland	0.06 mg/L	Unknown [a]
2011	Auckland	0.06 mg/L	Unknown [b]
2013	Auckland and Hamilton	0.06 mg/L	Unknown [c]
2014	Canterbury	0.06 mg/L	Unknown [d]
2014	Auckland and Wellington	Unknown (no viable culture samples available)	5% positive for mosaic *penA* [e]

[a b c d] ESR (2011, 2012, 2013, 2014) [e] Nicol *et al.* (2014)

The specific aim of this study is to determine the phenotypic characteristics, plus the presence of mutations in *penA*, *mtrR, ponA* and $porB_{IB}$ among 28 *Neisseria gonorrhoeae,* isolated in New Zealand, with elevated minimum inhibitory concentrations (MIC) to ceftriaxone. The aim is also to explore the association between the phenotypic and genotypic character of the strains.

CHAPTER TWO

METHODS AND MATERIALS

2. METHODS AND MATERIALS

2.1. Ethical Approval

This project was granted ethical approval by Massey University Human Ethics Committee (MUHEC) (No: 15/13) (Appendix A)

2.2. Isolates

N. gonorrhoeae atypical isolates used in this study were supplied by the Institute of Environmental Science and Research Limited (ESR), Porirua, New Zealand with all patient details removed. Twenty-eight *N. gonorrhoeae* isolates were sourced from laboratories in the Auckland (26), Wellington (1) and Taranaki (1) regions. These isolates were atypical since they exhibited a minimum inhibitory concentration (MIC) of ceftriaxone ranging from 0.03 mg/L to 0.12 mg/L when tested at ESR and thus, they were categorised as having a reduced susceptibility towards ceftriaxone (Whiley *et al.*, 2007a; Tapsall *et al.*, 2009a; Chen *et al.*, 2013; ESR, 2014). ESR sent these 28 *N. gonorrhoeae* isolates on supplemented chocolate agar slants (Fort Richard, Auckland). The list of *N. gonorrhoeae* isolates used in this study can be seen in **Table 2-1**.

Table 2-1 List of *Neisseria gonorrhoeae* isolates from the Institute of Environmental Science and Research Limited (ESR) and *N. gonorrhoeae* control strains

Isolates	Year of isolation	ESR number	Region	Ceftriaxone minimum inhibitory concentrations (MICs) (mg/L) [a]
WHO K	N/A	N/A	N/A	-
WHO F	N/A	N/A	N/A	-
WHO L	N/A	N/A	N/A	-
ATCC 49226	N/A	N/A	N/A	-
71	2012	ARL 12/71	Auckland	0.06
411	2012	ARL 12/411	Auckland	0.03
483	2012	ARL 12/483	Auckland	0.03
604	2012	ARL 12/604	Auckland	0.06
631	2012	ARL 12/631	Auckland	0.12
729	2012	ARL 12/729	Auckland	0.06
1526	2012	ARL 12/1526	Auckland	0.06
1530	2012	ARL 12/1530	Auckland	0.06
257	2013	ARL 13/257	Auckland	0.03
557	2013	ARL 13/557	Auckland	0.03
558	2013	ARL 13/558	Auckland	0.12
723	2013	ARL 13/723	Auckland	0.12
801	2013	ARL 13/801	Auckland	0.06
886	2013	ARL 13/886	Auckland	0.06
1380	2013	ARL 13/1380	Auckland	0.06
1641	2013	ARL 13/1641	Auckland	0.12
1026	2013	ARL 13/1026	Auckland	0.06
824	2013	ARL13/1026	Auckland	0.06
724	2013	ARL13/724	Auckland	0.06
263	2014	14NI/0263	Auckland	0.06

119	2014	14NI/0119	Auckland	0.06
264	2015	15AR0264	Auckland	0.06
792	2015	15AR0792	Wellington	0.12
893	2015	15AR0893	Auckland	0.03
896	2015	15AR0896	Taranaki	0.06
963	2015	15AR0963	Auckland	0.06
1332	2015	15AR1332	Auckland	0.12
1848	2015	15AR1848	Auckland	0.12

Note: The ceftriaxone MIC for all isolates except isolate 264, 792, 893, 896, 963 and 1332 were determined by Etest strips (bioMérieux, Solna, Sweden). For the rest of the stated six isolates, an agar dilution method was used.

2.2.1. Specimen culture

Upon arrival at Massey University, the 28 isolates of *N. gonorrhoeae* from ESR were sub-cultured on 60mm X 15mm supplemented chocolate agar plates (Fort Richard, Auckland) and into a brain-heart infusion (BHI) broth (Becton & Dickinson, USA). To create a 3% to 5% carbon dioxide (CO_2) atmosphere, the plates were arranged in a candle jar. The cap of the sterile bijou bottle that contained the BHI broth with cell suspension was slightly loose. A moist paper towel and a non-scented candle was placed on top of the plates. The candle was lit inside the jar, and the lid immediately sealed. After the candle had been extinguished, the jar was placed in a 36±1°C incubator room for 24 hours. After 24 hours of incubation, the plates were examined for growth. If no growth of bacterial colonies was observed, a new agar plate was inoculated using the overnight BHI broth culture of that isolate. The plates were incubated in 3% to 5% CO_2 at 37°C as described above.

2.2.2. Specimen storage

The *N. gonorrhoeae* isolates were preserved in BHI broth with 20% glycerol (BDH Chemicals) and were stored in a -70°C freezer (Thermo Fisher). The preparation of the BHI broth with 20% glycerol is shown in **Table E1**. Four sets of BHI + 20% glycerol stock were prepared for each isolate, and four reference isolates WHO K, WHO L, WHO F and ATCC 49226. A half to a full plate of overnight growth of each *N. gonorrhoeae* isolate was harvested with a sterile loop and suspended in the BHI-glycerol broth. The suspension was mixed using a vortex mixer (Labnet International) and frozen immediately at -70°c.

For purity checking, *N. gonorrhoeae* from the frozen culture stock at -70°C were removed from the freezer and put in an ice-box. A small scrapping of frozen culture was collected with a sterile loop and inoculated on supplemented chocolate agar. The tube was refrozen at -70°c immediately. The plates were incubated for 24 hours in 3% to 5% CO_2 in air at 37°C as described above.

After the incubation, the plates were observed for any sign of contamination. The purity of the culture was indicated by the uniformity of the colonies on the plate and consistency with the appearance of the original culture.

2.2.3. Reference isolates

N. gonorrhoeae reference isolates WHO K, WHO F, WHO L and ATCC 49226 were used as recommended for the antimicrobial susceptibility testing (ENTIRETY; Unemo *et al.*, 2009). The antimicrobial range and MIC data for the WHO reference isolate panel, and ATCC 49226 isolates can be seen in **Table 2-2**.

Reference isolates were sent from ESR as lyophilised cultures in glass ampoules. Upon receipt, the vials were broken aseptically and diluted with BHI broth (Fort Richard). A drop of the diluted culture was added to chocolate supplemented agar (Fort Richard) and streaked across the plate. The rest of the diluted culture was

aseptically added to a sterile glass bottle. Both plates and bottles were incubated for 24 hours in 3% to 5% CO_2 at 37°C.

After 24 hours of incubation, the agar plates were examined for growth. If no growth of bacteria was observed, a new chocolate supplemented agar plate was inoculated from the cultured BHI broth, and then the steps above repeated.

Table 2-2 Antimicrobial range and minimum inhibitory concentration (MIC) data for WHO reference strain panel and CLSI reference strain: ATCC 49226

Antimicrobial agent	Acceptable range for MIC (mg/L) [a]			
	WHO F	WHO K	WHO L	ATCC 49226
Ceftriaxone	<0.002 (S)	0.032-0.125 (NS)	0.064-0.25 (NS)	0.004-0.0016 (S)
Azithromycin	0.064-0.25 (S)	0.125-0.5 (S)	0.25-1.0 (I)	0.5-1.0 (I)
Ciprofloxacin	0.002-0.008 (S)	>32 (HLR)	>32 (HLR)	0.001-0.008 (S)
Tetracycline	≤0.25 (I)	≥2.0 (R)	≥2.0 (R)	0.25-1.0 (I)
Penicillin G	0.016-0.064 (S)	1.0-4.0 (CMRNG)	1.0-4.0 (CMRNG)	0.25-1.0 (I)

[a] Sources: For ATCC 49226 (Kirkcaldy *et al.*, 2010) ; for WHO F, WHO K, WHO L (Unemo *et al.*, 2009).

S: Susceptible; I: Indeterminate, NS: Non-susceptible (for ceftriaxone); HLR: High-level resistance; CMRNG: Chromosomally-mediated resistant *Neisseria gonorrhoeae*

2.3. Phenotypic Characterisation

2.3.1. β-lactamase production (nitrocefin test)

A nitrocefin disc (Remel) was placed on a clean glass slide using sterile forceps. A loopful of milli-Q water was added on top of the disc to moisten it. Next, with a sterile loop, five to six colonies of overnight (20- to 24-hours) pure culture of *N. gonorrhoeae* were collected and smeared on top of the moistened disc. The disc was incubated for five minutes at room temperature.

At the end of incubation, the nitrocefin result was read by observing the colour change of the disc. The result was considered positive for β-lactamase production if the pink or red colour was observed. The absence of a colour change indicated the isolate was negative for β-lactamase production.

2.3.2. Antimicrobial susceptibility test

MIC test strips were used to determine the antimicrobial susceptibility testing of *N. gonorrhoeae* in this study. MIC test strips from two different companies (Liofilchem s.r.l, Italy, and bioMérieux, Solna, Sweden) were used to determine the MICs of ceftriaxone, penicillin G, tetracycline, azithromycin and ciprofloxacin.

The antibiotic concentration range for both Liofilchem s.r.l MIC test strips and bioMérieux Etest strips for penicillin G (0.002-32 mg/L), tetracycline (0.016-256 mg/L), ciprofloxacin 90.002-32 mg/L), azithromycin (0.016-256 mg/L) and ceftriaxone (0.002-32 mg/L) were the same.

2.3.2.1. Sample culture:

Isolates were inoculated on a chocolate supplemented agar, placed in a candle jar to obtain 3% to 5% CO_2 and incubated in 36±1°C incubator room for 24 hours. After 24 hours of incubation, a pure colony of each isolate was sub-cultured onto a

second chocolate supplemented agar. The second chocolate supplemented agar was incubated as described above.

2.3.2.2. Inoculum preparation:

To prepare the inoculum, 1ml of 1X phosphate buffered saline (PBS) was pipetted into a sterile, clear glass tube. The PBS was prepared as shown in **Table E2**. Using a dacron swab (Copan Italia, Italy), four to five colonies of an overnight (20- to 24-hours) culture of the *N. gonorrhoeae* isolate were collected. The dacron swab was then dipped into the suspension to make a 0.5 McFarland turbidity suspension. This was done by comparing the suspension to a 0.5 McFarland Standard (Becton & Dickinson, USA) and holding the two tubes close to a Wickerham card in the presence of adequate lighting (Vetlab, 2014).

After achieving the standardised inoculum, a dacron swab was dipped into the suspension, and the excess liquid was removed by pressing the swab tip against the inside of the tube. The inoculum was initially spread across a GC II agar plate, and then the swab tip rotated 180 degrees and spread twice more to ensure even distribution. The inoculum was left to dry for approximately for 10 minutes. One MIC strip (Liofilchem s.r.l, Italy, or bioMérieux, Solna, Sweden) was applied to each agar plate using a sterile forceps, with the printed scale facing upward. The plates were inverted and incubated in a candle jar (3% to 5% CO_2 in air) at 36±1 °C (for Liofilchem MIC strips) (Diagnostici, L, 2015) and 35±2 °C (for bioMérieux Etest) (BioMérieux, 2012) for 24 hours.

2.3.2.3. Interpretation of the MIC results:

After incubation, the MIC values were read based on where the edge of the inhibition ellipse intersected the strip. The intersection between two scales segments was rounded up to the higher value. In the case of uneven intersection on the two sides of the strip, the higher value was considered the correct reading. The European Committee on Antimicrobial Susceptibility Testing (EUCAST)

breakpoint (taken from http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_5.0_Breakpoint_Table_01.pdf on 2 February 2015) was used for interpretation of MIC values of the isolates and to define their susceptibility categories, based on either resistant (R) or susceptible (S). As in the EUCAST guideline, the MICs that fell between the resistant and susceptible categories were categorised as indeterminate (I). A list of EUCAST susceptibility categories for ceftriaxone, penicillin G, azithromycin, ciprofloxacin and tetracycline for *N. gonorrhoeae* are shown in **Table 2-3**. For statistical analysis, the mean and 95% Confidence interval (CI) of the MIC was determined from page https://www.mccallum-layton.co.uk/tools/statistic-calculators/confidence-interval-for-mean-calculator/#confidence-interval-for-mean-calculator.

Table 2-3 MIC interpretative Standard according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST)

Antimicrobial agent	MIC interpretative standard (mg/L)		
	Susceptible	Resistant	Indeterminate [a]
Azithromycin	≤0.25	>0.5	0.5
Ceftriaxone [b]	≤ 0.125	>0.125	N/A
Ciprofloxacin	≤0.03	>0.06	0.06
Penicillin G	≤0.06	>1.0	0.12 - 1.0
Tetracycline	≤ 0.5	> 1.0	1.0

[a] To simplify the EUCAST tables, the indeterminate ranges are not listed in the EUCAST guidelines. (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_5.0_Breakpoint_Table_01.pdf). However, the indeterminate category is the MIC reading of each antimicrobial agent (except ceftriaxone) that fall between susceptible and resistant.

[b] MIC range for reduced susceptibility for ceftriaxone 0.03-0.12 mg/L (Whiley *et al.*, 2007a; Tapsall *et al.*, 2009a; Chen *et al.*, 2013; ESR, 2014).

2.3.2.4. Colony forming unit (CFU) of 0.5 McFarland Standard:

A standard inoculum was prepared as stated in Section 2.3.2 (Inoculum preparation).

To prepare the dilution inoculum for 0.5 McFarland Standard, 9 ml of 1X PBS was pipetted into a seven sterile, clear glass tube. A serial dilution was made by transferring 1 ml of the prepared 0.5 McFarland was pipetted into the first tube containing 9 ml of 1X PBS. The tube was mixed thoroughly. 1 ml of the recent suspension (tube 1) was pipetted into another test tube containing 9 ml PBS, and the tube was mixed thoroughly. The dilution process was repeated until the seventh tube. 1 ml of the suspension from the last tube was discarded.

Then, 100 µl of suspension from each dilution 10^{-4}, $10^{-5,}$ 10^{-6}, and 10^{-7} was pipetted onto the centre of the chocolate supplemented agar, respectively. A glass spreader was rinsed with 100% alcohol, and the excess of the alcohol was removed before sterilising the spreader by flaming. The glass spreader was cooled down by touching the spreader to the medium surface (without touching the bacteria suspension).

Once cooled down, the bacteria suspension was spread evenly over the surface with the spreader while slightly opening the lid of the plate. The same steps were applied to all plates with bacterial suspension. The petri dishes were incubated as mentioned in Section 2.3.2 (Sample Culture).

After the incubation, the number of discrete colonies from each plate was counted, and the CFU value of the sample was calculated. The CFU value was compared to the standard CFU value of 0.5 McFarland Standard (1.5×10^8 CFU/ml) to evaluate the accuracy of the inoculum preparation.

2.3.3. Comparison of two brands of MIC strips: Liofilchem s.r.l (Italy) MIC test strips and bioMérieux (Solna, Sweden) Etest

Two sets of comparisons were carried out to determine the MIC of penicillin G and ceftriaxone between two brands of MIC strips for 21 *N. gonorrhoeae* samples as according to **Table 2-4**.

Table 2-4 Comparison of MIC results for penicillin G and ceftriaxone between Liofilchem MIC test strips and bioMérieux Etest

Comparison Set	MIC test strips	Culture	Sub-culture	MIC test
Set 1	Penicillin (Liofilchem)	Choc Supplemented Agar	Choc Supplemented Agar	GC II Agar
	Penicillin (bioMérieux)	Choc Supplemented Agar	Choc Supplemented Agar	GC II Agar
Set 2	Ceftriaxone (Liofilchem)	Choc Supplemented Agar	Choc Supplemented Agar	GC II Agar
	Ceftriaxone (bioMérieux)	Choc Supplemented Agar	Choc Supplemented Agar	GC II Agar

2.3.4. Determination of the effect of using different media to culture *N. gonorrhoeae* prior to MIC test on the MIC result

For this study, 21 *N. gonorrhoeae* isolates were cultured on three different types of media: GCII agar, chocolate supplemented agar, and chocolate saponin agar (Fort Richard) prior to MIC test. This step of the experiment can be seen in **Table 2-5**.

Table 2-5 A test to compare the MIC results of penicillin G and ceftriaxone between Liofilchem MIC test strips and bioMérieux Etest

No.	Culture	Sub-culture [a]	MIC test	MIC strips [b]
1	GC II Agar	GC II Agar	GC II Agar	Penicillin
2	Chocolate supplemented agar	Chocolate supplemented agar	GC II Agar	
3	Chocolate saponin agar	Chocolate saponin agar	GC II Agar	
1	GC II agar	GC II agar	GC II Agar	Ceftriaxone
2	Chocolate supplemented agar	Chocolate supplemented agar	GC II Agar	
3	Chocolate saponin agar	Chocolate saponin agar	GC II Agar	

[a] 0.5 McFarland Standard was prepared from the sub-culture plates.

[b] Liofilchem MIC test strips were used in this study

2.3.5. Statistical analysis

The estimated fold difference of the dilution between the MIC results produce from Liofilchem MIC test strips and bioMérieux Etest was determined as the $\log_2$ difference. Agreement between MIC readings was defined as an MIC within ± 1 $\log_2$ of the comparison MIC as stated by Biedenbach and Jones (1996). The agreement was defined after rounding the MIC of both penicillin and ceftriaxone to the next approximately two-fold dilution value. The significant differences between the MIC readings of the two brands were determined using a two-tailed student's test from http://graphpad.com/quickcalcs/ttest1.cfm and statistical significance was set at $p < 0.05$.

Similarly, the estimated fold difference of the MICs between media was determined as the $\log_2$ difference. The agreement between these media was defined as an MIC within ± 1 $\log_2$ of the comparison MIC following the CDC GISP quality assurance guidelines (Biedenbach and Jones, 1996). The agreement was defined after rounding each MIC to the next appropriately two-fold dilution value. Secondly, the significant difference between the MIC results for different media

was determined using independent samples t-test calculator from http://www.socscistatistics.com/tests/studentttest/Default2.aspx, and the statistical significance was set at $p<0.05$.

2.4. Polymerase Chain Reaction (PCR) for mosaic *penA, penA, mtrR*, *porB$_{IB}$* and *ponA*

2.4.1. DNA extraction

The method used for the preparation of Chelex ® 100 (Bio-Rad) is shown in **Table E3**. For DNA extraction, 1ml of 2% Chelex ® 100 was pipetted into a microcentrifuge tube. Chelex® 100 must be adequately mixed since the solution tends to sediment at the bottom. Approximately 10 uniform colonies of overnight growth of *N. gonorrhoeae* isolates were harvested with a sterile loop and suspended in the solution. The suspension was vigorously mixed with vortex and brought to boil at 100°C for 10 minutes using a digital dry bath block (Labnet International).

Next, the tubes were spun in a microcentrifuge (Labnet International) for 1 minute at 12,500 g (13,000rpm) to ensure cell particles sediment at the bottom of the microcentrifuge tube. After that, 200µl of supernatant containing DNA was transferred to a clean microcentrifuge tube. The DNA templates were stored in a -70°C freezer (Thermo Fisher) for future use.

2.4.2. DNA concentration and purity

The DNA concentration and purity was determined by loading 2µl of the DNA sample onto a Nanodrop ND-1000 spectrophotometer (Thermo Fisher). Before the DNA was measured, a blank measurement was made using milli-Q water. Calculation and analysis of the aliquot were done using ND-1000 V3.3.0 software. The DNA concentrations and purity of samples are shown in **Table E4**.

2.4.3. Primers for *N. gonorrhoeae* mosaic *penA, mtrR, porB$_{IB}$* and *ponA* PCR

Conventional PCR was used to amplified the *penA, mtrR, porB$_{IB}$* and *ponA* gene. For detection of mosaic PBP2, the primers designed by *Ochiai et al.* (2008) based on the *penA* region of *N. cinerea* and *N. perflava* were used. However, in the current study, despite using the specific mosaic probe NG89-P1 (5'-ACCGATTTTGTAAGGCAGGG-3') suggested by Ochiai *et al.* (2008) to identify the presence of mosaic *penA*, the presence of mosaic PBP2 was confirmed through sequencing to determine the presence of I312M and V316T.

For *penA* PCR, the primers are divided into four parts to amplify the whole gene. *PenA* PCR was carried out for three purposes. The first purpose was to confirm the absence of mosaic *penA* (with I312M and V316T alterations) in isolates that were negative for mosaic *penA* PCR, by carrying out set B *penA* PCR. The second purpose was to identify other significant non-mosaic PBP2 alterations through using set D PCR**.** The third purpose, where funding is permitted, was that all four sets of *penA* PCR and sequencing should be carried out on the isolates to obtain complete information regarding the gene.

Primers were obtained from Integrated DNA Technologies (New Zealand). The specificity of the primers was checked using Basic Local Alignment Search Tool (BLAST). Working solution for primers (10μM) was prepared according to **Table E5**. The list of the primers can be seen in **Table 2-6**.

Table 2-6 List of primers used for mosaic *penA*, *penA*, *mtrR*, *porB$_{IB}$* and *ponA* PCR

PCR [a]	Set	Name of primers	Nucleotide sequence (5' to 3')	Amplify location [b]	Amplicon size
Mosaic *penA*	-	NG89-F2	GTTGGATGCCCGTACTGGG	801 to 1020	219 bp
		NG89-R1	ACCGATTTTGTAAGGCAGGG		
penA	A	PenA-FA1	CGGGCAATACCTTTATGGTGGAAC	8 to 676	668 bp
		PenA-RB1	AACCTTCCTGACCTTTGCCGTC		
	B [c]	PenA-FA2	AAAACGCCATTACCCGATGGG	597 to 1177	580 bp
		PenA-RB2	TAATGCCGCGCACATCCAAAG		
	C	PenA-FA3	GCCGTAACCGATATGATCGA	1003 to 1865	862 bp
		PenA-RB3	CGTTGATACTCGGATTAAGACG		
	D	PenA-FA4	AATTGAGCCTGCTGCAATTGGC	1376 to 1865	489 bp
		PenA-RB3	CGTTGATACTCGGATTAAGACG		
mtrR	-	MTR1	AACAGGCATTCTTATTTCAG	860 to 1775	915 bp
		MTR2	TTAGAAGAATGCTTTGTGTC		
porB$_{IB}$	-	PorB1	AAAGGCCAAGAAGACCTCGGC	160 to 917	757 bp
		PorB2	GAGAAGTCGTATTCCGCACCG		
ponA	-	PonA1	CGCGGTGCGGAAAACTGATATCGAT	955 to 2195	1240 bp
		PonA2	AGCCCGGATCGGTTACCATACGTT		

[a] Primers for mosaic *penA* (Ochiai *et al.*, 2008), *penA* (Ito *et al.*, 2005), *mtrR* (Mavroidi *et al.*, 2001), *porB$_{IB}$* (Tanaka *et al., 2006), ponA* (Ropp *et al.,* 2002)

[b] Amplify location inclusive the primers.

[c] Set B PCR was carried out to confirm the absence of mosaic *penA* sequence in isolates that were negative for mosaic *penA* PCR.

2.4.4. *N. gonorrhoeae* PCR protocol development

For mosaic *penA* the PCR kit used was the Platinum® Taq DNA Polymerase kit (Invitrogen NZ Ltd) and the deoxynucleotide triphosphates (dNTPs) used were from Promega NZ Ltd. The preparation of dNTPs for the mosaic *penA* PCR can be seen in **Table E6**. For *penA, mtrR, porB$_{IB}$* and *ponA* PCR the kit used was Kapa2G™ Robust PCR Kit, which includes a dNTPs mix (10mM each dNTP).

The optimization process was carried out for each PCR to determine the best protocol conditions and PCR master mix. The optimisation PCR protocol includes the necessary adjustment in the annealing step to reduce the number of nonspecific bands produced in the PCR product. The optimisation of the master mix was done by adjusting the concentration of primers and *Taq* polymerase.

Once the optimal PCR master mix was determined, a master mix with 20µL per reaction was calculated, allowing for the addition of 5µL of the DNA template. The tubes containing the PCR reactions were placed in a thermocycler (SensoQuest, Germany) and run using the selected protocol

For *penA*, *mtrR* and *ponA* the first protocol that was developed successfully produced a satisfactory result. No further optimisation was required for the PCR. The PCR protocol can be seen **Table 2-7** and **Table 2-8** (*penA*), **Table 2-9** and **Table 2-10** *(mtrR),* and **Table 2-11** and **Table 2-12** (*ponA*). For mosaic *penA* and *porB$_{IB}$* PCR, the optimised protocol was presented in the respective result section.

2.4.4.1. *PenA* PCR protocol

Table 2-7 Optimised PCR conditions for amplification of set A, set B, set C and set D of *penA*

PCR steps	Condition
Initial denaturation	1 cycle of 5 min at 95°C
Denaturation	40 cycles of 30 seconds at 95°C
Annealing	40 cycles of 15 seconds at 62°C
Extension	40 cycles of 30 seconds at 72°C
Final extension	1 cycle of 7 minutes at 72°C
Hold	At 10°C

Table 2-8 PCR master mix for amplification of set A, set B, set C and set D of *penA*

	Final concentration	Volume (µL)[1]
KAPA2G Robust DNA Polymerase	0.5U	0.1
5X KAPA2G Buffer [2]	1X	5
10mM dNTPs	200µM	0.5
10µM Primer_F [3]	0.5µM	1.25
10µM Primer_R [3]	0.5µM	1.25
Milli-Q Water		11.9
DNA template		5
		25

[1] Volumes for one PCR reaction.

[2] KAPA2G buffer contained 1.5mM $MgCl_2$ at 1X.

[3] Types of primer set are listed in **Table 2-6**.

2.4.4.2. *MtrR* PCR protocol

Table 2-9 PCR conditions for amplification of the *mtrR* gene

PCR steps	Condition
Initial denaturation	1 cycle of 5 min at 95°C
Denaturation	40 cycles of 30 seconds at 95°C
Annealing	40 cycles of 30 seconds at 55°C
Extension	40 cycles of 30 seconds at 72°C
Final extension	1 cycle of 7 minutes at 72°C
Hold	At 10°C

Table 2-10 PCR master mix used for amplification of the *mtrR* gene

	Final concentration	Volume (µL)[1]
KAPA2G Robust DNA Polymerase	0.5U	0.1
5X KAPA2G Buffer [2]	1X	5
10mM dNTPs	200µM	0.5
10µM Primer MTR1	0.5µM	1.25
10µM Primer MTR2	0.5µM	1.25
Milli-Q Water		11.9
DNA template		5
		25

[1] Volumes for one PCR reaction

[2] KAPA2G buffer contained 1.5mM $MgCl_2$ at 1X

2.4.4.3. *PonA* PCR protocol

Table 2-11 PCR conditions for amplification of the *ponA* gene

PCR steps	Condition
Initial denaturation	1 cycle of 5 min at 95°C
Denaturation	40 cycles of 30 seconds at 95°C
Annealing	40 cycles of 15 seconds at 62°C
Extension	40 cycles of 60 seconds at 72°C
Final extension	1 cycle of 7 minutes at 72°C
Hold	At 10°C

Table 2-12 PCR master mix used for amplification of the *ponA* gene

	Final Concentration	Volume (µL)[1]
KAPA2G Robust DNA Polymerase	0.5U	0.1
5X KAPA2G Buffer [2]	1X	5
10mM dNTPs	200µM	0.5
10µM Primer PonA1	0.2µM	0.5
10µM Primer PonA2	0.2µM	0.5
Milli-Q Water		13.4
DNA template		5
		25

[1] Volumes for one PCR reaction.

[2] KAPA2G buffer contained 1.5mM $MgCl_2$ at 1X.

2.4.5. Preparing the gel and visualising the PCR product

To observe the PCR product, 10µL of diluted PCR product plus 2µL loading dye that contains bromophenol blue (Bio-Rad) were added to the 1% agarose gel well (Invitrogen NZ). Similarly, the 10µl 100 kb DNA ladder (Invitrogen) was loaded in one of the well. The preparation of 1% agarose gel with safranin O (Intron Biotechnologist) is shown in **Table E7**. Safranin I was used over Ethidium Bromide (EtBr) because it is much safer and not carcinogenic.

The PCR products were run on 1% of an agarose gel (Invitrogen NZ) for 1 hour at 100V in an electrophoresis chamber (BRL, Life Technologies).

Once complete, the gel was then removed and placed in a UV light box (BioRad), with care taken to avoid creating bubbles underneath the gel. The Gel Doc XR system found in the Quantity One programme was used to capture a picture of the gel.

2.5. Sequencing

2.5.1. Preparation of DNA template for sequencing

For sequencing purposes, isolates that were positive for the target band were re-run again on 1% agarose gel for 1 hour 30 minutes at 40 V. This was to ensure that a clean and crisp band was obtained to prepare the DNA template for sequencing. The DNA band was cut out of the agarose gel using a sterile scalpel blade and put into a propylene tube. The gel was suspended in Elution Buffer (10mM TrisHCl, pH 8.0) and left overnight (22h-24 hours) at 4°C. The suspension was then spun for 10 minutes at 13,000 rpm (12,500g), and the supernatant, containing the eluted DNA, was used as a template for sequencing.

Two tubes were prepared for each isolate. 1µl of 10µM forward primer and 1µl of 10µM reverse primer for the particular PCR was added to each tube. Next, 20 µl of a solution containing PCR product, purified from the agarose gel, was added

to both of the tubes. The clean PCR product mixed with primer was sent to Massey Genome Service (MGS) for Sanger sequencing in both directions.

The nucleotide sequence of each isolate was determined through chromatograms. The sequence was determined from the base pair where a clean and crisp peak was observed. For reverse sequences, sequences were converted to their reverse-complement counterpart using Reverse Complement software (http://reverse-complement.com/). To convert to the amino acid sequence, the software package ExPasy (http://web.expasy.org/translate/) was used. Comparison of sequences was done using Multalin (http://multalin.toulouse.inra.fr/multalin/) and BLAST (Basic Local Alignment Search Tool).

2.5.2. Sequence comparison to confirm variations in amino acid sequence

To identify variations in each gene, comparisons of the sequences obtained from each isolate (from only one PCR run) were compared with the appropriate published control sequences. The list of the sequences used as controls to identify specific variations in each gene can be seen in **Table E8**.

For mosaic PBP2 *(penA)*, sequences of isolates were also compared to 20 published mosaic PBP2 sequences obtained from National Center for Biotechnology Information (NCBI). The amino acid sequences were also compared to *Neisseria perflava/sicca* 1654/1659 (GenBank accession no. X76422), *Neisseria cinerea* NCTC 10294 (GenBank accession no. X59540), Neisseria *flavescens* NCTC 8263 (GenBank accession no. M26645), and *Neisseria polysachharea* NCTC 11858 (GenBank accession no. X59626) to observe any variations that might rise from the *Neisseria* species. The mosaic PBP2 is known to be contributed by other *Neisseria* species through horizontal gene transfer (Ameyama *et al.*, 2002).

CHAPTER THREE

RESULTS

3.1. Phenotypic Results of *N. gonorrhoeae* isolates

3.1.1. Comparison between MIC results of ceftriaxone from ESR, MIC results using Liofilchem MIC test strips and bioMérieux Etest

Table 3-1 shows the MICs of ceftriaxone and penicillin G for *N. gonorrhoeae* isolates from ESR and the current study (using Liofilchem MIC test strips and bioMérieux Etest). The comparison of ceftriaxone MIC between two brands of MIC strips among 21 isolates indicated that 86% (18/21) of gonococcus isolates showed reduced susceptibility to ceftriaxone when both Liofilchem MIC and bioMérieux Etest strips were used (as illustrated in **Table F2**). The comparison between two brands of ceftriaxone MIC strips showed 100% agreement as illustrated in **Table F3**, where the differences in MIC reading were within ± 1 $\log_2$ dilution difference. There was no significant difference between MIC readings between these two brands (p=0.87) **(Table F3).**

Since there was no significant difference between the MIC values of the two MIC strips, ceftriaxone MIC results obtained with the Liofilchem MIC strips were used to represent the ceftriaxone MIC result of the current study.

In contrast to what has been reported by ESR where all 28 *N. gonorrhoeae* isolates showed reduced susceptibility to ceftriaxone (MIC from 0.03 mg/L to 0.12 mg/L), only 82% (23/28) of the isolates showed reduced susceptibility to ceftriaxone (MIC from 0.03 mg/L to 0.06 mg/L) in the current study, through using Liofilchem MIC test strips (as seen in **Table 3-1**). The comparison between these two set of results showed 86% agreement, where 24 isolates exhibited ± 1 $\log_2$ dilution differences in MIC value readings. A total of 14% (4/28) isolates showed more than 1 $\log_2$ dilution difference in the MIC reading, with higher MIC values yielded by ESR. We also observed a significant difference (p<0.05) between the MIC values of ceftriaxone from the current study and the MIC values of

ceftriaxone from ESR. The comparison is illustrated in **Table F1** and the agreement between the two sets of results is summarised in **Table F5**. A repeat MIC testing for ceftriaxone on isolates 963, 1026, 1380 and 1641 that showed the lowest ceftriaxone MIC (isolate 963: 0.008 mg/L, while isolate 1026, 1380 and 1641: 0.01 mg/L) yielded the same MIC results.

In comparison to ESR result, isolate 1641 showed the greatest discrepancy in MIC reading, with 3 $\log_2$ fold difference was observed. Isolate 1641 showed a change in the susceptibility category, which is from reduced susceptibility to ceftriaxone reported by ESR (0.12 mg/L) to fully susceptible to ceftriaxone (0.01 mg/L) in the current study. Another point to consider is that, isolates 558, 631, 723, 792 and 1848 with the highest ceftriaxone MIC from ESR (all 0.12mg/L) showed lower MIC level when the MIC test was repeated in the current study (as shown in **Table F4**).

On the other hand, the MICs of ceftriaxone for control strains WHOK, WHOF, WHOL and ATCC 49226 from the current study were all within the standard ranges based on the guideline stated in **Table 2-2** in Materials and Method section.

3.1.2. Comparison between MICs of penicillin G determined by Liofilchem MIC test strips and bioMérieux Etest

For penicillin G, 38% (8/21) isolates were resistant to the antibiotic when Liofilchem MIC strips were used, while 24% (5/21) isolates were resistant to penicillin G when bioMérieux Etest strips were used **(Table F2)**. As shown in **Table F3**, the comparison between two brands of MIC strips showed 96% of the isolates were in agreement, where the differences in MIC reading were within ± 1 $\log_2$ dilution difference. Regardless, no significant difference between penicillin G MIC readings was observed between these two brands (p=0.77) as shown in **Table F3**.

The penicillin G MIC results for control strains WHO K, WHO F, WHO L and ATCC 49226 in the study above were all in a valid range based on the guideline set by **Table 2-2**. The MIC results can be seen in **Table 3-1**. Similar with ceftriaxone, penicillin G MIC results obtained from using Liofilchem MIC test strips were used to represent the result of the current study since there was no significant difference between the MIC values of the two MIC strips.

3.1.3. Antimicrobial susceptibility testing (AST) result of penicillin G, tetracycline, ciprofloxacin, azithromycin and ceftriaxone of *N. gonorrhoeae* with reduced susceptibility to ceftriaxone

The results of the antimicrobial susceptibility testing (AST) for *N. gonorrhoeae* isolates for penicillin and ceftriaxone (based on result using Liofilchem MIC test strips) are shown in **Table 3-1**. For tetracycline, azithromycin and ciprofloxacin, the AST results are illustrated in **Table 3-2**. Based on the AST summary in **Table 3-3**, a total of 82% (23/28) isolates showed reduced susceptibility to ceftriaxone from this study, with MIC ranging from 0.03 to 0.06 mg/L. All isolates were resistant to ciprofloxacin, with MIC range between 2 mg/L to 32 mg/L. The rate of resistance (indeterminate susceptibility) of penicillin G, azithromycin, tetracycline

were 36% (64%), 7% (54%), and 25% (36%), respectively. Two isolates that were resistant to azithromycin had MICs of 12 mg/L (isolate 1380) and 4 mg/L (isolate 264).

A total of 21% (6/28) were positive for β-lactamase production **(Table 3-2)**. Isolate 729, 119 and 1641 were resistant to penicillin G, with MIC >32 mg/L, while isolates, 263, 724 and 824 had indeterminate resistance to penicillin G, with MIC ranges from 0.5 mg/L to 1.0 mg/L. The association of β-lactamase and MIC level of penicillin G can be seen in **Table F6**.

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Table 3-1 MIC of ceftriaxone and penicillin for *N. gonorrhoeae* isolates and control strains

	MIC (mg/L) [a]				
	Ceftriaxone [d]			Penicillin G [e]	
Isolate [b]	ESR [c]	Liofilchem	bioMérieux	Liofilchem	bioMérieux
729	0.06 (RS)	0.06 (RS)	0.06 (RS)	>32 (R)	>32 (R)
119	0.06 (RS)	0.06 (RS)	0.06 (RS)	>32 (R)	>32 (R)
1641	0.12 (RS)	0.01 /0.01(S)	0.01/0.01 (S)	>32 (R)	>32 (R)
631	0.12 (RS)	0.06 (RS)	0.12 (RS)	2 (R)	2 (R)
71	0.06 (RS)	0.06 (RS)	0.06 (RS)	2 (R)	2 (R)
604	0.06 (RS)	0.06 (RS)	0.03 (RS)	1 (I)	1 (I)
558	0.12 (RS)	0.06 (RS)	0.03 (RS)	1 (I)	1 (I)
723	0.12 (RS)	0.06 (RS)	0.03 (RS)	1 (I)	1 (I)
801	0.06 (RS)	0.06 (RS)	0.06 (RS)	1 (I)	1 (I)
1526	0.06 (RS)	0.03 (RS)	0.06 (RS)	2 (R)	1 (I)
1530	0.06 (RS)	0.03 (RS)	0.03 (RS)	2 (R)	1 (I)
1380	0.06 (RS)	0.01 /0.01(S)	0.01/0.01 (S)	1 (I)	1 (I)
411	0.03 (RS)	0.06 (RS)	0.03 (RS)	1 (I)	1 (I)
557	0.03 (RS)	0.06 (RS)	0.03 (RS)	0.5 (I)	1 (I)
886	0.06 (RS)	0.06 (RS)	0.03 (RS)	0.5 (I)	1 (I)
263	0.06 (RS)	0.03 (RS)	0.03 (RS)	1 (I)	1 (I)
724	0.06 (RS)	0.03 (RS)	0.03 (RS)	1 (I)	1 (I)
824	0.06 (RS)	0.03 (RS)	0.03 (RS)	0.5 (I)	0.5 (I)
257	0.03 (RS)	0.03 (RS)	0.06 (RS)	0.5 (I)	0.5 (I)
483	0.03 (RS)	0.03 (RS)	0.03 (RS)	0.5 (I)	0.5 (I)
1026	0.06 (RS)	0.01/0.01 (S)	0.01 /0.01(S)	2 (R)	0.5 (I)
264	0.06 (RS)	0.03 (RS)	-	0.25 (I)	-
792	0.12 (RS)	0.06 (RS)	-	0.5 (I)	-
893	0.03 (RS)	0.01 (S)	-	0.25 (I)	-
896	0.06 (RS)	0.06 (RS)	-	2 (R)	-
963	0.06 (RS)	0.008/0.008 (S)	-	0.25 (I)	-
1332	0.06 (RS)	0.03 (RS)	-	0.5 (I)	-
1848	0.12 (RS)	0.06 (RS)	-	2 (R)	-
WHO K	N/A	0.06 (RS)	0.06 (RS)	2 (R)	2.0 (R)

WHO L	N/A	0.06 (RS)	0.06 (RS)	2 (R)	2.0 (R)
WHO F	N/A	<0.002 (S)	<0.002 (S)	0.032 (S)	0.032 (S)
ATCC 49226	N/A	0.008 (S)	0.008 (S)	1 (I)	0.5 (I)

[a] Interpretative criteria for MIC test was determined using EUCAST guidelines **(Table 2-3).** Where the MIC result falls between resistant (R) and susceptible (S) range, the MICs were categorised as indeterminate (I). However for ceftriaxone, the MIC range for reduced susceptibility (RS) is from 0.03 mg/L to 0.12 mg/L (Whiley *et al.*, 2007a; Tapsall *et al.*, 2009a; Chen *et al.*, 2013; ESR, 2014). All of the MIC readings have been round up to the next doubling dilution before interpretation.

[b] MIC ranges of ceftriaxone and penicillin G for WHO K, WHO L, WHO F and ATCC 49226 can be seen in **Table 2-2** in Material & methods section.

[c] For strain 264, 792, 893, 896, 963 and 1332, ESR determined the MIC of ceftriaxone using the agar dilution method. While for other isolates, the MICs were determined using bioMérieux Etest strips.

[d] Second sampled from isolate 963, 1641, 1380 and 1026 were obtained from ESR for repeat testing. The MIC of ceftriaxone yielded the same result for both Liofilchem and bioMérieux.

N/A: Not applicable; (-): MIC test was not done

Table 3-2 MICs of tetracycline, ciprofloxacin, azithromycin, and nitrocefin results for *N. gonorrhoeae* isolates and control strains

Isolate [c]	MICs (mg/L) [a b]			Nitrocefin [d]
	Tetracycline	Ciprofloxacin [e]	Azithromycin	
729	32 (R)	16 (R)	0.25 (S)	+
119	0.5 (S)	16 (R)	0.25 (S)	+
1641	32 (R)	>32 (R)	0.12 (S)	+
631	2 (R)	>32 (R)	0.5 (I)	-
71	1 (I)	>32 (R)	0.5 (I)	-
604	2 (R)	>32 (R)	0.5 (I)	-
558	1 (I)	>32 (R)	0.5 (I)	-
723	1 (I)	>32 (R)	0.5 (I)	-
801	0.5 (S)	8 (R)	0.5 (I)	-
1526	2 (R)	>32(R)	0.5 (I)	-
1530	2 (R)	>32(R)	0.5 (I)	-
1380	1 (I)	>32(R)	12 (R)	-
411	2 (R)	>32(R)	0.5 (I)	-
557	1 (I)	>32(R)	0.5 (I)	-
886	0.5 (S)	2 (R)	0.5 (I)	-
263	1 (I)	8 (R)	0.25 (S)	+
724	1 (I)	>32 (R)	0.25 (S)	+
824	1 (I)	16 (R)	0.25 (S)	+
257	0.5 (S)	16 (R)	0.50 (I)	-
483	1 (I)	8 (R)	0.50 (I)	-
1026	0.5 (S)	2 (R)	0.50 (I)	-
264	0.5 (S)	4 (R)	4 (R)	-
792	0.5 (S)	2 (R)	0.25 (S)	-

893	0.5 (S)	8 (R)	0.50 (I)	-
896	1 (I)	>32 (R)	0.25 (S)	-
963	0.25 (S)	4 (R)	0.12 (S)	-
1332	0.5 (S)	2 (R)	0.25 (S)	-
1848	0.5 (S)	>32 (R)	0.25 (S)	-
WHO K	1 (I)	>32 (R)	0.25 (S)	-
WHO L	1 (I)	>32 (R)	0.25 (S)	-
WHO F	0.125 (S)	0.003 (S)	0.25 (S)	-
ATCC 49226	0.50 (I)	0.003 (S)	0.5 (S)	-

[a] All of the MIC readings have been round up to the next doubling dilution before interpretation. Interpretative criteria for MIC test was determined using EUCAST guidelines **(Table 2-3).** Where the MICs results fell between resistant (R) and susceptible (S) cut-off, the MICs was categorised as indeterminate (I).

[b] Liofilchem s.r.l (Italy) MIC strips were used to determine the susceptibility of gonococcus to tetracycline, ciprofloxacin and azithromycin.

[c] Published ranges of ceftriaxone and penicillin G for WHO K, WHO L, WHO F and ATCC 49226 can be seen in **Table 2-2** in the Materials and Methods section.

[d] (+) = Positive for β- lactamase; (-) = Negative for β-lactamase.

N/A: Not applicable; (-): MIC test was not done

Table 3-3 Antibiotic susceptibility of *N. gonorrhoeae* isolates based on The European Committee of Antimicrobial Susceptibility Testing (EUCAST) guidelines

Antimicrobial agents	EUCAST breakpoint	No. (%) of isolates showing:		
		S	I / RS [a]	R
Penicillin G	**S ≤ 0.06 / R > 1**	0	18 (64%)	10 (36%)
Tetracycline	**S ≤ 0.5 / R >1.0**	11 (39%)	10 (36%)	7 (25%)
Ciprofloxacin	**S ≤ 0.03 /R > 0.06**	0	0	28 (100%)
Azithromycin	**S ≤ 0.25/R > 0.5**	11 (39%)	15 (54%)	2 (7%)
Ceftriaxone [b]	**S ≤ 0.125/R > 0.125**	5 (18%)	23 (82%)	0

Note: S: Susceptible; I: Indeterminate; R: Resistant; RS: Reduced susceptibility

[a] The MIC range that falls between resistant (R) and susceptible (S) category was categorised as indeterminate (I). This rule applied for all antibiotics in this study except for ceftriaxone (which was categorised under reduced susceptibility (RS)).

[b] MIC for RS to ceftriaxone for *N. gonorrhoeae* is between 0.03 mg/L to 0.12 mg/L (Whiley *et al.*, 2007a; Tapsall *et al.*, 2009a; Chen *et al.*, 2013; ESR, 2014).

3.1.4. Antimicrobial susceptibility testing (AST) result comparison between European Committee of Antimicrobial Susceptibility Testing (EUCAST) Guideline and Clinical & Laboratory Standards Institute (CLSI) guideline

The antimicrobial susceptibility testing (AST) result comparison between European Committee of Antimicrobial Susceptibility Testing (EUCAST) Guideline and Clinical & Laboratory Standards Institute (CLSI) guideline can be seen in **Table 3-4**.

According to the CLSI MIC breakpoints, 36% (10/28) and 64% (18/28) isolates, respectively, showed resistance to or were indeterminate for penicillin G, similar to when the EUCAST guideline was used **(Figure 3-1)**.

For tetracycline, 4% (1/28), 71% (20/28) and 25% (7/28) isolates respectively showed susceptible, indeterminate and resistant MICs when using CLSI breakpoints, while 39% (11/28), 36% (10/28) and 25% (7/28) isolates showed susceptible, indeterminate and resistant level to tetracycline when EUCAST MIC breakpoints were used **(Figure 3-2)**.

For azithromycin, CLSI has not yet established breakpoints for *N. gonorrhoeae*. For the purpose of comparison, the breakpoints established by the CDC for the Gonococcal Isolate Surveillance Project (GISP) was used (Kirkcaldy *et al.*, 2015) to compared with the EUCAST guidelines. The study revealed that 93% (26/28) were susceptible to azithromycin when the guideline by CDC was used, while only 39% (11/28) isolates were found to be susceptible when EUCAST breakpoints were used **(Figure 3-3).** Additionally for azithromycin, none of the isolates were indeterminate for azithromycin when breakpoints established by CDC were used. However, 54% (15/28) isolates were indeterminate for azithromycin based on the EUCAST breakpoints for resistant (>0.5 mg/L) and

susceptible (≤0.25 mg/L). Both guidelines showed that 7% (2/28) of isolates were resistant to azithromycin since the two isolates that were resistant showed an MIC of ≥ 4mg/L.

For ciprofloxacin and ceftriaxone, results were similar for both EUCAST and CLSI guidelines. Even though the breakpoints for resistance and susceptible were different between the two guidelines, all isolates were resistant to ciprofloxacin while no isolates showed resistance to ceftriaxone.

The penicillin G, tetracycline, azithromycin MIC distributions with EUCAST and CLSI guideline are illustrated in **Figure 3-1**, **Figure 3-2**, and **Figure 3-3**, respectively.

Table 3-4 Comparison of antibiotic susceptibility of *N. gonorrhoeae* isolates using EUCAST and CLSI guideline

Antimicrobial agents	EUCAST breakpoints (mg/L)	No. (%) of isolates showing [a] :			CLSI breakpoints [d] (mg/L)	No. (%) of isolates showing [a] :		
		S	I /RS [b c]	R		S	I / RS	R
Penicillin G	**S ≤ 0.06 / R > 1**	0	18 (64%)	10 (36%)	**S ≤ 0.06 / I = 0.12-1.0/ R ≥ 2**	0	18 (64%)	10 (36%)
Tetracycline	**S ≤ 0.5 / R >1.0**	11 (39%)	10 (36%)	7 (25%)	**S ≤ 0.25 / I = 0.5-1.0/ R ≥ 2**	1(4%)	20 (71 %)	7 (25%)
Ciprofloxacin	**S ≤ 0.03 /R > 0.06**	0	0	28 (100%)	**S ≤ 0.06 / I = 0.12-0.5/ R ≥ 1**	0	0	28 (100%)
Azithromycin	**S ≤ 0.25/R > 0.5**	11 (39%)	15 (54%)	2 (7%)	**S≤1.0 /R ≥ 2** [e]	26 (93%)	0	2 (7%)
Ceftriaxone [b]	**S ≤ 0.125/R > 0.125**	5 (18%)	23 (82%)	0	**S ≤ 0.25/ R > 0.25**	5 (18%)	23 (82%)	0

[a] Percentage is converted to the nearest decimal point to ease comparison.

[b] Where EUCAST guidelines breakpoints do not stated the indeterminate (I) category, the MIC range that falls between resistant (R) and susceptible (S) category is categorised as indeterminate (I) (refer to Table 2-3). This rule applies for all antibiotics in this study except for ceftriaxone (which is categorised under reduced susceptibility (RS)). Refer to note c.

[c] MIC range for reduced susceptibility (RS) to ceftriaxone for *N. gonorrhoeae* is 0.03-0.12 mg/L (Whiley *et al.*, 2007a; Tapsall *et al.*, 2009a; Chen *et al.*, 2013; ESR, 2014)

[d] Source:(CLSI, 2015) accept for azithromycin (see d).

[e] Since CLSI has not yet established the breakpoints for azithromycin for gonococci, the breakpoint is adopted from CDC for Gonococcal Isolate Surveillance Project (Kirkcaldy et al., 2015)

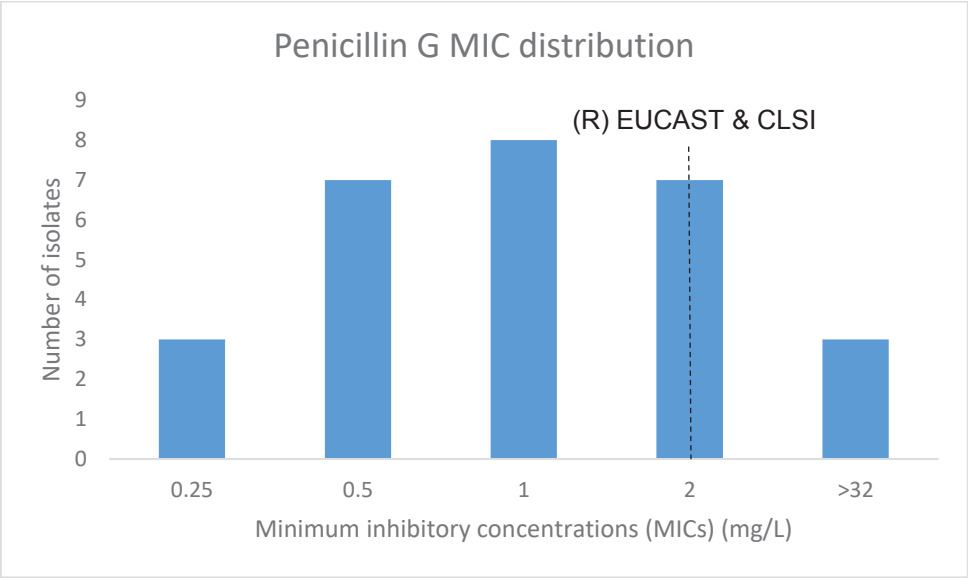


Figure 3-1Penicillin G MIC distributions with EUCAST and CLSI guidelines

Note:

1. Susceptible (S) cut-off (S < 0.06 mg/L) for both CLSI and EUCAST is out of range in this graph.
2. Resistance (R).
3. Dash lines denote the breakpoints of the guidelines: EUCAST (R > 1); CLSI (R ≥ 2).

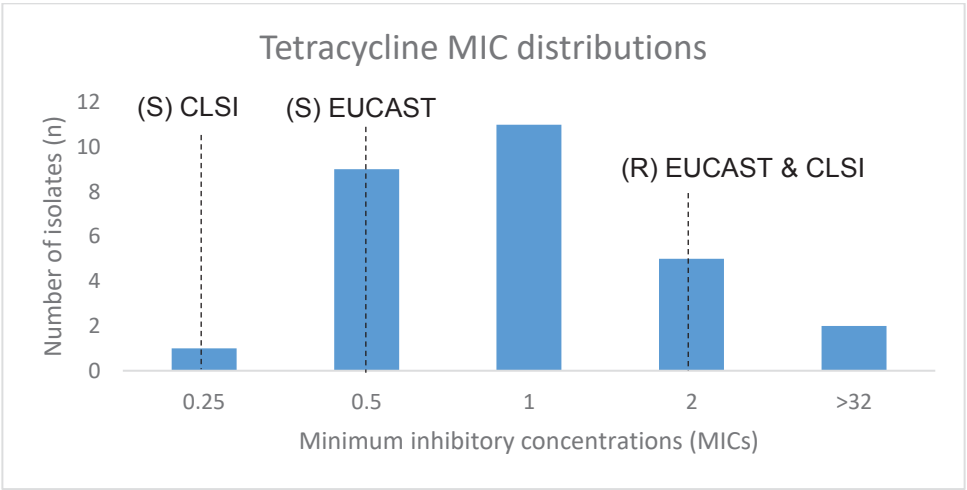


Figure 3-2 Tetracycline MIC distributions with EUCAST and CLSI guidelines

Note:

1. Susceptible (S) cut-off (S < 0.25 mg/L) for CLSI is out of range in this graph.
2. Resistance (R).
3. Dash lines denote the breakpoints of the guidelines: EUCAST (S ≤ 0.5, R > 1.0); CLSI (R ≥ 2).

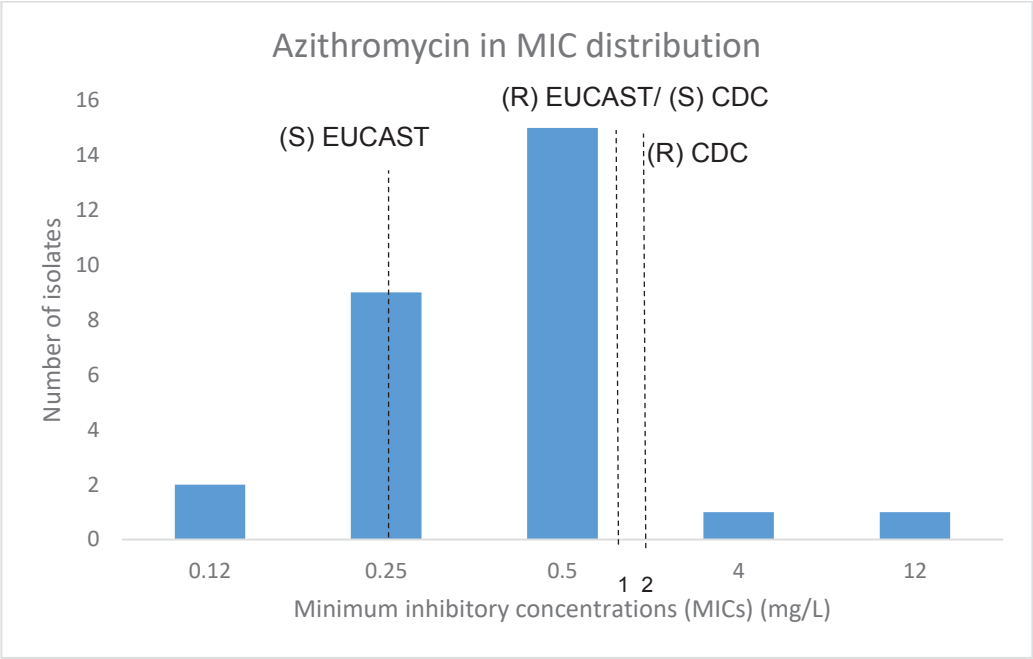


Figure 3-3 Azithromycin MIC distributions with EUCAST and CDC breakpoints established for GISP

Note: Dashed lines denote the breakpoints of the guidelines: EUCAST (S ≤ 0.25); CDC (S ≤ 1.0).

3.1.5. Effect of using GC II agar, chocolate supplemented agar, and GC saponin agar prior to MIC test for penicillin G and ceftriaxone MIC result

.

The comparison between MIC results of penicillin G for 25 *N. gonorrhoeae* that were grown on GC saponin agar, chocolate supplemented agar and GCII agar prior to MIC testing can be seen in **Table F7**. As summarised in **Table F9**, all 25 isolates grown on GC saponin agar and chocolate supplemented agar showed 100% agreement in the MIC level of penicillin G (± 1 $\log_2$ fold dilution). Similarly, the comparison between MIC results of penicillin G for *N. gonorrhoeae* that were grown on GC II agar and chocolate supplemented agar prior to MIC test showed 100% agreement, based on **Table F10**. There is no significant difference observed between MIC results produced between using GC saponin agar and chocolate supplemented agar (p=0.98) and between GC II agar and chocolate supplemented agar (p=1.0)

Similarly for ceftriaxone, the MIC comparison between *N. gonorrhoeae* that were grown on GC saponin agar and chocolate supplemented agar prior to MIC test can be seen in **Table F8**. Based on the summary in **Table F9**, only 100% (20/25) showed agreement between the two media where the MIC value are within ± 1 $\log_2$ fold dilution difference. There is no significant difference observed between MIC results produced from using different agar in this study (p = 1.00 (ceftriaxone)).

On the other hand, the comparison between MIC results of ceftriaxone for *N. gonorrhoeae* that were grown on GC II agar and chocolate supplemented agar prior to MIC test showed only 100% agreement of MIC value was reported, as shown in **Table F10**. Only 23 isolates culture on GC II agar and chocolate supplemented agar showed within ± $\log_2$ fold dilution difference while two isolates showed more than 1 $\log_2$ fold dilution difference, while two isolates was higher when GC II agar was used to grow the isolates prior to MIC test, instead of using

chocolate supplemented agar. Regardless, there is no significant difference observed in the MIC results when different media were used to grow the isolates (p = 1.00 (ceftriaxone)).

3.1.6. Comparison of ceftriaxone MIC of isolate 963 and WHO K control strain incubated with 3% CO_2 and 5% CO_2

The comparison between the MIC of ceftriaxone, colony forming unit and the colony morphologies of isolate 963 and WHO K can be seen in **Table F11** and F12. For both isolates, the size of colony was estimated to be 2 mm were from culture plate that were incubated in candle jar (with 3% CO_2) compared to the culture plate incubated in CO_2 incubator, which was 1mm.

Secondly, the count of colony forming unit of 0.5 McFarland standard was slightly higher from plates incubated in the candle jar compared to the plates incubated in CO_2 incubator. For isolate 963, the total colony forming units from candle jar incubation is 1.2 (±0.35) x 10^8 CFU/ml), while for CO_2 incubator is 1.0 (± 0.31) x 10^8. On the other hand for WHO K control strain, the total colony forming units from candle jar incubation is 1.1 (±0.3) x 10^8 CFU/ml), while for CO_2 incubator is 0.9 (± 0.3) x 10^8. All the previously mentioned CFU readings are slightly lower than the standard 0.5 McFarland standard colony forming unit that is 1.5 x 10^8.

Regardless the slight differences in the CFU readings and the morphology of the colonies, the MICs of ceftriaxone yielded through using candle jar and CO_2 incubator were the same for both isolate 963 and WHO K control strain. The MIC of ceftriaxone for isolate 963 is 0.008 mg/L while for WHO K control strain; the MIC of ceftriaxone is 0.05 mg/L.

3.2. *PenA* (PBP2) analysis of *N. gonorrhoeae* isolates

3.2.1. Mosaic *penA* PCR and sequencing

Three different protocols were evaluated during the optimisation process. The optimisation that involved adjusting the concentration of primer, *Taq* polymerase and improving the PCR protocol conditions, are included in **Table 3-5**, **Table 3-6**, **Table G1** and **Table G2** (and **Figure G1** and **Figure G2**). A gel photo of the amplified products can be seen in **Figure 3-4**. Protocol 2 was chosen since the protocol reduced the number of non-specific products without reducing the desired amplicon.

Table 3-5 Optimised PCR conditions for mosaic *penA* (Protocol 2)

PCR steps	Condition
Initial denaturation	1 cycle of 5 min at 95°C
Denaturation	40 cycles of 30 seconds at 95°C
Annealing	40 cycles of 15 seconds at 62°C
Extension	40 cycles of 30 seconds at 72°C
Final extension	1 cycle of 7 minutes at 72°C
Hold	At 10°C

Table 3-6 Optimised PCR master mix for mosaic *penA* PCR (Protocol 2)

	Final Concentration	Volume (µL) [a]
Platinum Taq DNA Polymerase	1U	0.2
10X PCR Buffer	1X	2.5
50mM $MgCl_2$	1.5mM	0.75
10mM dNTPs	200µM	2
10µM Primer_F	0.5µM	1.25
10µM Primer_R	0.5µM	1.25
Milli-Q Water		12.05
DNA Template		5
		25

[a] Volume for one PCR reaction.

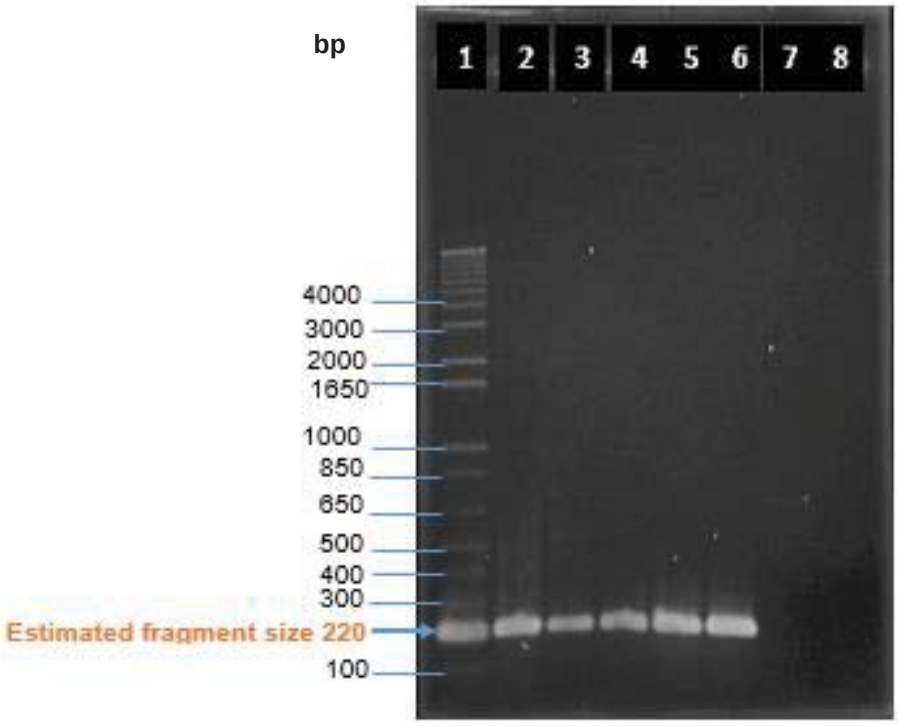


Figure 3-4 Gel electrophoresis of mosaic *penA* amplicons from DNA extracted from *N. gonorrhoeae* isolates. Lane 1: 100 bp molecular weight marker, plus WHO K (positive control). Lanes 2-6: mosaic *penA* amplicons from isolate 1026, 263, 119, 824, 724, respectively. Lane 7: ATCC 49226 (negative control). Lane 8: Blank.

Gel electrophoresis of mosaic *penA* products from the optimised PCR protocol can be seen in **Figure 3-4**. The gel migration of PCR product from mosaic

penA was measured (**Table G3**), and comparison made to a standard curve derived from the 100 bp molecular weight marker (**Figure G3**). The length of amplified products was estimated to be 220 bp.

3.2.2. Mosaic *penA* in *N. gonorrhoeae* isolates

A summary of the mosaic *penA* results can be seen in **Table 3-7** and **Table 3-8**. A total of 79% (22/28) of *N. gonorrhoeae* isolates were found to be positive for the 220 bp mosaic *penA* fragment. All of the amplified mosaic *penA* of the 22 positive isolates were leached using elution buffer (10mM TrisHCl, pH 8.0) and sequenced. Mosaic *penA* sequencing results obtained from Massey Genome Service (MGS) showed that 20 out of 22 isolates, plus WHO K strain, gave crisp and clean sequencing results for both forward and reverse sequences. However, for isolates 557 and 729, weak sequence results were produced for the reverse sequence. As a result, the forward sequence was used for mosaic *penA* analysis for the two isolates. The summary of the sequencing results **(Table I)** and the chromatograms for all isolates can be seen in "Mosaic *penA*" folder stored in the DVD enclosed at the end of the thesis.

The alignment of mosaic *penA* amplicon sequences (from 843 bp to 977 bp) of all 22 isolates and WHO K control showed 100% similar nucleotide sequences as shown in **Figure 3-5**.

Table 3-7 Mosaic *penA* PCR for *N. gonorrhoeae* isolates

Isolates	Mosaic *penA* PCR
1530	Positive
604	
483	
557	
729	
257	
886	
558	
1526	
483	
1848	
801	
411	
1641	
723	
631	
824	
724	
71	
1026	
263	
119	
963	Negative
893	
1380	
1332	
264	
792	
WHO F	
WHO L	
WHO K	Positive
ATCC 49226	Negative

Note: 1. Negative for mosaic *penA* PCR in both first and second run.

Table 3-8 Summary of mosaic *penA* PCR results for *N. gonorrhoeae* isolates

Mosaic *penA* (220 bp) [a]	*N. gonorrhoeae* with reduced susceptibility to ceftriaxone	
	Number of isolates (n)	Percentage (%)
Positive	22	79%
Negative	6	21%

[a] The gene was amplified between 843 to 977 bp

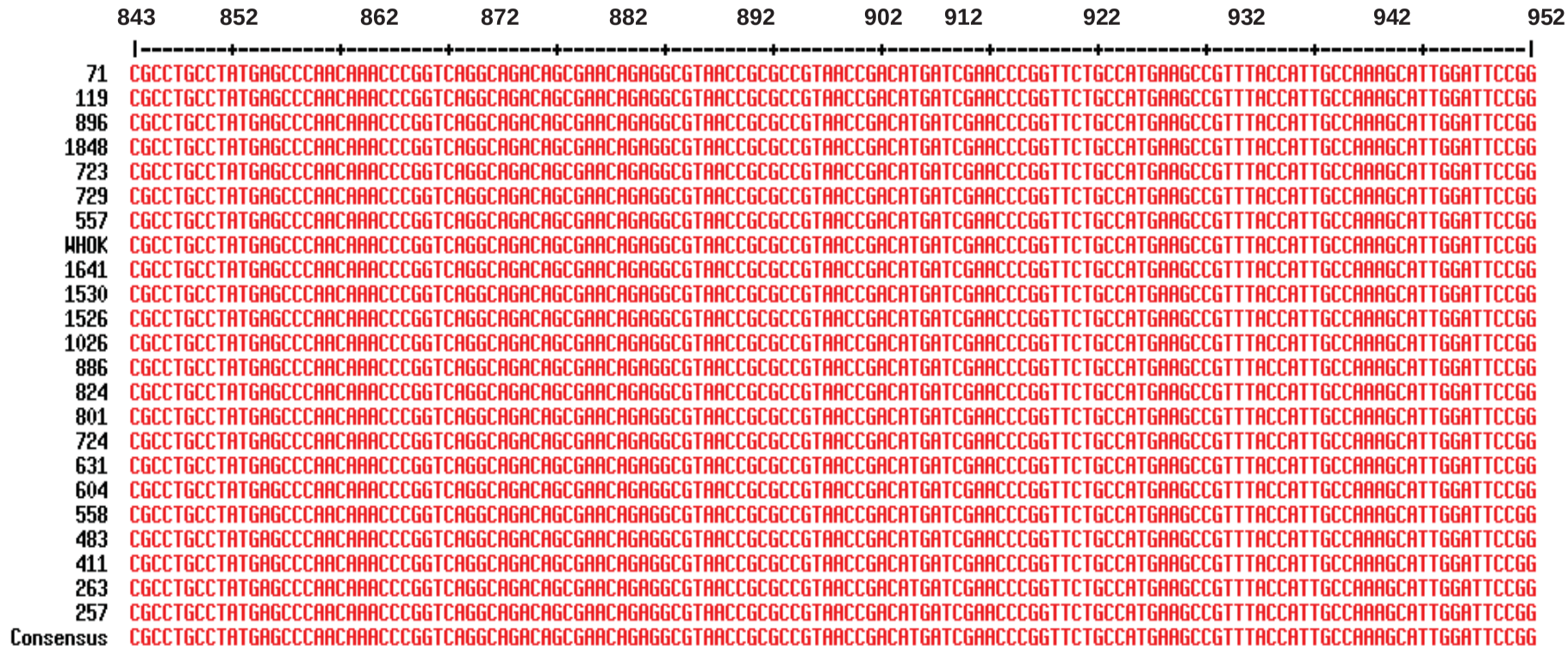


Figure 3-5 Alignment of mosaic *penA* of *N. gonorrhoeae* isolates and WHO K control (from 843 bp to 972 bp) produced using Multalin. Nucleotides that are completely conserved in all sequences in this population of genes are shown in red. Sequence numbering is based on *penA* of wildtype *N. gonorrhoeae* LM306 (GenBank accession no. M32091.1) (Ameyama *et al*., 2002).

Note: 1. Consensus sequence represents the nucleotide sequence agreement for all 22 *N. gonorrhoeae* isolates.

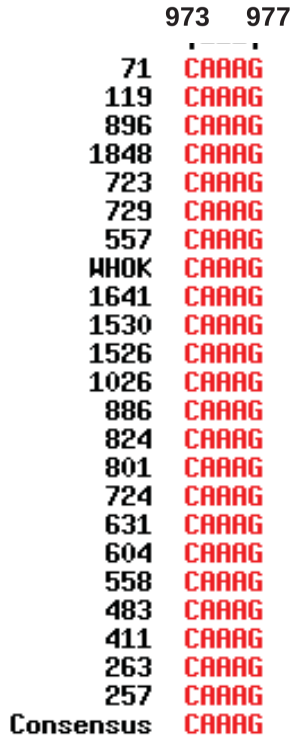


Figure 3-5 Continue Alignment of mosaic *penA* of *N. gonorrhoeae* isolates and WHO K control (from 973 bp to 977 bp) produced using Multalin. Nucleotides that are completely conserved in all sequences in this population of genes are shown in red. Sequence numbering is based on *penA* of wildtype *N. gonorrhoeae* LM306 (GenBank accession no. M32091.1) (Ameyama *et al.*, 2002).

Note: 1. Consensus sequence represents the nucleotide sequence agreement for all 22 *N. gonorrhoeae* isolates.

3.2.3. Comparison of mosaic *penA* (PBP2) of *N. gonorrhoeae* isolates with various published *penA* (PBP2) sequence

The consensus mosaic *penA* sequence (represents the mosaic *penA* sequence of 22 *N. gonorrhoeae* isolates) was compared to the *penA* sequence of *N gonorrhoeae* LM306 wild-type strain (GenBank accession no. M32091.1; Protein ID: AAA2543.1) and *N. gonorrhoeae* NG-3 which is the first published mosaic PBP2 sequence (GenBank accession no. AB071894; Protein ID: BAB86942.1) **(Figure 3-6)**. The mosaic *penA* consensus sequence (between 843 to 977 bp) was 100% identical to the sequence of *N. gonorrhoeae* NG-3. When compared to the wildtype LM306, 18% (24/134) nucleotides of the consensus mosaic *penA* sequence were different **(Table 3-9).**

Next, the comparison of consensus mosaic PBP2 amino acid sequence (276 to 329 amino acid residues) with the wildtype *N gonorrhoeae* LM306 **(Figure 3-7)**, showed that the mosaic PBP2 sequence in the current study harboured ten amino acid variations. As depicted in **Table 3-11**. the amino acid alterations include the key changes in mosaic PBP2: the I312M and V316T, plus eight other amino acid alterations, A279V, D285E, R288K, R291Q, A323S, T326V, L328A and N329T. The amino acid variations of the 22 gonococcus isolates were identical to that of cefixime-resistant and ceftriaxone-reduced susceptible strain *N. gonorrhoeae* NG-3. The list of the amino acid variations detected can be seen in **Table 3-10**.

Comparison with 19 published PBP2 sequences from the National Center for Biotechnology Information (NCBI**) (Figure G4)**, showed that the mosaic PBP2 sequence observed in the current study was 100% identical with 15 PBP2 sequences (Group A), including F89 strain (GenBank: JQ073701).

The mosaic PBP2 sequence of the 22 *N. gonorrhoeae* isolates was compared with *N. flavescens* NCTC 8263 (GenBank accession no. M26645), *N. cinerea*

NCTC 10924 (GenBank accession no. X59540), *N. polysaccharea* NCTC 11858 (GenBank accession no. X59626) and *N. perflava/sicca* 1654/1659 (GenBank accession no. X67442) as illustrated in Figure G5. A summarised in **Table G4**, the mosaic PBP2 of the consensus sequence was almost identical to *N.perflava/sicca* 1654/1659 (94.4% similarity), followed by *N. flavescens* (92.5% similarity), *N. polysaccharea* NCTC 11858 (87.3% similarity) and *N. cinerea* NCTC 10294 (85.5% similarity). The mosaic PBP2 in this study (the consensus sequence), *N. perflava/sicca* and *N. flavescens* harbour key alterations I312M and V316T.

The summary of the types of alteration found in the comparison with four *Neisseria* species is presented in **Table G5**. The comparison was made since *Neisseria* species was known to transfer part of their *penA* (PBP2) to *N. gonorrhoeae* through horizontal gene transfer.

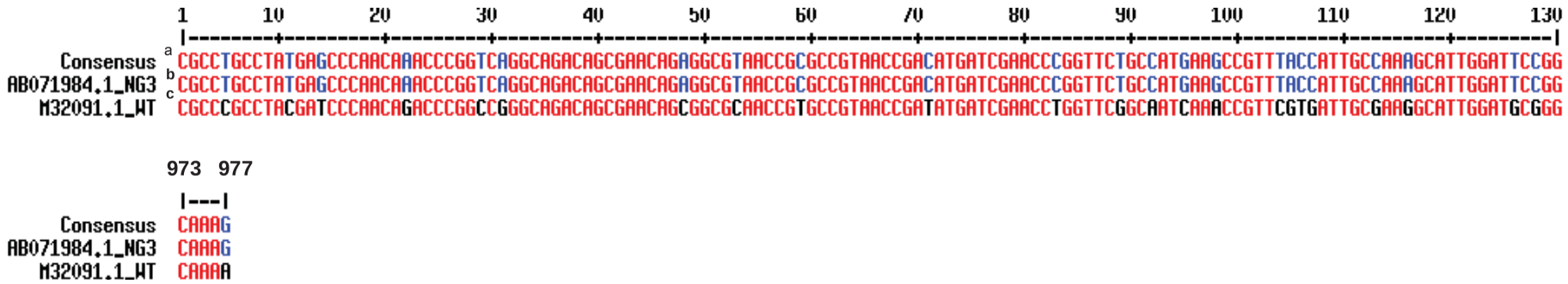


Figure 3-6 Comparison of consensus sequence for mosaic *penA* amplicons (843 bp to 977 bp) with wildtype *N. gonorrhoeae* LM306 (M32091.1_WT) (GenBank accession no. M32091.1) (Ameyama *et al.*, 2002) and *N. gonorrhoeae* NG-3 (GenBank accession no. AB071984.1) (Ameyama *et al.*, 2002) using Multalin. Sequence numbering is based on wildtype *N. gonorrhoeae* LM306 (GenBank accession no. M32091.1). Nucleotides that are completely conserved in all sequences in this population of genes are shown in red. Nucleotides that appear blue or black indicate variation in the sequences, with nucleotides in blue having greater consensus than the nucleotides in black.

[a] Consensus sequence represents the nucleotide sequence agreement for all 22 *N. gonorrhoeae* isolates.

[b] *N. gonorrhoeae* strain NG-3 (AB071984.1_NG3) is resistant to cefixime and shows reduced susceptibility to ceftriaxone and has mosaic *PenA* pattern X. It is the first mosaic *penA* sequence published (Ameyama *et al.*, 2002).

[c] Wild-type *N. gonorrhoeae* LM 306 (M32091.1) (Ameyama *et al.*, 2002).

Table 3-9 Nucleotides comparison between mosaic *penA* of *N. gonorrhoeae* isolates with NG-3 and LM306 strain

Strain	Different nucleotides (%)	Identical nucleotides (%)
***N. gonorrhoeae* NG-3**	0 (0)	134 (100)
***N. gonorrhoeae* LM306**	24 (17.9)	110 (81.2)

Note: Total number of nucleotides :134bp

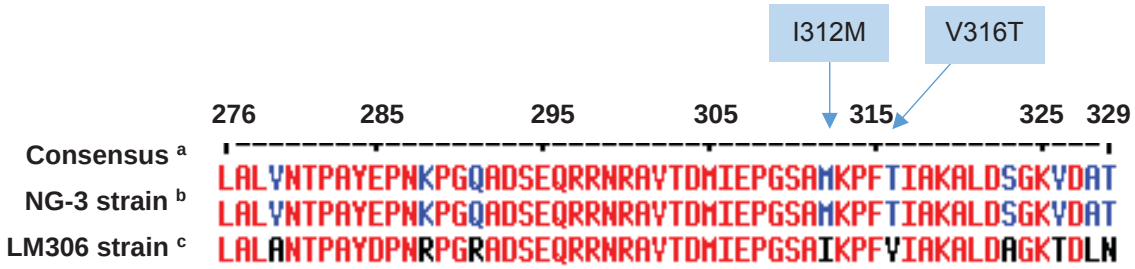


Figure 3-7 Comparison of the mosaic PBP2 sequence (residues 276 to 329) *of N. gonorrhoeae* isolates with the PBP2 sequence of wildtype *N. gonorrhoeae* LM306 (Protein ID: AAA25463.1) and *N. gonorrhoeae* NG-3 (Protein ID: BAB86942.1) using Multalin. Sequence numbering is based on wildtype *N. gonorrhoeae* LM306 (GenBank accession no. M32091.1) (Ameyama *et al*., 2002). Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black.

[a] Consensus sequence represents the protein sequence agreement for all 28 *N. gonorrhoeae* isolates.

[b] *N. gonorrhoeae* strain NG-3 has been associated with resistance to cefixime and reduced susceptibility to ceftriaxone, and is the first mosaic *penA* sequence published (Ameyama *et al.*, 2002).

[c] Wild-type *N. gonorrhoeae* LM 306 (M32091.1) (Ameyama *et al.*, 2002)

Table 3-10 Amino acid changes detected in the mosaic PBP2 sequence of *N. gonorrhoeae* isolates (consensus sequence) compared to wild type *N. gonorrhoeae* LM306

Location of variation in amino acid sequence	Amino acid in wildtype *N. gonorrhoeae* LM306	Amino acid in consensus sequence and NG-3	Mutation in consensus sequence and NG-3
279	Alanine (A)	Valine (V)	A279V
285	Aspartic Acid (D)	Glutamic Acid (E)	D285E
288	Arginine (R)	Lysine (K)	R288K
291	Arginine (R)	Glutamine (Q)	R291Q
312	**Isoleucine (I)**	**Methionine (M)**	**I312M**
316	**Valine (V)**	**Threonine (T)**	**V316T**
323	Alanine (A)	Serine (S)	A323S
326	Threonine (T)	Valine (V)	T326V
328	Leucine (L)	Alanine (A)	L328A
329	Asparagine (N)	Threonine (T)	N329T

Note: I312M and V316T have been verified as key alterations in mosaic *penA* (Takahata *et al.*, 2006)

Table 3-11 Amino acid differences between the consensus mosaic PBP2 sequence of *N. gonorrhoeae* isolates and with NG-3 and LM306 strain

Published strains	Different amino acids (%)	Identical amino acid sequence (%)
***N. gonorrhoeae* NG-3 strain**	0 (0)	53 (100)
***N. gonorrhoeae* LM306**	10 (18.8)	43 (81.2)

Note: Total number of amino acid: 53 residues

3.2.4. Confirmation of nonmosaic *penA* for six *N. gonorrhoeae* isolates that were negative for mosaic *penA* PCR

The amplified part B *penA* of isolates 1380, 963, 1332, 893, 264 and 792, ATCC 49226 were leached using elution buffer (10mM TrisHCl, pH 8.0) and sequenced for further analysis. The length of the DNA is estimated to be 580 bp when it is based on a standard curve derived from the 100 bp molecular weight marker **(Figure G3).** The length of *penA* region amplified from strain 1380 and ATCC 49226 is 500 bp, while, for other isolates, the sequence length was between 200 to 350 bp. The shorter products still enabled interpretation of the nucleotide sequences and did not interfere with the analysis at positions 312 and 316 of the PBP2 amino acid sequence. Hence, the sequences were still able to be used to confirm the presence of nonmosaic *penA*.

The summary of sequencing results **(Table II)**, the chromatograms and the alignment of. part B *penA* sequences (615 to 1110 bp) of isolates 1380, 963, 1332, 893, 264 and 792, ATCC 49226 and *N. gonorrhoeae* NG-3 **(Figure I)** can be seen in “PartB *penA* folder” folder stored in the DVD enclosed at the end of the thesis.

Based on the alignment of the Part B of PBP2 sequence (residues 183 to 357), of consensus sequence (which represents the six isolates) with of NG-3 and ATCC 49226 strains, no changes were observed at position 312 and 316 of the PBP2 sequence of the six isolates **(Figure 3-8).** Changes at position 312 and 316 of PBP2 is commonly observed in mosaic PBP2 sequence. Additionally, the *penA* (PBP2) sequence of the six isolates was 100% identical to of ceftriaxone susceptible ATCC 49226. The amino acid changes in PBP2 are summarised in **Table 3-12**.

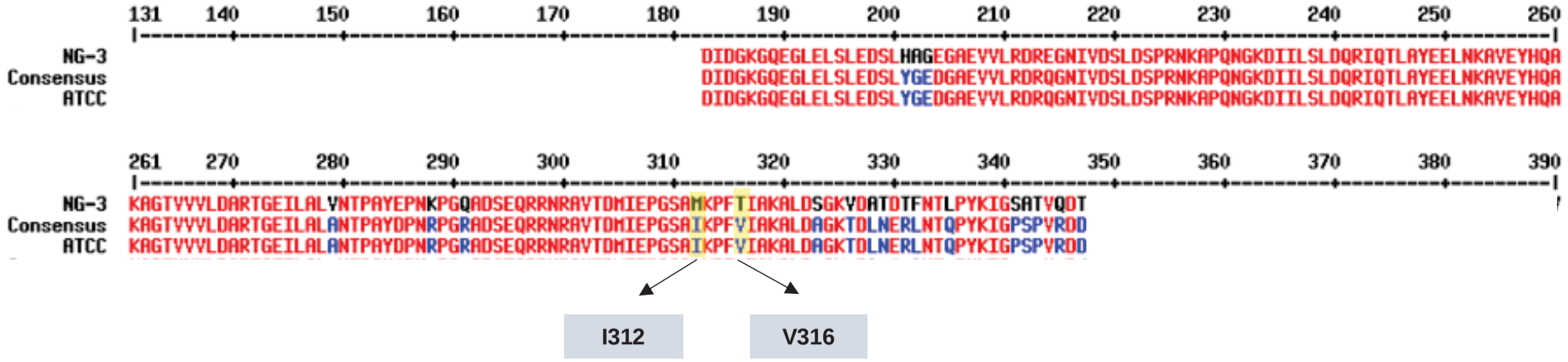


Figure 3-8 Alignment of part B of PBP sequence (residues 183 to 347) of six *N. gonorrhoeae* isolates that were negative for mosaic *penA* (consensus sequence), ATCC 49226 strain and *N. gonorrhoeae* NG-3 produced using Multalin. The numbering of the sequence is based on the PBP2 sequence of *N. gonorrhoeae* NG-3. The yellow shaded box indicate the location of key mosaic PBP2 alterations, I312 and V316. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black.

Table 3-12 Amino acid variation detected in part B of PBP2 (residues 183 to 347) for *N. gonorrhoeae* isolates that were negative for mosaic *penA*

Location of variation in amino acid of PBP	Amino acid in ATCC 49226 [a]	Amino acid in the consensus sequence [b]	Amino acid in *N. gonorrhoeae* NG-3 [c]
201	Y		H
202	G		A
203	E		G
279	A		V
288	R		K
291	R		Q
312	**I**		**M**
316	**V**		**T**
323	A		S
326	T		V
328	L		A
329	N		T
331	R		T
332	L		F
335	Q		L
341	P		S
342	S		A
343	P		T
345	R		Q
346	D		T

[a] ATCC 49226 is ceftriaxone susceptible *N. gonorrhoeae* strain.

[b] Consensus sequence: Sequence agreement of isolates 1380, 963, 1332, 893, 264 and 792 that were negative for mosaic *penA.*

[c] The bolded section showed the key amino acid alteration of mosaic PBP2 at position 312 (I312M) and 316 (V316T) in PBP2 of *N. gonorrhoeae* NG-3 strain.

3.2.5. Identification of various PBP2 alterations in part D PBP2 (residues 430 to 555) associated with elevated MIC of ceftriaxone

The amplified part D *penA* of 28. *N. gonorrhoeae* isolates plus WHO K and WHO L were leached using elution buffer (10mM TrisHCl, pH 8.0) and sequenced for further analysis. The length of the DNA is estimated to be 489 bp based on a standard curve derived from the 100 bp molecular weight marker in **Table G3**. All the sequencing results of part D *penA* for *N. gonorrhoeae* isolates plus WHO K and WHO L strain indicated good quality sequence, with the length of the sequences was not less than 350 bp. The summary of the quality of the part D *penA* **(Table III),** the chromatograms, and the alignment of part D *penA* for *N. gonorrhoeae* isolates, WHO K, WHO L strain with wild-type *penA* sequence of *N. gonorrhoeae* LM306 **(Figure II)** can be seen in "Part D *PenA*" folder stored in the DVD enclosed at the end of the thesis.

Comparison between part D PBP2 sequence (residues 430 to 560) of *N. gonorrhoeae* isolates, WHO K, WHO L strain, and wild-type *N. gonorrhoeae* LM306 can be seen in **Figure 3-9**. A total of 24 various amino acid alteration in the PBP2 sequence **(Table G6).** The changes include seven key PBP2 alterations, the A501V, A501T, N512Y, G542S, G545S, P551S and P551L. The alignment in **Figure 3-9** also showed that WHO K harbour G545S alteration in the PBP2, while WHO L harbour the A501V as described by Unemo *et al.* (2009). In addition to these, WHO K also appears to harbour N512Y, while WHO L harbours G542S.

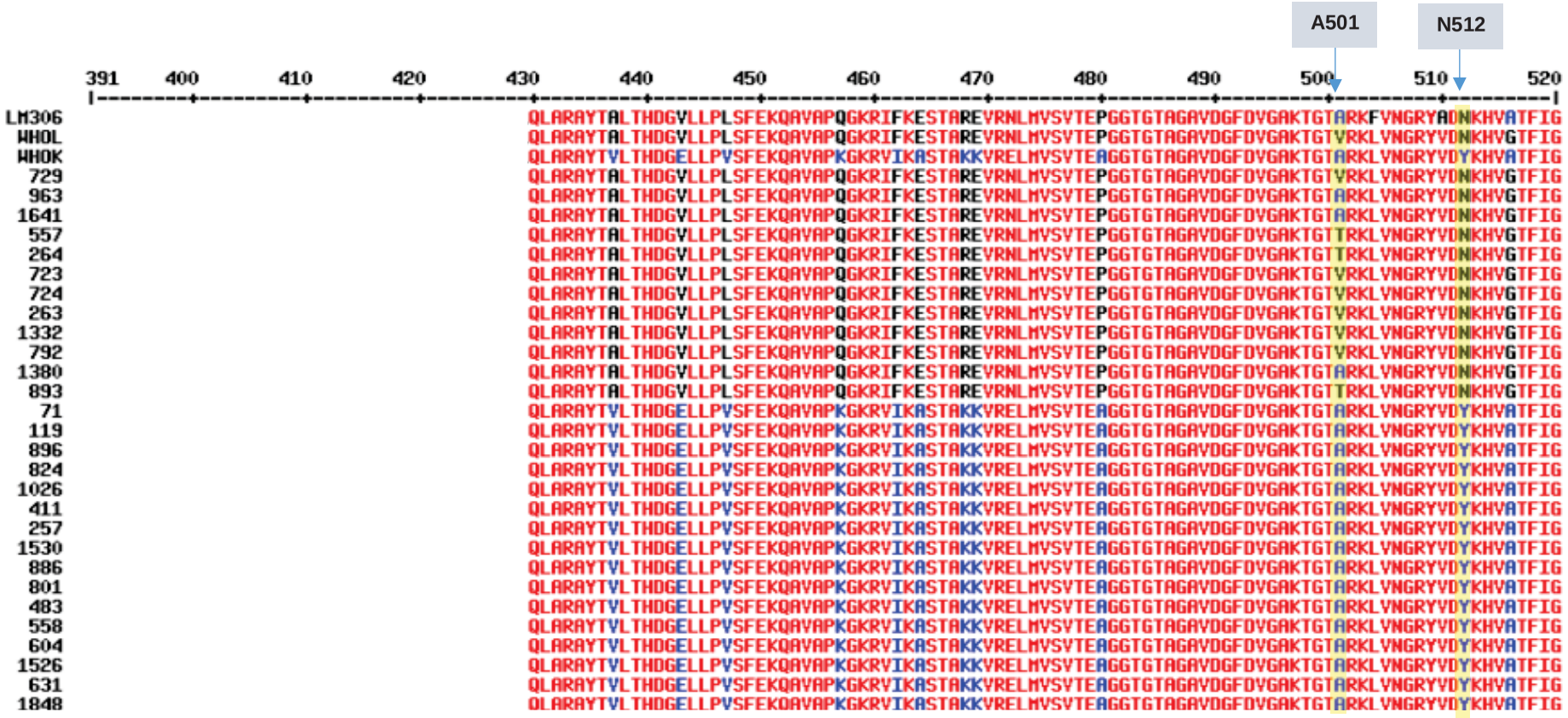


Figure 3-9 Alignment of part D of PBP2 sequence (residues 430 to 520) of *N. gonorrhoeae* isolates, WHO K and WHO L control isolates, with wild-type *N. gonorrhoeae* LM306 produced with multalin. Alterations at amino acid position 501 and 512, (highlighted in the figure) of PBP2 that have been reported to associate with elevated MIC of ceftriaxone in *N. gonorrhoeae* isolates (Tomberg *et al.*, 2010), was observed in several isolates. The numbering of the sequence is based on the sequence of *N. gonorrhoeae* LM306. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black.

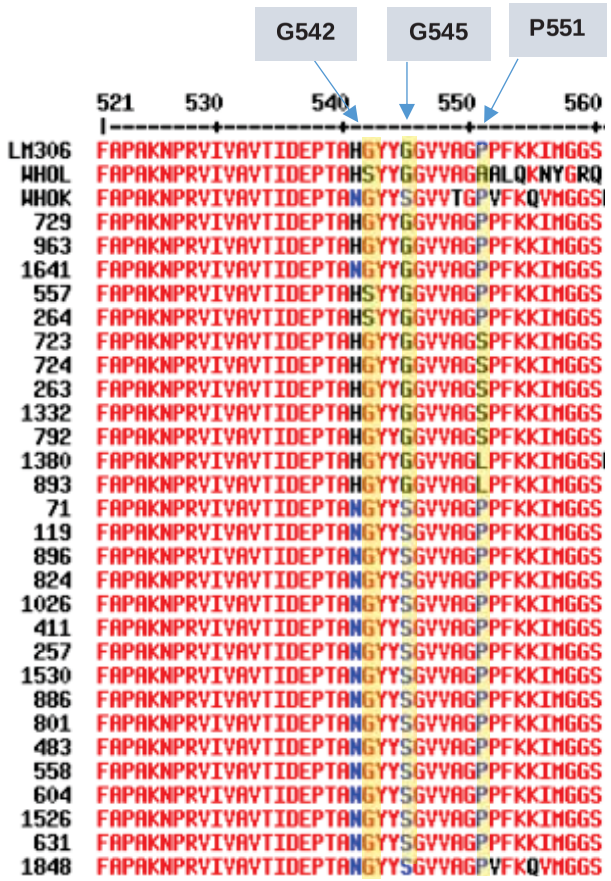


Figure 3-9 (Cont.) Alignment of part D of PBP2 sequence (residues 521 to 560) of *N. gonorrhoeae* isolates, WHO K and WHO L control isolates, with wild-type *N. gonorrhoeae* LM306 produced with multalin. Alterations at amino acid position 542, 545 and 551, (highlighted in the figure) of PBP2 that have been reported to associate with elevated MIC of ceftriaxone in *N. gonorrhoeae* isolates (Takahata *et al.*, 2006; Shimuta *et al.*, 2013), were observed in several isolates. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black.

3.2.6. The overall pattern of PBP2 in *N. gonorrhoeae* isolates

Overall analysis of PBP2 alterations in *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone, based on amplification of part B PBP2 (residues 183 to 347) and part D PBP2 sequence (residues 430 to 555) revealed two main PBP2 patterns. A total of 79% (22/28) isolates harbour various mosaic PBP2 patterns (M-1, M-2, M-3, M-4 and M-5), while 21% (6/28) harbour nonmosaic PBP2 alterations NM-1, N-2, NM-3, NM-4 and NM-5. The summary of various PBP2 patterns can be seen in **Table G7** and summarised in **Table 3-13**.

From 22 *N. gonorrhoeae* isolates that harboured mosaic PBP2, sixteen isolates harboured mosaic PBP2 type 1 (M-1), with 25 amino acid alterations observed which includes key alterations I312M, V316T, G545S and N512Y. However, isolate 1848, despite having mosaic PBP2 M-1, harbours two additional amino acid changes, the P552V and K555Q, making a total of 27 amino acid mutations in the PBP2 sequence. Mosaic PBP2 type (M-2) with 15 mutations was observed in three gonococcus isolates, while three isolates respectively harbour mosaic PBP2 type 3 (M-3) (15 amino acid mutations), mosaic PBP2 type 4 (M-4) (14 amino acid mutations) and mosaic PBP2 typed 5 (M-5) mosaic (13 amino acid mutations). PBP2 M-5 pattern, besides harbouring I312M and V316T, retain no additional key mutations.

For six isolates that harbour nonmosaic PBP2 pattern, two isolates harbour nonmosaic type 1 (NM-1) with the presence of five mutations that include A501V and P551S. The remaining four isolates harboured NM-2 PBP2 patterns (5 amino acid mutations), NM-3 pattern (5 amino acid mutations), NM-4 pattern (4 amino acid mutations) and NM-5 pattern (3 amino acid mutations), respectively. The NM-5 PBP2 did not harbour any additional key PBP2 alterations besides F504L and A501V.

When the five mosaic PBP2 sequences were compared to the selected published mosaic PBP2 sequence X (GenBank accession no. AB071984) (Ameyama *et al.*, 2002) and XXXIV (GenBank accession no. ADE2248.1) (Pandori *et al.*, 2009) as shown in **Figure G6**, M-1 showed 100% identity with the mosaic PBP2 XXXIV for amino acid residues 276 to 329 (part B PBP2) and 430 to 560 (part D PBP2). On the other hand, the mosaic PBP2 M-2, M-3, M-4 and M-5 do not match with either mosaic PBP2 X or XXXIV.

When the mosaic PBP2 sequences from residues 430 to 560 (Part D) of the five mosaic PBP2 were compared to of *N. gonorrhoeae* LM306 wildtype strain (as illustrated in **Figure G7**), the PBP2 M-1 harboured 15 amino acid changes. In contrast, PBP2 M-2, M-3, M-4 and M-5 do not harbour ten amino acid changes observed in both mosaic M-1 and mosaic PBP2 XXXIV. The summary of amino acid changes in the part D region of PBP2 can be seen in **Table G8**.

A comparison of nonmosaic PBP2 in the current study with the published nonmosaic PBP2 sequences by Whiley *et al*. (2007) and Ohnishi *et al.* (2011) revealed that the nonmosaic NM-1 was 100% identical to the pattern XIII, NM-2 with pattern XVIII, NM-3 with pattern XI, NM-4 with pattern IX and NM-5 with pattern XVI. Since the FASTA format for these published nonmosaic PBP2 sequences was not able to be obtained due to lack of information regarding the GenBank accession number, the comparison between the nonmosaic PBP2 sequences were done manually. A summary of the comparison is shown in **Table 3-14**.

Table 3-13 Summary of PBP2 patterns in *N. gonorrhoeae* isolates

PBP2 patterns [a]	PBP types	Number of isolates	Number of amino acid alterations [b]	Key PBP2 alterations reported to associate with elevated MIC of ceftriaxone in *N. gonorrhoeae* [c]
M-1	Mosaic	16	25 (27 [d])	I312M, V316T, N512Y, G545S
M-2	Mosaic	3	15	I312M, V316T, A501V, P551S
M-3	Mosaic	1	15	I312M, V316T, A501T, G542S
M-4	Mosaic	1	14	I312M, V316, A501V
M-5	Mosaic	1	13	I312M, V316T
NM-1	Nonmosaic	2	5	A501V, P551S
NM-2	Nonmosaic	1	5	A501T, G542S
NM-3	Nonmosaic	1	5	A501T, P551L
NM-4	Nonmosaic	1	4	P551L
NM-5	Nonmosaic	1	1	None

[a] M: Mosaic PBP2 pattern; NM: Nonmosaic PBP2 pattern

[b] Total amino acid changes count based on alterations in part B PBP2 region (residues 276 to 329) and part D PBP2 region (residues 430 to 520).

[c] Association of I312M & V316T with elevated MIC of ceftriaxone (Takahata *et al.*, 2006); A501V/T/P (Tomberg *et al.*, 2010; Unemo *et al.*, 2012), N512Y (Tomberg *et al.*, 2010), G542S (Whiley *et al.*, 2010a), G545S (Takahata *et al.*, 2006), P551S/L (Shimuta *et al.*, 2013).

[d] Total amino acid changes for isolate 1848 that has pattern M-1 is 27 due to two additional alterations P552V and K555Q.

Table 3-14 Comparison of PBP2 sequence observed in *N. gonorrhoeae* isolates with published mosaic PBP2 sequences in *N. gonorrhoeae* isolates

PBP2 pattern observed in the current study	Pattern similarity with other published PBP2 [a] (Country Originate)	Source of sequence
Mosaic		
M-1	Pattern XXXIV (United States) [b]	(Pandori *et al.*, 2009)
M-2	Unknown	-
M-3	Unknown	-
M-4	Unknown	-
M-5	Unknown	-
Nonmosaic [c]		
NM-1	Pattern XIII (Australia)	(Whiley *et al.*, 2007b)
NM-2	Pattern XVIII (Australia)	(Whiley *et al.*, 2007b)
NM-3	Pattern XI (Australia) [d]	(Whiley *et al.*, 2007b)
NM-4	Pattern IX (Australia)	(Whiley *et al.*, 2007b)
NM-5	Pattern XVI (Australia)	(Whiley *et al.*, 2007b)

[a] Comparison is based on part B PBP2 region (residues 276 to 329), and part D PBP2 region (residues 430 to 520).

[b] Pattern XXXIV was originally described by Pandori *et al.,* (2009) but with a different pattern name SF-A (Allen *et al.*, 2011). Refer to **Figure G6**.

[c] The alignment of each nonmosaic (NM) PBP2 sequence with their respectively published sequences was not able to be done due to the lack of information of the GenBank accession no. of the published sequences. However, the sequences of the PBP2 nonmosaic XIII, XVIII, XI, IX and XVI are published in the studies by Whiley *et al*. (2007b) and Ohnishi *et al.* (2011).

[d] NM-3 resembles pattern XI. However, instead of A501T, A501V alteration was observed at position 501 of PBP2 sequence.

3.2.7. Association of different PBP2 patterns with MIC of ceftriaxone of *N. gonorrhoeae* isolates in New Zealand

Table 3-15 shows the distribution of mosaic PBP2 and nonmosaic PBP2 patterns among *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone (MIC: 0.03 mg/L to 0.06 mg/L) and *N. gonorrhoeae* isolates that were fully susceptible to ceftriaxone (0.008 to 0.01 mg/L). Generally, a total of 87% (20/23) *N. gonorrhoeae* isolates harboured various mosaic PBP2 sequence and conveyed reduced susceptibility to ceftriaxone in the current study. Mosaic PBP2 M-1 was the most common PBP2 pattern observed among *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone, with 15 out of 23 of the isolates harbouring the PBP2 pattern.

On the other hand, 3 out of 23 of *N. gonorrhoeae* isolates that conveyed reduced susceptibility to ceftriaxone in this study harbour nonmosaic PBP2. The three isolates harboured nonmosaic NM-1 (2 isolates) and NM-2 (1 isolate) pattern. One isolate that was fully susceptible to ceftriaxone, with the lowest ceftriaxone MIC of 0.008 mg/L (grouped in NM-5) harboured no key alterations in PBP2, as shown in **Table 3-16**. The list of *N. gonorrhoeae* isolates with PBP2 alterations and ceftriaxone MIC can be seen in **Table G9**.

Table 3-16 and **Figure 3-10** showed that although the range of MICs of ceftriaxone between the mosaic PBP2 group (0.01 to 0.06 mg/L) and the nonmosaic PBP2 groups (0.008 to 0.06 mg/L) overlaps, the MIC range shown by the mosaic PBP2 group was elevated.

Table 3-15 PBP2 patterns observed in *N. gonorrhoeae* isolates

PBP2 pattern	PBP2 alterations that were linked to elevated MICs of ceftriaxone	*N. gonorrhoeae* isolates	
		Reduced susceptibility to ceftriaxone (n=23)[a]	Susceptible to ceftriaxone (n=5) [b]
M-1	I312M, V316T, N512Y, G545S	15	1
M-2	I312M, V316T, A501V, P551S	3	0
M-3	I312M, V316T, A501T, G542S	1	0
M-4	I312M, V316T, A501V	1	0
M-5	I312M, V316T	0	1
Total for mosaic PBP2 = n (%)		**20 (71)**	**2 (7)**
NM-1	A501V, P551S	2	0
NM-2	A501T, G542S	1	0
NM-3	A501T, P551L	0	1
NM-4	P551L	0	1
NM-5	None	0	1
Total for nonmosaic PBP2 =n (%)		**3 (10.7)**	**3 (10.7)**
Total = n (%) [c]		**23 (82)**	**5 (18)**

[a] MIC observed within this study that indicate reduced susceptibility to ceftriaxone: 0.03 mg/L to 0.06 mg/L

[b] MIC observed within this study that showed full susceptibility to ceftriaxone: 0.008 mg/L to 0.01 mg/L

Table 3-16 Summary of ceftriaxone susceptibility and different types of alterations in PBP2 of *N. gonorrhoeae* isolates used in the study

PBP2 patterns [a]	Alterations that have been linked to elevated MICs of ceftriaxone	MIC of ceftriaxone (mg/L) [a]				Range (mg/L)
		0.008	0.01	0.03	0.06	
M-1	I312M, V316T, N512Y, G545S		1	4	11	
M-2	I312M, V316T, A501V, P551S			2	1	
M-3	I312M, V316T, A501T, G542S				1	
M-4	I312M, V316T, A501V				1	
M-5	I312M, V316T		1			
Mosaic PBP2			**2**	**6**	**14**	**0.01 to 0.06**
NM-1	A501V, P551S			1	1	
NM-2	A501T, G542S			1		
NM-3	A501T, P551L		1			
NM-4	P551L		1			
NM-5	None	1				
Nonmosaic PBP2		**1**	**2**	**2**	**1**	**0.008 to 0.06**

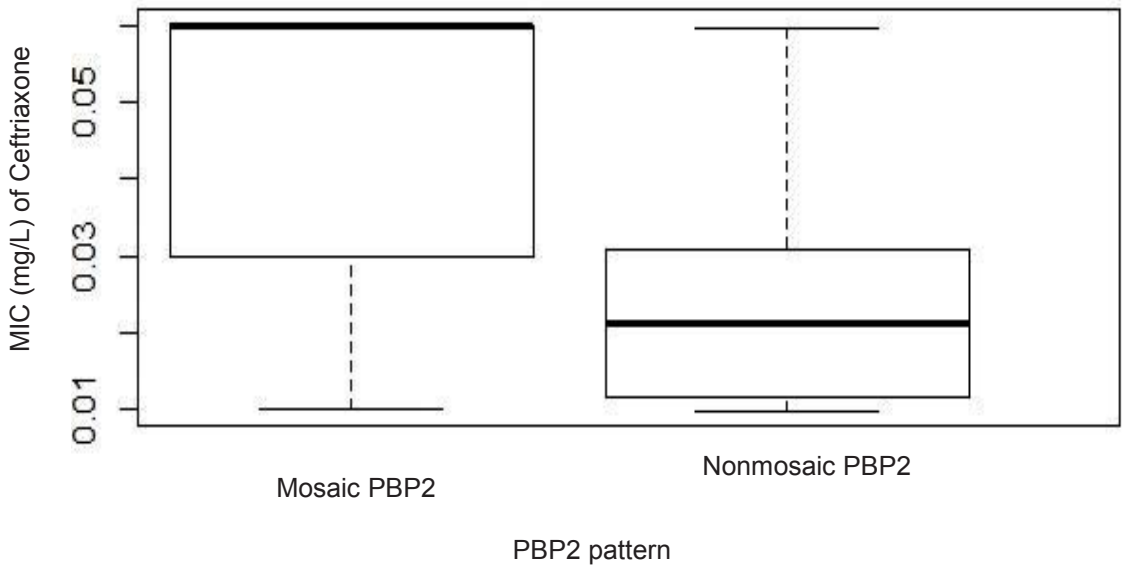


Figure 3-10 Ceftriaxone MIC distribution between isolates of *N. gonorrhoeae* with mosaic PBP2 or nonmosaic PBP2

3.2.8. Analysis of full length of *penA* of WHO K, WHO L, ATCC 49226 and strain 1380

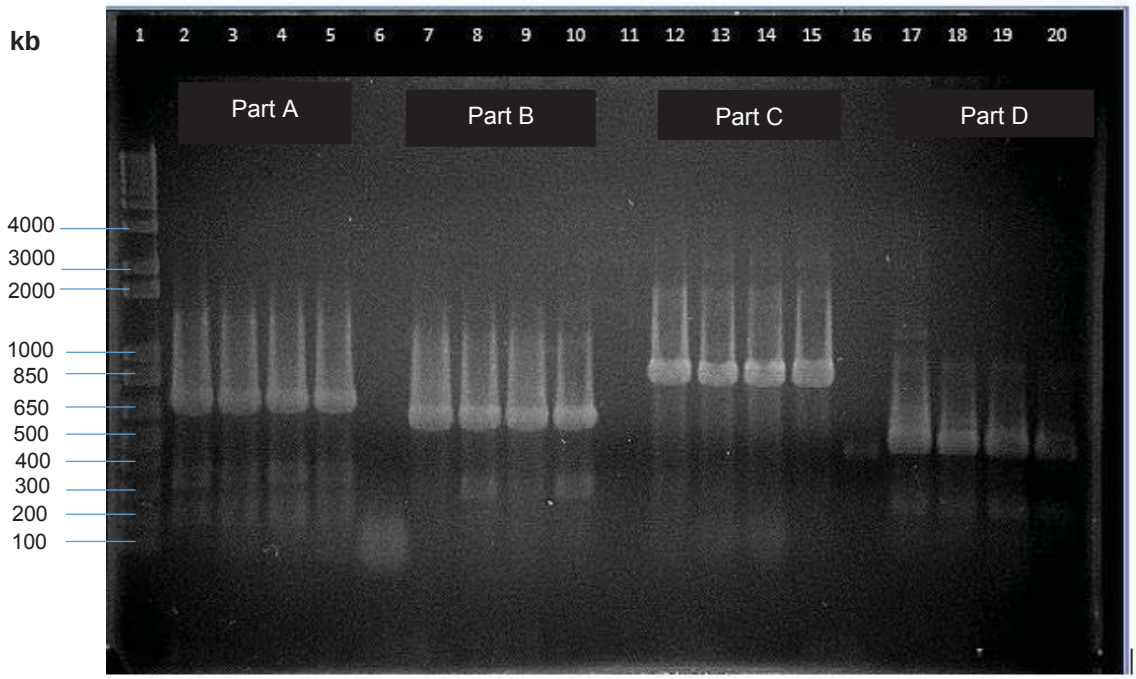


Figure 3-11 Gel electrophoresis of full length of *penA* PCR products amplified from DNA extracted from *N. gonorrhoeae* WHO K, WHO L, ATCC 49226 control strain, and NZ isolate 1380. The full length of *penA* was amplified in four parts, part A (from 8 to 676 bp), part B (from 597 to 1177 bp), part C (from 1003 to 1865 bp) and part D (from 1376 to 1865 bp).

Lane 1:100 bp ladder. WHO K, WHO L, ATCC 49226 and isolate 1380 were tested for each of the PCRs - Lanes 2-5: products of part A of *penA* PCR, lanes 7-10: products of part B of *penA* PCR, lanes 12-15: part C of *penA* PCR products, and lanes 17-20: products of part D of *penA* PCR.

Gel electrophoresis of *penA* PCR products can be seen in **Figure 3-11**. Based on the standard curve derived from the 100 bp molecular weight marker in **Table G3**, the length of amplified products was estimated to be 668 bp (Set A), 580 bp (Set B), 862 bp (Set C) and 489 bp (Set D).

The sequencing of the PCR products showed good quality results. The summary of the sequencing results **(Table IV),** the chromatograms and the alignment of full length of *penA* (consists of all four set of *penA* fragments) for WHO K, WHO L, ATCC 49226, isolate 1380, wild-type *N. gonorrhoeae* strain LM306 and *N. gonorrhoeae* strain NG-3 **(Figure III)** can be seen in folder "Full *penA*" in the attached DVD enclosed at the end of thesis

Based on the comparison with wildtype LM306 strain **(Figure 3-12)**, five PBP2 alterations were observed for 1380 isolate (summarised in **Table G16**)**.** The five amino acid alterations are the insertion of aspartic acid (D) at position 346, substitution of phenylanalanine (F) with leucine (L) at position F505, substitution of alanine (A) to valine (V) at position 511, alanine (A) to glycine (G) and position 517, glycine (G) to serine (S) at position 543, and substitution of proline (P) to leucine (L) a position 552. On the other hand, comparison to WHO K strain and the NG-3 strain showed that isolate 1380 does not harbour I312M and V316T alterations. These alterations are summarised in **Table 3-17**.

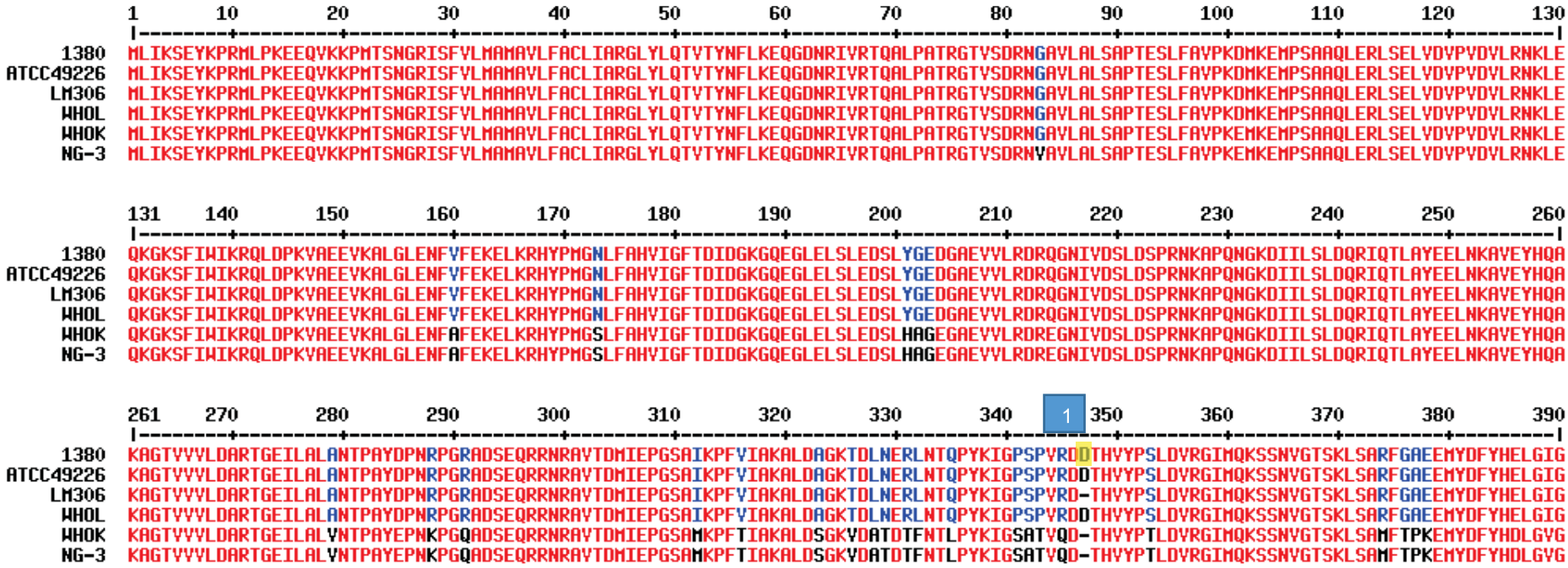


Figure 3-12 Alignment of PBP2 sequence (residues 1 to 390) of 1380, ATCC 49226, WHO L and WHO K, with *N. gonorrhoeae* LM306 and NG-3 strain. The numbering of the sequence is based on the sequence of *N. gonorrhoeae* LM306. Yellow shaded amino acid showed changes in 1380 strain (1: Insertion of aspartic acid (D) at position 347). The change was determined based on the comparison to the wildtype LM306 PBP2 sequence. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black.

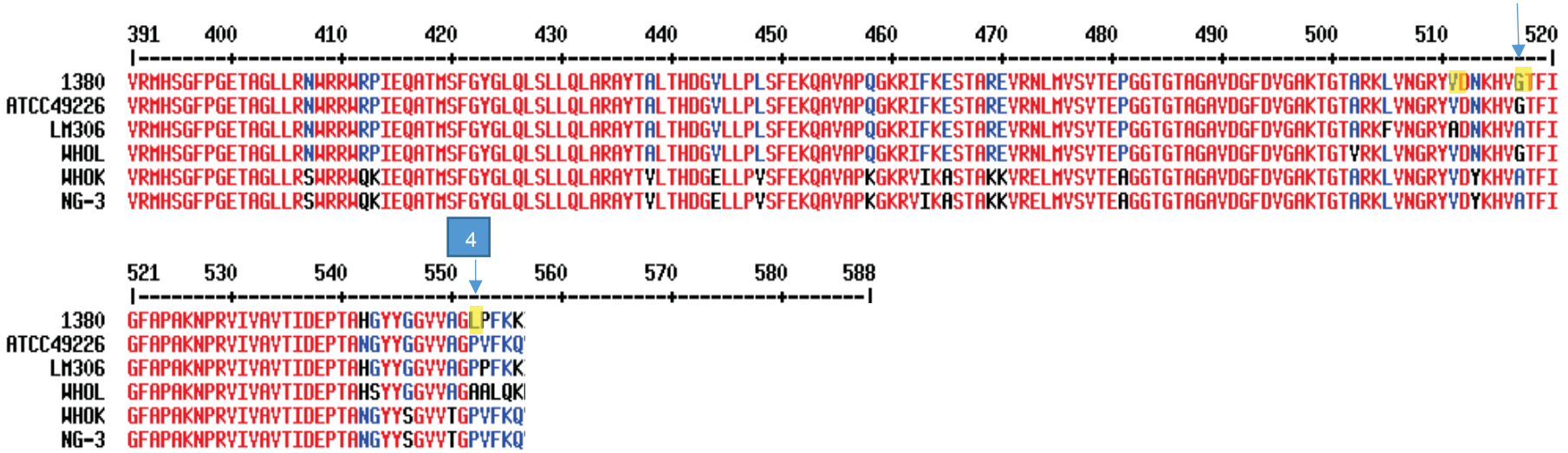


Figure 3-12 (Cont.) Alignment of PBP2 sequence (residues 391 to 556) of 1380, ATCC 49226, WHO L and WHO K, with *N. gonorrhoeae* LM306 and NG-3 strain. The numbering of the sequence is based on the sequence of *N. gonorrhoeae* LM306. Yellow shaded amino acid showed changes in 1380 strain (2: Alanine to valine at position 511; 3: Alanine to guanine at position 517; 4: Proline to leucine at position 552). The changes were determined based on the comparison to the wildtype LM306 PBP2 sequence. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black.

Table 3-17 Amino acid alterations of PBP2 of WHO K, NG-3 *N. gonorhoeae* strain, WHO L, ATCC 49226 and 1380 isolate

Amino acid mutation in *penA* [a]	WHO K	NG-3 [b]	WHO L	ATCC 49226	1380
G83V	-	/	-	-	-
D101E	/	/	-	-	-
V160A	/	/	-	-	-
N173S	/	/	-	-	-
Y201H	/	/	-	-	-
G202A	/	/	-	-	-
E203G	/	/	-	-	-
A279V	/	/	-	-	-
D285E	/	/	-	-	-
R288K	/	/	-	-	-
R291Q	/	/	-	-	-
I312M	/	/	-	-	-
V316T	/	/	-	-	-
A323S	/	/	-	-	-
T326V	/	/	-	-	-
L328A	/	/	-	-	-
N329T	/	/	-	-	-
E330D	/	/	-	-	-
R331T	/	/	-	-	-
T332F	/	/	-	-	-
Q335L	/	/	-	-	-
P341S	/	/	-	-	-
S342A	/	/	-	-	-
P343T	/	/	-	-	-
R345Q	/	/	-	-	-
Insertion D at 346			/	/	/
S352T	/	/	-	-	-
R373M	/	/	-	-	-
G375T	/	/	-	-	-
A376P	/	/	-	-	-
E377K	/	/	-	-	-
E385D	/	/	-	-	-
I388V	/	/	-	-	-
N406S	/	/	-	-	-
R411Q	/	/	-	-	-
P412K	/	/	-	-	-
A437V	/	/	-	-	-
V443E	/	/	-	-	-
L447V	/	/	-	-	-
Q457K	/	/	-	-	-

Amino acid mutation in *penA*	WHO K	NG-3	WHO L	ATCC 49226	1380
I461V	/	/	-	-	-
F462I	/	/	-	-	-
E464A	/	/	-	-	-
R468K	/	/	-	-	-
E469K	/	/	-	-	-
N472E	/	/	-	-	-
P480A	/	/	-	-	-
A501V	-	-	/	-	-
F505L	/	/	/	/	/
A511V	/	/	/	/	/
N513Y	/	/	-	-	-
A517G			/	/	/
H542N	/	/	-	-	-
G543S	-	-	/	-	-
G546S	/	/	-	-	-
A550T	/	/	-	-	-
P552L	-	-	-	-	/
P552A	-	-	/	-	-
P553A	-	-	/	-	-
P553V	/	/	-	/	-
F554L	-	-	/	-	-

[a] The grey shaded area highlight the PBP2 changes involve in isolate 1380. Due to the shift of the PBP2 fragments when all the isolates (1380, NG-3, WHO K, WHO L) are aligned with *N. gonorrhoeae* LM306 strain, the numbering of the amino acid alterations change. Insertion of aspartic acid (D) observed in this study was between position 346 and 347 instead of the published insertion between position 345 and 346 (Brannigan *et al.*, 1990). Similarly, A505L is A504L (Sun *et al.*, 2010), A511V is A510V (Zhao *et al.*, 2009), A517G is A516G (Ito *et al.*, 2005), and P552L is P551L (Whiley *et al.*, 2010a).

[b] *N. gonorrhoeae* strain NG-3 is resistant to cefixime and has reduced susceptibility to ceftriaxone and it is the first ever mosaic *penA* sequence published (Ameyama *et al.*, 2002)

3.3. *MtrR* analysis of *N. gonorrhoeae* isolates

3.3.1. *MtrR* PCR and sequencing

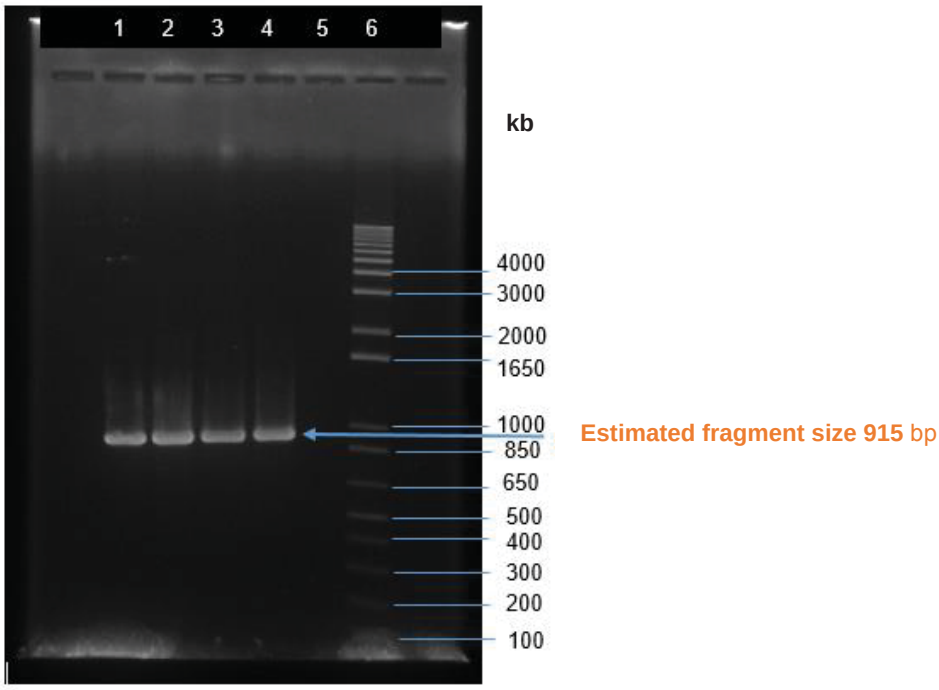


Figure 3-13 Gel electrophoresis of *mtrR* PCR products from DNA extracted from *N. gonorrhoeae* isolates. Lane 1 to 4: WHO K, ATCC 49226, NZ isolate 1380 and WHO L respectively. Lane 5: Blank. Lane 6: 100 bp molecular weight marker.

Gel electrophoresis of *mtrR* products can be seen in **Figure 3-13**. The migration of PCR product was measured and, based on a standard curve derived from the 100 bp molecular weight marker **(Table G3)**, the length of the amplified products were estimated to be 915 bp. All of the amplified products were sequenced to detect the presence of an adenine (A) deletion in the *mtrR* 13 bp inverted repeat sequence of the promoter region, and the change from glycine (G) to aspartic acid (D) at position 45 (G45D) in the amino acid sequence of MtrR repressor.

Sequencing results obtained from Massey Genome Service (MGS) showed usable quality. The Alignment of the *mtrR* nucleotides sequence of all *N. gonorrhoeae* isolates, plus WHO K and WHO F control isolates with the published

mtrR sequence of *N. gonorrhoeae* (Pan and Spratt, 1994) (GenBank Accession Number: Z25796.1; Protein ID: CAA81045.1) confirmed that the products amplified were within the *mtrR* gene The summary of the quality of the sequencing result **(Table V)**, the sequence alignment **(Figure IV)** and the chromatograms for all isolates can be seen in "MtrR sequence" folder stored in the attached DVD. However, for isolate 1641 and control isolate WHO F, unsatisfactory sequence results were produced for the forward sequence, and reverse sequence, respectively. As a result, the reverse sequence was used for result interpretation for isolate 1641, while the forward sequence was used for WHO F.

3.3.2. Identification of adenine (A) deletion in *mtrR* promoter, G45D in the MtrR coding region, and other alterations in MtrR protein

Based on the alignment of partial *mtrR* sequences (1 to 130 bp residues) of *N. gonorrhoeae* isolates with 13 bp inverted repeat *mtrR* promoter sequence (**Figure 3-14**), and the alignment of the MtrR protein sequence of the same set can be seen in **Figure 3-15**, five type of alterations in *mtrR* gene were observed. As summarised in **Table H1**, the A deletion in the *mtrR* promoter is the most common alteration with 93% (26/28) gonococcus isolates shown to harbour this variation. On the other hand, H105Y, G45D, A39T and T86A alterations in MtrR coding region were seen in 68% (19/28), 18% (5/28), 11% (3/28), and 7% (2/28) of gonococcus isolates in the current study.

In addition to the above results, the published *mtrR* sequence of *N. gonorrhoeae* (Genbank accession number: Z25796.1) harboured a single A deletion in the promoter sequence, but not the G45D alteration. WHO K isolate was positive for both alterations. On the other hand, WHO F isolate was negative for both alterations. The summary of the *mtrR* alterations can be seen in **Table 3-18**.

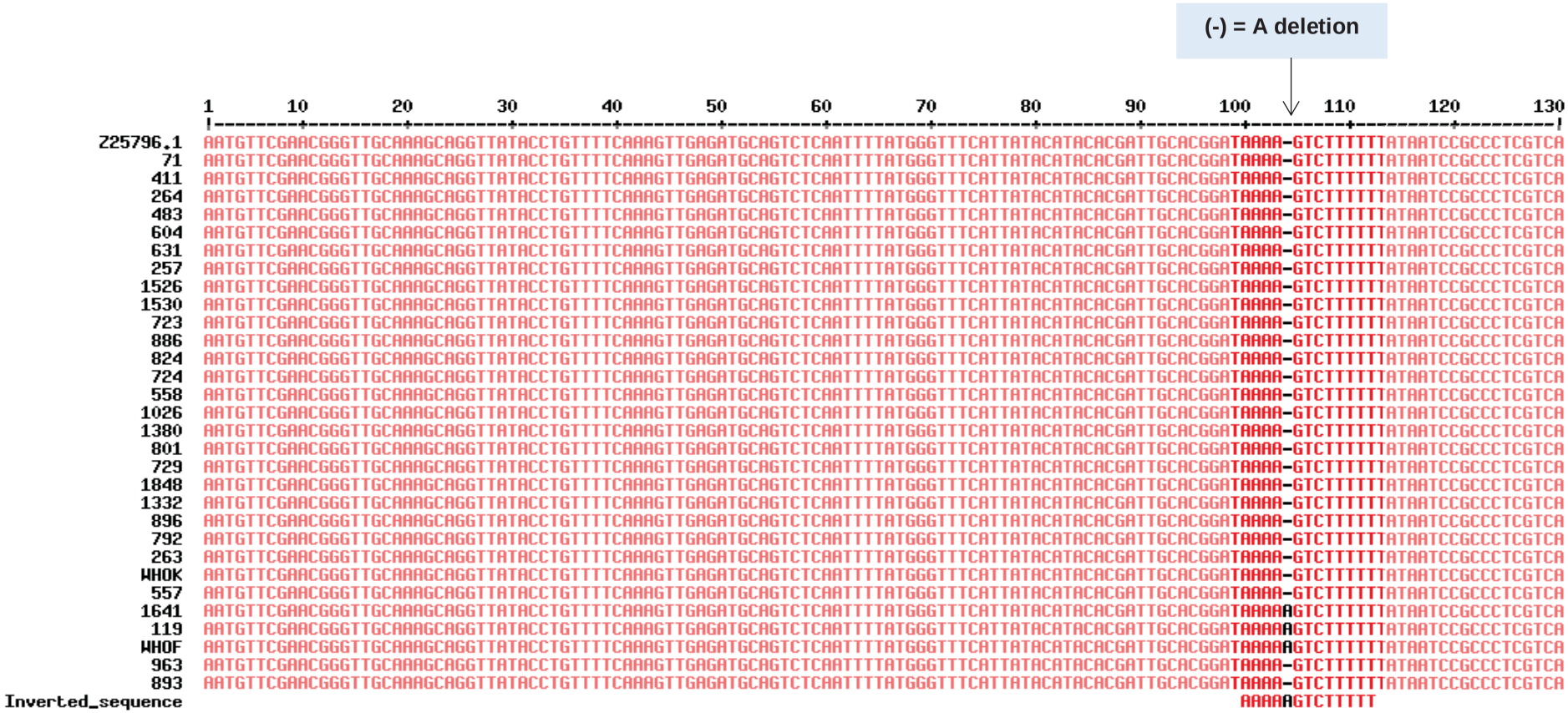


Figure 3-14 Alignment of partial *mtrR* sequences (1 to 130 bp residues) of *N. gonorrhoeae* isolates, published *mtrR* of *N. gonorrhoeae* (GenBank accession no.: Z25796.1) (Pan and Spratt, 1994), WHO K and WHO F control with 13 bp inverted repeat *mtrR* promoter sequence (Hagman *et al.*, 1995) using Multalin. Dash symbol (-) represents adenine (A) deletion. Nucleotides that are completely conserved in all sequences in this population of genes are shown in red**.**

Note: Full length of *mtrR* nucleotide sequence alignment is stored in the attached DVD in “MtrR sequence” folder

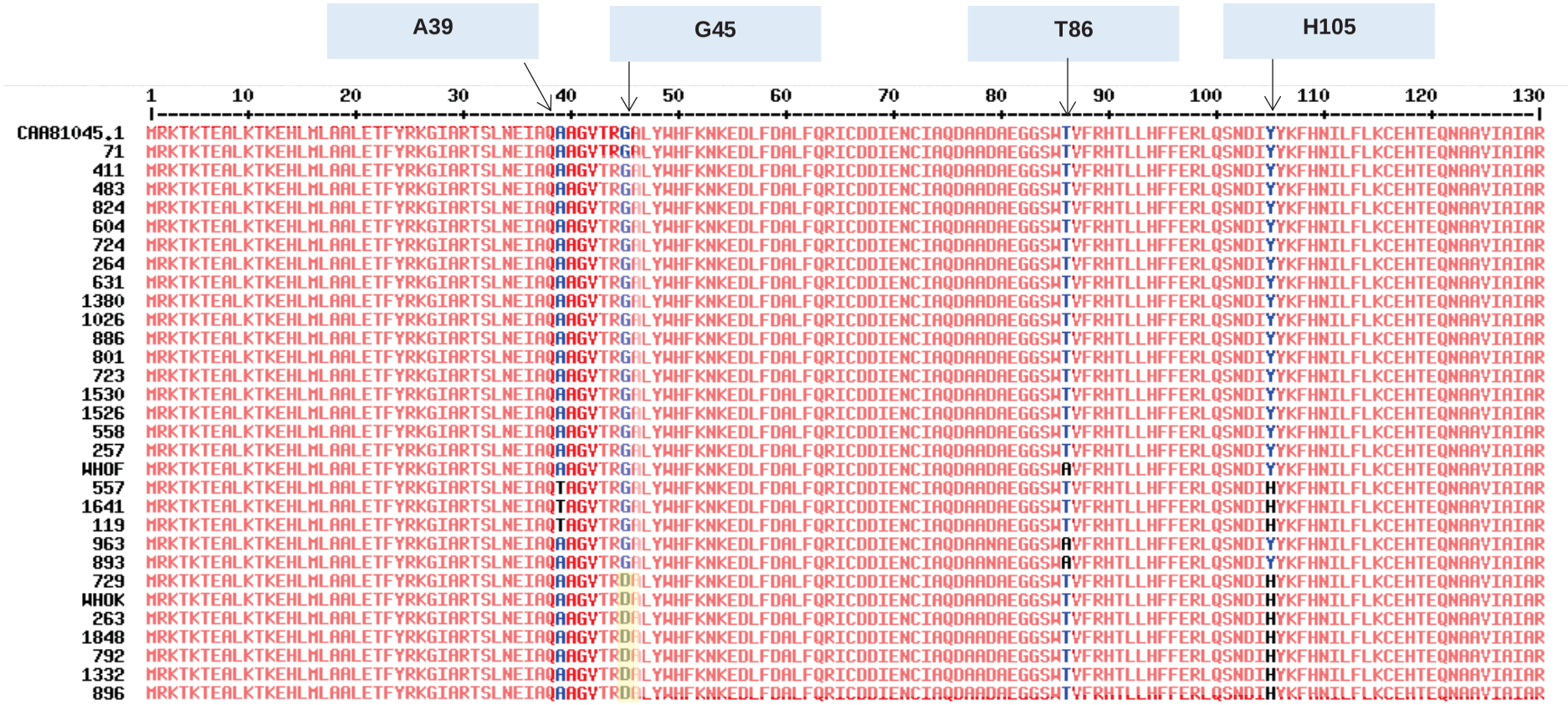


Figure 3-15 Alignment of MtrR protein sequences (1 to 130 residues) of *N. gonorrhoeae* isolates with published MtrR protein sequence of *N. gonorrhoeae* (protein ID: CAA81045.1) (Pan and Spratt, 1994), positive control WHO K and wildtype WHO F using Multalin. Alteration from glycine (G) to aspartic acid (D) at position 45 can be seen in six isolates (yellow shaded area). Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black.

Table 3-18 Alterations in *mtrR* promoter and MtrR coding region of *N. gonorrhoeae* isolates

<table>
<tr><th rowspan="2">Isolate</th><th colspan="3">Alterations</th></tr>
<tr><th>Adenine (A) deletion in mtrR promoter</th><th>G45D alteration in MtrR coding region</th><th>Other alterations in MtrR coding region</th></tr>
<tr><td>Z25796.1 strain [a]</td><td>Present</td><td>Not present</td><td rowspan="10">Not present</td></tr>
<tr><td>WHO K [b]</td><td colspan="2">Present</td></tr>
<tr><td>WHO F [c]</td><td colspan="2">Not present</td></tr>
<tr><td>729</td><td colspan="2" rowspan="5">Present</td></tr>
<tr><td>792</td></tr>
<tr><td>896</td></tr>
<tr><td>1848</td></tr>
<tr><td>1332</td></tr>
<tr><td>893</td><td rowspan="24">Present</td><td rowspan="25">Not present</td><td rowspan="2">T86A, H105Y</td></tr>
<tr><td>963</td></tr>
<tr><td>71</td><td rowspan="23">H105Y</td></tr>
<tr><td>604</td></tr>
<tr><td>558</td></tr>
<tr><td>723</td></tr>
<tr><td>801</td></tr>
<tr><td>411</td></tr>
<tr><td>886</td></tr>
<tr><td>1526</td></tr>
<tr><td>1530</td></tr>
<tr><td>724</td></tr>
<tr><td>824</td></tr>
<tr><td>257</td></tr>
<tr><td>264</td></tr>
<tr><td>483</td></tr>
<tr><td>1380</td></tr>
<tr><td>1026</td></tr>
<tr><td>631</td></tr>
<tr><td>557</td><td>A39T</td></tr>
<tr><td>119</td><td rowspan="2">Not present</td><td rowspan="2">A39T</td></tr>
<tr><td>1641</td></tr>
<tr><td>263</td><td>Present</td><td>Not present</td></tr>
</table>

[a] *N. gonorrhoeae* published by Pan and Spratt (1994) (GenBank accession no. Z25796.1; Protein ID: CAA81045.1).

[b] WHO K: Verified to have an A deletion in the *mtrR* promoter sequence and G45D alteration in MtrR coding region (Unemo *et al.*, 2009).

[c] WHO F: Verified to have wildtype *mtrR* sequence (Unemo *et al.*, 2009).

3.3.3. Association of alterations in *mtrR* promoter and MtrR coding region with susceptibility level of ceftriaxone

Five sets of *mtrR* alterations were observed in the current study. A total of 96% (22/23) of gonococcus isolates with reduced susceptibility to ceftriaxone harboured an A deletion in *mtrR* promoter, either with or without additional missense mutation. The combination of A deletion in *mtrR* promoter with H105Y MtrR mutation is observed commonly among *N. gonorrhoeae* with reduced susceptibility to ceftriaxone. A total of 65% (15/23) of *N. gonorrhoeae* with reduced ceftriaxone susceptibility harboured this set of mutations. The A deletion in *mtrR* promoter couples with G45D/A39T alterations was the second common *mtrR* pattern, observed in 26% (6/23) *N. gonorrhoeae* isolates that showed reduced susceptibility to ceftriaxone. Two *N. gonorrhoeae* with reduced susceptibility to ceftriaxone was also found to harbour only A deletion in the *mtrR* promoter, and A39T mutation in the MtrR coding region, respectively.

The A deletion in the *mtrR* promoter was also observed in four isolates that were fully susceptible to ceftriaxone. The summary of the result can be seen in **Table 3-19**.

Table 3-19 Summary of alterations in *mtrR* promoter and MtrR coding region of *N. gonorrhoeae* isolates

Alterations in *mtrR*	Number of isolates (n)	Reduced susceptibility to ceftriaxone (n=23) [a]	Susceptible to ceftriaxone (n=5) [b]
Adenine (A) deletion in *mtrR* promoter (+G45D/A39T) [c]	6	6	0
A deletion in *mtrR* promoter (+ H105Y + T86A)	2	0	2
A deletion in *mtrR* promoter (+ H105Y)	17	15	2
A deletion in *mtrR* promoter only	1	1	0
A39T only	2	1	1
Total=n (%)	**28 (100%)**	**23 (82%)**	**5 (18%)**

[a] MIC observed within this study that indicated reduced susceptibility to ceftriaxone: 0.03 mg/L to 0.06 mg/L

[b] MIC observed within this study that showed full susceptibility to ceftriaxone: 0.008 mg/L to 0.01 mg/L

[c] Adenine (A) deletion in *mtrR* promoter + A39T (n=1)

3.4. *PorB$_{IB}$* analysis of *N. gonorrhoeae* isolates

3.4.1. *PorB$_{IB}$* amplification & sequencing

Four different PCR protocols were evaluated during the optimisation process. The optimisation involved adjusting the concentration of primers and dNTPs. The optimisation processes (protocol 4) are included in **Table 3-20** and **Table 3-21**, and **Table I1**. Protocol 4 was chosen since it reduced the number of non-specific products without causing a reduction in the *porB$_{IB}$* amplicon.

Table 3-20 PCR conditions for amplification of the *porB$_{IB}$* gene of *N. gonorrhoeae* (protocol 4)

PCR steps	Condition
Initial denaturation	1 cycle of 5 min at 95°C
Denaturation	40 cycles of 30 seconds at 95°C
Annealing	40 cycles of 15 seconds at 62°C
Extension	40 cycles of 30 seconds at 72°C
Final extension	1 cycle of 7 minutes at 72°C
Hold	At 10°C

Table 3-21 Optimised PCR master mix for *porB$_{IB}$* PCR (protocol 4)

	Final Concentration	Volume (µL)[1]
KAPA2G Robust DNA Polymerase	0.5U	0.1
5X KAPA2G Buffer[2]	1X	5
10mM dNTPs	200µM	0.5
10µM Primer PorB1	0.2µM	0.5
10µM Primer PorB2	0.2µM	0.5
Milli-Q Water		13.4
DNA template		5
		25

[1] Volumes for one PCR reaction.

[2] KAPA2G Buffer contains 1.5mM $MgCl_2$ at 1X.

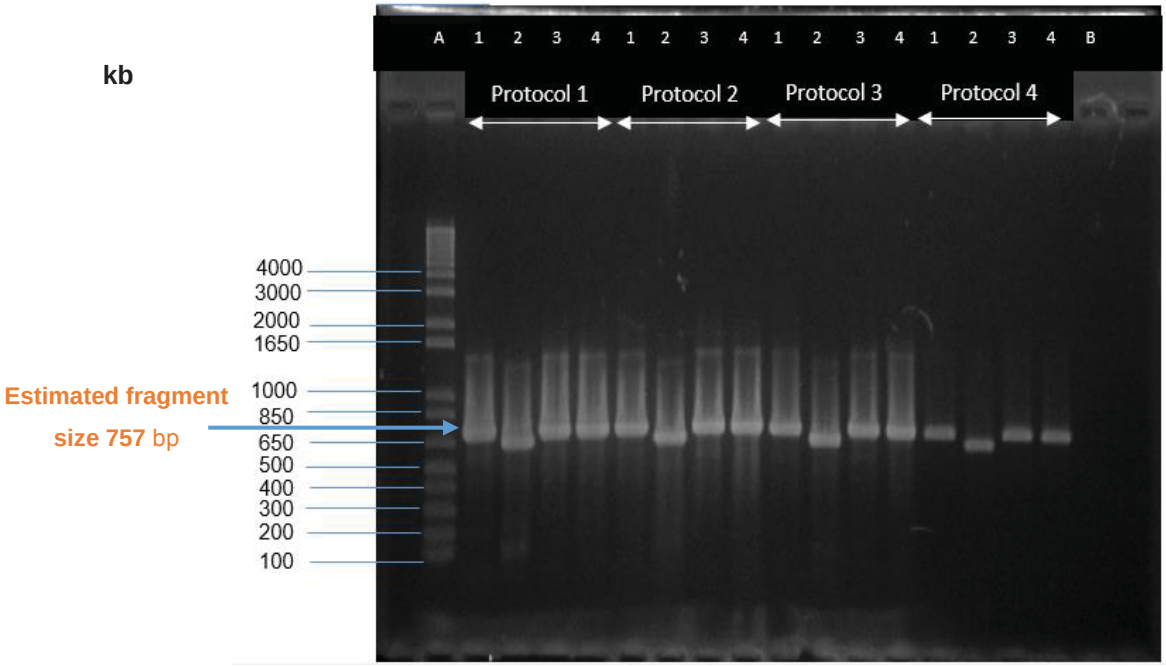


Figure 3-16 Gel electrophoresis of *porB$_{IB}$* PCR products consists of four protocols, amplified from DNA extracted from *N. gonorrhoeae* isolates WHOK, ATCC 49226, isolate 729 and 824. Lane 1 to 4: WHO K, ATCC 49226, NZ isolate 729 and 824 respectively. Lane A : 100 bp molecular weight marker. Lane B: Blank.

Gel electrophoresis of the *porB$_{IB}$* PCR product can be seen in **Figure 3-16**. The migration of PCR product was measured and comparison made to a standard curve **(Figure G3)**. The length of the amplified products was estimated to be 757 bp.

Based on the chromatograms, the sequences of most isolates were usable for both forward and reverse sequence. However, for isolates 724, 729, 801 and 1526, the quality of the forward sequence was unsatisfactory since the length of the nucleotide sequence was half of the total length of *porB$_{IB}$* (757 bp). For these isolates, the reverse sequence was used for *porB$_{IB}$* analysis. The alignment of the *porB$_{IB}$* nucleotide sequence of *N. gonorrhoeae* isolates, WHO K strain and published *porB$_{IB}$* sequence of *N. gonorrhoeae* H1-2 (Gill *et al.*, 1998) (GenBank accession no.: AJ004943.1) confirmed that the products amplified during PCR were within the *porB$_{IB}$* gene. The PCR amplified roughly from nucleotide position 160 to 917. The summary of the sequencing results **(Table VI),** the chromatograms, and the alignment of the *porB$_{IB}$* nucleotide sequences **(Figure V)** can be seen in "PorB sequence" folder in the attached DVD.

3.4.2. PorBIB alterations and association ceftriaxone susceptibility of *N. gonorrhoeae* isolates

The alignment of the PorBIB protein sequence (residues 81 to 300) of *N. gonorrhoeaea* isolates plus WHO K strain and *N. gonorrhoeae* H1-2 strain showed that 75% (21/28) harboured double substituitions at codons 120 and 121 **(Figure 3-17). Table 3-23** showed three main G120 and A121 alterations observed in *N. gonorrhoeae* isolates in the current study. A total nine out of 28 isolates harboured the G120K/A121D PorBIB mutations, eight out of 28 isolates harboured the G120K/A121N, while four out of 28 isolates harboured the G120K/A121G. The WHO K strain was positive for harbouring G120K and A121D alterations, as verified by Unemo *et al*. (2009).

Overall, 18 out of 23 (78%) *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone harboured PorBIB mutations, with the most common PorBIB alterations being G120K/A121D (8 isolates) and G120K/A121N (7 isolates). Three gonococcus isolates with reduced susceptibility to ceftriaxone harboured G120K/A121G alterations. On the contrary, five *N. gonorrhoeae* isolates with reduced ceftriaxone susceptibility showed no changes in the codons 120 and 121 of PorBIB. The association between the PorBIB alterations and susceptibility level of ceftriaxone can be seen in **Table 3-23**.

Besides alterations at codon 120 and 121, 37 type of other PorBIB changes (excluding alterations at G120 and A121) were observed in 4% to 100% of *N. gonorrhoeae* isolates. The list of the PorBIB changes can be see in **Table I6**.

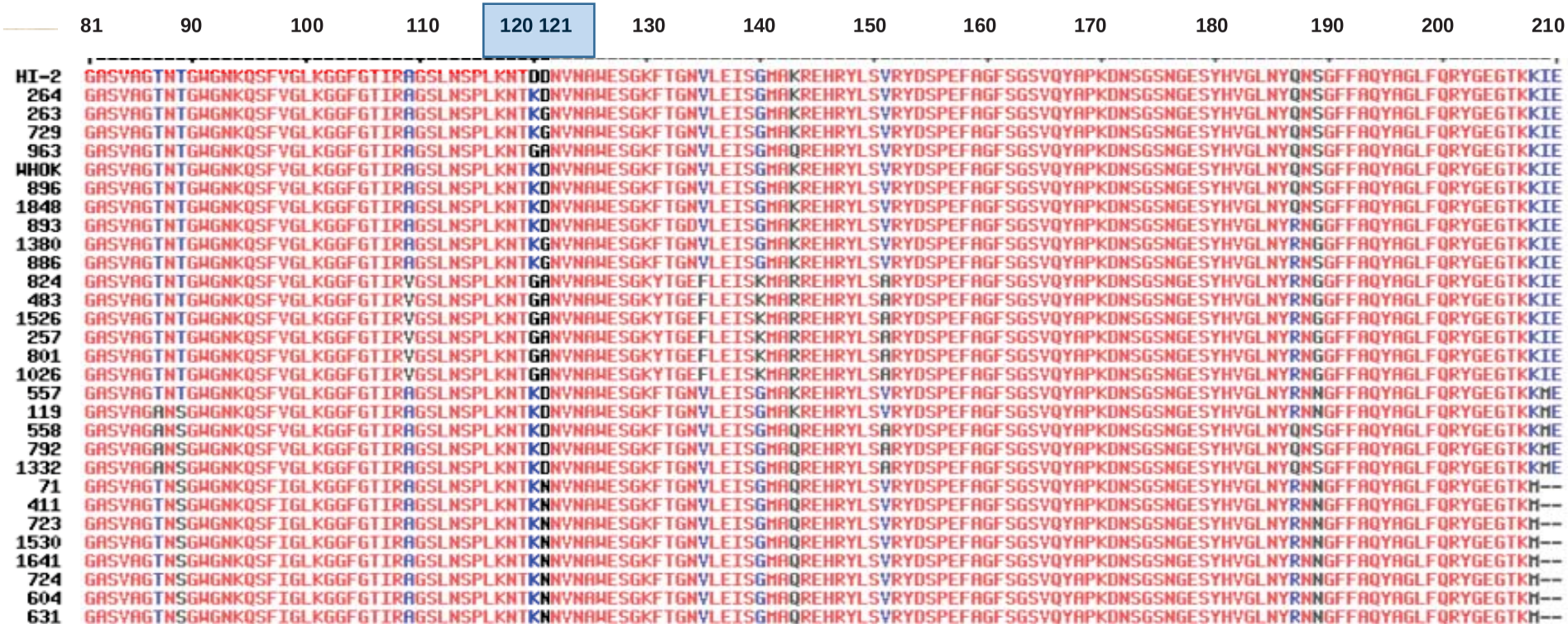


Figure 3-17 Alignment of partial PorBIB amino acid sequence (81 to 210 residues) of *N. gonorrhoeae* isolates, published wild-type PorBIB sequence of *N. gonorrhoeae* H1-2 (Genbank Protein ID: CAA06234.1) and WHO K control strain using Multalin. Alterations in PorBIB of G120 and A121 are marked. The numbering of the sequence is based on that of the PorBIB amino acid sequence of *N. gonorrhoeae* H1-2. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black. A dash symbol (-) represents an amino acid deletion, which cause a gap in the sequence.

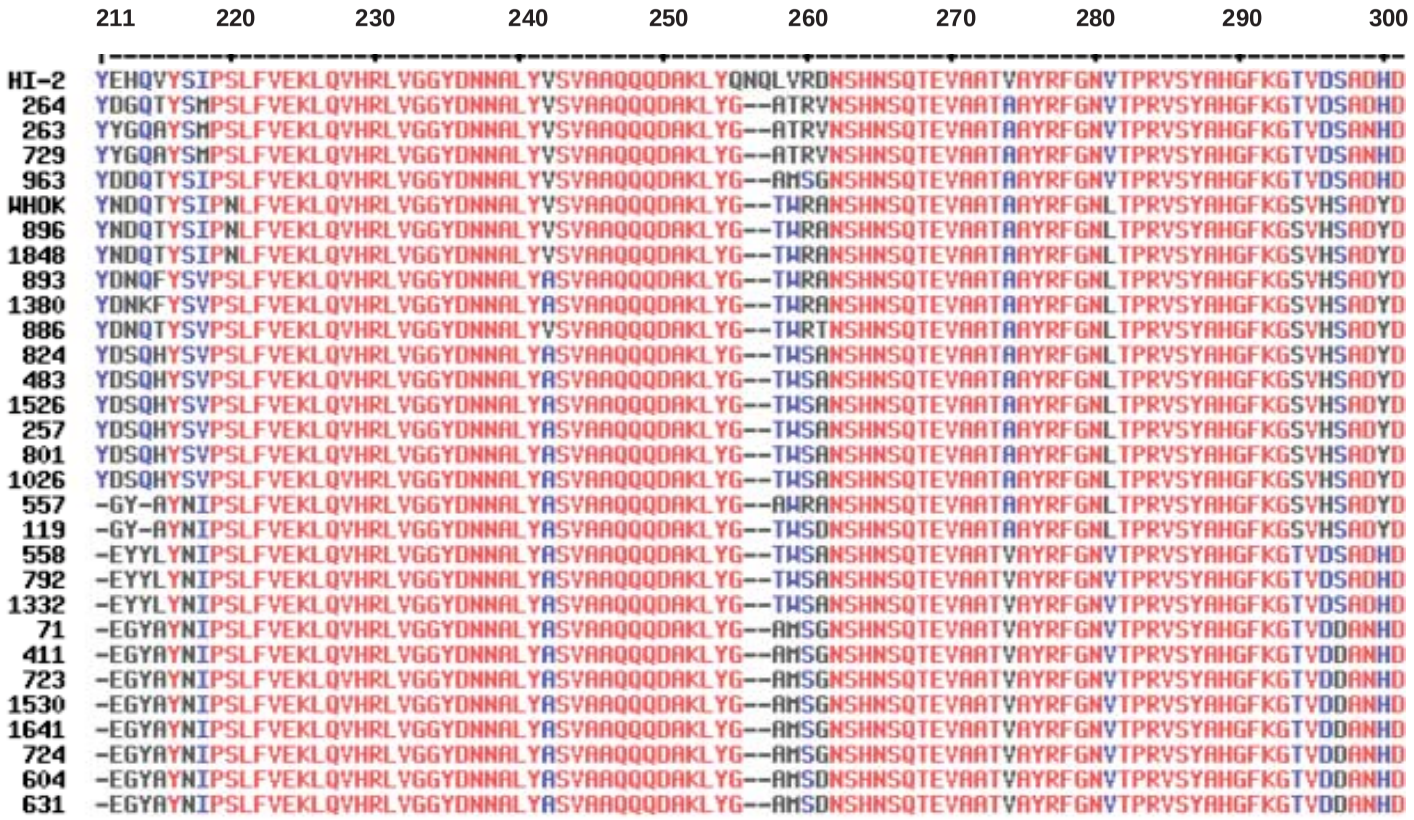


Figure 3-16 (Cont.) Alignment of partial PorBIB amino acid sequence (211 to 300 residues) of *N. gonorrhoeae* isolates, published wild-type PorB sequence of *N. gonorrhoeae* H1-2 and WHO K control strain using Multalin. The numbering of the sequence is based on that of the PorBIB amino acid sequence of *N. gonorrhoeae* H1-2. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the amino acids in black. A dash symbol (-) represents an amino acid deletion, which cause a gap in the sequence. A dash symbol (-) represents an amino acid deletion, which cause a gap in the sequence.

Table 3-22 G120 and A121 PorBIB alterations of *N. gonorrhoeae* isolates

Isolate	PorBIB alterations	
	G120	A121
N. gonorrhoeae isolate HI-2	Aspartic Acid (D)	Aspartic Acid (D)
WHO K [a]	Lysine (K)	Aspartic Acid (D)
1848		
896		
264		
893		
119		
557		
792		
558		
1332		
71	Lysine (K)	Asparagine (N)
724		
1530		
411		
1641		
723		
631		
604		
886	Lysine (K)	Glycine (G)
263		
729		
1380		
483	-	-
1026		
1526		
801		
824		
257		
963		

[a] WHO K control isolate: Verified for having G120K and A121D alterations in PorBIB amino acid sequence (Unemo *et al.*, 2009).

(-): No changes observed

Table 3-23 PorBIB alterations in *N. gonorrhoeae* isolates

Alterations of PorBIB	Number of isolates (n) (%)	Reduced susceptibility to ceftriaxone (n=23) (%) [a]	Susceptible to ceftriaxone (n=5) (%) [b]
G120K/A121D	9	8	1
G120K/A121N	8	7	1
G120K/A121G	4	3	1
Total	**21 (75)**	**18 (78)**	**3 (60)**
No change at G120 and A121	7 (25)	5 (22)	2 (40)

[a] MIC observed within this study that indicate reduced susceptibility to ceftriaxone: 0.03 mg/L to 0.06 mg/L

[b] MIC observed within this study that showed full susceptibility to ceftriaxone: 0.008 mg/L to 0.01 mg/L

3.5. *PonA* analysis of *N. gonorrhoeae* isolates

3.5.1. *PonA* amplification and sequencing

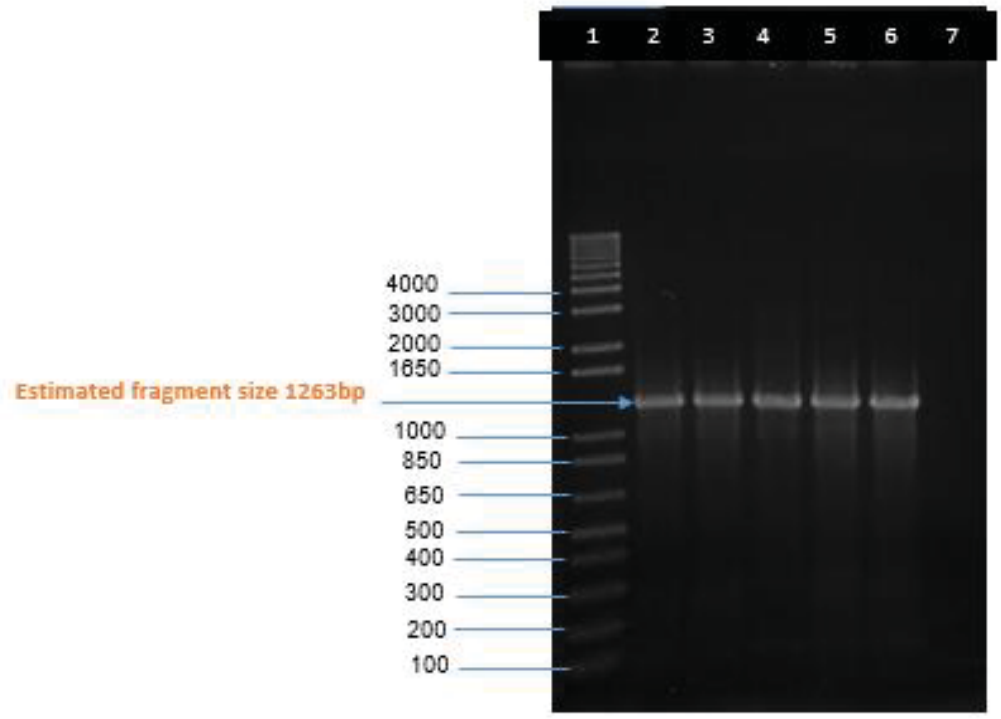


Figure 3-18 Gel electrophoresis of the *ponA* PCR products amplified from DNA extracted from *N. gonorrhoeae* isolates. Lane 1:100 bp molecular weight marker, Lane 2: WHO K, NZ isolate 71, 119, 557 and 1380 respectively. Lane 9: Blank

Gel electrophoresis of the *ponA* PCR product can be seen in **Figure 3-18**. The migration of PCR product was measured, and comparison made to a standard curve derived from the 100 bp ladder **(Figure G3)**. The length of the amplified products was estimated to be 1263 bp.

Based on the chromatograms, the sequencing results showed good quality for both reverse and forward sequences. However, for isolates 263, 264, 558, 729, 792, 963, 1332, 1526, and 1848, the quality of the *ponA* forward sequence was

unsatisfactory due to the short length of the sequence (<600 bp). For these isolates, the reverse sequence was used for *ponA* analysis. The alignment of the nucleotide sequences of the *N. gonorrhoeae* isolates, WHO K strain and published *ponA* sequence of *N. gonorrhoeae* NG00085 (Takahashi *et al.*, 2013) (GenBank accession no.: AB727713.1) confirmed that the products amplified during PCR were within the *ponA* gene. The PCR amplified roughly from nucleotide positions 1070 until 1950. Also, all sequences had 100% identical sequence to the WHO K control strain. The summary of the sequencing results **(Table VI),** the chromatograms and the sequence alignment **(Figure VI)** is stored in "PonA sequence folder" in the attached DVD.

The summary of the sequencing results and the chromatograms for all isolates can be seen in "*PonA* sequence" folder stored in the DVD enclosed at the end of the thesis.

3.5.2. Identification of L421P alteration in PBP1 and the association with susceptibility level of ceftriaxone.

For the amino acid sequence of PBP1, the substitution corresponds with the alteration of leucine (L) to proline (P) at position 421 (L421P). The mutation was seen in all 28 (100%) *N. gonorrhoeae* isolates, including the WHO K control strain **Figure 3-19**. In other words, both isolates that were found to convey reduce ceftriaxone susceptibility and those fully susceptible to ceftriaxone harboured the L42P mutation. See **Table 3-24.**

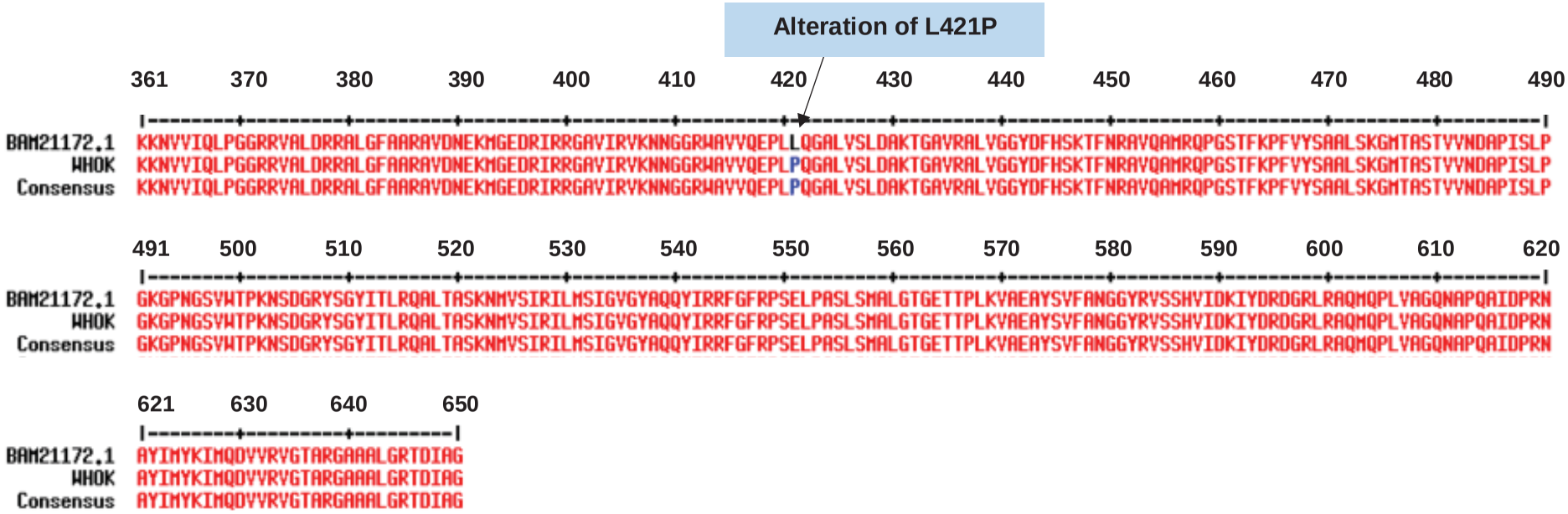


Figure 3-19 Alignment PBP1 amino acid sequence (residues 361 to 650) of *N. gonorrhoeae* isolates, the published wild-type PBP1 sequence of *N. gonorrhoeae* NG00085 (GenBank accession no.: AB727713.1, Protein ID: BAM21172.1) (Takahashi *et al.*, 2013) and the WHO K control strain using Multalin. The key alteration of PBP1 is the L421P. The numbering of the sequence is based on the number of the PBP1 amino acid sequence of *N. gonorrhoeae* NG00085. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with nucleotides in blue having greater consensus than the nucleotides in black.

Note: [a] Consensus sequence represents amino acid sequences of 28 *N. gonorrhoeae* with reduced susceptibility to ceftriaxone

Table 3-24 *PonA* (PBP1) alteration in *N. gonorrhoeae* isolates

Mutations in PBP1 (*ponA)*	*N. gonorrhoeae* isolates in New Zealand	
	Reduced susceptibility to ceftriaxone [a]	Susceptible to ceftriaxone [b]
L421P	23 (82)	5 (18)

[a] MIC observed within this study that indicate reduced susceptibility to ceftriaxone: 0.03 mg/L to 0.06 mg/L

[b] MIC observed within this study that showed full susceptibility to ceftriaxone: 0.008 mg/L to 0.01 mg/L

3.6. Association of various combination of mutated *penA, mtrR*, *porB$_{IB}$* and *ponA* with susceptibility level of ceftriaxone

The current study showed that mosaic *penA* was present in 87% (20/23) of *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone obtained from 2012 to 2015. Nonmosaic *penA* was found in 13% (3/23) isolates with reduced susceptibility to ceftriaxone. The A deletion in *mtrR* promoter was observed in 97% (22/23) *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone, while 78% (18/23) isolates were positive for G120 and A121 PorBIB alterations. Last but not least, the L421P mutations in PBP2 (*ponA*) was observed in all 23 *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone. The summary of the presence of key mutations in 23 *N. gonorrhoeae* with reduced susceptibility to ceftriaxone can be seen in **Table 3-25**.

Based on the presence of the mutated *penA, mtrR, porB$_{IB}$* and *ponA* among *N. gonorrhoeae* isolates, five main groups, were observed in this study, and this can be seen in **Table 3-26**, and summarised in **Table 3-27**. A total of 50% (14/28) isolates belonging to Group I harbouring four mutated genes, the mosaic *penA*, *mtrR, porB$_{IB}$* and *ponA,* and all of the isolates conveyed reduced susceptibility to

ceftriaxone based on the MIC results from the current study using Liofilchem MIC test strips.

The second most common pattern observed in this study is Group II, where 21% (6/28) gonococcus isolates were found to harbour three mutated genes, the mosaic *penA, mtrR* and *ponA*. Five out of six *N. gonorrhoeae* isolates were found to show reduced susceptibility to ceftriaxone. Group IV is the third most common group of resistance gene determinants, where 18% (5/28) of *N. gonorrhoeae* isolates harboured four mutated genes, the nonmosaic *penA*, *ponA*, *mtrR*, and $porB_{IB}$. Three gonococcus isolates that harboured these resistance gene mutations showed reduced susceptibility to ceftriaxone.

A total of 7% (2/28) of *N. gonorrhoeae* isolates belonged to Group III harbour mutations in three genes, the mosaic *penA*, $porB_{IB}$ and *ponA*. One of the isolates in this group (isolate 119) showed reduced susceptibility to ceftriaxone. Finally, isolate 963 (Group V) harboured only two resistance gene determinants, the A deletion in *mtrR* promoter and the *ponA* mutation. Isolate 963 was fully susceptible to ceftriaxone with MIC of 0.008 mg/L.

Table 3-28 and **Figure 3-20** showed that the involvement of four mutated genes mosaic *penA, mtrR,* $porB_{IB}$ and *ponA* (Group I) led to a 50 to 100 fold increase of ceftriaxone MIC in comparison with *N. gonorrhoeae* FA19 wildtype strain published by Zhao *et al.* (2009) with ceftriaxone MIC of 0.0006 mg/L. Compared to Group IV that also showed four mutated genes, but with nonmosaic *penA*, *mtrR,* $porB_{IB}$ and *ponA,* the observed ceftriaxone MIC fold increase was between 16.6 to 83.3 fold.

For Group II and Group III with three mutated genes mosaic *penA, mtrR* or $porB_{IB}$ *and ponA*, the ceftriaxone MIC was 16.6 to 83.3 fold higher than the *N. gonorrhoeae* FA19 strain. For isolate that harbour only two mutated genes *mtrR*

and *ponA* (Group V), 13 fold increase of ceftriaxone MIC was observed in comparison to *N. gonorrhoeae* FA19 strain.

Table 3-25 Summary of mutations *penA, mtrR, porB$_{IB}$* and *ponA* that are associated with elevated ceftriaxone MIC in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone

Gene (protein)	Key mutations in protein	Number present in isolates (%) (n=23) [a]
Mosaic *penA* (PBP2)	**I312M, V316T, G545S/A501V/A501T**	20 (87%)
Nonmosaic *penA* (PBP2)	**A501V/A501T**	3 (13%)
***MtrR* (MtrR)**	**A deletion (in *mtrR* promoter)**	22 (96%)
***PorB$_{IB}$* (PorBIB)**	**G120 and A121 alterations**	18 (78%)
***ponA* (PBP1)**	**L421P**	23 (100%)

[a] *N. gonorrhoeae* isolates that conveyed reduce ceftriaxone susceptibility from the current study (MIC: 0.03 to 0.06 mg/L)

Table 3-26 Susceptibility to ceftriaxone, and key alterations identified in PBP1, PBP2, MtrR and PorBIB in *N. gonorrhoeae* isolates

		Amino acid alterations [a]																	
		MIC of ceftriaxone (mg/L)				PBP2							MtrR [b]		PorBIB		PBP1		
Group	Strain	0.008	0.01	0.03	0.06	I312	V316	A501	N512	G542	G545	P551	A del	G45D	G120	A121	L421P		
	896				/	M	T	.	Y	.	S	.	Yes	Yes	K	D	Yes		
	1848				/	M	T	.	Y	.	S	.	Yes	Yes	K	D	Yes		
	1530			/		M	T	.	Y	.	S	.	Yes	.	K	N	Yes		
	631				/	M	T	.	Y	.	S	.	Yes	.	K	N	Yes		
I (n= 14)	71				/	M	T	.	Y	.	S	.	Yes	.	K	N	Yes		
Mosaic *penA*	604				/	M	T	.	Y	.	S	.	Yes	.	K	N	Yes		
+ *mtrR* +	411				/	M	T	.	Y	.	S	.	Yes	.	K	N	Yes		
porB$_{IB}$ +	558				/	M	T	.	Y	.	S	.	Yes	.	K	D	Yes		
ponA	886				/	M	T	.	Y	.	S	.	Yes	.	K	G	Yes		
	723				/	M	T	V	.	.	.	S	Yes	.	K	N	Yes		
	263			/		M	T	V	.	.	.	S	Yes	.	K	G	Yes		
	724			/		M	T	V	.	.	.	S	Yes	.	K	N	Yes		
	557				/	M	T	T	.	S	.	.	Yes	.	K	D	Yes		
	729				/	M	T	V	.	.	.	.	Yes	Yes	K	G	Yes		

Group	Strain	MIC of ceftriaxone (mg/L)				PBP2							MtrR [b]		PorBIB		PBP1
		0.008	0.01	0.03	0.06	I312	V316	A501	N512	G542	G545	P551	AdeI	G45D	G120	A121	L421P
	801				/	M	T	.	Y	.	S	.	Yes	.	.	.	Yes
	1526			/		M	T	.	Y	.	S	.	Yes	.	.	.	Yes
Group II (n= 6)	824			/		M	T	.	Y	.	S	.	Yes	.	.	.	Yes
Mosaic *penA* +	257			/		M	T	.	Y	.	S	.	Yes	.	.	.	Yes
mtrR* + *ponA	483			/		M	T	.	Y	.	S	.	Yes	.	.	.	Yes
	1026		/			M	T	.	Y	.	S	.	Yes	.	.	.	Yes
Group III (n= 2)	119				/	M	T	.	Y	.	S	.	.	.	K	D	Yes
Mosaic *penA* +	1641		/			M	T	.	.	.	.	.	.	.	K	N	Yes
porB*$_{IB}$ + *ponA																	

Group	Strain	MIC of ceftriaxone (mg/L)				PBP2							MtrR [b]		PorBIB		PBP1
		0.008	0.01	0.03	0.06	I312	V316	A501	N512	G542	G545	P551	AdeI	G45D	G120	A121	L421P
Group IV (n= 5) Nonmosaic *penA* + *mtrR*+ *porB*$_{IB}$ + *ponA*	792				/	.	.	V	.	.	.	S	Yes	Yes	K	D	Yes
	1332			/		.	.	V	.	.	.	S	Yes	Yes	K	D	Yes
	264			/		.	.	T	.	S	.	.	Yes	.	K	D	Yes
	893		/			.	.	T	.	.	.	L	Yes	.	K	D	Yes
	1380		/			.	.	.	.	.	.	L	Yes	.	K	G	Yes
Group V (n= 1) *mtrR* + *ponA*	963	/				.	.	.	.	.	.	.	Yes	.	.	.	Yes

[a] Amino acid alterations in PBP2, MtrR, PorBIB and PBP1 associate with reduced susceptibility to ceftriaxone

[b] A del: Adenine (A) deletion in *mtrR* promoter

(/): represents the ceftriaxone MIC showed by the respective *N. gonorrhoeae* isolates.

(.) : represents no alterations

Table 3-27 Association of mutated *penA, mtrR, porB$_{IB}$* and *ponA* with *N. gonorrhoeae* isolates

Group	Mutated genes combination	*N. gonorrhoeae* isolates		
		Reduced susceptibility to ceftriaxone (n=23) (%) [a]	Susceptible to ceftriaxone (n=5) (%) [b]	Total (n=28) (%)
Group I	Mosaic *penA* + *mtrR* + *porB$_{IB}$* + *ponA*	14 (50)	0 (0.0)	14 (50)
Group II	Mosaic *penA* + *mtrR* + *ponA*	5 (18)	1 (3.6)	6 (21)
Group III	Mosaic *penA* + *porB$_{IB}$* + *ponA*	1 (3.6)	1 (3.6)	2 (7)
Group IV	Nonmosaic *penA* + *mtrR*+ *porB$_{IB}$* + *ponA*	3 (11)	2 (7)	5 (18)
Group V	*mtrR* + *ponA*	0 (0)	1 (3.6)	1 (3.6)
Total		**23 (82)**	**5 (18)**	**28 (100)**

[a] MIC observed within this study that showed reduced susceptibility to ceftriaxone (MIC : 0.03 mg/L to 0.06 mg/L)

[b] MIC observed within this study that showed full susceptibility to ceftriaxone (0.008 mg/L to 0.01 mg/L)

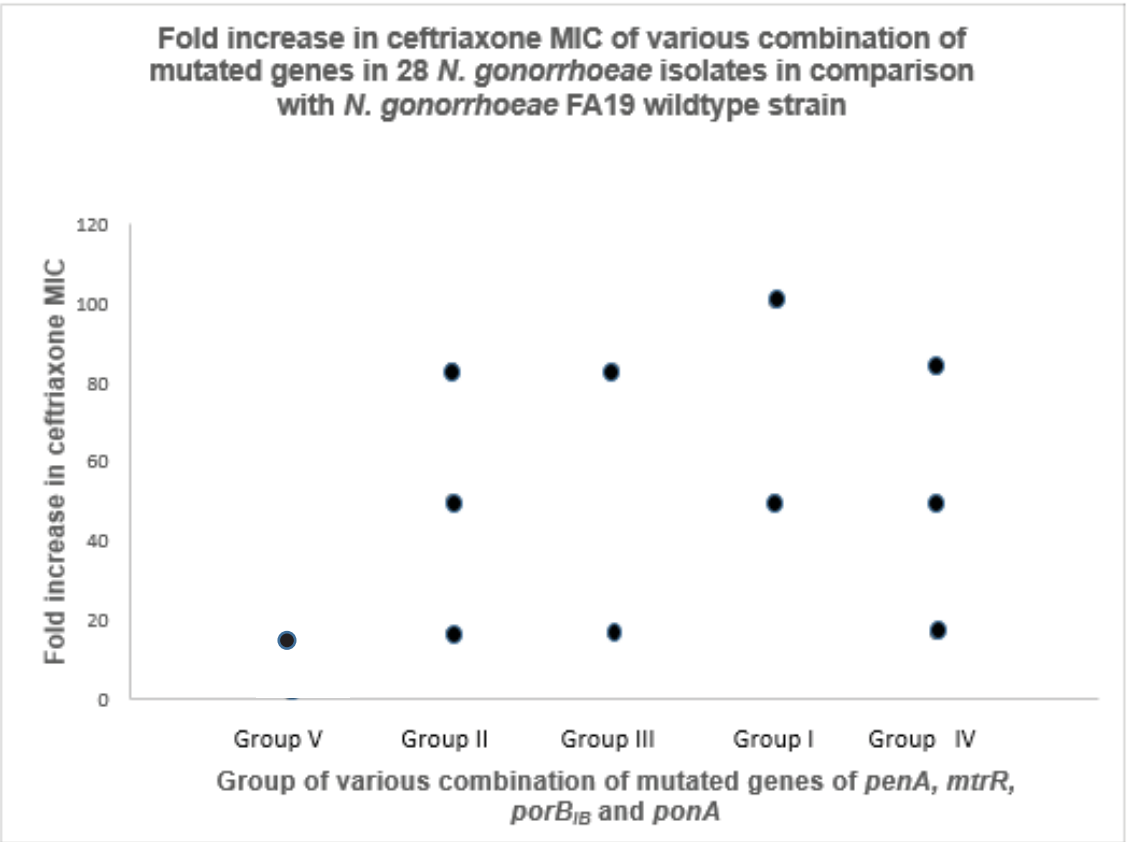
Table 3-28 Association of mutated *penA, mtrR, porB$_{IB}$* and *ponA* with ceftriaxone MIC level of *N. gonorrhoeae* isolates

Group	Mutated genes combination	Ceftriaxone MIC (mg/L) [a] [b]				Fold increase [c]
		0.008	0.01	0.03	0.06	
Group V	***mtrR* + *ponA***	1				13 fold
Group II	**Mosaic *penA* + *mtrR* + *ponA***		1	4	1	16.6 to 83.3 fold
Group III	**Mosaic *penA* + *porB$_{IB}$* + *ponA***		1		1	16.6 to 83.3 fold
Group I	**Mosaic *penA* + *mtrR* + *porB$_{IB}$* +*ponA***			3	11	50 to 100 fold
Group IV	**Nonmosaic *penA* + *mtrR* + *porB$_{IB}$* + *ponA***		2	2	1	16.6 to 83.3 fold

[a] The MIC was based from the results from the currrent study

[b] The grey shaded area represent reduced susceptibility range of ceftriaxone MIC (0.03 to 0.06 mg/L).

[c] The fold increase of ceftriaxone MIC was determined in comparison with wild type *N. gonorrhoeae* FA19 strain, with MIC of 0.0006 mg/L (Zhao *et al.*, 2009).



Note:

a) Group I - Mosaic *penA* + *mtrR* + $porB_{1B}$ +*ponA*

Group II - Mosaic *penA* + *mtrR* + *ponA*

Group III - Mosaic *penA* + $porB_{1B}$ + *ponA*

Group IV- Nonmosaic *penA* + *mtrR* + $porB_{1B}$ + *ponA*

Group V - *mtrR* + *ponA*

b) Black dot represents the increase of ceftriaxone MIC conveyed in each group of various combination of mutated genes when compare to the wild type *N. gonorrhoeae* FA19 strain, with MIC of 0.0006 mg/L (Zhao *et al.*, 2009).

Figure 3-20 Effect of different groups of gene mutations on the susceptibility of ceftriaxone

CHAPTER FOUR

DISCUSSION

4. DISCUSSION

4.1. Effect of Media and brands of antibiotic gradient strips on MIC results

Liofilchem MIC test strips were chosen instead of bioMérieux Etest strips to determine the MIC readings of antimicrobial agents in the current study since they were cheaper and there was no significant difference in the MIC results of penicillin G and ceftriaxone (p=0.77 (penicillin G); p=0.87 (ceftriaxone)) **(Table F3)**. The differences in the MIC readings of ceftriaxone and penicillin G between the two kits might be due to the slight variation in the production of the concentration gradient of antibiotic on the strips. The different materials used for the antibiotic gradient strips might influence the rate of the antibiotic elution into the media, causing variation in the MIC reading. bioMérieux Etest strips were made of plastic material while Liofilchem MIC test strips were made of high-quality paper.

The current study also revealed a significant difference between the MIC results produced from the current study, and the MIC results produced by ESR ($p < 0.01$; agreement: 86%) **(Table F5).** Even after fresh isolates were obtained, the MIC discrepancy remained **(Table 3-1).** All 28 *N. gonorrhoeae* isolates were originally determined by ESR to show reduced susceptibility to ceftriaxone, with MIC from 0.03 mg/L to 0.12 mg/L. However, in the current study, only 82% (23/28) of *N. gonorrhoeae* isolates showed reduced susceptibility to ceftriaxone (MIC from 0.03 to 0.06 mg/L) **(Table 3-3).** ESR used both agar dilution methods and Etest strips (bioMérieux) to determine the MIC of ceftriaxone of the isolates, which might partially explain the discrepancy. For the five isolates where the agar dilution method was used to determine the MIC of ceftriaxone, the results were consistently higher than those of the current study. The finding is in agreement with other studies that report, although the MIC test strips method (in this study Etest was used) correlates well with the agar dilution method, the method produces a slightly lower MIC reading (Van Dyck *et al.*, 1994; Daly *et al.*, 1997; Jones *et al.*, 2000). Variation in the incubation temperature has been out ruled

out as the cause of discrepancies in this study, since all the culture plates were incubated at the recommended temperature 36±1°C. Similarly, the variation in the CO_2 supply (3% and 5%) during the culture incubation was also excluded as the factor that might affect the MIC readings. The current study showed that difference in the CO_2 supply does not affect the ceftriaxone MIC readings **(Table F11)** and this is in agreement with a study carried by James-Holmquest *et al.* (1973). In the current study, a candle jar which produced 3% carbon dioxide was used to grow *N. gonorrhoeae* cultures and to incubate plates for MIC testing. The candle jar extinction method has been confirmed by Spink & Keefer, 1947 (as cited by Martin, Armstrong and Smith, 1973) and Holmquest *et al.* (1973) to supply adequately CO_2 for gonococcus growth. Although the use of candle extinguishing method decreases the oxygen pressure due to the candle combustion, reducing oxygen pressure did not significantly affect the growth of gonococci, unlike the enhancement of growth by increased CO_2 concentration (Ferguson, 1945; James-Holmquest *et al.*, 1973). Nonetheless, the CO_2 incubator, used by ESR, might provide better conditions for the growth of the gonococcus.

It is difficult to prepare consistent suspensions of *N. gonorrhoeae* to match the 0.5 McFarland standard but, counts showed that the colony forming unit (CFU) did not vary much from the standard 1.5 X 10^8 CFU/ml **(Table F11)**. In fact, the slight variation in the CFU also did not affect the MIC of ceftriaxone. Hence, this parameter has been out ruled as a cause of the MIC discrepancy. For the future investigation, an alternative antimicrobial susceptibility testing (AST) method such as the broth dilution method might help to decide the true value of the MICs.

In this study, the effect of growing *N. gonorrhoeae* on different media, prior to conducting MIC tests, was assessed for both penicillin and ceftriaxone. GC saponin agar and GC II agar can be considered as a second option for the culture of *N. gonorrhoeae* before the ceftriaxone MIC test, since the agreement in the MIC values with the recommended chocolate supplemented agar was high (100%) **(Table F9 and F10)**. This observation is supported by Gose *et al.* (2013).

In their study, GC II agar showed as good a performance as medium to culture *N. gonorrhoeae* prior to MIC test since ceftriaxone MICs obtained using chocolate supplemented agar and GC II agar showed a high level of agreement (Gose *et al.*, 2013a). On the other hand, GC saponin agar and GC II agar might also be adequate to grow *N. gonorrhoeae* for the penicillin MIC test (**Table F10** and **Table F11**). Although the chocolate supplemented agar is the suggested medium to grow *N. gonorrhoeae* isolates, this study revealed that other, slightly cheaper media could be used to grow the bacteria.

4.2. Antimicrobial resistance in *N. gonorrhoeae*

The current study showed that 23 out of 28 (82%) of the atypical *N. gonorrhoeae* isolates that were obtained from Auckland (21 isolates), Wellington (1 isolate) and Taranaki (1 isolate) from 2012 to 2015 showed reduced susceptibility to ceftriaxone, with MIC between 0.03 to 0.06 mg/L **(Table 3-3)**. Previously, ESR has reported *N. gonorrhoeae* with reduced susceptibility to ceftriaxone from Auckland, Hamilton and Canterbury with MIC of 0.06 mg/L **(Table 1-9)**. In other countries, although not outright resistant to ceftriaxone, *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone have been previously reported to be associated with ceftriaxone treatment failures **(Table 1-8)**. However, until the current time, no ceftriaxone treatment failures have been reported in New Zealand.

This study also revealed that the penicillin-resistance rate (36% resistant; **Table 3-3)** can be compared with that for the Auckland region since more than 50% of the *N. gonorrhoeae* isolates were from Auckland region. In 2010 and 2011, the penicillin-resistance rates for Auckland were 23.2% and 28.7%, respectively (ESR, 2011; ESR, 2012). Even though the current study showed a 7% increase in the resistant rate over the ESR reports, the number of isolates in the current study is very low, and *N. gonorrhoeae* with reduced susceptibility to ceftriaxone tend to show resistance to penicillin. The character of the sample contributes to

a higher resistant rate. Overall, a rate of 36% resistant to penicillin G among the *N. gonorrhoeae* isolates is consistent with the rate reported for Auckland.

Additionally, the presence of 21% penicillinase producing *N. gonorrhoeae* (PPNG) in the current study, including *N. gonorrhoeae* isolates that showed a high level of penicillin G resistance (>32 mg/L) signalled the involvement of the bla_{TEM-1} gene on a plasmid. The gene is responsible for encoding a penicillinase called TEM-1-type β-lactamase (Unemo and Shafer, 2014). The presence of the PPNG is higher than was been reported by ESR in 2002 (9.0%) (Heffernan *et al.*, 2004), but once again, the number of samples in the current study is too low and biased for a valid comparison. On the other hand, the other 22 non-PPNG *N. gonorrhoeae* isolates that were indeterminate or resistant to penicillin G, suggested that different resistance determinants might be involved, such as an insertion of aspartate acid (D) at position 345 (D345), or other changes like F504L, A510V, A516G and P551S of the PBP2 sequence (Powell *et al.*, 2009). Although D345 identification in PBP2 was not carried out for all isolates, one non-PPNG isolate, 1380, was found to harbour the D345A alteration, and the alteration might contribute to its indeterminate susceptibility to penicillin G (MIC:1 mg/L). In future investigations, it would be beneficial to determine the prevalence of D345A alterations among *N. gonorrhoeae* isolates for the purpose of surveillance and understanding its association with high MIC of penicillin in this country. The current study also showed the presence of A504L and A510V in all the *N. gonorrhoeae* isolates, and A516G in 43% of the samples **(Table G-6)**, so these mutations could have played a part in elevating the penicillin G MIC. On the other hand, the presence of mutated *mtrR*, $porB_{IB}$ and *ponA* **(Table 3-25)** might also have contributed to the elevated penicillin G MIC.

For tetracycline, although the isolates are not representative of the whole of New Zealand, the finding that just 25% (7/28) of the *N. gonorrhoeae* isolates were resistant to tetracycline **(Table 3-3)** is slightly less than in 2008 when the last report of a tetracycline resistance rate was 31.4% for Auckland (ESR, 2008a). It is predicted that plasmid mediated tetracycline resistance gene (*tetM*) might be

responsible for two isolates that exhibited the highest tetracycline MIC in the current study (24 mg/L and 32 mg/L). In 2002, Heffernan and colleagues reported 6.5% of tetracycline-resistant *N. gonorrhoeae* (TRNG) (MICs ≥ 16 mg/L) strains in New Zealand, while 21.3% of the gonococcus isolates showed low-level resistance to tetracycline (MICs 1-8 mg/L). The current result suggests that there has not been a drastic increase in the proportion of TRNG isolates, however, there is an increase in the presence of gonococcus isolates showing low-level resistance to tetracycline in the current study. Additionally, two TRNG isolates and three isolates with low-level resistance to tetracycline were also PPNG strains. Although the number of the isolates is low and biased to isolates with elevated ceftriaxone MIC, the findings are in agreement with the Brett *et al.* (1992) study where PPNG strains were found to be resistant to tetracycline.

Between 2010 and 2014, the ciprofloxacin resistance rate in *N. gonorrhoeae* from the Auckland region ranged from 31.4% to 43.5% (national rate from 35.4% to 43.5%). In the current study, the resistance rate was 100% **(Table 3-3)**, which may not be representative of ceftriaxone-susceptible isolates as noted above. In most studies of *N. gonorrhoeae* with high MIC of ESCs, the strains were also resistant to ciprofloxacin (Ohnishi *et al.*, 2011; Unemo *et al.*, 2011a; Camara *et al.*, 2012; Unemo *et al.*, 2012; Lewis *et al.*, 2013).

For the first time, azithromycin-resistant *N. gonorrhoeae* were found in New Zealand. However, the 7% (2/28) rate of azithromycin-resistant *N. gonorrhoeae* isolates **(Table 3-3)** needs to be interpreted cautiously due to the small sample size and the very biased set of *N. gonorrhoeae* isolates. Worryingly, one azithromycin resistant isolates (isolate 264) also showed reduced ceftriaxone susceptibility, with MIC of 0.03 mg/L. The presence of two azithromycin-resistant *N. gonorrhoeae* alongside 15 isolates that showed indeterminate resistance of azithromycin indicates the emergence of *N. gonorrhoeae* with reduced susceptibility to azithromycin in this country. An association between elevated azithromycin and ESC MICs has been reported in several countries such as United States (CDC, 2013b), United Kingdom (PHE, 2013), Australia (Lahra,

2014) and Canada (Martin *et al.*, 2016). It is an important finding since azithromycin is currently administered alongside ceftriaxone as a dual antimicrobial therapy regime of uncomplicated gonorrhoeae infection in this country (NZSHS, 2015).

N. gonorrhoeae resistance to azithromycin is strongly associated with an adenine (A) deletion in the *mtrR* promoter (Zarantonelli *et al.*, 1999). However, in this study, the association of the A deletion in the *mtrR* promoter with high MIC of azithromycin was weak. The A deletion in the *mtrR* promoter was also present in *N. gonorrhoeae* isolates that are fully susceptible to azithromycin. Although it is possible that the A deletion in the *mtrR* promoter could partially contribute to the increased MIC to azithromycin, other factors such as mutation in the 23S rRNA, involvement of *erm* genes, MacAB or *mef*-efflux pump might also be involved in increasing the MIC of azithromycin (Unemo and Shafer, 2014). With the emergence of *N. gonorrhoeae* with elevated azithromycin MIC in this country, a further study that elucidates the phenotypic and genotypic character of the strains and, that involves a higher number of unbiased isolates is required.

On the other hand, this study showed that the AST interpretation using CLSI and EUCAST breakpoints gave different rates in the susceptibility categorisation for tetracycline. More isolates were found to show indeterminate susceptibility to tetracycline when the CLSI guideline was used compared to when using the EUCAST guidelines (71% CLSI vs 39% EUCAST). On the other hand, no changes in the susceptibility categorisation for penicillin since both guidelines have the same breakpoints. For azithromycin where CDC breakpoint was used, no isolates showed indeterminate resistance, while 50% isolates showed indeterminate resistance with EUCAST guidelines **(Table 3-4)**. Until the current time, CLSI has not yet established azithromycin breakpoint for *N. gonorrhoeae* (Kirkcaldy *et al.*, 2015).

Even though there were no changes in the susceptibility categorisation for ciprofloxacin and ceftriaxone, the resistant breakpoints set by CLSI for these antibiotics are also higher than EUCAST. The different in the breakpoints could be due to the way these guidelines were established by both standard in order to meet their own objectives. CLSI include the viewpoints of industry, government and the healthcare professions in a consensus-driven process to establish their breakpoints, while EUCAST was set up with the main objective to harmonize the susceptibility testing and setting breakpoints in Europe (Brown *et al.*, 2016). The difference in susceptibility categorisation in this study particularly for azithromycin and tetracycline indicates a need for the establishment of one international set of breakpoints for AST interpretation that can be used in laboratories worldwide. The differences in breakpoints between the different guidelines, particularly the CLSI and EUCAST, might complicate the international surveillance of antimicrobial resistance, and could cause a false comparison of resistance rates in different countries (Kahlmeter and Brown, 2002).

4.3. *N. gonorrhoeae* DNA leaching using elution buffer (10mM TrisHCl, pH 8.0)

The use of an elution buffer to leach *N. gonorrhoeae* DNA was found to be reliable since it successfully produced a usable sequencing result for most of the sequences in this study (as summarised in **Table I** to **Table VII**). Tables of sequence summary are stored in the respective gene folder in the attached DVD). Leaching DNA using an elution buffer could be a good substitute to obtain DNA from *N. gonorrhoeae* for the purpose of sequencing rather than other more expensive kits. Moreover, the method requires minimal resources in the laboratory, is more convenient, much safer and more environmentally-friendly compared to methods requiring material such as phenol (Casali, 2003).

However, in comparison with other specialised DNA extraction kit in the market, the method requires a manual cutting of the agarose gel to obtain the DNA, which can be time-consuming and needs good technical skills. Secondly, the method is

not a rapid method since it involved an overnight preparation of the DNA template. Some sequences in this study showed a low quality of the sequencing result for either forward or the reverse sequence probably due to the poor amplification during PCR. This did not cause any issues in analysing the sequence since either the forward or the reverse sequence with a good quality sequencing result was available for the analysis.

4.4. Association of mosaic PBP2 alterations in *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone

The current study revealed that the mosaic *penA* is the most common *penA* observed in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone in this country (87%) **(Table 3-25).** The key mosaic PBP alterations, I312M and V316T, can be seen in all of the sequences, confirming the presence of the sequence. The current study is in agreement with a study by Osaka *et al.* (2008) that mosaic PBP2 is one of the primary contributors to reduced susceptibility to ceftriaxone since the multiple alterations cause conformational alteration of the β-lactam-binding pocket. The mosaic *penA* (PBP2) sequence largely resembles that of *N. peflava/sicca* and *N.flavescens*, followed by *N. cinerea* and *N. polysacchareae* **(Table G4).** Hence, some of the mutations observed in the sequence have been reported in various *Neisseria* commensals **(Table G5)**. In agreement with the finding by Ameyama *et al.* (2002) that the mosaic *penA* sequence resembles the *N. perflava/sicca*, *N. cinerea, N. flavescens* and *N. meningitidis*, the mosaic PBP2 observed in *N. gonorrhoeae* in New Zealand is probably a product of intraspecies exchanges of the *penA* fragment with more than one *Neisseria* commensal. The presence of mosaic *penA* in *N. gonorrhoeae* isolates in this country is in agreement with the finding by Nicol *et al.* (2014), where 5% of the residuals DNA of *N. gonorrhoeae* was found to be positive for the mosaic gene.

Based on the comparison with the PBP2 wild-type sequence of *N. gonorrhoeae* LM306 strains, the current study revealed that 14 out of 23 *N. gonorrhoeae*

isolates with reduced susceptibility to ceftriaxone harboured the mosaic PBP2 M-1, with I312M, V316T, G545S and N512Y mutations **(Table 3-15)**. The finding indicates it is the most common patterns found for the PBP2 mosaic among *N. gonorrhoea*e isolates with reduced susceptibility to ceftriaxone in this country and the significant impact of this pattern on ceftriaxone MIC. The combination of I312M, V316T and G545S alterations in mosaic PBP2 is common and has been widely reported, and these variations together have been verified to cause a significant increase in ceftriaxone MIC in *N. gonorrhoeae* (Takahata *et al.*, 2006; Tomberg *et al.*, 2010). On the other hand, the presence of N512Y in gonococcus isolates with elevated ceftriaxone MIC has also been published (Takahata *et al.*, 2006). Among these isolates, isolate 631 showed the highest MIC of ceftriaxone in this study (0.06 mg/L), which further supports the impact of the three PBP2 mutations together. Additionally, besides the I312M, V316T and G545S combination, and the participation of N512Y mutation, the presence of a high number of mutations (25 to 27 mutations) in the mosaic PBP2 M-1 might also contribute to more isolates showing reduced susceptibility to ceftriaxone **(Table 3-13)**. The I312M and V316T need other PBP2 changes to enhance the ceftriaxone MIC to a higher level, a phenomenon called epistasis, described by Tomberg *et al.* (2010).

The current study also highlights the possibility that an important mosaic PBP2 is present among *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone in this country. Although the PBP2 sequence was not complete, it is highly likely that the mosaic M-1 belongs to the significant mosaic PBP2 pattern XXXIV (**Figure G7** and **Table G8**) that has been associated with two ceftriaxone-resistant *N. gonorrhoeae* in France (Unemo *et al.*, 2012) and Spain (Camara *et al.*, 2012) in 2012. However, in both cases, an additional A501P mutation was observed. Although the A501P mutation was not observed in the current study, the occurrence of this mutation in the future in conjunction with the mosaic M-1 might threaten the current gonorrhoea treatment in the country. Even without the A501P mutation, the mosaic PBP2 XXXIV has been reported to be associated with ceftriaxone treatment failures for *N. gonorrhoeae* in countries such as Slovenia (Unemo *et al.*, 2011b), Sweden (Golparian *et al.*, 2014) and Australia

(Chen *et al.*, 2013). The ceftriaxone MIC conveyed by these isolates (between 0.03 mg/L to 0.125 mg/L) overlapped the ceftriaxone MIC range shown by gonococcus isolates that harbour the mosaic M-1 **(Table G9)** in the current study (0.03 mg/L to 0.06 mg/L). In addition, it is worth mentioning that one isolate (1848) harboured the mosaic M-1 with two additional alterations, the P552V and K555Q **(Table G7)**, that were not observed in the published mosaic XXXIV, but have been seen in other mosaic PBP 2 pattern (Ohnishi *et al.*, 2011). However, the additional mutations in that case did not lead to any further increase in ceftriaxone MIC.

It is interesting that, based on the PBP2 region amplified, the mosaic patterns M-2, M-3, M-4 and M-5 do not match with any of the published mosaic PBP2 patterns. Unlike mosaic M-1, these mosaic PBP2 seems to be missing at least ten common mosaic PBP2 mutations usually observed in published mosaic PBP2 (other than I312M and V316T) (**Figure G7** and **Table G8)** (Ohnishi *et al.*, 2011). However, although not 100% identical, the mosaics M-2, M-3, M-4 and M-5 resemble the mosaic PBP2 XXXV published by Ohnishi *et al.* (2011) since the ten PBP2 changes were also absent from this published sequence. Additionally, M-2, M-3 and M-4 patterns were observed in isolates with reduced ceftriaxone susceptibility (three isolates with M-2, one isolate each with M-3 and M-4) **(Table 3-13).** Regardless, the finding of these mosaics PBP2 pattern in the current study might indicate the presence of unique mosaic PBP2 patterns among *N. gonorrhoeae* isolates in this country. Further work should be done to confirm the novelity of these patterns, which involve investigating the full *penA* sequence of the mosaic patterns. The investigation is important since mosaic M-2, M-3 and M-4 were observed in isolates with reduced ceftriaxone susceptibility.

This study revealed that the presence of other key PBP2 mutations, such as A501V/T, P551S, G542S and G545S in addition to mosaicism (I312M and V316T), might be important to futher enhance the ceftriaxone MIC. Mosaic M-5 which was found in isolate 1641 that is susceptible to ceftriaxone in the current study (MIC: 0.01 mg/L) did not harbour any of the additional mutations stated

above, except for the mutations I312M and V316T **(Table 3-16)**. Although this isolate was originally found by ESR to have reduced susceptibility to ceftriaxone, it is possible that the presence of only I312M and V316T is not sufficient to cause an increase in ceftriaxone MIC, and these results match those in other studies (Zhao *et al.*, 2009; Tomberg *et al.*, 2010). The A501V/T, P551S, G542S and G545S mutations are believed to be gonococcal-specific alterations selected by antibiotics and are not acquired from *Neisseria* commensals (Takahata *et al.*, 2006; Tomberg *et al.*, 2010; Whiley *et al.*, 2010a). The current study is in agreement with the study by Tomberg *et al.* (2010), Whiley *et al.* (2010a) and Takahata *et al*. (2006) that the A501V/T, P551S, G542S and G545S might be needed to elevate ceftriaxone MIC to a higher level.

It is also worth noting that the significance of the A501V/T mutation can be seen in this study **(Table 3-16)**. The presence of A501V/T in mosaic PBP2 is rare, and has been observed only in mosaic pattern-4 (pattern XXVI) (Takahata *et al.*, 2006; Ohnishi *et al.*, 2010) and pattern XXX (Ohnishi *et al.*, 2010). Although the G545S mutation was not seen in mosaics M-2, M-3 and M-4, the ceftriaxone MIC shown by most of the isolates that harbour the A501V/T mutation are similar to the ceftriaxone MIC conveyed by most isolates that harbour mosaic M-1, which is 0.06 mg/L. These findings support previous research carried out by Tomberg *et al.* (2010) who found that, like G545S, alterations at position 501 of PBP2, particularly A501V together with mosaic PBP2, causes a substantial increase in ceftriaxone MIC. Osaka *et al.* (2008) have also described the point A501V mutation (in the absence of the mosaic *penA*) as one of the main contributors to reduced susceptibility to ceftriaxone in *N. gonorrhoeae,* since it causes conformational change in the β-lactam-binding pocket of PBP2.

4.5. Association of nonmosaic PBP2 alterations in *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone

Although the presence of nonmosaic PBP2 was not as common as the mosaic PBP2, the importance of nonmosaic PBP2 can be seen in this study since the ceftriaxone MIC overlapped the ceftriaxone MIC of isolates that harboured

mosaic PBP2 **(Table 3-16)**. Once again, the impact of the A501V/T can be seen, where this time it was observed in nonmosaic PBP2 sequences (NM-1, NM-2 and NM-3). This PBP2 sequence was observed in three *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone **(Table 3-16)**. The finding is in agreement with the finding by Tomberg *et al.* (2010) that alteration of A501V/T in nonmosaic PBP2 causes a 2-fold increase in ceftriaxone MIC. Furthermore, the nonmosaic PBP2 with A501V mutation (NM-1) resembles nonmosaic PBP2 pattern XIII, originally found in Australia (Whiley *et al.*, 2007b), which was associated with ceftriaxone treatment failure case in 2009 (Tapsall *et al.*, 2009a).

Although the number of samples was low, the study also showed that the presence of P551S or G542S alongside the A501V/T, observed in nonmosaic NM-1 and NM-2, might elevate the ceftriaxone MIC further. This result is in agreement with the finding by Whiley *et al.* (2007) where patterns XI and XVIII, that resemble the nonmosaic PBP2 NM-1 and NM-2 respectively, were observed in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone (MIC: 0.06 to 0.12 mg/L). Both the G542S and P551S changes are frequently observed among *N. gonorrhoeae* isolates with elevated ceftriaxone MIC (Whiley *et al.*, 2007b; Whiley *et al.*, 2010a).

On the other hand, although the P551L alterations might contribute to the elevation of the ceftriaxone MIC in the current study, the mutation might not be sufficient to cause *N. gonorrhoeae* to express reduced susceptibility to ceftriaxone, either alone (NM-4) or in the presence of A501T mutation (NM-3) **(Table 3-16)**. The combination of amino acid substitutions at codons 501 and 551 of PBP2 have been reported to lead to reduced susceptibility to ceftriaxone in *N. gonorrhoeae* but in the presence of *mtrR* and *penB* mutations (Whiley *et al.*, 2010a). However in the current study, isolate 893 and 1380, that harboured nonmosaic NM-3 and nonmosaic NM-4 respectively, also harboured the mutated *mtrR, porB*$_{IB}$ and *ponA* **(Table 3-26)**. Hence the results of this study still contradict the reported by Whiley *et al*. (2010a). Similarly, Whiley *et al.* (2007) reported that a nonmosaic PBP2 (pattern XI) that resembles pattern NM-3 was seen in *N.*

gonorrhoeae with reduced susceptibility to ceftriaxone. It is possible that other unknown mutations are required to further increase the ceftriaxone MIC. However, the ceftriaxone MIC expressed by isolate with the NM-4 PBP2 is in agreement with the finding by Whiley *et al.* (2007). The nonmosaic PBP2 pattern IX (resembling the pattern NM-4) with P551L mutation was also observed in a *N. gonorrhoeae* strain that was fully susceptible to ceftriaxone (MIC ≤0.008 mg/L and 0.016 mg/L). This suggests that nonmosaic PBP2 pattern NM-4 is not associated with reduced susceptibility to ceftriaxone *in N. gonorrhoeae*.

Regardless, the current study showed that PBP2 alterations such as A501V/T, P551S/L, G542S and other unknown mutations might be required to elevate the ceftriaxone MIC. This was proven by the one NM-5 isolate, 963, that showed the lowest ceftriaxone MIC reading in the current study (0.008 mg/L) **(Table 3-16)**. The isolate does not have any additional key PBP2 alterations reported to cause an elevated MIC of ceftriaxone. The nonmosaic PBP2 NM-5 resembles pattern XVI from the study by Whiley *et al.* (2007), where isolates that harbour the pattern XVI also showed very low ceftriaxone MIC ≤0.008 mg/L.

Additionally, it is worth noting that PBP2 mutations A311V, T316P, A328T and the T484S associated with the ceftriaxone-resistant strain H041 in Japan (Ohnishi *et al.*, 2011) were not observed in this study. These alterations have been verified to enhance the ceftriaxone MIC to a clinical resistance level (Tomberg *et al.*, 2013).

The full length *penA* PCR was carried out on only one isolate (isolate 1380) due to the limited resources. Isolate 1380 was chosen since it was one of the earliest isolates that did not harbour a mosaic PBP2, and showed a discrepancy of ceftriaxone MIC between ESR (0.06 mg/L) and the current study (0.01 mg/L). As well as a nonmosaic NM-4 pattern, isolate 1380 also harboured four PBP2 changes related to a high penicillin G MIC. These were an aspartic acid (D) insertion between amino acids 345 and 346 (346 and 347 in the current study

due to the PBP2 frame shift caused by alignment with *N. gonorrhoeae* LM306 strain), F504L, A510V and A516G **(Table 3-17)**. Since the MIC of penicillin G of isolate 1380 is 1 mg/L, which is categorised indeterminate resistance **(Table 3-1),** it is possible that the PBP2 changes contributed to this. The insertion of D345 can cause a 4-fold increase in penicillin G MIC (Brannigan *et al.*, 1990) while the rest of the mutations cause an increase of 1.3 to 1.8 fold of penicillin MIC (Powell *et al.*, 2009). Isolate 1380 lacked all mutations in PBP2 reported to increase the ceftriaxone MIC, apart from P551L.

4.6. Association of *mtrR* alterations in *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone

The adenine (A) deletion in the *mtrR* promoter, with or without various other alteration in MtrR coding region was the predominant *mtrR* alteration observed among *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone **(Table 3-19)**. It was observed in 22 out of 23 isolates that expressed reduced susceptibility to ceftriaxone (MIC: 0.03 to 0.06 mg/L). This A deletion in the *mtrR* promoter is the primary *mtrR* mutation reported among gonococcus strains with reduced susceptibility to ceftriaxone (Unemo *et al.*, 2002; Tanaka *et al.*, 2006; Warner *et al.*, 2008; Liao *et al.*, 2011). Importantly, the mutation was also observed among ceftriaxone-resistant *N. gonorrhoeae* strains reported in France (Unemo *et al.*, 2012) and Spain (Camara *et al.*, 2012). This finding is consistent with other research where the A deletion in the *mtrR* promoter might contribute to further elevate the ceftriaxone MIC .

However, the A deletion in was also observed in *N. gonorrhoeae* isolates that were fully susceptible to ceftriaxone, including isolate 963 which showed the lowest ceftriaxone MIC in this study (0.008 mg/L). The finding is in agreement with the suggestion of Zhao *et al*. (2009) that *mtrR* mutations require other mutated allelles such as *penA* and $porB_{IB}$ to elevate the ceftriaxone MIC to reduced suseptibility range. The four isolates (963, 893, 1380, 1026) that were

fully susceptibile to ceftriaxone despite harbouring A deletion in *mtrR* promoter, lacked important PBP2 and PorBIB alterations **(Table 3-26)**.

In the current study, 25 gonococcus isolates that harboured the A deletion also harboured various other point mutations in MtrR such as the A39T, G45D, T86A and H105Y. All six isolates that harboured the A deletion and G45D/A39T alteration showed reduced susceptibility to ceftriaxone (MIC: 0.03 to 0.05 mg/L) **(Table 3-19)**, which is in agreement with a study carried out by Tanaka *et al.* (2006). The impact of the A deletion in the *mtrR* promoter, and G45D alteration in the MtrR coding region, on an increase in ceftriaxone MIC has been verified by Zhao *et al*. (2009). It is believed that the location of both G45D and A39T within the helix-turn-helix (HTH) of the DNA-binding site might increase the helical characteristics of the region, affecting the repressor activity of the *MtrR* and enhancing expression of the *mtrCDE* (Hagman *et al.*, 1995; Unemo *et al.*, 2002). However, the presence of either A39T and G45D alone only causes an intermediate level of resistance (Hagman *et al.* 1995). Regardless, Tanaka *et al*. (2006) proposed that the presence of a double alteration in the MtrR coding region might lead to more efficient efflux pump production in *N. gonorrhoeae,* leading to further decreased susceptibility to ceftriaxone. It is worth noting that one *N. gonorrhoeae* isolate with reduced susceptibility to ceftriaxone (isolate 119) was found to harbour only an A39T mutation in the MtrR coding region; however, its elevated ceftriaxone MIC might be due to the presence of mosaic PBP2 M-1 **(Tables 3-18 & 3-26)**.

The combination of both an A deletion in the *mtrR* promoter and an H105Y alteration in the coding region was the most common, where 15 out of 17 isolates with these mutations showed reduced susceptibility to ceftriaxone **(Table 3-19)**. Unlike G45D and A39T alterations, H105Y is situated outside the HTH DNA-binding site region. Mutations that are situated downstream of the HTH region could affect the tertiary structure or sub-unit interactions that are important in DNA-binding (Shafer *et al.*, 1995) and, based on the location, the T86A alteration might also cause a similar effect. The current study showed that H105Y mutation

was observed in both *N. gonorrhoeae* with reduced susceptibility to ceftriaxone and fully susceptible to ceftriaxone. On the other hand, the T86A mutation was seen only *in N. gonorrhoeae* that were fully susceptible to ceftriaxone. Liao *et al.* (2011) found the single H105Y mutation among *N. gonorrhoeae* that were fully susceptible to ceftriaxone.However, the single T86A alteration was only observed in gonococci that showed reduced susceptibility to ceftriaxone by Liao *et al.* (2011), which contrasts to what was observed in the current study. It is hard to determine the effect of these mutations on the ceftriaxone MIC in the current study since the A deletion in the *mtrR* promoter was present in most isolates. Although the mutations might affect the ceftriaxone MIC, unlike the mutations in the MtrR coding region, the impact of the T86A and H105Y mutations with the A deletion in the *mtrR* promoter on the ceftriaxone MIC was never verified.

4.7. Association of G120 and A121 alterations of PorBIB with *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone

In this study, both G120 and A121 PorBIB alterations were found in 18 out of 23 isolates with reduced susceptibility to ceftriaxone **(Table 3-23)**. These findings mirror those of previous studies that found PorBIB alterations in *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone (Takahata *et al.*, 2006; Tanaka *et al.*, 2006; Warner *et al.*, 2008; Allen *et al.*, 2011; Lee *et al.*, 2015).

However, unlike the mutated A deletion of *mtrR* promoter which was commonly found in *N. gonorrhoeae* with reduced susceptibility to ceftriaxone (22 out of 23 isolates), there was no clear relationship between the reduced susceptibility to ceftriaxone in the gonococcus isolates and PorBIB alterations. The reason is that five *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone did not harbour these alterations **(Table 3-23)**. Conversely, despite retaining PorBIB alterations, three *N. gonorrhoeae* isolates in this study were still fully susceptible to ceftriaxone. However, it is worth noting that the three isolates were originally found to show reduced susceptibility to ceftriaxone by ESR. Although the PorBIB

alterations might contribute to an elevated ceftriaxone MIC, it is possible that the presence of the mutated gene alone might not be sufficient to cause reduced susceptibility to ceftriaxone in *N. gonorrhoeae* as Zhao and colleagues (2009) have noted. Other mutated genes such as *mtrR* mutations, are required to increase the ceftriaxone MIC to a higher level. Additionally, the absence of alterations in PorBIB in five *N. gonorrhoeae* with reduced susceptibility to ceftriaxone signalled that other resistance gene determinants might have more impact on the ceftriaxone MIC compared to PorBIB. Regardless, the presence of the G120 and A121 alterations are still required to elevate the ceftriaxone MIC, in conjunction with other mutated genes. The absence of G120 and A121 PorBIB alterations in isolate 963 **(Table 3-22)**, which showed the lowest ceftriaxone MIC of 0.008 mg/L in this study, hints at the possible requirement for the G120 and A121 alterations for an increase in ceftriaxone MIC in *N. gonorrhoeae*.

Three predominant PorBIB alterations were observed among *N. gonorrhoeae* isolates in New Zealand, the G120K A121D, the G120K A121N and the G120K A121G **(Table 3-22)**. The presence of G120K A121D and G120K A121N were equally common in the current and other studies. The presence of G120K A121D mutant combination is one of the most common PorBIB alterations found in the majority of gonococcus isolates with reduced susceptibility to cephalosporins (MICs ranging from 0.06 to 0.12 mg/L) (Lindberg *et al.*, 2007; Warner *et al.*, 2008; Lee *et al.*, 2015). The G120K A121N has been frequently observed in *N. gonorrhoeae* associated with cefixime treatment failures in Canada (Allen *et al.*, 2013). It was also observed in two ceftriaxone-resistant *N. gonorrhoeae* strains isolated in France (Unemo *et al.*, 2012) and Spain (Camara *et al.*, 2012), and ceftriaxone treatment failure in Australia (Chen *et al.*, 2013). Two *N. gonorrhoeae* isolates that have been associated with treatment failure in Australia express reduced susceptibility to ceftriaxone (MIC: 0.03 to 0.06 mg/L), and harbour the PorBIB alterations. These studies suggest the probability of the G120K A121N association with ESCs treatment failures in *N. gonorrhoeae* in many countries. On the other hand, the G120K A121G PorBIB alterations **(Table 3-23)** have been commonly reported among *N. gonorrhoeae* isolates with reduced susceptibility to

ceftriaxone in China (Liao *et al.*, 2011; Li *et al.*, 2014) and Canada (Martin *et al.*, 2012).

In addition, 37 other PorBIB alterations were found in the current study **(Table I2)**. The presence of these alterations was observed in 4% to 100% of the gonococcus isolates, which reflects the high variability of the PorBIB sequence. The high diversity of PorBIB changes is a useful molecular epidemiological tool for investigating the genetic relationship between the *N. gonorrhoeae* strains (Unemo *et al.*, 2002). Together with the sequence of the *tbp* gene, the genotypic character of *N. gonorrhoeae* can be further investigated using *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) to elucidate the sequence type (ST) of *N. gonorrhoeae* that is present in New Zealand, and to determine the presence of important *N. gonorrhoeae* clone such as NG-MAST ST1407 and NG-MAST ST4220.

4.8. Association of *ponA* (L421P) mutation with decreased susceptibility of ceftriaxone in *N. gonorrhoeae* in New Zealand

The mutation in *ponA* which results in the substitution of leucine (L) with proline (P) at codon 421 (L421P) of PBP1 has been reported to reduce the binding affinity of the PBP1 and β-lactam drugs by 3 to 4-fold (Ropp *et al.*, 2002), causing *N. gonorrhoeae* to become less susceptible to β-lactam drugs such as cephalosporins. Previous studies have discovered that the *ponA* mutation is associated with *N. gonorrhoeae* that expressed reduced susceptibility to ceftriaxone (Lindberg *et al.*, 2007; Lee *et al.*, 2010). However, in this study, the association of *ponA* mutation with *N. gonorrhoeae* with reduced susceptibility to ceftriaxone is weak. The PBP1 L421P alteration was found in all *N. gonorrhoeae* isolates, which included isolates that showed reduced susceptibility and were fully susceptible to ceftriaxone based on the AST results from the current study **(Table 3-24).** A similar finding was observed by Shigemura *et al*. (2005) where *ponA* mutants showed MICs ranging from fully susceptible (<0.004 mg/L) to reduced susceptibility to ceftriaxone (0.063 mg/L). However, the *ponA* mutants

showed a much higher ceftriaxone MIC and a wider MIC range compared to the non-*ponA* mutants (<0.004 mg/L) in that study, indicating the *ponA* mutation does play a part in enhancing the ceftriaxone MIC. Regardless, since all *N. gonorrhoeae* isolates were earlier determined by ESR to show reduced susceptibility to ceftriaxone, with MICs from 0.03 mg/L to 0.12 mg/L, the contribution of the *ponA* mutation is difficult to determine. The result in the current study does, in some ways, support the work of Zhao *et al*. (2009), who found the insertion of mutated *ponA* in FA19 *N. gonorrhoeae* wildtype strain with mutated mosaic *penA, mtrR, and penB (porB$_{IB}$)* did not cause any change in ceftriaxone MIC. Another mutated allele, such as the *pilQ* with a D526 alteration might be needed, since the mutated gene has been reported to significantly enhance the effect of the *ponA* mutation (Whiley *et al.*, 2010b).

4.9. Combinations of mutated *penA, mtrR, porB$_{IB}$* and *ponA* as markers to identify *N. gonorrhoeae* with reduced susceptibility to ceftriaxone

The four mutations: the mosaic PBP2, the A501V/A501T nonmosaic PBP2 mutation, the A deletion in *mtrR* promoter and the G120 A121 alterations of PorBIB do appear to be good indicators of *N. gonorrhoeae* with reduced susceptibility to ceftriaxone. These mutations were observed in 78% to 96% *of N. gonorrhoeae* isolates with MICs from 0.03 mg/L to 0.06 mg/L in the current study **(Table 3-25).** On the other hand, although these mutations were also observed in isolates that were fully susceptible to ceftriaxone based on the AST results from the current study, these isolates were originally found to have reduced susceptibility to ceftriaxone by ESR. It is important to highlight that the presence of these mutations in the current study was made based on a small sample size that is biased to isolates with elevated ceftriaxone MIC. For the future investigation, a bigger sample size of fully ceftriaxone-susceptible *N. gonorrhoeae* would help to establish the true prevalence of these mutated genes among the isolates.

Although each of the mutated genes might play a part in the elevation of the ceftriaxone MIC, it is the cumulative effect of mutations in *penA, mtrR, porB$_{IB}$* and *ponA,* plus the synergism between *mtrR* and *porB$_{IB,}$* which has caused the incremental increases in the ceftriaxone MIC in *N. gonorrhoeae* **(Tables 3-26 and 3-27)**. *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone in the current study were found to harbour three or four genes with mutations, including mutations in *penA*. Although four isolates that were fully susceptible to ceftriaxone were also observed to harbour three or four genes with mutation, the absence of certain significant mutation in those genes might have led to the low ceftriaxone MIC.

In Group I and Group IV, the cumulative effect of all four mutated genes together can be seen, where 17 out of 23 *N. gonorrhoeae* with reduced susceptibility to ceftriaxone harbour the mosaic or nonmosaic PBP2 (with A501V/T), mutated *mtrR,* mutated *porB$_{IB}$* and *ponA* **(Table 3-27)**.

The current study also showed that six isolates that showed reduced susceptibility to ceftriaxone harboured only three mutated genes, namely the mosaic *penA*, *mtrR*/*porB$_{IB}$* and *ponA* (**Table 3-27**; (Groups II and III)). However, unlike Group I where 100% of the gonococci showed reduced susceptibility to ceftriaxone due to the presence of all mutated genes, these set of mutations were also observed in two isolates that were fully susceptible to ceftriaxone. It is predicted that the absence of either one of the mutated *mtrR* or *porB$_{IB,}$* might contribute to this condition. As seen in Group I where both mutated *mtrR* and *porB$_{IB,}$* were present, the ceftriaxone MIC is elevated higher, suggesting the importance of the synergism between *mtrR* and *porB$_{IB.}$* The importance of the synergism between *mtrR* and *porB$_{IB}$* for increased ceftriaxone MIC previously has been suggested by Zhao *et al.* (2009). In this study, the insertion of *mtrR35* into the *FA19penA35* strain caused little to no increase in the ceftriaxone level in the transformant. However, upon the insertion of *penB35*, which contains PorBIB alterations, the ceftriaxone MIC increased by 2.5-fold (Zhao *et al.*, 2009) **(Table 1-3)**. Similarly, Olesky *et al*. (2006) hypothesised that PorBIB variants required

the efflux pump activity of the MtrCDE system, and the two work together through porin regulation and efflux pump activity to decrease the periplasmic concentration of antibiotics. PorBIB alterations alone do not significantly affect the permeation rate of β-lactam drugs (Olesky *et al.*, 2006). Regardless, for five *N. gonorrhoeae* isolates that showed reduced susceptibility to ceftriaxone but lacked either *porB1B* or *mtrR* mutations, it is likely that the mosaic *penA* or other unknown mutations plays a bigger role in increasing the ceftriaxone MIC.

The significant contribution of mosaic and nonmosaic *penA* (A501V/T), plus the synergism between the mutated *mtrR* and $porB_{IB}$ in causing reduced susceptibility to ceftriaxone is further suggested by one isolate that harboured none of these mutations. Isolate 963 had the lowest ceftriaxone MIC (MIC of 0.008 mg/L) and harboured only the mutated *mtrR* and *ponA* **(Table 3-26).**

CHAPTER FIVE

CONCLUSION

5. CONCLUSION

In summary, this study has provided an information on *N. gonorrhoeae* isolates in New Zealand, particularly those that exhibit reduced susceptibility to the last drug available for empirical therapy, ceftriaxone. A total of 23 (82%) out of 28 *N. gonorrhoeae* isolates collected from 2012 to 2015 in New Zealand showed reduced susceptibility to ceftriaxone, with MICs from 0.03 mg/L to 0.06 mg/L, at least, when tested in a candle jar. By contast, ESR has found that all isolates showed reduced susceptibility to ceftriaxone (0.03 mg/L to 0.12 mg/L). The

finding is concerning because *N. gonorrhoeae* with reduced susceptibility to ceftriaxone have been associated with ceftriaxone treatment failures in countries such as Sweden (Golparian *et al.,* 2014; Unemo *et al.,* 2011a), Slovenia (Unemo *et al.,* 2011b) and Australia (Whiley *et al.,* 2007b; Tapsall *et al.,* 2009a). These isolates with ceftraxone MICs that do not meet the resistant threshold are still capable of causing treatment failures, particularly in the pharyngeal site.

The current study has shown that the 28 *N. gonorrhoeae* isolates consisted of MDR *N. gonorrhoeae*, with 36% of the isolates resistant to penicillin G, 25% to tetracycline, 100% to ciprofloxacin and 7% to azithromycin. This is the first time azithromycin resistance *N. gonorrhoeae* is reported in New Zealand. One of the two isolates that was resistant to azithromycin (isolate 264) also exhibited reduced ceftriaxone susceptibility. In addition the presence of 15 *N. gonorrhoeae* isolates with reduced susceptibility to azithromycin is worrying. Since the current practice for gonorrhoea treatment involves administering azithromycin (1 g) alongside 500 mg ceftriaxone as a dual therapy for uncomplicated urogenital, anorectal and pharyngeal gonorrhoea in this country, the emergence of these strains is a concern. In the future, a study should be conducted to elucidate the character of these strains.

Although mosaic PBP2 has previously been reported among *N. gonorrhoeae* in New Zealand, due to the lack of culture sample, the gene was not fully studied. The current study revealed that mosaic PBP2 (with G545S/A501V) and nonmosaic PBP2 (A501V/T) might be the primary contributors to the reduced susceptibility to ceftriaxone in *N. gonorrhoeae* isolates since these mutations were observed in all 23 *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone. Another significant finding was that the *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone in New Zealand harbour recognised PBP2 patterns resembling the mosaic PBP2 XXXIV, and also the nonmosaic PBP2 XIII (with A501V). Mutations in PBP2 can be highly varied, and the fact that the partial sequences are identical with the published sequence showed that the significant

mosaic and nonmoasic PBP2 might be circulating in this country. However, full sequence analysis would need to be done to validate the PBP2 pattern.

The study has also revealed unique mosaic PBP2 patterns, M-2, M-3, M-4 and M-5, which do not resemble any published mosaic PBP2 sequences, signalling the possible emergence of new mosaic PBP2. The finding will serve as a basis for future studies to confirm the novelty of the patterns.

This is the first time the presence of mutated *mtrR, porB$_{IB}$* and *ponA* have been reported among *N. gonorrhoeae* isolates in New Zealand. Although it is unclear if the mutated genes do contribute to reduced susceptibility to ceftriaxone due to the small number and bias of the isolates, the mutated genes were common among *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone. It is suspected that other known (*pilQ*) and unknown resistance gene determinant(s) might be present among *N. gonorrhoeae* with reduced susceptibility to ceftriaxone in this country. The association of *pilQ* alterations with high ceftriaxone MIC should be investigated in future studies.

The analysis of the PorBIB sequence of 28 *N. gonorrhoeae* isolates in this study revealed a highly variable sequence, which will be useful as a molecular epidemiological tool to investigate the genetic relationship between the *N. gonorrhoeae* isolates.

The cumulative effect of four mutated genes, *penA (*mosaic PBP2 G545S/A501V or nonmosaic PBP2 with A501V/T) with *mtrR, porB$_{IB}$* and *ponA* together can be seen in this study, where this group of mutation was frequently observed among *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone. Six other *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone were found to harbour three mutated genes, the mosaic *penA* mutated *mtrR* or *porB$_{IB}$,* and *ponA*. The type of mutations in PBP2 (besides the alteration at codons 312 and

316, a part of mosaic PBP2 alterations) which involve changes in codon 545 and 501, plus the synergism of *mtrR and* $porB_{IB}$ might play an important role in elevating the ceftriaxone MIC to reduced susceptibility to ceftriaxone level.

Once again it important to note that these findings are based on a relatively small number of isolates that have elevated ceftriaxone MIC as pretested by the ESR. A higher number of unbiased samples should be examined in the future to determine the true phenotypic character of *N. gonorrhoeae* isolates, plus the true prevalence of these mutated genes. Nevertheless, the current study for the first time has shown the presence of these mutated genes among *N. gonorrhoeae* isolates with elevated ceftriaxone MIC.

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6. REFERENCES

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APPENDIX

7.1. APPENDIX A: Massey University Human Ethics Committee (MUHEC) application

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

COPY FOR YOUR INFORMATION

Norshuhaidah bt Mohd Jamaludin
110B Linton Street
West End
PALMERSTON NORTH 4410

Dear Aeda

Re: HEC: Southern A Application – 15/13
Phenotypic and molecular characterization of the *Neisseria gonorrhoeae* with reduced susceptibility to ceftriaxone in New Zealand

Thank you for your letter dated 16 March 2015.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Mr Jeremy Hubbard, Acting Chair
Massey University Human Ethics Committee: Southern A

cc A/Prof Mary Nulsen
Institute of Food Science & Technology
PN434

Massey University Human Ethics Committee
Accredited by the Health Research Council

Research Ethics Office, Research and Enterprise
Massey University, Private Bag 11222, Palmerston North 4442, New Zealand T 06 3505573; 06 3505575 F 06 350 5622
E humanethics@massey.ac.nz; animalethics@massey.ac.nz; gtc@massey.ac.nz www.massey.ac.nz

7.2. APPENDIX B: The Hawkes Bay Medical Research Foundation (HBMRF) Funding

THE HAWKES BAY MEDICAL RESEARCH FOUNDATION INC.

P.O. BOX 596
NAPIER
NEW ZEALAND
www.hbmrf.org.nz

HBMRF

27th February 2015

Mary Nulsen,
Associate Professor Medical Microbiology
Programme Director, Medical Laboratory Science,
Massey University,
Private Bag 11222,
PALMERSTON NORTH. 4442

Dear Mary,

Re: Application for funding. (Ms Norshuhaidah bt Mohd Jamaludin – Project: 'Phenotypic and molecular characterization of Neisseria gonorrhoeae with reduced susceptibility to ceftriaxone in N.Z.

We are happy to report that at the executive meeting of the Foundation held last Monday evening your project, as above was approved to $4,000 to be put towards Ms Nirshuhaidah bt Mohd Jamaludin Studentship funding.

Enclosed please find our 'Research Agreement' form to be filled in and returned. (Just the first page). (Details of student).
Ethical approval will need to be applied, and please send us a copy of the ethical approval once received.

Once we have all the documentation, we can organise for payment of the grant.

Please fill in bank details if requiring direct debiting to your account.

In the meantime, good luck with the research.

Kind regards.

J. M. Baxter

Judith M Baxter (Secretary HBMRF)
Phone 06 8799199

7.3. APPENDIX C: School of Food Nutrition, Massey University Funding

Gmail by Google

aeda chucky <aidasg83@gmail.com>

More funds for your project

4 messages

Mary Nulsen <M.F.Nulsen@massey.ac.nz> Thu, Apr 30, 2015 at 9:46 AM
To: aidasg83@gmail.com
Cc: Jackie Benschop <j.benschop@massey.ac.nz>, JulieCollinsEmmerson <J.M.Collins-Emerson@massey.ac.nz>

Hello Aeda

Here is some excellent news re funding for your project! i.e. $3000 from the School of Food and Nutrition!

Best wishes
Mary

-------- Forwarded Message --------
Subject: SFN Postgraduate Research Support Outcome for: JAMALUDIN, Ms Norshuhaidah bt Mohd, 14036407
Date: Thu, 30 Apr 2015 09:29:40 +1200
From: Busby, Miria <M.Busby@massey.ac.nz>
To: Nulsen, Mary <M.F.Nulsen@massey.ac.nz>
CC: Duxfield, Matthew <M.J.Duxfield@massey.ac.nz>, Heyes, Julian <J.A.Heyes@massey.ac.nz>

Good morning Mary

On behalf of the School Food and Nutrition Postgraduate Study Award Committee, I am pleased to inform you of the approval of NZ$3,000.00 for MSc (Full-Time) for Ms Norshuhaidah bt Mohd Jamaludin.

Please contact Matt Duxfield (SFN Finance Division) to discuss the fund transfer arrangement and other details.

Regards

Miria Busby (on behalf Professor Julian Heyes)

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

THE ENGINE OF THE NEW NEW ZEALAND

Miria Busby | Academic Administrator | Massey Institute of Food Science & Technology | School of Food and Nutrition | Massey University Manawa
PN 452 | +6 951 6329 | Internal ext 83329

7.4. APPENDIX D: Institute of Veterinary, Animal and Biomedical Sciences (IVABS) Funding

Gmail by Google

aeda chucky <aidasg83@gmail.com>

IVABS Research Fund

Hill, Debbie <D.M.Hill@massey.ac.nz> Wed, Apr 9, 2014 at 12:55 PM
To: "aeda chucky (aidasg83@gmail.com)" <aidasg83@gmail.com>
Cc: "Nulsen, Mary" <M.F.Nulsen@massey.ac.nz>

Dear Norshuhaidah

Thank you for your application to the IVABS Postgraduate Research Fund to support postgraduate student research. Your application has been considered and I am pleased to advise that $1,000 has been awarded to you to assist with the expenses outlined in your budget for your project " Phenotypic and molecular characterisation of the Neisseria gonorrhoeae with reduced susceptibility to ceftriaxone in NZ ".

Please use account number RM16192 NORSH when you want to purchase something – please let me know if you need any assistance with this or if you want the money moved to the College of Health please let me know.

Kind regards

Debbie

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

THE ENGINE OF THE NEW NEW ZEALAND

Debbie Hill

Postgraduate and Research Administrator

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Mail IVABS, Private Bag 11-222, Palmerston North, New Zealand 4442

Website http://ivabs.massey.ac.nz | http://vet-school.massey.ac.nz

7.5. APPENDIX E: Methods and Materials

Table E1 Storage of *N. gonorrhoeae* isolates in BHI + 20% glycerol broth

Ingredients	**Amount**
Brain heart infusion broth powder (Becton & Dickinson, USA)	3.7g
Glycerol (BDH Chemicals)	20 ml
Milli-Q water	80 ml

All of the ingredients were mixed in a 150 ml screw cap bottle. The mixture was autoclaved at 121°C for 15 minutes. After the autoclave process, the mixture was left to cool to room temperature. Once cool, 1.0 ml of the mixture was aseptically dispensed into a 1.5 ml screw-cap propylene cryotubes using a sterile pipette. The mixture in tubes was then stored at 4°C. The tubes containing BHI + 20% glycerol were brought to room temperature before use.

Table E2 Preparation of 1L of 1X Phosphate Buffered Saline (PBS)

Ingredients [a]	**Amount**
Sodium Chloride	8 g
Potassium Chloride	0.2 g
Disodium Phosphate	1.44 g
Potassium Phosphate	0.24
Milli-Q water	80 ml

[a] All chemical ingredients are from BDH Chemicals.

All of the ingredients were mixed in a 150 ml screw cap bottle. The pH of the solution was adjusted to 7.4 using a pH meter (MeterLab). After the adjustment, the volume of the solution was adjusted to 1 L with additional milli-Q water. The pH of the solution was again adjusted to 7.4. Once adjusted, the solution was sterilized by autoclaving at 121°C at 15 minutes.

Table E3 Preparation of 20% Chelex® 100 suspensions for DNA extraction

Ingredients	Amount
Chelex® 100 (Bio-Rad)	10 g
Milli-Q water	50 ml
Disodium Phosphate	1.44 g
Potassium Phosphate	0.24 g

To prepare a 20% Chelex, all of the ingredients were mixed in a 150 ml screw cap bottle using a sterile stir bar. The solution was then stored at room temperature. To prepare 2% Chelex as a working stock for the use of DNA extraction, 3ml of 20% Chelex® 100 suspension was added to 27 of milli-Q water and mixed.

Table E4 DNA purity and concentration measurement using Nanodrop ND-1000 spectrophotometer.

No	Sample	ng/µl	Abs 260	Abs 280	260/280	260/320	Constant
1	71	30.02	0.600	0.330	1.82	0.85	50
2	411	44.02	0.880	0.521	1.69	1.02	50
3	483	33.2	0.664	0.405	1.64	0.85	50
4	604	49.07	0.981	0.699	1.40	1.41	50
5	631	37.21	0.744	0.431	1.73	0.83	50
6	729	51.73	1.035	0.627	1.65	0.91	50
7	1526	34.86	0.697	0.403	1.73	0.99	50
8	1530	45.77	0.915	0.554	1.65	0.95	50
9	257	22.58	0.452	0.270	1.67	0.82	50
10	557	40.03	0.801	0.475	1.68	1.06	50
11	558	37.16	0.743	0.448	1.66	0.98	50
12	723	38.38	0.768	0.415	1.85	0.87	50
13	801	51.42	1.028	0.581	1.77	0.90	50
14	886	43.95	0.879	0.552	1.59	0.99	50
15	1380	48.06	0.961	0.534	1.80	1.02	50
16	1641	37.37	0.747	0.433	1.72	0.82	50
17	1026	58.67	1.173	0.622	1.89	1.03	50
18	263	13.81	0.276	0.143	1.94	1.28	50
19	119	9.88	0.198	0.115	1.72	1.31	50
20	824	44.79	0.896	0.481	1.86	1.04	50
21	724	27.73	0.555	0.286	1.94	1.09	50
22	264	74.43	1.489	0.784	1.90	0.95	50
23	792	131.06	2.621	1.397	1.88	0.97	50
24	893	55.15	1.103	0.589	1.87	0.86	50
25	896	57.47	1.149	0.645	1.78	0.90	50
26	963	59.80	1.196	0.664	1.80	0.91	50
27	1332	50.41	1.008	0.606	1.66	0.81	50
28	1848	51.82	1.036	0.552	1.88	1.03	50
29	WHO K	23.84	0.477	0.294	1.62	0.89	50
30	WHOL	25.4	0.508	0.300	1.69	0.77	50
31	WHOF	33.39	0.668	0.361	1.85	0.94	50
32	ATCC	41.92	0.838	0.467	1.79	0.85	50

Abs: Absorbance

Table E5 Preparation of working solution for primers (10µM)

Ingredients	Amount
100µM of primer (Integrated DNA Technologies)	20 µl
Milli-Q water	180 ml

Working solution was prepared by adding two ingredients together in a sterile polypropylene tube. The working solution was gently mixed and kept at -20^{0}C.

Table E6 Preparation of working solution dNTPs (10mM) (Promega NZ)

Ingredients	Amount
dATP (100mM)	5 µl
dTTP (100mM)	5 µl
dCTP (100mM)	5 µl
dGTP (100mM)	5 µl
Milli-Q water	180 µl

Working solution was prepared by adding all ingredients together in a sterile polypropylene tube. The working solution was gently mixed and kept at -20^{0}C.

Table E7 Preparation of 1% agarose gel with safranin O

Ingredients	Amount
Agarose gel powder (Invitrogen NZ)	2 g
0.5 Tris-Borate-EDTA (TBE)	200 ml
Safranin O (Intron Biotechnologist)	0.75ml (for 20 ml agarose gel)

To prepare 1% of agarose gel, agarose gel powder and 0.5 TBE were mixed in a 500 ml screw cap bottle. The content was swirled, and the bottle was loosely closed. The mixture was microwaved for 1 to 3 minutes until the gel melted completely. The melted gel was left cooled on the benchtop for five minutes. To prepare a small and short gel, 20 ml of agarose gel was poured into a separate screw cap bottle. Safranin O was added to the molten gel, and the bottle was

swirled to mix the safranin with the melting gel. The mixture was poured into a tray with the designated comb. The gel was left for 20 minutes to harden. The comb was removed once the gel hardens.

Table E8 Published control sequence used to identify variations in amino acid sequences in each resistance gene

Variations in amino acid residue	Published control sequence	Reference
a) **Mosaic PBP2 *(penA)*** • **I312M** • **V316T**	Positive sequence • WHO K • *N. gonorrhoeae* NG-3 strain (GenBank accession no. AB071984; Protein ID: BAB86942.1) Negative sequence • Wild-type *N. gonorrhoeae* strain LM306 (GenBank accession no. M32091; Protein ID: AAA25463.1)	 (Unemo *et al.*, 2009) (Ameyama *et al.*, 2002) (Ameyama *et al.*, 2002)
b) Non-mosaic PBP2 *(penA)* • **Absence of I312M and V316T alterations**	Positive sequence • ATCC 49226 Negative sequence • *N. gonorrhoeae* NG-3 strain	 (CDC, 2005) (Ameyama *et al.*, 2002)
c) Other PBP2 *(penA)* alterations • **Alteration at A501** • **Alteration at N512** • **Alteration at G542**	Positive sequence • WHO L (for A501, G542 and P551) • WHO K (for N512 and G545)	 (Unemo *et al.*, 2009)

• **Alteration atG545** • **Alteration at P551**	Negative sequence • Wild-type *N. gonorrhoeae* strain LM306	(Ameyama *et al.*, 2002)
d) mtrR • **T/A deletion in 13 bp inverted sequence of *mtrR* promoter** • **G45D mutation in MtrR coding sequence**	Positive sequence • WHO K • 13 bp inverted sequence of *mtrR* promoter Negative sequence • WHO F • *N. gonorrhoeae* with wild-type *mtrR* sequence (GenBank accession no. Z25796.1; Protein ID:CAA81046.1)	(Unemo *et al.*, 2009) (Pan and Spratt, 1994) (Unemo *et al.*, 2009) (Hagman *et al.*, 1995)
e) PorBIB *($porB_{IB}$)* • **Alteration at G120** • **Alteration at A121**	Positive sequence • WHO K Negative sequence • *N. gonorrhoeae* H1-2 (GenBank accession no. AJ004943; Protein ID: CAA06234.1)	(Unemo *et al.*, 2009) (Gill *et al.*, 1998)
***f)* PBP1 (*ponA*)** • **L421P**	Positive sequence • WHO K Negative sequence • *N. gonorrhoeae* NG00085 (GenBank accession no. AB727713.1; Protein ID: BAM21172.1)	(Unemo *et al.*, 2009) (Takahashi *et al.*, 2013)

7.6. APPENDIX F: Phenotypic Testing

Table F1 Estimated fold difference in dilution (log_2) between Liofilchem s.r.l (Italy) and bioMérieux (Sweden) for ceftriaxone (CRO) and penicillin G

Isolate [a]	MIC (mg/L) for CRO [b]		Estimated fold difference in dilution (log_2)	MIC (mg/L) for PG [c]		Estimated fold difference in dilution (log_2)
	Lio	Bio		Lio	Bio	
729	0.06 (RS)	0.06 (RS)	0	>32 (R)	>32 (R)	0
119	0.06 (RS)	0.06 (RS)	0	>32 (R)	>32 (R)	0
631	0.06 (RS)	0.12 (RS)	-1	2 (R)	2 (R)	0
71	0.06 (RS)	0.06 (RS)	0	2 (R)	2 (R)	0
604	0.06 (RS)	0.03 (RS)	1	1 (I)	1 (I)	0
558	0.06 (RS)	0.03 (RS)	1	1 (I)	1 (I)	0
723	0.06 (RS)	0.03 (RS)	1	1 (I)	1 (I)	0
801	0.06 (RS)	0.06 (RS)	0	1 (I)	1 (I)	0
1526	0.03 (RS)	0.06 (RS)	-1	2 (R)	1 (I)	1
1530	0.03 (RS)	0.03 (RS)	0	2 (R)	1 (I)	1
1380	0.01 (S)	0.01 (S)	0	1 (I)	1 (I)	0
411	0.06 (RS)	0.03 (RS)	1	1 (I)	1 (I)	0
557	0.06 (RS)	0.03 (RS)	1	0.5 (I)	1 (I)	-1
886	0.06 (RS)	0.03 (RS)	1	0.5 (I)	1 (I)	-1
263	0.03 (RS)	0.03 (RS)	0	1 (I)	1 (I)	0
724	0.03 (RS)	0.03 (RS)	0	1 (I)	1 (I)	0
824	0.03 (RS)	0.03 (RS)	0	0.5 (I)	0.5 (I)	0
257	0.03 (RS)	0.06 (RS)	-1	0.5 (I)	0.5 (I)	0
483	0.03 (RS)	0.03 (RS)	0	0.5 (I)	0.5 (I)	0
1026	0.01 (S)	0.01 (S)	0	2 (R)	0.5 (I)	2
1641	0.01 (S)	0.01 (S)	0	>32 (R)	>32 (R)	0
WHO K	0.06 (RS)	0.06 (RS)	0	2 (R)	2 (R)	0
WHO L	0.06 (RS)	0.06 (RS)	0	2 (R)	2 (R)	0
WHO F	<0.002 (S)	<0.002 (S)	0	0.032 (S)	0.032 (S)	0
ATCC 49226	0.008 (S)	0.008 (S)	0	1 (I)	0.5 (I)	1

Note:

1. Grey shaded areas represent isolate that showed changes in the susceptible category between Liofilchem MIC test strips and bioMérieux Etest.

2. Lio: Liofilchem s.r.l (Italy); Bio: bioMérieux (Sweden)

[a] Due to insufficient bioMérieux Etest strips, comparison was done only for 21 isolates and four controls.

[b] For (CRO) ceftriaxone: RS: Reduced susceptibility: 0.03 mg/L to 0.12 mg/L; S: Susceptible: < 0.03 mg/L (Whiley *et al.*, 2007a; Tapsall *et al.*, 2009a; Chen *et al.*, 2013; ESR, 2014).

[c] For (PG) penicillin G: R: Resistant >1, **S**: Sensitive ≤ 0.06 (EUCAST Guidelines)

Table F2 Susceptibility classification of penicillin G and ceftriaxone for 21 *N. gonorrhoeae* isolates using Liofilchem and bioMérieux

Antimicrobial agents	Susceptibility Level	Number of isolates (%)	
		Liofilchem	bioMérieux
Ceftriaxone (CRO)	Reduced susceptibility (RS)	18 (86)	18 (86)
	Susceptible (S)	3 (14)	3 (14)
Penicillin G (PG)	Resistant (R)	8 (38.1)	5 (23.8)
	Indeterminate (I)	13 (61.9)	16 (76.2)

Table F3 MIC results agreement between Liofilchem MIC test strips and bioMérieux Etest (used and suggested by ESR) of *N. gonorrhoeae* isolates plus WHO K, WHO L, WHO F and ATCC 49226.

Antibiotic	Number of isolates [a]	Number of isolates (between MIC using Liofilchem MIC test strips and bioMérieux Etest) that differ by the following number of ± log_2 dilutions [b]			Student t-test, $p <$ 0.05 of MIC means		Significant	% agreement [c]
		-2 to -1 < n	-1 ≤ n ≤ 1	n > 1 to 2	t	*p*		
Penicillin G	25	0	24	1	0.19	0.77	No	96%
Ceftriaxone	25	0	25	0	0.25	0.87	No	100%

[a] Number of isolates that have MIC result obtain from using both Liofilchem MIC test strips and bioMérieux Etest and can, therefore, be compared. The number includes four reference strains WHO K, WHO L, WHO F and ATCC 49226.

[b] Shaded areas indicate isolates in agreement (i.e. ±1 log_2 dilution difference).

[c] Agreement was defined as an MICs differences within ± 1 log_2 of the comparison MIC (Biedenbach and Jones, 1996).

Table F4 Estimated fold difference in dilution (log_2) between ceftriaxone MIC test result from the current study and the Institute of Environmental Science and Research (ESR)

Isolate	MIC (mg/L) for ceftriaxone		Estimated fold difference in dilution (log_2)
	ESR [a]	Current study [b]	
729	0.06 (RS)	0.06 (RS)	0
119	0.06 (RS)	0.06 (RS)	0
1641	0.12 (RS)	0.01 (S)	-3
631	0.12 (RS)	0.06 (RS)	-1
71	0.06 (RS)	0.06 (RS)	0
604	0.06 (RS)	0.06 (RS)	0
558	0.12 (RS)	0.06 (RS)	-1
723	0.12 (RS)	0.06 (RS)	-1
801	0.06 (RS)	0.06 (RS)	0
1526	0.06 (RS)	0.03 (RS)	-1
1530	0.06 (RS)	0.03 (RS)	-1
1380	0.06 (RS)	0.01 (S)	-2
411	0.03 (RS)	0.06 (RS)	1
557	0.03 (RS)	0.06 (RS)	1
886	0.06 (RS)	0.06 (RS)	-1
263	0.06 (RS)	0.03 (RS)	-1
724	0.06 (RS)	0.03 (RS)	-1
824	0.06 (RS)	0.03 (RS)	-1
257	0.03 (RS)	0.03 (RS)	0
483	0.03 (RS)	0.03 (S)	0
1026	0.06 (RS)	0.01 (S)	-2
264	0.06 (RS)	0.03 (RS)	-1
792	0.12 (RS)	0.06 (RS)	-1
893	0.03 (RS)	0.01 (S)	-1
896	0.06 (RS)	0.06 (RS)	0
963	0.06 (RS)	0.008 (S)	-3
1332	0.06 (RS)	0.03 (RS)	-1
1848	0.12 (RS)	0.06 (RS)	-1

Note: 1. Grey shaded areas represent *N. gonorrhoeae* isolates that showed changes in the ceftriaxone susceptible category.

[a] All gonococcus isolates showed reduced susceptibility (RS) to ceftriaxone based on results send by ESR. For strain 264, 792, 893, 896, 963 and 1332, the MIC of ceftriaxone was determined.by ESR using agar dilution method. Whereas the rest of the isolates were determined by ESR using bioMérieux (France) Etest.

[b] Since there is no significant difference between ceftriaxone MIC result using Liofilchem MIC test strips and bioMérieux Etest, MIC result using Liofilchem MIC test strips was chosen as the official result in this study. Furthermore, all twenty-eight isolates were able to be tested using Liofilchem MIC test strips compared to bioMérieux Etest (due to the insufficient number of the strips).

Table F5 Agreement between MIC of ceftriaxone of the current study and result from ESR

Antibiotic	Number of isolates [a]	Number of isolates (between MIC result from the current study and ESR) that differ by the following number of ± 1 log_2 dilutions [b]			Student t-test, $p <$ 0.05 of MIC means		Significant	% agreement [c]
		-2 to -1 < n	-1 ≤ n ≤ 1	n > 1 to 2	t	*P*		
Ceftriaxone	28	4	24	0	4.4	<0.01	Yes	86%

[a] Number of isolates that have ceftriaxone MIC results using both Liofilchem MIC test strips and bioMérieux Etest and can, therefore, be compared.

[b] Shaded areas indicate isolates in agreement (i.e. ± 1 log_2 dilution difference).

[c] Agreement was defined as an MICs within ± 1 log_2 of the comparison MIC (Biedenbach and Jones, 1996).

Table F6 Association of β-lactamase expression with susceptibility level of penicillin G

Strains with β-lactamase	Minimum inhibitory concentrations (MICs) to penicillin G (mg/L)		
	0.5 (I)	1.0 (I)	>32 (R)
729			/
119			/
1641			/
263		/	
724		/	
824	/		

Note: I: Indeterminate; R: Resistant.

Table F7 MIC results of penicillin G where isolates were grown on GC II agar, GC saponin agar and chocolate supplemented agar prior to MIC test

Isolate	MIC of penicillin G (mg/L) Media used to grow the gonococcus prior to the MIC test [ab]		
	GC II Agar	GC Saponin Agar	Chocolate Supplemented Agar
729	>32	>32	>32
119	>32	>32	>32
1641	>32	>32	>32
631	2	2	2
71	2	2	2
604	1	1	1
558	2	2	1
723	1	1	1
801	1	1	1
1526	2	2	2
1530	1	2	2
1380	1	1	1
411	1	1	1
557	1	0.5	0.5
886	1	1	0.5
263	1	1	1
724	1	1	1
824	0.5	0.5	0.5
257	0.5	0.5	0.5
483	0.5	0.5	0.5
1026	1	1	2
WHO K	2	2	2
WHO L	2	2	2
WHO F	0.016	0.032	0.032
ATCC 49226	1	1	1

[a] All MIC test were carried out on GC II Agar

[b] Liofilchem (Italy) MIC test strips were used to determine the MIC of penicillin G

Table F8 MIC results of ceftriaxone where isolates were grown on GC II agar, GC saponin agar and chocolate supplemented agar prior to MIC test strips

Isolate	MIC of ceftriaxone (mg/L) Media used to grow the gonococcus prior to the MIC test strips [ab]		
	GC II Agar	GC Saponin Agar	Chocolate Supplemented Agar
729	0.06	0.06	0.06
119	0.06	0.06	0.06
1641	0.01	0.03	0.01
631	0.06	0.06	0.06
71	0.06	0.03	0.06
604	0.03	0.03	0.06
558	0.06	0.06	0.06
723	0.03	0.03	0.06
801	0.06	0.06	0.03
1526	0.03	0.03	0.03
1530	0.06	0.03	0.03
1380	0.03	0.01	0.01
411	0.03	0.03	0.06
557	0.06	0.03	0.06
886	0.06	0.06	0.06
263	0.03	0.03	0.03
724	0.03	0.01	0.03
824	0.03	0.03	0.03
257	0.06	0.03	0.03
483	0.03	0.03	0.03
1026	0.01	0.03	0.01
WHO K	0.06	0.06	0.06
WHO L	0.12	0.06	0.06
WHO F	<0.002	<0.002	<0.002
ATCC 49226	0.008	0.008	0.008

[a] All MIC test was carried out on GC II Agar.

[b] Liofilchem (Italy) MIC test strips were used to determine the MIC of ceftriaxone in this study.

Table F9 Evaluation of the use of GC saponin agar and chocolate supplemented agar (recommended for *N. gonorrhoeae* culture) to grow *N. gonorrhoeae* prior to MIC test for penicillin G and ceftriaxone

Antibiotic	Number of isolates [a]	Number of isolates (between MIC result from the current study and ESR) that differ by the following number of ± 1 log_2 dilutions in comparison to chocolate supplemented agar			Independent sample t-test, $p < 0.05$ of MIC means		Significant	% agreement [c]
		-2 to -1 < n	-1 ≤ n ≤ 1 [b]	n > 1 to 2	t	*p*		
Penicillin G	25	0	25	0	0.00	0.99	No	100%
Ceftriaxone	25	0	25	0	0.00	1.00	No	100%

[a] Number of gonococcus isolates tested for this study.

[b] Shaded areas indicate isolates in agreement (i.e. ± 1-fold difference).

[c] Agreement was defined as an MICs within ± 1 log_2 of the comparison MIC (Biedenbach and Jones, 1996).

Table F10 Evaluation of the used of GC II agar and chocolate supplemented agar (suggested for *N. gonorrhoeae* culture) to grow *N. gonorrhoeae* prior to MIC test for penicillin G and ceftriaxone

Antibiotic	Number of isolates [a]	Number of isolates (of strains that grown GC II agar and chocolate supplemented agar prior to MIC test) that differ by the following number of ± 1 log_2 dilution in comparison to chocolate supplemented agar [b]			Independent sample t-test, $p < 0.05$ of MIC means		Significant	% agreement [c]
		-2 to -1 < n	-1 < n < 1	n > 1 to 2	t	*p*		
Penicillin G	25	0	25	0	0.00	1.0	No	100%
Ceftriaxone	25	0	25	0	0.00	1.0	No	100%

[a] Number of gonococcus isolates that were tested for thus study.

[b] Shaded areas indicate isolates in agreement (i.e. minimum and maximum of ± 1 log_2 fold difference).

[c] Agreement was defined as an MICs within ± 1 log_2 of the comparison MIC (Biedenbach and Jones, 2010).

Table F11 Ceftriaxone MIC (mg/L), colony forming unit (CFU) and colony morphologies of strain 963 incubated in candle jar and car carbon dioxide (CO_2) incubator

	WHO K				Isolate 963			
	Candle jar (with 3% CO_2) Temperature : 37°C		Carbon dioxide incubator (with 5% CO_2) Temperature : 37°C		Candle jar (with 3% Carbon dioxide) Temperature : 37°C		Carbon dioxide incubator (with 5% Carbon dioxide) Temperature : 37°C	
	First Run	Second Run	First Run	Second Run	First Run	Second Run	First Run	Second Run
Ceftriaxone MIC (mg/L)	0.05	0.05	0.05	0.05	0.008	0.008	0.008	0.008
	0.5 McFarland Standard (CFU/ml)				0.5 McFarland Standard (CFU/ml)			
Dilution Factor	First Run	Second Run	First Run	Second Run	First Run	Second Run	First Run	Second Run
10^4	270	280	262	292	276	257	240	253
10^5	58	64	53	77	42	42	33	33
10^6	12	12	9	10	10	12	9	9
10^7	1	2	1	3	5	5	1	1
1 x 10^8 (Estimated)	**1.2 (± 0.35) x 10^8**		**1.0 (± 0.31) x 10^8**		**1.1 (± 0.3) x 10^8**		**0.9 (± 0.3) x 10^8**	
Colony morphologies	Bigger single colony (2mm)		Pin point colony (1mm)		Bigger single colony (2mm)		Pin point colony (1mm)	

7.7. APPENDIX G: *PenA* (PBP2) analysis

Table G1 Mosaic *penA* PCR method development (Protocol 1 and Protocol 3)

	Protocol 1			Protocol 1		
Steps	**Temperature**	**Time**	**Cycle**	**Temperature**	**Time**	**Cycle**
Initial denaturation	95°C	5 minutes	1 cycle	95°C	5 minutes	1 cycle
Denaturation	95°C	30 seconds	40 cycle	95°C	30 seconds	40 cycle
Annealing	60°C	15 seconds	40 cycle	65°C	15 seconds	40 cycle
Extension	72°C	30 seconds	40 cycle	72°C	30 seconds	40 cycle
Final extension	72°C	7 minutes	1 cycle	72°C	7 minutes	1 cycle
Hold	10°C	Infinite	1 cycle	10°C	Infinite	1 cycle

Table G2 Master Mix for mosaic *penA* PCR (Protocol 1 and Protocol 3)

	Protocol 1		Protocol 1	
Reagent	**Final Concentration**	**Volume (µL) [1]**	**Final Concentration**	**Volume (µL) [1]**
Platinum Taq DNA Polymerase	1U	0.2	0.5U	0.1
10X PCR Buffer	1X	2.5	1X	2.5
50mM MgCl2	1.5mM	0.75	1.5mM	0.75
10mM dNTPs	200µM	2	200µM	2
10µM Primer_F	1µM	2.5	1µM	1.25
10µM Primer_R	1µM	2.5	1µM	1.25
Milli-Q Water		9.55		12.15
		25		25

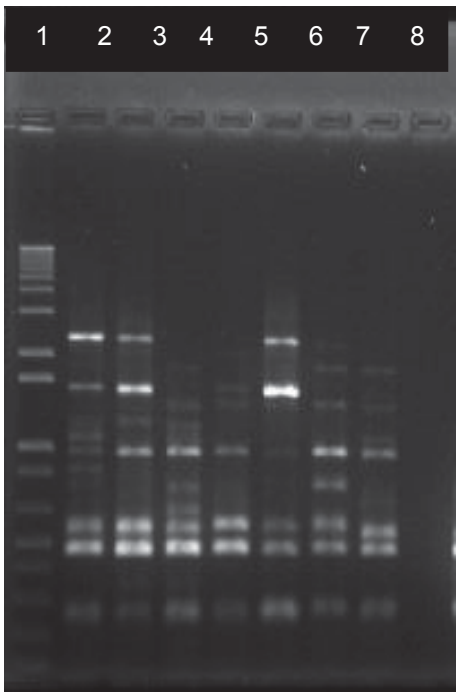

Figure G1 Gel electrophoresis of mosaic *penA* amplicons (Protocol 1). Lane 1: 100 bp ladder. Lane 2-8: mosaic *penA* amplicons from isolate 558, 631, 723, 1641, WHO K (positive control), WHO L, WHO F. Lane 8: ATCC 49226. Lane 9: Blank.

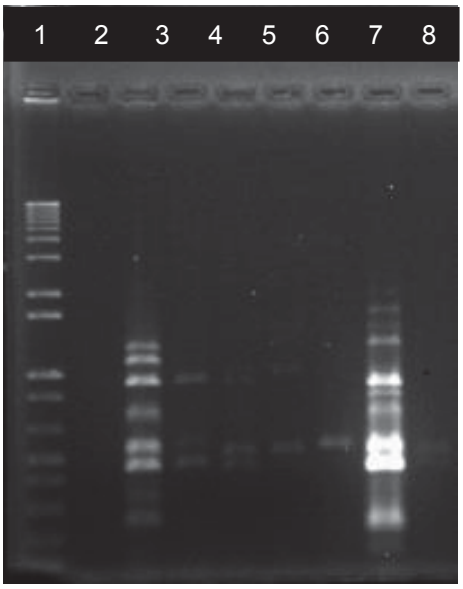

Figure G2 Gel electrophoresis of mosaic *penA* amplicons (Protocol 3). Lane 1: 100 bp ladder. Lane 2-8: mosaic *penA* amplicons from isolate 558, 631, 723, 1641, WHO K (positive control), WHO L, WHO F. Lane 8: ATCC 49226. Lane 9: Blank.

Table G3 Fragment migration and estimated size of *N. gonorrhoeae* mosaic *penA* PCR product

	Fragment Size (bp)	Log $_{10}$ Fragment Size	Migration (mm)
	400	2.602	66
	300	2.477	68
	200	2.301	72
	100	2.000	75
PCR Product:	220 [1]		70

1 Estimated fragment size was derived from the standard curve (Appendix Figure 3)

2 Example was given for mosaic *penA*, however, the same method was used to estimate the fragment size of other PCR products such as *penA PCR, mtrR, porB$_{IB}$* and *ponA*.

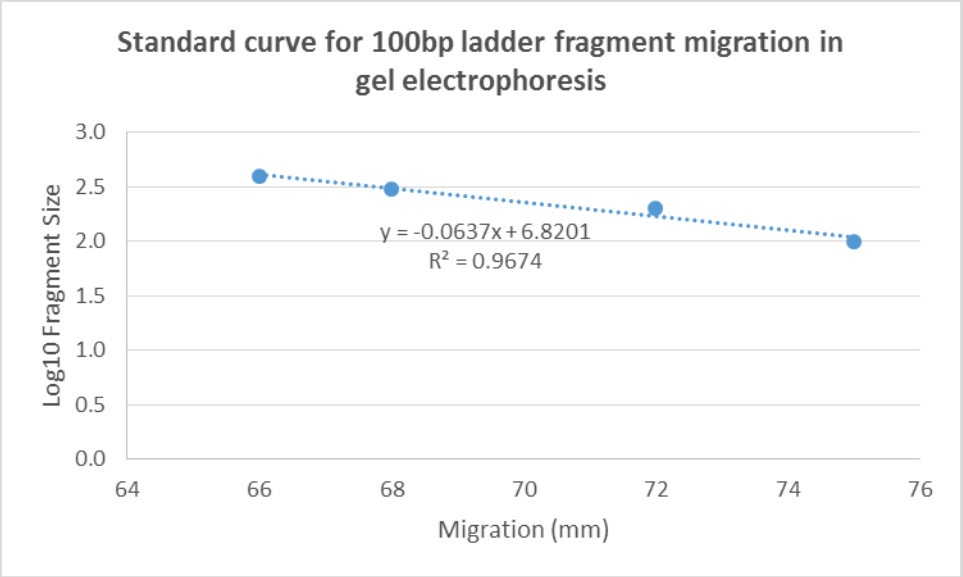

Figure G3 Standard curve for migration of mosaic *penA* PCR product

Note : Example was given for mosaic *penA*, however, the standard curve was derived in the same way to estimate the fragment size of other PCR products such as *penA PCR, mtrR,* $porB_{IB}$ and *ponA*.

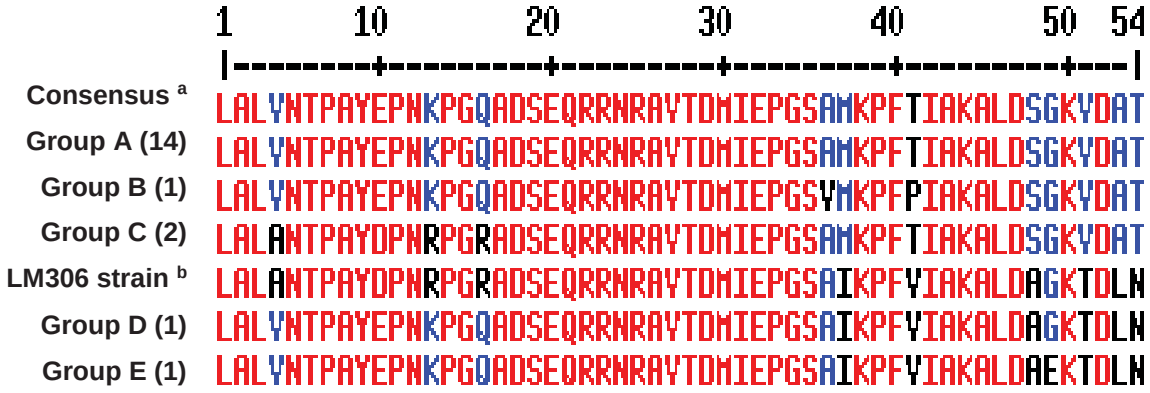

Figure G4 Comparison of the mosaic PBP2 amino acid sequence (residues 276 to 329) of *N. gonorrhoeae* isolates with the PBP2 sequence of wildtype *N. gonorrhoeae* strain LM306 (Protein ID: AAA25463.1) and 19 published PBP2 sequences from NCBI using Multalin. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the nucleotides in black.

[a] Consensus sequence represents the nucleotide sequence agreement for all 22 *N. gonorrhoeae* isolates.

[b] Wild-type *N. gonorrhoeae* LM 306 (M32091.1) (Ameyama *et al.*, 2002)

* Variation at residues 285 of PBP2 sequence.

Group A : i) GenBank Accession no. AB071984 – *N. gonorrhoeae* NG-3 strain (Ameyama *et al.*, 2002)

ii) GenBank Accession no. HQ204563, HQ204560, HQ204559, HQ204558, HQ204556, HQ204555, HQ204554, HQ204553, HQ204552, HQ204557 (Allen *et al.*, 2013)

iii) GenBank Accession no. GU732422 (Pandori *et al.*, 2009)

iv) GenBank Accession no. JQ073701 – *N. gonorrhoeae* F89 strain (Unemo *et al.*, 2012)

v) GenBank Accession no. KM403401 (Johnson *et al.*, 2014)

Group B: i) GenBank Accession no. AB546858- *N. gonorrhoeae* H041 strain (Fully resistant to ceftriaxone) (Ohnishi *et al.*, 2011)

Group C : i) GenBank Accession no. HQ- 204565 (Allen *et al.*, 2011)

ii) GenBank Accession no. KC192769 (Gose *et al.*, 2013b)

Group D: i) GenBank Accession no. HQ204563 (Allen *et al.*, 2011)

Group E: i) GenBank Accession no. AB608050- mosaic *penA* XXXVI (Ohnishi *et al.*, 2011)

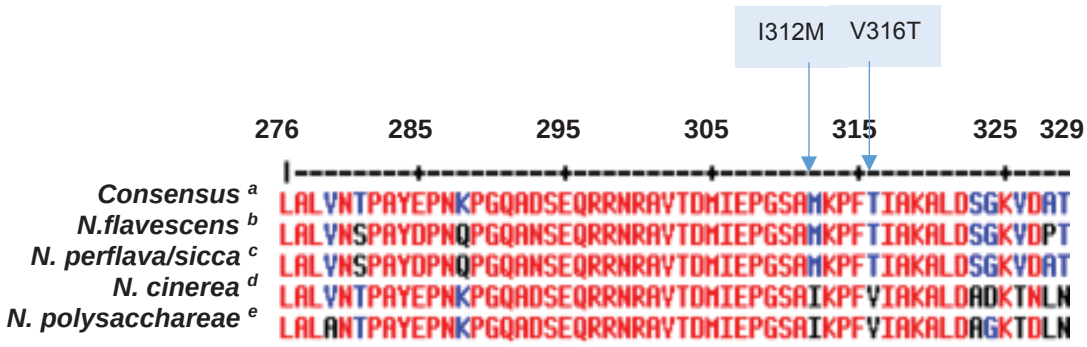

Figure G5 Comparison of the mosaic PBP2 sequence of *N. gonorrhoeae* isolates (residues 276 to 329) with the PBP2 sequences of other *Neisseria* species using Multalin. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the nucleotides in black.

[a] The consensus sequence represents the 22 *N. gonorrhoeae* isolates in this study

[b] *N. flavescens* NCTC 8263 (GenBank accession no. M26645)

[c] *N. perflava/sicca* 1654/1659 (GenBank accession no. X76422)

[d] *N. cinerea* NCTC 10294 (GenBank accession no. X59540)

[e] *N. polysaccharea* NCTC 11858 (GenBank accession no. X59626)

Table G4 Amino acid differences between mosaic PBP2 (residues 276 to 329) of 22 *N. gonorrhoeae* isolates and other *Neisseria* species

Strain (GenBank accession no.)	Different amino acids (%)	Identical amino acid sequence (%)
***N. flavescens* NCTC 8263 (M26645)**	4 (7.5)	49 (92.5)
N. perflava/sicca 1654/1659 (X76422)	3 (5.6)	53 (94.4)
***N. cinerea* NCTC 10294 (X59540)**	7 (13.2)	47 (86.8)
***N. polysaccharea* NCTC 11858 (X59626)**	8 (15)	48 (85)

Number of amino acid residues: 53

Table G5 Comparison of PBP2 alterations in consensus sequence with *N. perflava/sicca*, *N. cinerea* and *N. polysacchareae*

Mosaic PBP2 mutation found in consensus sequence [a]	*N. flavescens*	*N. perflava/sicca*	*N. cinerea*	*N. polysacchareae*
A279V	Yes	Yes	Yes	-
D285E	-	Yes	Yes	Yes
R288K	-	-	Yes	Yes
R291Q	Yes	Yes	-	Yes
I312M	Yes	Yes	-	-
V316T	Yes	Yes	-	-
A323S	Yes	Yes	-	-
T326V	Yes	Yes	-	-
L328A	-	Yes	-	-
N329T	Yes	Yes	-	-

[a] The consensus sequence represents the 22 *N. gonorrhoeae* isolates in this study

Table G6 Summary of amino acid alterations in part D of PBP2 (residues 430 to 555) of *N. gonorrhoeae* isolates

Alterations of part D of PBP2	Number of isolates (n)	Percentage (%)
Key alteration links to high MIC of ceftriaxone	3	11
A501T		
A501V	6	21
N512Y	16	57
G542S	2	7
G545S	16	57
P551S	5	18
P551L	2	7
Other alterations [a]		
A437V	16	57
V443E	16	57
L447V	16	57
Q457K	16	57
I462F	16	57
E464A	16	57
R468K	16	57
E469K	16	57
N472E	16	57
P480A	16	57
F504L	28	100
A510V	28	100
A516G	12	43
H541N	17	61
P552V	1	4
P554Q	1	4
K555Q	1	4

[a] PBP2 alterations not associated with decreased susceptibility to ceftriaxone.

Table G7 Summary of PBP2 alterations of *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone

Isolates	PBP patterns Residues 276-329	A437V	V443E	L447V	Q457K	I462F	E464A	R468K	E469K	N472E	P480A	T484S	A501T	A501V	F504L	A510V	N512Y	A516G	H541N	G542S	G545S	A549T	P551S	P551L	P552V	F553L	K554Q	K555Q
631	Mosaic PBP2 (M-1)	/	/	/	/	/	/	/	/	/	/	-	-	-	/	/	/	-	/	-	/	-	-	-	-	-	-	-
119																												
71																												
558																												
604																												
801																												
411																												
886																												
896																												
1526																												
1530																												
824																												
257																												
483																												
1026																												
1848																									/			/

Isolates	PBP patterns	A437V	V443E	L447V	Q457K	I462F	E464A	R468K	E469K	N472E	P480A	T484S	A501T	A501V	F504L	A510V	N512Y	A516G	H541N	G542S	G545S	A549T	P551S	P551L	P552V	F553L	K554Q	K555Q
723	M-2	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	-	/	-	-	-	-	/	-	-	-	-	-
263																												
724																												
557	M-3	-	-	-	-	-	-	-	-	-	-	-	/	-	/	/	-	/	-	/	-	-	-	-	-	-	-	-
729	M-4	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	-	/	-	-	-	-	-	-	-	-	-	-
1641	M-5	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	-	/	-	-	-	-	-	-	-	-	-	-
792	Nonmosaic (NM-1)	-	-	-	-	-	-	-	-	-	-	-	-	/	/	/	-	/	-	-	-	-	/	-	-	-	-	-
1332																												
264	NM-2	-	-	-	-	-	-	-	-	-	-	-	/	-	/	/	-	/	-	/	-	-	-	-	-	-	-	-
893	NM-3	-	-	-	-	-	-	-	-	-	-	-	/	-	/	/	-	/	-	-	-	-	-	/	-	-	-	-
1380	NM-4	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	-	/	-	-	-	-	-	/	-	-	-	-
963 [d]	NM-5	-	-	-	-	-	-	-	-	-	-	-	-	-	/	/	-	/	-	-	-	-	-	-	-	-	-	-

Note:

1. Green shaded area represents amino acid alterations that are associated with a high MIC of ceftriaxone in *N. gonorrhoeae.* A501V/T/ (Tomberg *et al*., 2010; Unemo *et al*., 2012), N512Y (Tomberg *et al*., 2010), G542S (Whiley *et al.*, 2007b), G545S (Takahata *et al*., 2006), P551S/L (Shimuta *et al*., 2013).
2. (-) : No alteration

[a] M: Mosaic PBP2 pattern; [b] NM: Nonmosaic PBP2 pattern

[b] Mosaic PBP2 sequence in this study represent 10 mutations: A279V, D285E, R288K, R291Q, I312M, V316T, A323S, T326V, L328A and N329T

[c] For strain 1848, although it harbours additional mutations at 552 and 555, it is classified under M-1 because it still harbour the main mutations set of M-1.

[d] Isolate 963 does not harbour any key alterations in PBP2 that are related to high MIC of ceftriaxone.

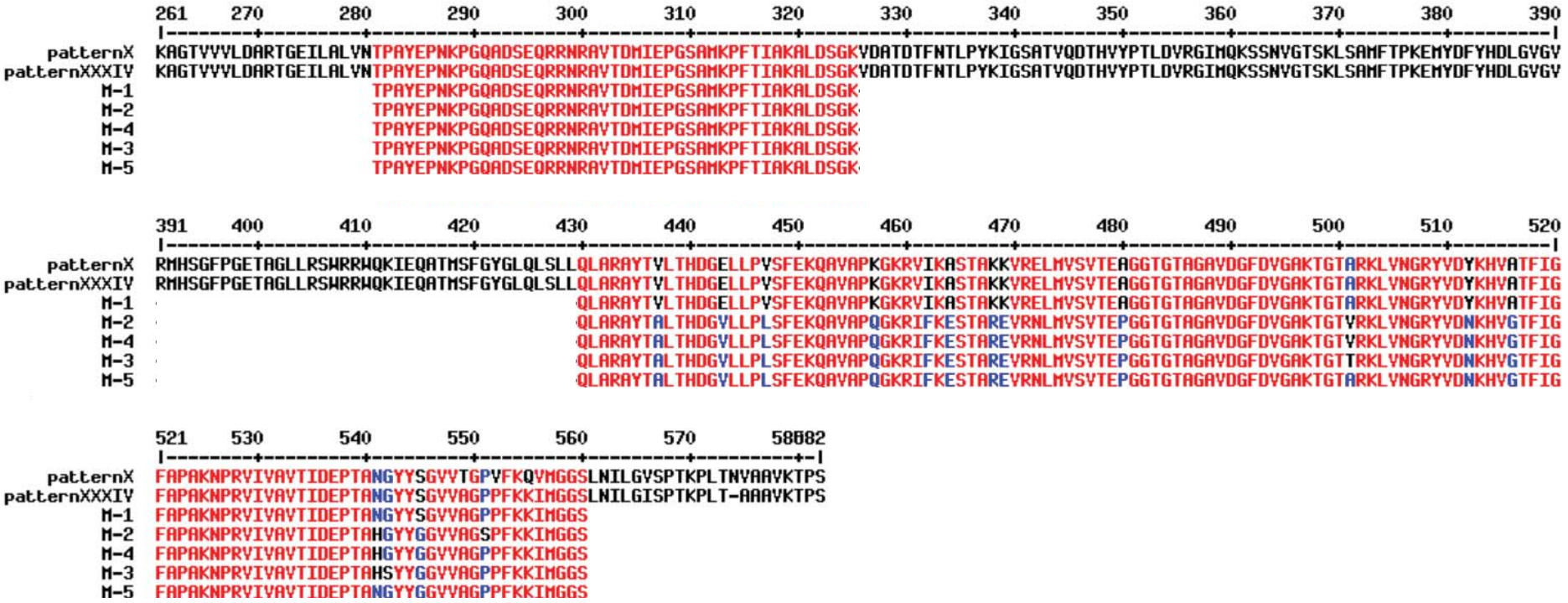

Figure G6 Comparison of partial mosaic PBP2 M-1, M-2, M-3, M-4 and M-5 with mosaic PBP2 X (GenBank accession no. AB071984) (Ameyama *et al.*, 2002), and XXXIV (GenBank accession no. ADE2248.1) (Pandori *et al.*, 2009). The numbering of the sequence is based on the sequence of mosaic PBP2 XXXIV. Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the nucleotides in black.

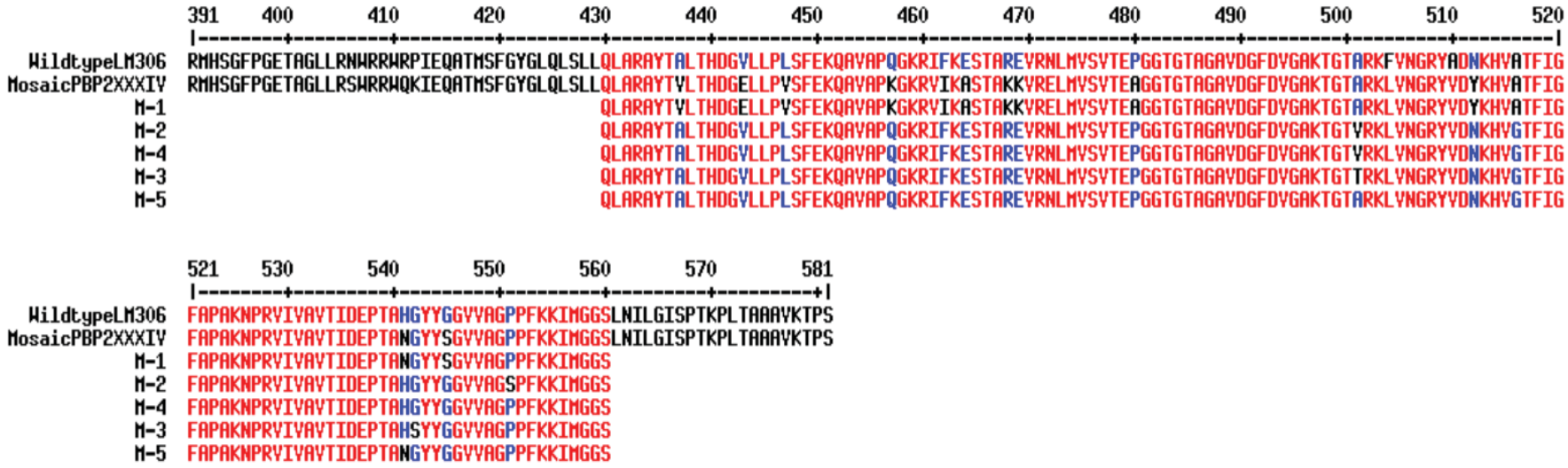

Figure G7 Comparison of part D PBP2 of mosaic PBP2 pattern M-1, M-2, M-3, M-4 and M-5 with mosaic PBP2 pattern XXXIV and *N. gonorrhoeae* LM306 strain. The numbering of the sequence is based on the sequence of *N. gonorrhoeae* LM306**.** Amino acids that are completely conserved in all sequences in this population of genes are shown in red. Amino acids that appear blue or black indicate variation in the sequences, with amino acids in blue having greater consensus than the nucleotides in black.

Table G8 Comparison of part D PBP2 of mosaic PBP2 sequences M-1, M-2, M-3, M-4 and M-5 with mosaic PBP2 XXXIV and wildtype PBP2 of *N. gonorrhoeae* LM306

Amino acid changes observed	PBP2 sequence (residues 281 to 560)						
	LM306 (Wildtype)	Mosaic PBP2 XXXIV	M-1	M-2	M-3	M-4	M-5
A437V	-	/	/	-	-	-	-
V443E	-	/	/	-	-	-	-
L447V	-	/	/	-	-	-	-
Q457K	-	/	/	-	-	-	-
F462I	-	/	/	-	-	-	-
E464A	-	/	/	-	-	-	-
R468K	-	/	/	-	-	-	-
E469K	-	/	/	-	-	-	-
N472E	-	/	/	-	-	-	-
P480A	-	/	/	-	-	-	-
A501V/T	-	-	-	/	/	/	-
F504L	-	/	/	/	/	/	/
A510V	-	/	/	/	/	/	/
N512Y	-	/	/	-	-	-	-
A516G	-	-	-	/	/	/	/
H541N	-	/	/	-	-	-	/
G542S	-	-	-	-	/	-	-
G545S	-	/	/	-	-	-	-
P551S	-	-	-	/	-	-	-

Note : (/): Indicate the presence of the PBP2 alterations; (-): Indicate the absence of the PBP2 alterations

Table G9 Susceptibility to ceftriaxone and PBP2 alterations in *N. gonorrhoeae* isolates

PBP2 patterns (n)	Alterations that were linked to high MICs of ceftriaxone	Isolates	MIC of ceftriaxone (mg/L) [a]				
			0.008	0.01	0.03	0.04	0.06
Mosaic (M-1) (15 isolates)	I312M V316T N512Y G545S	631					1
		119					1
		71					1
		604					1
		558					1
		801					1
		411					1
		886					1
		896					1
		1526			1		
		1530			1		
		824			1		
		257			1		
		483			1		
		1026		1			
		1848					1

PBP2 patterns (n)	Alterations that were linked to high MICs of ceftriaxone	Isolates	MIC of ceftriaxone (mg/L) [a]					
			0.008	0.01	0.03	0.04	0.06	
Mosaic M-2 (3 isolates)	I312M V316T A501V P551S	**723**					1	
		263			1			
		724			1			
Mosaic -3 (1 isolate)	I312M V316T A501T G542S	**557**					1	
Mosaic M-4 (1 isolate)	I312M V316T A501V	**729**					1	
Mosaic M-5 (1 isolate)	I312M V316T	**1641**		1				
Nonmosaic NM-1 (2 isolates)	A501V P551S	**792**					1	
		1332			1			

PBP2 patterns (n)	Alterations that were linked to high MICs of ceftriaxone	Isolates	MIC of ceftriaxone (mg/L) [a]				
			0.008	0.01	0.03	0.04	0.06
Nonmosaic (NM-2) (1 isolate)	A501T G542S	**264**			1		
Nonmosaic (NM-3) (1 isolate)	A501T P551L	**893**		1			
Nonmosaic (NM-4) (1 isolate)	P551L	**1380**		1			
Nonmosaic (NM-5) (1 isolate)	No key alterations for PBP2	**963**	1				

[a] MIC of ceftriaxone results are based on MIC result from the Liofilchem MIC test strips.

Shaded area represents reduced susceptibility level of ceftriaxone (0.03 – 0.06 mg/L)

7.8. APPENDIX H: *MtrR* analysis

Table H1 Types of alterations observed in *mtrR* promoter and MtrR coding region of *N. gonorrhoeae* isolates

Type of *mtrR* alteration observed	Number (n)	Percentage (%)
A deletion in *mtrR* promoter	26	93
G45D alteration in MtrR coding region	5	18
A39T alteration in MtrR coding region	3	11
T86A alteration in MtrR coding region	2	7
H105Y alteration in MtrR coding region	19	68

7.9. APPENDIX I: PorBIB analysis

Table I1 *$PorB_{IB}$* PCR protocol development

	Protocol 1 (Master Mix 1)		Protocol 2 (Master Mix 2)		Protocol 3 (Master Mix 3)	
Reagent	**Final Concentration**	**Volume (µL) [1]**	**Final Concentration**	**Volume (µL) [1]**	**Final Concentration**	**Volume (µL) [1]**
KAPA2G Robust DNA Polymerase	0.5U	0.1	0.5U	0.1	1U	0.1
5X KAPA2G Buffer [2]	1X	5	1X	2.5	1X	5
10mM dNTPs	200µM	1	1.5mM	0.75	1.5mM	0.5
10µM PorB1 (Forward)	0.5µM	1.25	200µM	2	200µM	0.5
10µM PorB2 (Reverse)	0.5µM	1.25	1µM	1.25	1µM	0.5
Milli-Q Water		11.4		1.25		16.4
Template		5		12.15		2
		25		25		25

Note: PCR conditions for all three master mixes are similar to the PCR protocol stated in Results Section Table 3-20

[1] Volumes for one PCR reaction

[2] KAPA2G Buffer contain 1.5mM $MgCl_2$ at 1X

Table I2 Summary of various amino acid alterations in PorBIB protein sequence of *N. gonorrhoeae* isolates

Alterations of PorBIB [a]	Number of isolates (n)	Percentage (%)
T89S	12	43%
A109V	6	21%
V135F	6	21%
G140K	6	21%
Alteration of 143	18	64%
G145E	28	100%
L147R	28	100%
V151A	9	32%
Q187R	18	64%
Alteration of 189	18	64%
Deletion of 208	8	29%
Deletion 209 to 211	10	36%
Deletion of 209	15	54%
K209M	3	11%
K210E	3	11%
Alteration 212	15	54%
Alteration 213	28	100%
Alteration 214	14	50%
Alteration 215	28	100%
S217N	13	46%
Alteration 218	13	46%
S220N	1	4%
V242A	21	75%
Q255G	28	100%
Deletion of 256 & 257	28	100%
Alteration of 258 & 259	28	100%
R260S	19	68%
Alteration of 261	26	93%
V274A	17	61%
V281L	12	43%
T294S	12	43%
D296H	12	43%
S297D	8	29%
H299Y	12	43%

Note: [a] These alterations were not published to be associate with ceftriaxone susceptibilities.