

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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

A Thesis Submitted to the College of Health  
in partial fulfilment of the requirements for the Master of Science in Microbiology  
at  
Massey University  
New Zealand  
By

**Norshuhaidah bt Mohd Jamaludin**

# 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<sub>IB</sub>* 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<sub>IB</sub>* 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<sub>IB</sub>* 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  $\beta$ -lactamase. The 23 isolates that conveyed reduced ceftriaxone susceptibility were found to harbour three or four mutated genes (*penA*, *mtrR* and/or *porB<sub>IB</sub>* 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

## ACKNOWLEDGEMENT

Alhamdulillah, all praises to Allah S.W.T and to Him alone for the strengths and His blessings in completing this thesis.

I would like to express the deepest gratitude to my main supervisor Dr. Mary Nulsen for her full support, expert guidance, understanding, and encouragement throughout my stay in New Zealand, my study and research. Without her incredible patience and timely wisdom and counsel, my thesis work would not be successful. In addition, I express my deepest appreciation to Dr. Jackie Benschop and Dr. Julie Collins-Emerson for the support, passion in this project and having served on my committee. Their thoughtful questions and comments were valued greatly.

I would also like to thank Dr. Kristene Gedye for her hard work, valuable advice and dedication that makes this project successful. I am so grateful to have you on the team.

To New Zealand Aid programme, I express thanks from deep within my heart and show my gratitude for granting me the New Zealand Aid Commonwealth Scholarship. I sincerely thank you for providing me the chance to pursue my postgraduate studies.

Special thanks go to my numerous beloved friends for the never ending support and encouragement and make my life in New Zealand is one of the amazing experience. You know who you are.

Finally, I would like to thank my parents, brothers and sisters, the rest of my families, and friends back home for their unconditional love and support during the last two years. I would not have been able to complete this thesis without their endless love and encouragement.

# Table of Contents

List of Tables.....	ix
List of Figures .....	xiv
Abbreviations .....	xvii
Nucleotides abbreviations .....	xix
Amino acid abbreviations .....	xx
<b>1. CHAPTER ONE : INTRODUCTION.....</b>	<b>2</b>
1.1.    Gonorrhoea: Epidemiology and Health Impact.....	3
1.2. <i>Neisseria gonorrhoeae</i> morphology and traits .....	5
1.3.    Methods to diagnose gonorrhoea .....	7
1.4.    Chronology of multidrug-resistant (MDR) <i>Neisseria gonorrhoeae</i> .....	14
1.5.    Extended-spectrum cephalosporins (ESCs), the current treatment for gonorrhoea, and the emergence of <i>N. gonorrhoeae</i> with reduced susceptibility to ESCs .....	21
1.6.    Chromosomal gene mutations in <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> cause reduced susceptibility to ESCs .....	24
1.6.1.    Penicillin-binding protein 2 ( <i>penA</i> ) gene .....	29
1.6.2.    The role of mosaic <i>penA</i> (PBP2) in causing reduced susceptibility to ESCs .....	30
1.6.3.    Significant involvement of mosaic PBP2 (I312M, V316T and G545S alterations) .....	33
1.6.1.    Other PBP2 alterations and their role in reduced susceptibility to ESCs .....	35
1.7.    Introduction to the multiple transferable resistance ( <i>mtr</i> ) locus .....	42
1.7.1.    Mutations in the <i>mtrR</i> cause <i>N. gonorrhoeae</i> to become resistant to hydrophobic agents, penicillin and azithromycin .....	43
1.7.2.    Mutations in the <i>mtrR</i> region enhanced the reduced susceptibility to ceftriaxone in <i>N. gonorrhoeae</i> .....	45
1.8.    Introduction to <i>penB</i> and <i>porB<sub>IB</sub></i> .....	47
1.8.1.    The alterations of loop 3 area of PorB ( <i>porB<sub>IB</sub></i> ) .....	48
1.8.2.    Mutations in the <i>porB<sub>IB</sub></i> cause reduced susceptibility to ESCs in <i>N. gonorrhoeae</i> .....	50

1.9.	Introduction to <i>ponA</i> .....	52
1.9.1.	Mutation in <i>ponA</i> which causes <i>N. gonorrhoeae</i> to become resistant to ESCs.	54
1.10.	Treatment failure of infections due to <i>N. gonorrhoeae</i> with reduced susceptibility to ceftriaxone .....	55
1.11.	Susceptibility of New Zealand isolates of <i>N. gonorrhoeae</i> to ceftriaxone.....	60
<b>2.</b>	<b>CHAPTER TWO: METHODS AND MATERIALS .....</b>	<b>63</b>
2.1.	Ethical Approval .....	63
2.2.	Isolates .....	63
2.2.1.	Specimen culture.....	65
2.2.2.	Specimen storage .....	66
2.2.3.	Reference isolates.....	66
2.3.	Phenotypic Characterisation .....	68
2.3.1.	$\beta$ -lactamase production (nitrocefin test).....	68
2.3.2.	Antimicrobial susceptibility test (Etest).....	68
2.3.3.	Comparison of two Etest brands: Liofilchem s.r.l (Italy) and bioMérieux (Solna, Sweden).....	72
2.3.4.	Determination of the effect of using different media to culture <i>N. gonorrhoeae</i> prior to Etest on the MIC result .....	72
2.3.5.	Statistical analysis.....	73
2.4.	Polymerase Chain Reaction (PCR) for mosaic <i>penA</i> , <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> .	74
2.4.1.	DNA extraction .....	74
2.4.2.	DNA concentration and purity .....	74
2.4.3.	Primers for <i>N. gonorrhoeae</i> mosaic <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> PCR .....	75
2.4.4.	<i>N. gonorrhoeae</i> PCR protocol development .....	77
2.4.5.	Preparing the gel and visualising the PCR product .....	81
2.5.	Sequencing .....	81
2.5.1.	Preparation of DNA template for sequencing .....	81
2.5.2.	Sequence comparison to confirm variations in amino acid sequence .....	82

<b>3. CHAPTER THREE: RESULT</b> .....	<b>84</b>
3.1. Phenotypic Result of <i>N. gonorrhoeae</i> isolates .....	84
3.1.1. Comparison between MIC results of ceftriaxone from ESR, Liofilchem Etest strips and bioMérieux Etest strips .....	84
3.1.2. Comparison between MICs of penicillin G determined by Liofilchem Etest strips and bioMérieux Etest strips .....	86
3.1.3. Antimicrobial susceptibility testing (AST) result of penicillin G, tetracycline, ciprofloxacin, azithromycin and ceftriaxone of <i>N. gonorrhoeae</i> with reduced susceptibility to ceftriaxone .....	86
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 .....	93
3.1.5. Effect of using GC II agar, chocolate supplemented agar, and GC saponin agar prior to Etest for penicillin G and ceftriaxone Etest result .....	98
3.1.6. Comparison of ceftriaxone MIC of isolate 963 and WHO K control strain incubated with 3% CO <sub>2</sub> and 5% CO <sub>2</sub> .....	99
3.2. <i>PenA</i> (PBP2) analysis of <i>N. gonorrhoeae</i> isolates .....	100
3.2.1. Mosaic <i>penA</i> PCR and sequencing .....	100
3.2.2. Mosaic <i>penA</i> in <i>N. gonorrhoeae</i> isolates .....	102
3.2.3. Comparison of mosaic <i>penA</i> (PBP2) of <i>N. gonorrhoeae</i> isolates with various published <i>penA</i> (PBP2) sequence .....	107
3.2.4. Confirmation of nonmosaic <i>penA</i> for six <i>N. gonorrhoeae</i> isolates that were negative for mosaic <i>penA</i> PCR .....	112
3.2.5. Identification of various PBP2 alterations in part D PBP2 (residues 430 to 555) associated with elevated MIC of ceftriaxone .....	115
3.2.6. The overall pattern of PBP2 in <i>N. gonorrhoeae</i> isolates .....	118
3.2.7. Association of different PBP2 patterns with MIC of ceftriaxone of <i>N. gonorrhoeae</i> isolates in New Zealand .....	122
3.2.8. Analysis of full length of <i>penA</i> of WHO K, WHO L, ATCC 49226 and strain 1380 .....	126
3.3. <i>MtrR</i> analysis of <i>N. gonorrhoeae</i> isolates .....	132
3.3.1. <i>MtrR</i> PCR and sequencing .....	132
3.3.2. Identification of adenine (A) deletion in <i>mtrR</i> promoter, G45D in the <i>MtrR</i> coding region, and other alterations in <i>MtrR</i> protein .....	133



3.3.3.	Association of alterations in <i>mtrR</i> promoter and MtrR coding region with susceptibility level of ceftriaxone .....	137
3.4.	<i>PorB<sub>IB</sub></i> analysis of <i>N. gonorrhoeae</i> isolates.....	139
3.4.1.	<i>PorB<sub>IB</sub></i> amplification & sequencing .....	139
3.4.2.	<i>PorB<sub>IB</sub></i> alterations and association ceftriaxone susceptibility of <i>N. gonorrhoeae</i> isolates .....	141
3.5.	<i>PonA</i> analysis of <i>N. gonorrhoeae</i> isolates .....	141
3.5.1.	<i>PonA</i> amplification and sequencing .....	147
3.5.2.	Identification of L421P alteration in PBP1 and the association with susceptibility level of ceftriaxone.....	148
3.6.	Association of various combination of mutated <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> with susceptibility level of ceftriaxone .....	150
<b>4.</b>	<b>CHAPTER FOUR: DISCUSSION.....</b>	<b>160</b>
4.1.	Effect of Media and Etest strips on MIC results .....	160
4.2.	Antimicrobial resistance in <i>N. gonorrhoeae</i> .....	162
4.3.	<i>N. gonorrhoeae</i> DNA leaching using elution buffer (10mM TrisHCl, pH 8.0) .....	166
4.4.	Association of mosaic PBP2 alterations in <i>N. gonorrhoeae</i> isolates with reduced susceptibility to ceftriaxone.....	167
4.5.	Association of nonmosaic PBP2 alterations in <i>N. gonorrhoeae</i> isolates with reduced susceptibility to ceftriaxone.....	170
4.6.	Association of <i>mtrR</i> alterations in <i>N. gonorrhoeae</i> isolates with reduced susceptibility to ceftriaxone .....	173
4.7.	Association of G120 and A121 alterations of <i>PorB<sub>IB</sub></i> with <i>N. gonorrhoeae</i> isolates with reduced susceptibility to ceftriaxone .....	175
4.8.	Association of <i>ponA</i> (L421P) mutation with decreased susceptibility of ceftriaxone in <i>N. gonorrhoeae</i> in New Zealand .....	177
4.9.	Combinations of mutated <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> as markers to identify <i>N. gonorrhoeae</i> with reduced susceptibility to ceftriaxone .....	178
<b>5.</b>	<b>CHAPTER FIVE: CONCLUSION .....</b>	<b>181</b>
<b>6.</b>	<b>REFERENCES .....</b>	<b>185</b>

<b>7. APPENDIX</b> .....	212
7.1. APPENDIX A: Massey University Human Ethics Committee (MUHEC) application	212
7.2. APPENDIX B: The Hawkes Bay Medical Research Foundation (HBMRF) Funding	213
7.3. APPENDIX C: School of Food Nutrition, Massey University Funding .....	214
7.4. APPENDIX D: Institute of Veterinary, Animal and Biomedical Sciences (IVABS) Funding.....	215
7.5. APPENDIX E: Methods and Materials .....	216
7.6. APPENDIX F: Phenotypic Testing .....	222
7.7. APPENDIX G: <i>PenA</i> (PBP2) analysis.....	234
7.8. APPENDIX H: <i>MtrR</i> analysis.....	251
7.9. APPENDIX I: PorBIB analysis.....	252

# LIST OF TABLES

## CHAPTER ONE: INTRODUCTION

Table 1-1 Classification of antibiotics used to treat <i>N. gonorrhoeae</i> .....	15
Table 1-2 The development antimicrobial resistance in <i>N. gonorrhoeae</i> .....	16
Table 1-3 Stepwise transformation of <i>N. gonorrhoeae</i> FA19 strains with mosaic <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> of <i>N. gonorrhoeae</i> 35/02 (adapted from Zhao <i>et al.</i> (2009)) .....	25
Table 1-4 Susceptibility to ceftriaxone and mutated <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> observed in <i>N. gonorrhoeae</i> in published journals .....	28
Table 1-5 Verified mutations within PBP2 that contribute the decreased susceptibility to ceftriaxone .....	38
Table 1-6 Mutations within the <i>mtrR</i> region that contribute to the decreased susceptibility to hydrophobic agents, penicillin and ceftriaxone .....	45
Table 1-7 Published PorBIB mutations that contribute to reduced susceptibility to ESCs (e.g. ceftibuten, cefpodoxime, cefuroxime, cefotaxime, and ceftriaxone) .....	51
Table 1-8 Published ceftriaxone-resistant <i>N. gonorrhoeae</i> strains, and <i>N. gonorrhoeae</i> isolates with reduced susceptibility to ceftriaxone associate with ceftriaxone treatment failures .....	58
Table 1-9 <i>N. gonorrhoeae</i> with reduced susceptibility to ceftriaxone in New Zealand and the presence of mosaic <i>penA</i> .....	60

## CHAPTER TWO: METHODS AND MATERIALS

Table 2-1 List of <i>Neisseria gonorrhoeae</i> isolates from the Institute of Environmental Science and Research Limited (ESR) and <i>N. gonorrhoeae</i> control strains .....	64
Table 2-2 Antimicrobial range and minimum inhibitory concentration (MIC) data for WHO reference strain panel and CLSI reference strain: ATCC 49226 .....	67
Table 2-3 MIC interpretative Standard according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) .....	70
Table 2-4 Comparison of MIC results for penicillin G and ceftriaxone between Liofilchem MIC test strips and bioMérieux Etest .....	72
Table 2-5 A test to compare the MIC results of penicillin G and ceftriaxone between Liofilchem MIC test strips and bioMérieux Etest .....	73
Table 2-6 List of primers used for mosaic <i>penA</i> , <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> PCR .....	76
Table 2-7 Optimised PCR conditions for amplification of set A, set B, set C and set D of <i>penA</i> .....	78
Table 2-8 PCR master mix for amplification of set A, set B, set C and set D of <i>penA</i> .....	78
Table 2-9 PCR conditions for amplification of the <i>mtrR</i> gene .....	79
Table 2-10 PCR master mix used for amplification of the <i>mtrR</i> gene .....	79
Table 2-11 PCR conditions for amplification of the <i>ponA</i> gene .....	80
Table 2-12 PCR master mix used for amplification of the <i>ponA</i> gene .....	80

### CHAPTER THREE: RESULTS

Table 3-1 MIC of ceftriaxone and penicillin for <i>N. gonorrhoeae</i> isolates and control strains .....	88
Table 3-2 MICs of tetracycline, ciprofloxacin, azithromycin, and nitrocefin results for <i>N. gonorrhoeae</i> isolates and control strains .....	90
Table 3-3 Antibiotic susceptibility of <i>N. gonorrhoeae</i> isolates based on The European Committee of Antimicrobial Susceptibility Testing (EUCAST) guidelines .....	92
Table 3-4 Comparison of antibiotic susceptibility of <i>N. gonorrhoeae</i> isolates using EUCAST and CLSI guideline .....	95
Table 3-5 Optimised PCR conditions for mosaic <i>penA</i> (Protocol 2) .....	100
Table 3-6 Optimised PCR master mix for mosaic <i>penA</i> PCR (Protocol 2) .....	101
Table 3-7 Mosaic <i>penA</i> PCR for <i>N. gonorrhoeae</i> isolates .....	103
Table 3-8 Summary of mosaic <i>penA</i> PCR results for <i>N. gonorrhoeae</i> isolates .....	104
Table 3-9 Nucleotides comparison between mosaic <i>penA</i> of <i>N. gonorrhoeae</i> isolates with NG-3 and LM306 strain .....	110
Table 3-10 Amino acid changes detected in the mosaic PBP2 sequence of <i>N. gonorrhoeae</i> isolates (consensus sequence) compared to wild type <i>N. gonorrhoeae</i> LM306 .....	111
Table 3-11 Amino acid differences between the consensus mosaic PBP2 sequence of <i>N. gonorrhoeae</i> isolates and with NG-3 and LM306 strain .....	111
Table 3-12 Amino acid variation detected in part B of PBP2 (residues 183 to 347) for <i>N. gonorrhoeae</i> isolates that were negative for mosaic <i>penA</i> .....	114
Table 3-13 Summary of PBP2 patterns in <i>N. gonorrhoeae</i> isolates .....	120
Table 3-14 Comparison of PBP2 sequence observed in <i>N. gonorrhoeae</i> isolates with published mosaic PBP2 sequences in <i>N. gonorrhoeae</i> isolates .....	121
Table 3-15 PBP2 patterns observed in <i>N. gonorrhoeae</i> isolates .....	123
Table 3-16 Summary of ceftriaxone susceptibility and different types of alterations in PBP2 of <i>N. gonorrhoeae</i> isolates used in the study .....	124
Table 3-17 Amino acid alterations of PBP2 of WHO K, NG-3 <i>N. gonorrhoeae</i> strain, WHO L, ATCC 49226 and 1380 isolate .....	130
Table 3-18 Alterations in <i>mtrR</i> promoter and MtrR coding region of <i>N. gonorrhoeae</i> isolates .....	136
Table 3-19 Summary of alterations in <i>mtrR</i> promoter and MtrR coding region of <i>N. gonorrhoeae</i> isolates .....	138
Table 3-20 PCR conditions for amplification of the <i>porB<sub>IB</sub></i> gene of <i>N. gonorrhoeae</i> (protocol 4) .....	139
Table 3-21 Optimised PCR master mix for <i>porB<sub>IB</sub></i> PCR (protocol 4) .....	140
Table 3-22 G120 and A121 PorBIB alterations of <i>N. gonorrhoeae</i> isolates .....	145
Table 3-23 PorBIB alterations in <i>N. gonorrhoeae</i> isolates .....	146
Table 3-24 <i>PonA</i> (PBP1) alteration in <i>N. gonorrhoeae</i> isolates .....	150
Table 3-25 Summary of mutations <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> that are associated with elevated ceftriaxone MIC in <i>N. gonorrhoeae</i> with reduced susceptibility to ceftriaxone .....	152

Table 3-26 Susceptibility to ceftriaxone, and key alterations identified in PBP1, PBP2, MtrR and PorBIB in <i>N. gonorrhoeae</i> isolates.....	153
Table 3-27 Association of mutated <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> with <i>N. gonorrhoeae</i> isolates .....	156
Table 3-28 Association of mutated <i>penA</i> , <i>mtrR</i> , <i>porB<sub>IB</sub></i> and <i>ponA</i> with ceftriaxone MIC level of <i>N. gonorrhoeae</i> isolates.....	157

## APPENDIX E

Table E1 Storage of <i>N. gonorrhoeae</i> isolates in BHI + 20% glycerol broth.....	217
Table E2 Preparation of 1L of 1X Phosphate Buffered Saline (PBS).....	218
Table E3 Preparation of 20% Chelex® 100 suspensions for DNA extraction .....	218
Table E4 DNA purity and concentration measurement using Nanodrop ND-1000 spectrophotometer .....	219
Table E5 Preparation of working solution for primers (10µM) .....	220
Table E6 Preparation of working solution dNTPs (10mM) (Promega NZ).....	220
Table E7 Preparation of 1% agarose gel with safranin O .....	220
Table E8 Published control sequence used to identify variations in amino acid sequences in each resistance gene .....	221

## APPENDIX F

Table F1 Estimated fold difference in dilution (log <sub>2</sub> ) between Liofilchem s.r.l (Italy) and bioMérieux (Sweden) for ceftriaxone (CRO) and penicillin G.....	223
Table F2 Susceptibility classification of penicillin G and ceftriaxone for 21 <i>N. gonorrhoeae</i> isolates using Liofilchem and bioMérieux .....	224
Table F3 MIC results agreement between Liofilchem and bioMérieux Etest strips of <i>N. gonorrhoeae</i> isolates plus WHO K, WHO L, WHO F and ATCC 49226.....	225
Table F4 Estimated fold difference in dilution (log <sub>2</sub> ) between ceftriaxone MIC test result from the current study and the Institute of Environmental Science and Research (ESR).....	226
Table F5 Agreement between MIC of ceftriaxone of the current study and result from ESR... ..	228
Table F6 Association of β-lactamase expression with susceptibility level of penicillin G .....	229
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 .....	230
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 .....	231
Table F9 Evaluation of the use of GC saponin agar and chocolate supplemented agar to grow <i>N. gonorrhoeae</i> prior to Etest for penicillin G and ceftriaxone.....	232
Table F10 Evaluation of the used of GC II agar and chocolate supplemented agar to grow <i>N. gonorrhoeae</i> prior to Etest for penicillin G and ceftriaxone.....	233

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 <sub>2</sub> ) incubator .....	234
---	-----

## APPENDIX G

Table G1 Mosaic <i>penA</i> PCR method development (Protocol 1 and Protocol 3) .....	235
Table G2 Master Mix for mosaic <i>penA</i> PCR (Protocol 1 and Protocol 3) .....	236
Table G3 Fragment migration and estimated size of <i>N. gonorrhoeae</i> mosaic <i>penA</i> PCR product .....	237
Table G4 Amino acid differences between mosaic PBP2 (residues 276 to 329) of 22 <i>N. gonorrhoeae</i> isolates and other <i>Neisseria</i> species .....	240
Table G5 Comparison of PBP2 alterations in consensus sequence with <i>N. perflava/sicca</i> , <i>N. cinerea</i> and <i>N. polysacchareae</i> .....	241
Table G6 Summary of amino acid alterations in part D of PBP2 sequence of <i>N. gonorrhoeae</i> isolates with reduced susceptibility to ceftriaxone .....	242
Table G7 Summary of PBP2 alterations of <i>N. gonorrhoeae</i> isolates with reduced susceptibility to ceftriaxone .....	243
Table G8 Comparison of part D PBP2 (residues 430 to 560) of mosaic sequences M-1, M-2, M-3, M-4 and M-5 with mosaic PBP2 pattern XXXIV and wildtype PBP2 of <i>N. gonorrhoeae</i> LM306 .....	248
Table G9 Susceptibility to ceftriaxone and PBP2 alterations in <i>N. gonorrhoeae</i> isolates .....	249

## APPENDIX H

Table H1 Types of alterations observed in <i>mtrR</i> promoter and MtrR coding region of <i>N. gonorrhoeae</i> isolates with reduced susceptibility to ceftriaxone .....	252
--	-----

## APPENDIX I

Table I1 <i>PorB<sub>I</sub></i> PCR protocol development .....	253
Table I2 Summary of various amino acid alterations in PorBIB protein sequence of <i>N. gonorrhoeae</i> isolates .....	254

## STORED IN DVD

Table I Summary of sequencing results OF mosaic <i>penA</i> amplicons from <i>N. gonorrhoeae</i> isolates and WHO K control	
Table II Summary of sequencing result for part B <i>penA</i> (nonmosaic confirmation) amplicons for nonmosaic <i>N. gonorrhoeae</i> isolates and ATCC 49226 control strain	

Table III Summary of sequencing result for part D of *penA* (reverse sequence) amplicons for *N. gonorrhoeae* isolates, WHO K and WHO L control strains

Table IV Summary of sequencing result for full length of *penA* (forward sequence) amplicons for WHO K, WHO L, ATCC 49226 and isolate 1380

Table V Summary of sequencing results for *mtrR* amplicons from 28 *N. gonorrhoeae* isolates and WHO K control

Table VI Summary of sequencing results for *porB<sub>IB</sub>* amplicons 757 bp products for *N. gonorrhoeae* isolates and WHO K control strain

Table VII Summary of sequencing results for *ponA* amplicons for *N. gonorrhoeae* isolates and WHO K control strains

# LIST OF FIGURES

## CHAPTER ONE: INTRODUCTION

Figure 1-1 Alignment of published mosaic PBP2 patterns X, XXXV and XXXVIII, XXXIV and XXXII with the wild-type sequence of <i>N. gonorrhoeae</i> LM306.....	32
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 <i>et al.</i> (2002), Ito <i>et al.</i> (2004), Osaka <i>et al.</i> (2008) .....	37
Figure 1-3 Organisation of the <i>mtrCDE</i> region in gonococci, adapted from Kayla <i>et al.</i> (1995, 1997), Veal <i>et al.</i> (1998) and Warner <i>et al.</i> (2008).. .....	43
Figure 1-4 Amino acid sequences from PorBIB of <i>N. gonorrhoeae</i> strains adapted from Gill <i>et al.</i> (1998) and Olesky <i>et al.</i> (2002). .....	51
Figure 1-5 Organisation of <i>ponA</i> in gonococci, adapted from Ropp and Nicholas (1997), and Ropp <i>et al.</i> (2001).. .....	53
Figure 3-1 Penicillin G MIC distributions with EUCAST and CLSI guidelines .....	96
Figure 3-2 Tetracycline MIC distributions with EUCAST and CLSI guidelines .....	96
Figure 3-3 Azithromycin MIC distributions with EUCAST and CDC breakpoints established for GISP .....	97
Figure 3-4 Gel electrophoresis of mosaic <i>penA</i> amplicons from DNA extracted from <i>N. gonorrhoeae</i> isolates.....	101
Figure 3-5 Alignment of mosaic <i>penA</i> of <i>N. gonorrhoeae</i> isolates and WHO K control (from 843 bp to 972 bp). .....	105
Figure 3-6 Comparison of consensus sequence for mosaic <i>penA</i> amplicons (843 bp to 977 bp) with wildtype <i>N. gonorrhoeae</i> LM306 and <i>N. gonorrhoeae</i> NG-3.....	109
Figure 3-7 Comparison of the mosaic PBP2 sequence (residues 276 to 329) of <i>N. gonorrhoeae</i> isolates with the PBP2 sequence of wildtype <i>N. gonorrhoeae</i> LM306. ....	110
Figure 3-8 Alignment of part B of PBP sequence (residues 183 to 347) of six <i>N. gonorrhoeae</i> isolates that were negative for mosaic <i>penA</i> (consensus sequence), ATCC 49226 strain and <i>N. gonorrhoeae</i> NG-3 .....	113
Figure 3-9 Alignment of part D of PBP2 sequence (residues 430 to 520 ) of <i>N. gonorrhoeae</i> isolates, WHO K and WHO L control isolates, with wild-type <i>N. gonorrhoeae</i> LM306.....	116
Figure 3-10 Ceftriaxone MIC distribution between isolates of <i>N. gonorrhoeae</i> with mosaic PBP2 or nonmosaic PBP2 .....	125
Figure 3-11 Gel electrophoresis of full length of <i>penA</i> PCR products amplified from DNA extracted from <i>N. gonorrhoeae</i> WHO K, WHO L, ATCC 49226 control strain, and NZ isolate 1380.....	126
Figure 3-12 Alignment of PBP2 sequence (residues 1 to 390) of 1380, ATCC 49226, WHO L and WHO K, with <i>N. gonorrhoeae</i> LM306 and NG-3 strain. The numbering of the sequence is based on the sequence of <i>N. gonorrhoeae</i> LM306.. .....	128



Figure 3-13 Gel electrophoresis of <i>mtrR</i> PCR products from DNA extracted from <i>N. gonorrhoeae</i> isolates..	132
Figure 3-14 Alignment of partial <i>mtrR</i> sequences (1 to 130 bp residues) of <i>N. gonorrhoeae</i> isolates, published <i>mtrR</i> of <i>N. gonorrhoeae</i> (GenBank accession no.: Z25796.1), WHO K and WHO F control with 13 bp inverted repeat <i>mtrR</i> promoter sequence.....	134
Figure 3-15 Alignment of MtrR protein sequences (1 to 130 residues) of <i>N. gonorrhoeae</i> isolates with published MtrR protein sequence of <i>N. gonorrhoeae</i> (protein ID: CAA81045.1), positive control WHO K and wildtype WHO F.....	135
Figure 3-16 Gel electrophoresis of <i>porB<sub>IB</sub></i> PCR products consists of four protocols, amplified from DNA extracted from <i>N. gonorrhoeae</i> isolates WHOK, ATCC 49226, isolate 729 and 824.. ....	140
Figure 3-17 Alignment of partial PorBIB amino acid sequence (81 to 210 residues) of <i>N. gonorrhoeae</i> isolates, published wild-type PorBIB sequence of <i>N. gonorrhoeae</i> H1-2 (Genbank Protein ID: CAA06234.1) and WHO K control strain .....	143
Figure 3-18 Gel electrophoresis of the <i>ponA</i> PCR products amplified from DNA extracted from <i>N. gonorrhoeae</i> isolates.....	147
Figure 3-19 Alignment PBP1 amino acid sequence (residues 361 to 650) of <i>N. gonorrhoeae</i> isolates, the published wild-type PBP1 sequence of <i>N. gonorrhoeae</i> NG00085 (GenBank accession no.: AB727713.1, Protein ID: BAM21172.1) and the WHO K control strain .....	149
Figure 3-20 Effect of different groups of gene mutations on the susceptibility of ceftriaxone ..	158

## APPENDIX G

Figure G1 Gel electrophoresis of mosaic <i>penA</i> amplicons (Protocol 1).....	235
Figure G2 Gel electrophoresis of mosaic <i>penA</i> amplicons (Protocol 3).....	236
Figure G3 Standard curve for migration of mosaic <i>penA</i> PCR product.....	237
Figure G4 Comparison of the mosaic PBP2 amino acid sequence of <i>N. gonorrhoeae</i> isolates with the PBP2 sequence of wildtype <i>N. gonorrhoeae</i> strain LM306 and published PBP2 sequences..	238
Figure G5 Comparison of the mosaic PBP2 sequence of <i>N. gonorrhoeae</i> isolates with the PBP2 sequences of other <i>Neisseria</i> species.....	239
Figure G6 Comparison of partial mosaic PBP2 M-1, M-2, M-3, M-4 and M-5 with mosaic PBP2 X and XXXIV.....	241
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 <i>N. gonorrhoeae</i> LM306 strain.....	242

## STORED IN DVD

Figure I Alignment of the part B <i>penA</i> nucleotide sequence of six nonmosaic <i>N. gonorrhoeae</i> isolates, ATCC 49226 strain and <i>N. gonorrhoeae</i> NG-3	
--	--

Figure II Alignment of part D *penA* sequence of 28 *N. gonorrhoeae* isolates, WHO K, WHO L, and *N. gonorrhoeae* LM306

Figure III Alignment of *penA* nucleotide sequence of WHO L, ATCC 49226, 1380 and WHO K strain, with *N. gonorrhoeae* LM306 and NG-3 strain. The numbering of the sequence is based on *N. gonorrhoeae* LM306

Figure IV Alignment of *mtrR* sequences of *N. gonorrhoeae* isolates, *mtrR* sequence of published *N. gonorrhoeae*, WHO K and WHO F control strain

Figure V Alignment of partial *porB<sub>B</sub>* nucleotide sequence of *N. gonorrhoeae* isolates with published wild-type *porB<sub>B</sub>* sequence of *N. gonorrhoeae* HI-2 and WHO K control strain using Multalin

Figure VI Alignment of partial *ponA* nucleotide sequences of *N. gonorrhoeae* isolates, published wild-type *ponA* sequence of *N. gonorrhoeae* NG00085 and WHO K control strain

## 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 <i>Neisseria gonorrhoeae</i>
CO <sub>2</sub>	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 <sub>2</sub>	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	<i>Neisseria gonorrhoeae</i> 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 <i>Neisseria gonorrhoeae</i>
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 <i>Neisseria gonorrhoeae</i>
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