MECHANISMS OF CEFTRIAXONE RESISTANCE IN *NEISSERIA GONORRHOEAE* ISOLATED FROM THAI PATIENTS

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กลไกการดื้อยา ceftriaxone ในเชื้อ *Neisseria gonorrhoeae* ที่แยกได้จากผู้ป่วยไทย

นายนริศ เกื้อกุลพัฒนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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นริศ เกื้อกุลพัฒนา : กลไกการดื้อยา ceftriaxone ในเชื้อ *Neisseria gonorrhoeae* ที่แยกได้จากผู้ป่วย
ไทย. (MECHANISMS OF CEFTRIAXONE RESISTANCE IN *NEISSERIA
GONORRHOEAE* ISOLATED FROM THAI PATIENTS) อ.ที่ปรึกษาหลัก : ดร.ธนิษฐา ฉัตรสุวรรณ

  N. gonorrhoeae เป็นสาเหตุของโรคหนองใน ซึ่งเป็นหนึ่งในโรคติดต่อทางเพศสัมพันธ์ที่สำคัญ การดื้อยาหลายขนานในเชื้อ
N. gonorrhoeae ได้มีรายงานเพิ่มสูงขึ้นทั่วโลก วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาความชุกและ กลไกการดื้อยา ceftriaxone ในเชื้อ
N. gonorrhoeae ที่แยกได้จากผู้ป่วยไทย และศึกษาการเสริมฤทธิ์กันของยาต้านจุลชีพต่อเชื้อ *N. gonorrhoeae* ที่มีความไวลดลงหรือดื้อ
ต่อยา ceftriaxone จากผลการศึกษาในเชื้อ *N. gonorrhoeae* จำนวน 134 สายพันธุ์ ที่แยกได้จากผู้ป่วยที่มารับการรักษาที่คลินิกนิรนาม
สภากาชาดไทยและโรงพยาบาลจุฬาลงกรณ์ พบว่าอัตราการดื้อต่อยา ciprofloxacin, tetracycline, penicillin G, gentamicin,
azithromycin, ertapenem, fosfomycin, cefixime, และ ceftriaxone คิดเป็น 90.30%, 82.09%, 73.13%, 59.70%, 13.43%, 1.49%,
0%, 0%, และ 0%, ตามลำดับ พบเชื้อ *N. gonorrhoeae* ที่ดื้อยาหลายขนานสูงถึง 82.84% ถึงแม้ว่าเชื้อ *N. gonorrhoeae* ทุกสายพันธุ์ไว
ต่อยา ceftriaxone (ค่า MIC อยู่ในช่วง 0.001-0.125 mg/L MIC$_{50}$ และ MIC$_{90}$ เท่ากับ 0.002 และ 0.015 mg/L ตามลำดับ) แต่พบเชื้อ 2
สายพันธุ์ (1.49%) ที่มีความไวลดลงต่อยา ceftriaxone (MIC=0.125 mg/L) ผลการศึกษาด้วยวิธี *N. gonorrhoeae* multi-antigen
sequence typing พบว่าตัวแทนเชื้อ *N. gonorrhoeae* จำนวน 31 สายพันธุ์มีความหลากหลายของสายพันธุ์ (29 STs) การศึกษานี้เป็น
รายงานแรกในประเทศไทยที่พบเชื้อ *N. gonorrhoeae* ที่มีความไวลดลงต่อยา ceftriaxone ในประเทศไทย คือ สายพันธุ์ NG-083
(ST7235) และ NG-091 (new ST) โดยทั้งสองสายพันธุ์พบ PBP2 pattern XXXIV ซึ่งมีความสัมพันธ์กับการลดลงของความไวต่อยา
ceftriaxone พบการเปลี่ยนลำดับกรดอะมิโนของ PBP1 ที่ตำแหน่ง L421P พบการขาดหายไปของ adenine ภายใน 13-bp inverted
repeat sequence บริเวณ *mtrR* promoter พบการเปลี่ยนลำดับกรดอะมิโนของ MtrR repressor ที่ตำแหน่ง H105Y และพบการเปลี่ยน
ลำดับกรดอะมิโนภายใน PorB porin ที่ตำแหน่ง G120K กับ A121N การศึกษาการใช้ของยาต้านจุลชีพร่วมกันของ ceftriaxone ร่วมกับ
azithromycin, fosfomycin, gentamicin, หรือ ertapenem ด้วยวิธี checkerboard ต่อเชื้อ *N. gonorrhoeae* 2 สายพันธุ์ที่มีความไวลดลง
ต่อยา ceftriaxone พบว่าไม่พบการเสริมฤทธิ์กันของทุกคู่ยาที่ใช้ทดสอบ โดยคู่ยา ceftriaxone กับยา azithromycin ให้ผล additive และมี
ค่า FICI ต่ำที่สุดจึงถูกนำมาทดสอบยืนยันโดยวิธี time-kill ซึ่งผลการทดสอบไม่พบการเสริมฤทธิ์กัน แต่มี bactericidal activity จากผล
การศึกษานี้แสดงให้เห็นถึงอัตราการดื้อยาต้านจุลชีพที่สูงของยาที่เคยถูกใช้ในการรักษา ได้แก่ penicillin G, tetracycline และ
ciprofloxacin อย่างไรก็ตาม ไม่พบเชื้อ *N. gonorrhoeae* ที่ดื้อยา ceftriaxone แสดงให้เห็นว่ายานี้ยังมีฤทธิ์ต่อเชื้อ *N. gonorrhoeae* ที่
แยกได้จากประเทศไทย การกลายพันธุ์ในยีน *penA, mtrR* promoter, *mtrR,* และ *porB* มีความสัมพันธ์กับการมีความไวลดลงต่อยา
ceftriaxone ดังนั้นการเฝ้าระวังเชื้อ *N. gonorrhoeae* ที่ดื้อยาต้านจุลชีพและการวินิจฉัยที่รวดเร็วมีความสำคัญต่อการควบคุมการอุบัติขึ้น
และแพร่กระจายของเชื้อ *N. gonorrhoeae* ที่ดื้อยา

สาขาวิชา	จุลชีววิทยาทางการแพทย์	ลายมือชื่อนิสิต ....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

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KEYWORD: NEISSERIA GONORRHOEAE, CEFTRIAXONE RESISTANCE MECHANISM, ANTIBIOTIC COMBINATION

Naris   Kueakulpattana   :   MECHANISMS OF CEFTRIAXONE RESISTANCE IN *NEISSERIA GONORRHOEAE* ISOLATED FROM THAI PATIENTS. Advisor: Tanittha Chatsuwan, Ph.D.

N. gonorrhoeae is the causative agent of gonorrhea, which is one of the most important sexually transmitted disease. The multidrug resistance in *N. gonorrhoeae* has been increasingly reported worldwide. The objectives of this study were to investigate the prevalence and the mechanisms of ceftriaxone resistance in *N. gonorrhoeae* Thai isolates, and to investigate the synergistic activities of antibiotic combinations against *N. gonorrhoeae* isolates with reduced susceptibility or resistance to ceftriaxone. A total of 134 *N. gonorrhoeae* isolates were included in this study. The resistance rates of ciprofloxacin, tetracycline, penicillin G, gentamicin, azithromycin, ertapenem, fosfomycin, cefixime, and ceftriaxone were 90.30%, 82.09%, 73.13%, 59.70%, 13.43%, 1.49%, 0%, 0%, and 0%, respectively. The high prevalence of multidrug-resistant *N. gonorrhoeae* was found in 82.84%. Although all isolates were susceptible to ceftriaxone (MIC range of 0.001 to 0.125 mg/L with MIC_{50} and MIC_{90} of 0.002 and 0.015 mg/L, respectively.), two (1.49%) of them displayed reduced susceptibility to ceftriaxone. This is the first report of 2 *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone, NG-083 (ST723) and NG-091 (new ST) in Thailand. Both isolates had mosaic PBP2 pattern XXXIV, L421P substitution in PBP1, an adenine deletion in the 13-bp inverted repeat sequence of the *mtrR* promoter region, a H105Y substitution in the MtrR repressor, and G120K and A121N substitutions in PorB porin. The result from NG-MAST showed the heterogeneous populations (29 STs) among 31 representative strains of *N. gonorrhoeae* . The activities of ceftriaxone plus azithromycin, fosfomycin, gentamicin, or ertapenem were evaluated by checkerboard method against the 2 isolates with reduced susceptibility to ceftriaxone. There was no synergistic activity of these antibiotic combinations. The best combination was ceftriaxone plus azithromycin, which showed the lowest FICIs of additive and was confirmed by time-kill method. The results showed that this combination had no synergistic activity but had bactericidal activity. This study showed high resistance rates of antibiotics previously used for treatment of gonorrhea, e.g., penicillin G, tetracycline, and ciprofloxacin. However, no isolates were resistant to ceftriaxone, suggesting that it is still active against *N. gonorrhoeae* Thai isolates. Mutations in, *penA*, *mtrR* promoter*, mtrR*, and *porB* genes were associated with reduced susceptibility to ceftriaxone. Therefore, antimicrobial resistance surveillance and rapid diagnosis are important to limit the emergence and spread of antibiotic-resistant *N. gonorrhoeae* isolates.

Field of Study:	Medical Microbiology	Student's Signature	
Academic Year:	2018	Advisor's Signature	

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Naris Kueakulpattana

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LIST OF ABBREVIATIONS

°C	Degree Celsius
μL	Microliter
Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
ATCC	American type culture collection
AZT	Azithromycin
bp	Base pairs
carA	Carbamoyl-phosphate synthase subunit A
CDC	Centers for Disease Control and Prevention
CFM	Cefixime
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CRO	Ceftriaxone
del	Deletion
DNA	Deoxynucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
et al.	Et alii
ESCs	Extended-spectrum cephalosporins
ETP	Ertapenem
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FB	Fastidious broth
FICI	Fractional inhibitory concentration index
FOS	Fosfomycin

g	Grams
GEN	Gentamicin
Gly (G)	Glycine
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
His (H)	Histidine
I	Intermediate
L	Liter
Lbp	Lactoferrin receptor
Leu (L)	Leucine
LOS	Lipooligosaccharides
Lys (K)	Lysine
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
mol	Mole
mL	Milliliter
MLST	Multilocus sequence typing
mm	Millimeter
mM	Millimolar
MtrR	Multiple transfer resistance repressor
MSM	Men who have sex with men
NAD	Nicotinamide adenine dinucleotide
NARST	National Antimicrobial Resistance Surveillance Center, Thailand
NCBI	National Center for Biotechnology Information
ND	No data
NG-MAST	*Neisseria gonorrhoeae* multi-antigen sequence typing
NG-STAR	*Neisseria gonorrhoeae* sequence typing for antimicrobial resistance

orf1	Open reading frame 1 gene
PBPs	Penicillin binding proteins
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEN	Penicillin G
Por	Porin
Pro (P)	Proline
R	Resistant
Rmp	Reduction-modifiable protein
S	Susceptible
ST	Sequence type
STI	Sexually transmitted infection
TBE	Tris-borate-EDTA
Tbp	Transferrin receptors
tbpB	Transferrin binding protein unit B
TET	Tetracycline
Thr (T)	Threonine
Tris-HCl	Tris-(hydroxymethyl)-aminomethane-hydrochloride
Tyr (Y)	Tyrosine
WHO	World Health Organization
WT	Wild type

CHAPTER I

INTRODUCTION

Neisseria gonorrhoeae is a Gram-negative kidney-shaped diplococci and non-motile bacteria. It causes gonorrhea, a sexually transmitted infection (STI), and is transmitted by human-to-human through the sexual contact with the anus, rectum, cervix, urethra and throat. Gonorrhea can be transmitted vertically from mother to newborn baby during delivery. Gonorrhea typically presents as cervicitis in women and urethritis in men but when left untreated, it can cause severe complications such as urethral stricture and prostatitis in males and pelvic inflammatory disease in females, leading to infertility and ectopic pregnancy. Mother-to-child transmission of gonorrhea during birth can cause ophthalmia neonatorum (1). The World Health Organization (WHO) estimated that there were 106 million new gonorrhea cases worldwide in 2008, which represented 21% increase since 2005 (2, 3). In China, 95,263 cases of gonorrhea were reported in 2012, making gonorrhea the first most common STI (4). In the United States, gonorrhea was the second most common STI and there were 820,000 new cases of gonorrhea reported in 2015 (5, 6). The morbidity rate of gonorrhea cases in Thailand increased from 4.89 in 2001 to 12.56 in 2011 per 100,000 population (7). In 2016, gonorrhea cases were reported to be 14.72 per 100,000 population (8), followed by syphilis, non-gonococcal urethritis and chancroid (9).

Ceftriaxone is recommended as the first-line drug for treatment of gonorrhea (10). Ceftriaxone is the third-generation cephalosporin, which is a bactericidal agent that can inhibit bacterial cell wall synthesis. It acts on penicillin-binding proteins (PBPs) that catalyzes the cross-linking of the peptidoglycan polymers forming the bacterial cell wall. The inhibition of PBPs activity leads to the formation of defective cell wall and eventually to cell death (11). Ceftriaxone-susceptible *N. gonorrhoeae* isolates are defined by a minimal inhibitory concentration (MIC) of ≤ 0.25 mg/L (as described by Clinical and Laboratory Standards Institute (CLSI) guideline) (12). Treatment outcome data from

Toronto, Canada, suggested that ceftriaxone MIC of ≥ 0.125 mg/L of *N. gonorrhoeae* isolates correlated well with treatment failures when ceftriaxone was used alone (13). Treatment failures by ceftriaxone have been reported in Australia (14), Japan (15), Slovenia (16) and Sweden (17). Definition of reduced ceftriaxone susceptibility by the Centers for Disease Control and Prevention (CDC) was ceftriaxone MIC of 0.125-0.25 mg/L (18). However, the spread of multidrug-resistant and ceftriaxone-resistant *N. gonorrhoeae* isolates (MIC of ≥ 0.5 mg/L) were reported worldwide including Japan (19), France (20), Spain (21), Canada (22), Denmark (23), Argentina (24), and China (25). In Thailand, the data from Bangrak STIs Center showed that *N. gonorrhoeae* isolates had ceftriaxone MIC of 0.032 mg/L in 2016 (26). These suggest that ceftriaxone-resistant *N. gonorrhoeae* isolates has not been reported in Thailand (27). Currently, CDC recommends that ceftriaxone plus azithromycin is the first-line treatment for gonorrhea (28).

Mechanisms of ceftriaxone resistance in *N. gonorrhoeae* are mediated by mutations in chromosomal genes including *penA, mtrR, porB,* and *ponA* (29-33). Penicillin binding protein 2 (PBP2), encoded by *penA*, is the major target of β-lactam antibiotics in *N. gonorrhoeae*. Mutation in *penA* containing aspartic acid insertion at position 345 in the transpeptidase domain of PBP2, namely, nonmosaic, leading to reduced binding affinity of β-lactam antibiotics to PBP2 and resulted in resistance to penicillin (30, 34, 35). The mosaic *penA* alleles containing up to 70 amino acid alterations (32, 36) which were considered to emerge by recombination with partial *penA* genes from commensal *Neisseria* species (37-39). Some mosaic *penA* patterns X and XXXIV (40) were reported to be associated with reduced susceptibility or resistance to ceftriaxone. Furthermore, specific alterations of the *penA* gene at positions A501, G542, and P551 (34, 41) resulted in reduced susceptibility to extended-spectrum cephalosporins (ESCs) (42). Mutation in *mtrR* promoter by an adenine deletion in the 13-bp inverted repeat sequence between -10 to -35 bp sequence of *mtrR* promoter and/or amino acid substitutions at positions 32-53, especially A39T and G45D mutations, in a DNA-binding-domain coding regions of the *mtrR* gene which encoded MtrR repressor

that bind to *mtrCDE* promoter, resulted in overexpression of the mtrCDE efflux pump, which increased efflux for exports of the ceftriaxone out of the cell (34, 43, 44). Mutation in PorB porin by amino acid substitutions at position 120 and/or 121 within constriction loop of *porB*, resulted in reduced permeability of the outer membrane porin to ceftriaxone (34, 44, 45). The L421P substitution in PBP1, encoded by *ponA*, resulted in reduced affinity for penicillin and contributed to penicillin resistance (36, 46). There was an association of various resistance determinants (*penA*, *ponA*, *mtrR*, and *porB*) and decreased ESC susceptibility (32, 42). Genetic resistance mechanisms of ceftriaxone in *N. gonorrhoeae* have been reported in many countries such as China (34), Korea (44), Japan (40), Canada (45), France (20) and Spain (21). However, there are few studies on the mechanisms of ceftriaxone resistance in *N. gonorrhoeae* in Thailand (27).

Although ceftriaxone plus azithromycin is used for treatment for *N. gonorrhoeae* infection, treatment failure has been reported in the United Kingdom (47). Combination therapy is the most effective strategy to combat multidrug-resistant *N. gonorrhoeae* (48). There are few studies on antibiotic combinations among ceftriaxone–resistant *N. gonorrhoeae* isolates in Thailand.

Nowadays, the spread of multidrug-resistant *N. gonorrhoeae* represents a major public health concern. Ceftriaxone is considered a drug of choice for treatment. At present, ceftriaxone–resistant *N. gonorrhoeae* is emerging. However, there are few studies on molecular mechanism of ceftriaxone resistance and the genetic relationship among ceftriaxone–resistant *N. gonorrhoeae* isolates in Thailand. Therefore, the purposes of this study were to investigate (i) the prevalence of ceftriaxone resistance, (ii) the ceftriaxone resistance mechanisms, and (iii) the *in vitro* activities of ceftriaxone combination with azithromycin, fosfomycin, gentamicin, and ertapenem against *N. gonorrhoeae* isolates with reduced susceptibility or resistance to ceftriaxone by checkerboard and time-kill methods.

CHAPTER II

OBJECTIVES

1) To investigate the prevalence of ceftriaxone resistance in *N. gonorrhoeae* clinical isolates in Thailand.

2) To characterize the mechanisms of ceftriaxone resistance in *N. gonorrhoeae* clinical isolates in Thailand.

3) To investigate the synergistic activities of antibiotic combinations against clinical *N. gonorrhoeae* Thai isolates with reduced susceptibility or resistance to ceftriaxone.

CHAPTER III

LITERATURE REVIEW

1.1 *Neisseria gonorrhoeae*

N. gonorrhoeae is Gram-negative diplococci, approximately 0.8 μm in diameter, with the adjacent sides flattened (typically kidney bean shaped), non-capsule and non-motile bacteria belonging to the Phylum Proteobacteria, Class Betaproteobacteria, Order Neisseriales, and Family Neisseriaceae (49). They can grow on chocolate agar, produce oxidase and catalase (50). It cause gonorrhea, one of the most important sexually transmitted diseases in worldwide. *N. gonorrhoeae* is transmitted by human-to-human through the sexual contact with the anus, rectum, cervix, urethra, mouth, and throat. Gonorrhea can be transmitted vertically from mother to newborn baby during delivery. *N. gonorrhoeae* infects only human mucosal surfaces and causes urethritis in men that can be severe complicated by urethral stricture when left untreated. In women, it presents as cervicitis but when left untreated, it can cause severe complication by pelvic inflammatory disease, leading to infertility and ectopic pregnancy (1, 51-53). *N. gonorrhoeae* infections can have a predominantly asymptomatic prostatitis and pharyngitis, especially in men who have sex with men (MSM) (54). However, these diseases can occur in both sex, depending on sexual activity and number of sexual partner. *N. gonorrhoeae* can also infect to conjunctiva, joints, and blood system which cause conjunctivitis, septic arthritis and disseminated in blood stream (51). Mother-to-child transmission of gonorrhea during birth through the eye can cause ophthalmia neonatorum, which results in blindness in the newborn. Transmission of *N. gonorrhoeae* is associated with other sexually transmitted diseases including chlamydia (*Chlamydia trachomatis* infection), syphilis (*Treponema pallidum* infection), and the human immunodeficiency virus (HIV) (52-55).

N. gonorrhoeae is fastidious organism, relatively fragile, susceptible to temperature changes, and other environmental stresses. *N. gonorrhoeae* requires

supplements containing hemoglobin, nicotinamide adenine dinucleotide (NAD), or yeast extract in culture media for growth at 35 $^{\circ}$C in an atmosphere containing 5% CO_2 (50).

1.2 Pathogenesis and virulence factor of *N. gonorrhoeae*

N. gonorrhoeae virulence factor utilizing helps them to penetrate host cells and escapes the phagocytosis of the immune system. *N. gonorrhoeae* is antigenically heterogeneous and capable of changing its surface structures. The major virulence factors and/or antigenic variation are summarized in Table 1 (56-58).

1.2.1 Pili (Fimbriae)

Pili are the hairlike appendages that extend up from the surface of *N. gonorrhoeae*. The initial of infection in *N. gonorrhoeae* is adhesion by using pili, which enhance attachment to mucosal cell surfaces of host cells and also are resistant to phagocytosis. Pili of almost all strains of *N. gonorrhoeae* are antigenically different, some strain can make many antigenically distinct forms of pili.

1.2.2 Lipooligosaccharides (LOS)

N. gonorrhoeae can produce LOS, which are endotoxin. The structure of LOS is similar to glycosphingolipids, which is one of human cell membrane. The LOS and the glycosphingolipids of the same structure respond to the same monoclonal antibody which results in helps for evasion of immune recognition. The structure of LOS is shown in Figure 1 (58).

1.2.3 Reduction-modifiable protein (Rmp; Protein III)

Rmp is antigenically conserved protein which found in all *N. gonorrhoeae* isolates. When Rmp is in a reduced state, it can changes its apparent molecular weight. It usually associates with Por in the formation of pores in the cell surface of *N. gonorrhoeae*.

1.2.4 Por

Por protein extends through cell membrane of *N. gonorrhoeae*. It forms pores in the surface through some nutrients or antimicrobial agents enter the cell. Por proteins of *N. gonorrhoeae* have impact intracellular killing by prevention phagosome-lysosome fusion from neutrophil. The best characterized invasion pathway for primary human cervical cells occurs through interactions between the complement receptor three integrin or cell adhesion molecules, and a complex formed by Por porin, Rmp, and LOS.

1.2.5 Opa proteins

Opa proteins are proteins function in adhesion of *N. gonorrhoeae* within colonies and attachment between *N. gonorrhoeae* to host cell receptor including epithelium cell CD66, and heparin-related compounds. One portion of the Opa is exposed on the surfaces, and the rest is in the outer membrane of *N. gonorrhoeae*.

1.2.6 Transferrin receptors (Tbp1 and Tbp2) and lactoferrin receptor (Lbp)

N. gonorrhoeae obtains iron from host during growth, which is necessary to support bacterial invasion. These Tbp1, Tbp2, and Lbp proteins can also extract iron from heme and hemoglobin of host cells.

Table 1. Surface components of *N. gonorrhoeae* (57)

Designation	Location	Contribution
PilE	major fimbrial protein	initial binding to epithelial cells
P.II (Opa)	outer membrane protein	contributes to invasion
P.I (Por)	outer membrane porin	may prevent phagolysosome formation in neutrophils and/or reduce oxidative burst
LOS	outer membrane lipooligosaccharide	elicits inflammatory response, triggers release of TNF
P.III (Rmp)	outer membrane protein	elicits formation of ineffective antibodies that block bactericidal antibodies against P.I and LOS
Tbp1, Tbp2	outer membrane receptors for transferrin	iron acquisition for growth
Lbp	outer membrane receptor for lactoferrin	iron acquisition for growth
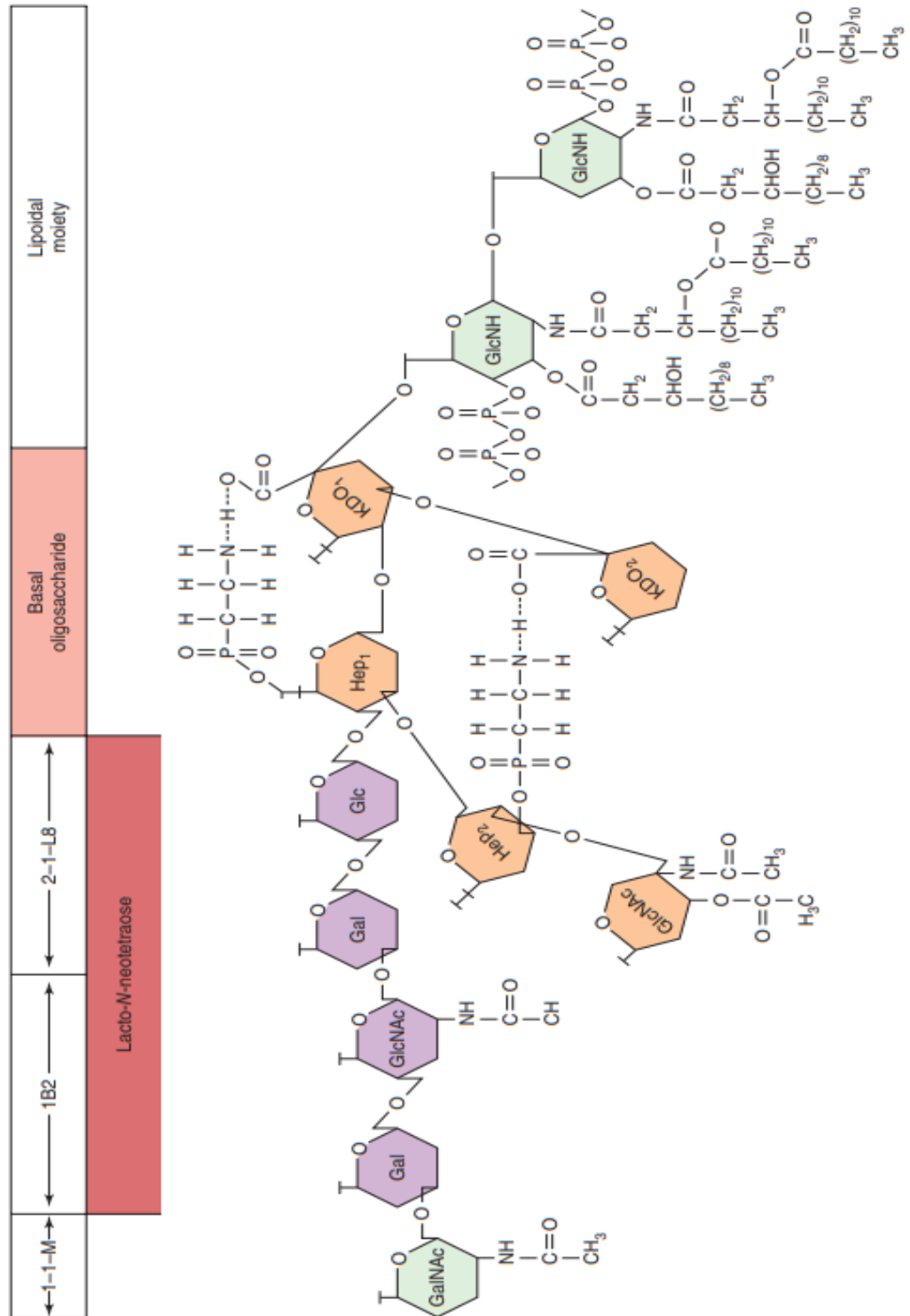
Figure 1. Structure of lipooligosaccharides in *N. gonorrhoeae*

The basal oligosaccharide is in light red, and the lacto-N-neotetraose is in dark red (58).

1.3 Diagnosis of *N. gonorrhoeae* infections

The diagnosis of *N. gonorrhoeae* infections is established by microscopy for Gram stained smear, cultivation, molecular methods, or nucleic acid amplification tests (NAATs) (50, 59, 60).

Characterization of urethral discharge by Gram stain shows Gram-negative intracellular diplococci. This method yields rapid results and high sensitivity and specificity. However, Gram stain is not used for diagnosis of gonorrhea from cervical, pharyngeal, rectal swab, or asymptomatic diseases because of low sensitivity and it depends on the experience of microscopist for characterization.

Cultivation is gold standard method in laboratory diagnosis of *N. gonorrhoeae* (as shown in Figure 2). Selective media for cultivation of *N. gonorrhoeae* including Thayer–Martin (TM) medium, modified Thayer–Martin (MTM) medium, Martin–Lewis (ML) medium, and New York City (NYC) medium (Table 2). This method shows high sensitivity, almost 100% specificity, and capacitate to antimicrobial resistance testing. However, culture is relatively slow and it needs optimization for the condition, temperature, sample collection, storage, and sensitivity to external environmental factor.

An in-house polymerase chain reaction (PCR) method was developed and evaluated for the detection of *N. gonorrhoeae*. The high specificity and sensitivity with low cost and rapidity of the in-house PCR method described here can serve as a promising diagnostic method for detecting of gonococcus directly from clinical swab samples. PCR was performed using specific primers for carbamoyl-phosphate synthase subunit A (*carA*) gene (59) and open reading frame 1 (*orf1*) gene (60).

NAATs also can detect nonviable, all specimens, particularly for pharyngeal, and rectal specimens. This method is rapid and has more sensitivity than all other methods. However, NAATs do not determine antimicrobial resistance testing. Moreover, in-house NAATs show different specificities for detection of *N. gonorrhoeae* because commensal *Neisseria* species frequently found in pharynx and rectum have genetic homology with *N. gonorrhoeae*, and might be cross-react, resulting in false-positive NAATs results.

Table 2. Selective media for cultivation of *N. gonorrhoeae* (50)

Selective media	Antimicrobial agents					
	vancomycin	colistin	nystatin	trimethoprim	anisomycin	amphotericin B
Thayer–Martin (TM) medium	+	+	+	-	-	-
modified Thayer–Martin (MTM) medium	+	+	+	+	-	-
Martin–Lewis (ML) medium	+	+	-	+	+	-
New York City (NYC) medium	+	+	-	+	-	+
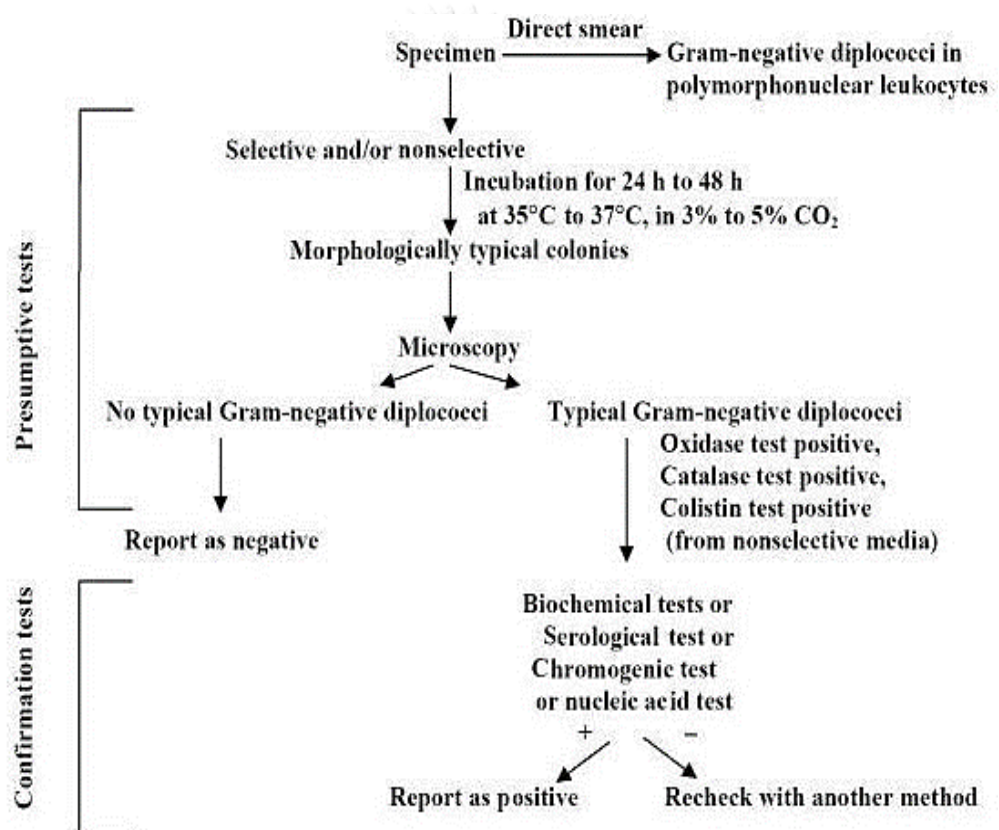
Figure 2. Algorithm for cultivation and identification of *N. gonorrhoeae* (50)

1.4 Treatment of *N. gonorrhoeae* infections

N. gonorrhoeae has developed resistance to antibiotics including sulfonamides, penicillin, tetracycline, and ciprofloxacin. Fluoroquinolones have been recommended to treat gonorrhea since 1992, due to high rates of resistance to sulfonamides, penicillin and tetracycline (61). However, prevalence of ciprofloxacin resistance in *N. gonorrhoeae* increased from 9.4% in 2005 to 13.3% in 2006, in the United States (62). There were 28% of *N. gonorrhoeae* isolates resistant to ciprofloxacin in Canada in 2006 (63). The problem of antimicrobial resistance in *N. gonorrhoeae* has been increasing worldwide. High prevalence (>5%) of multidrug-resistant *N. gonorrhoeae* isolates which were resistant to penicillin, tetracycline, and fluoroquinolones was reported in 2007 (62). Moreover, the resistance rates of penicillin, tetracycline, and ciprofloxacin have been reported in many countries. The antimicrobial resistance rates of *N. gonorrhoeae* isolates are shown in Table 3.

Table 3. The reports of the antimicrobial resistance rates of *N. gonorrhoeae* isolates

Year of report	Country	Resistance rates (%)			References
		penicillin	tetracycline	ciprofloxacin	
2011	Canada	89.2%	72.3%	29.0%	(45)
2013	Vietnam	48%	82%	98%	(64)
2013	India, Pakistan, Bhutan	68%	55%	94%	(65)
2014	China	84.2%	71.3%	96.3%	(66)
2014	Switzerland	85.3%	88.2%	73.5%	(67)
2014	Hungary	79.5%	85.5%	67.2%	(68)
2017	Thailand	85.7%	89.1%	88.0%	(27)
2017	Laos	89.9%	99.4%	84.8%	(69)
2017	Thailand	ND	83.5%	92.2%	(26)
2010	Taiwan	71.1%	60.0%	77.8%	(70)
2017	China	73.8%	81.7%	100%	(71)
2018	New Zealand	98%	68%	32%	(72)

Note: ND; No data

Cefixime is oral cephalosporins which CDC no longer recommends cefixime as the first-line treatment for gonorrhea. Treatment failure by cefixime had been reported in Norway (73), Austria (74), England (75), and Canada (13). Now, ceftriaxone is recommended as the first-line drug for treatment of gonorrhea (10). However, the first high level of ceftriaxone resistance isolates was reported in Japan (19) and treatment failure by ceftriaxone has been reported in Australia (14), Japan (15), Slovenia (16), and Sweden (17). Currently, CDC recommends that ceftriaxone 250 mg as a single intramuscular dose plus azithromycin 1 gram orally in a single dose is the first-line treatment for gonorrhea (10).

The history of recommended antimicrobials and evolution of resistance determinants in *N. gonorrhoeae* are shown in Figure 3 (30).
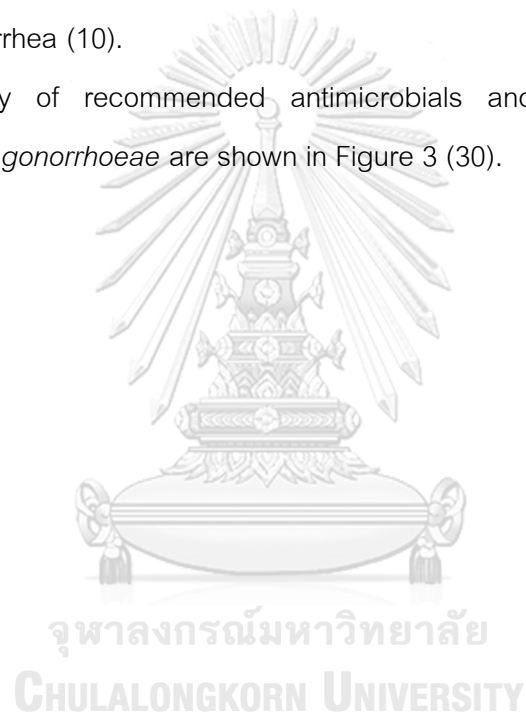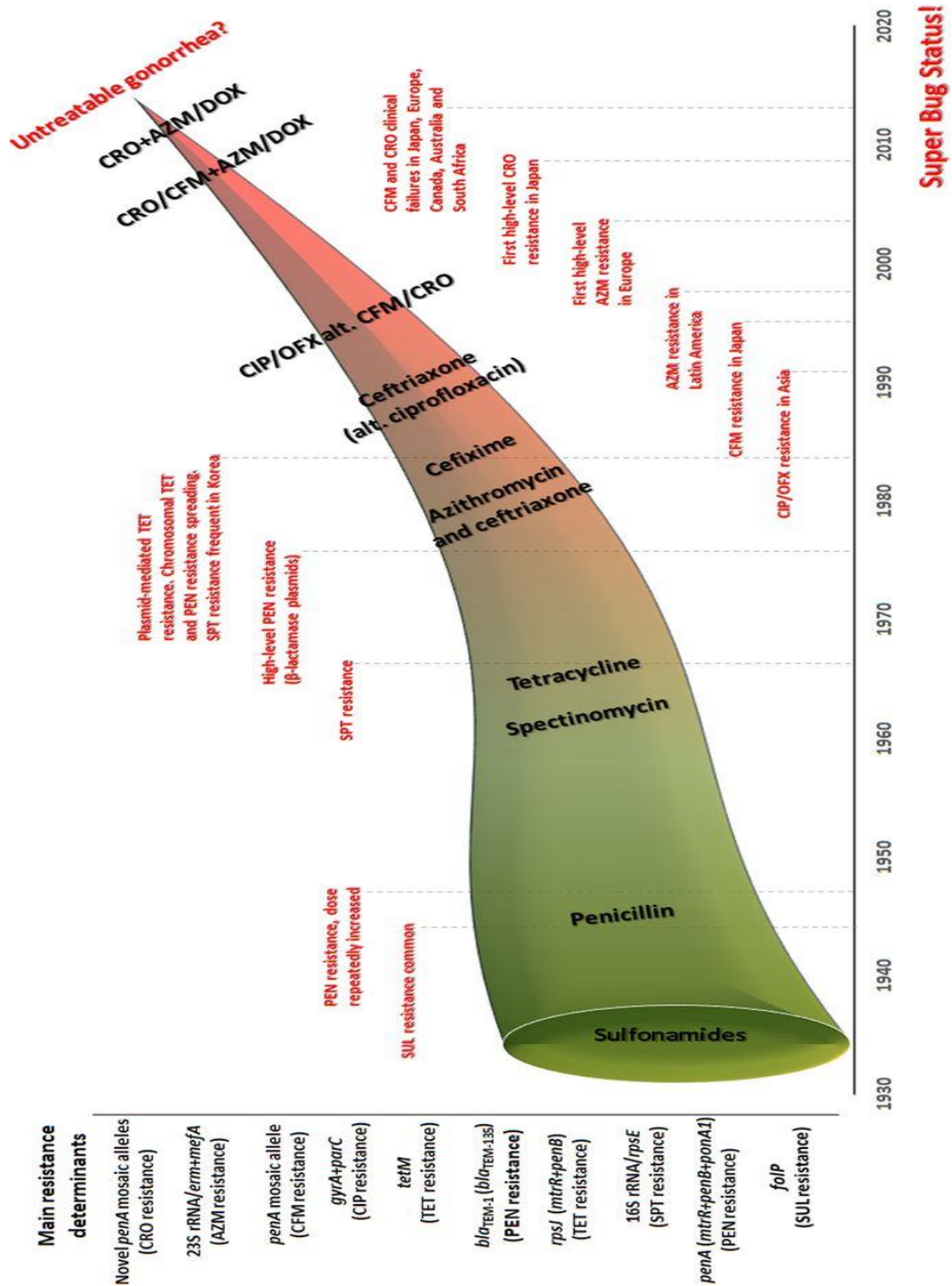
Figure 3. History of recommended antimicrobials and evolution of resistance determinants in *N. gonorrhoeae* (30)

Note: AZT, azithromycin; CFX, cefixime; CRO, ceftriaxone; CIP, ciprofloxacin; DOX, doxycycline; OFX, ofloxacin; PEN, penicillin G; TET, tetracycline; SPT, spectinomycin; SUL, sulfonamides.

1.5 Ceftriaxone

Ceftriaxone [Formula of $C_{18}H_{18}N_8O_7S_3$ (Figure 4) and Molar mass of 554.58 g/mol] is a third-generation cephalosporin, which is β-lactam antibiotic group used in the treatment of bacterial infections. Ceftriaxone is a bactericidal agent that can inhibit cross-linking of the peptidoglycan which can also interfere with bacterial cell wall synthesis and cell division. It binds to transpeptidases in the bacterial cytoplasmic membrane and acts on penicillin-binding proteins (PBPs) that catalyzes the cross-linking of the peptidoglycan polymers forming the bacterial cell wall. The inhibition of PBPs by ceftriaxone activity leads to the formation of defective cell wall and eventually to cell death (11). Ceftriaxone is stable against hydrolysis of beta-lactamases including penicillinases, cephalosporinases, and extended spectrum β-lactamases (76).
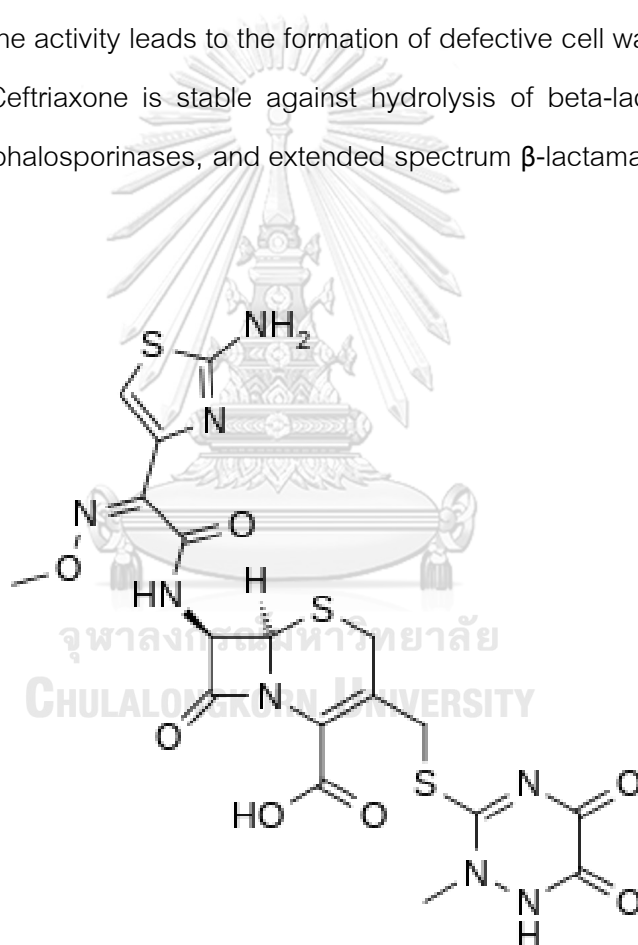
Figure 4. Structure of ceftriaxone (11)

1.6 Ceftriaxone resistance in *N. gonorrhoeae*

Ceftriaxone-susceptible *N. gonorrhoeae* isolates are defined by a minimal inhibitory concentration (MIC) of ≤ 0.25 mg/L as described by Clinical and Laboratory Standards Institute (CLSI) guideline (12). Currently, surveillance of antimicrobial susceptibility of *N. gonorrhoeae* in many countries has reported clinical isolates with ceftriaxone MIC of > 0.25 mg/L. The ceftriaxone-resistant *N. gonorrhoeae* strains are shown in Table 4.

Table 4. The reports of ceftriaxone-resistant *N. gonorrhoeae* strains

Year of isolation	Strains	Country	CRO MIC (mg/L)	NG-MAST	Reference
2000-01	GP853	Japan	0.5	ND	(77)
2003	GU030113	Japan	0.5	ST668	(40)
2007	Chinese	China	0.5	ST2288	(66)
2009	H041	Japan	2-4	ST4220	(19)
2009	A8806	Australia	0.5	ST4015	(78)
2010-11	F89	France, Spain	1-2	ST1407	(20, 21)
2011	GU110095	Japan	0.5	ST3935	(40)
2011	GU110332	Japan	0.5	ST13194	(40)
2011	GU110362	Japan	0.5	ST5687	(40)
2014	GU140106	Japan	0.5	ST6543	(79)
2014	ND	Argentina	0.5	ST13064	(24)
2015	FC428	Japan	0.5	ST3435	(5)
2015	FC460	Japan	0.5	ST3435	(5)
2017	GK124	Denmark	0.5	ST1614	(23)
2017	47707	Canada	0.5	ST1614	(22)
2017	A7846	Australia	0.5	ST1614	(80)
2017	A7536	Australia	0.5	ST15925	(80)

Note: CRO; ceftriaxone, NG-MAST; *N. gonorrhoeae* multi-antigen sequence typing, ST; Sequence type, ND; No data

Report of Lahra *et al.* (80) showed substantive similarity of gonococcal clones from Japan (5), Canada (22), Denmark (23), and Australia (80). F89 strains in France (20) and Spain (21), there had been no reports of substantive similarity of these

gonococcal clones identified globally (80). As shown in Figure 5, among the FC428 and FC460 from Japan, A7536 and A7846 from Australia, and 47707 from Canada were closely genetic relatedness and belonged to the same clone, ST223 from NG-STAR or ST1903 from MLST. Moreover, 47707 from Canada and A7846 from Australia belonged to the same clone, ST1614 from NG-MAST. These 5 isolates were distinct from F89, A8806, and H041. Therefore, these report indicates that strains from Japan, Australia, and Canada are the successful gonococcal clones with ceftriaxone MIC of resistance levels that have been spread globally (80).

	Country, Date	PenA	NG-STAR	MLST	NG-MAST
FC428	Japan, 2015	PenA60	233	1903	3435
FC460	Japan, 2015	PenA60	233	1903	3435
A7536	Australia, Aug 2017	PenA60	233	1903	15925
47707	Canada, Jan 2017	PenA60	233	1903	1614
A7846	Australia, Apr 2017	PenA60	233	1903	1614
F89	France, 2010	PenA42	16	1901	1407
A8806	Australia, 2009	PenA64	227	7363	4015
H041	Japan, 2009	PenA37	226	7363	4220
FA1090 (2003)					

Figure 5. Phylogenetic tree of ceftriaxone-resistant *N. gonorrhoeae* isolates (80)

Note: NG-STAR, *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance; MLST, Multilocus sequence typing; NG-MAST, *Neisseria gonorrhoeae* multi-antigen sequence typing.

Treatment failure by using ceftriaxone alone has been reported in Australia (14), Japan (15), Slovenia (16), and Sweden (17). Treatment outcome data from Toronto, Canada, suggested that ceftriaxone MIC of $\geq$ 0.125 mg/L of *N. gonorrhoeae* isolates correlated well with treatment failures when ceftriaxone was used alone (13). Definition of reduced ceftriaxone susceptibility by CDC was ceftriaxone MIC of 0.125-0.25 mg/L

(18). *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone were reported in Korea (44), Japan (37), China (81), Canada (45), and Switzerland (67). Currently, CDC recommends that ceftriaxone plus azithromycin is the first-line treatment for gonorrhea (17). However, combination of ceftriaxone plus azithromycin treatment failure was reported in the United Kingdom in 2014. These *N. gonorrhoeae* isolates belonged to ST6800 which had ceftriaxone MIC of 0.25 mg/L and azithromycin MIC of 1mg/L (47). However, ceftriaxone-resistant *N. gonorrhoeae* strains has not been reported in Thailand (34). The data from Bangrak STIs Center showed high ceftriaxone MIC of 0.032 mg/L in 2016 (26).

Development of ceftriaxone resistance in *N. gonorrhoeae* through specific mutations or gene transfer by transformation and subsequent recombination into the genome. Ceftriaxone resistance in *N. gonorrhoeae* has been associated with mainly mutation in penicillin-binding protein 2 (PBP2), but also in overexpression of MtrCDE efflux pumps, loss or reduction of outer membrane porins and mutation in PBP1 as described below (30, 32).

1. Mutation in PBP2

The primary ceftriaxone resistance determinants in *N. gonorrhoeae* are specific alterations of *penA* gene, encoding penicillin-binding protein 2 (PBP2), which is the major target of ceftriaxone. Ceftriaxone resistance is mostly found in the mosaic alleles that contains up to 70 amino acid alterations. The mosaic alleles do not find aspartic acid insertion at position 345 (32). The mosaic *penA* alleles were considered to emerge by recombination with partial *penA* gene from commensal *Neisseria* species including *N. cinerea, N. flavescens, N. perflava, N. polysaccharea, N. sicca* (38, 45), and *N. meningitidis* (82). Mutations in mosaic alleles in PBP2 at following positions; A311V, I312M, V316T/P, T483S, A501P/T/V, N512Y, and G545S were found to contribute to the significantly increased ceftriaxone MIC and decreased ceftriaxone susceptibility (30, 83). The additional A501V/P/T and N512Y mutations into a mosaic PBP2 have been reported mainly in *N. gonorrhoeae* isolates with decreased ceftriaxone and cefixime susceptibility, leading to increase MICs of ceftriaxone and cefixime 2- to 4-fold and the

wild type belonged to resistance strains (33, 39, 44). Furthermore, the G542S, P551S/L mutations have also been associated with increased MIC of ceftriaxone in *N. gonorrhoeae* (41). The A311V and T483S substitutions are responsible for high-level resistance to the third-generation cephalosporins (84).

The amino acid sequences of *penA* gene of ceftriaxone-resistant strain are shown in Figure 6. Key amino acid alternations causing the ceftriaxone resistance including the A311V and T483S substitutions were found in ceftriaxone-resistant strains FC428 (5), GU140106 (79), and A8806 (78). The A501P substitution in mosaic PBP2 pattern XXXIV was found in ceftriaxone-resistant strain F89 (20, 21). The A311V, T316P, and T483S substitutions were found in ceftriaxone-resistant strain H041 (15).

Figure 6. Amino acid sequences in PBP2 of ceftriaxone-resistant *N. gonorrhoeae* (80)

The *ponA* gene, encoding penicillin-binding protein 1 (PBP1) is the second penicillin target. Mutations in *ponA* by the amino acid substitution at position L421P resulted in reduced penicillin acylation of PBP1 and decreased penicillin MIC to 2- to 4-fold. These mutations do not appear to affect the MIC of ceftriaxone. However, most strains with decreased susceptibility or resistance to ceftriaxone contained the L421P substitution (29, 36).

2. Overexpression of efflux pump

Bacterial efflux pumps are one of the mechanisms responsible for the antimicrobial resistance and can be found in almost all bacterial species. Efflux pumps are grouped into five families containing the ATP-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the major facilitator (MF) family, the resistance-nodulation-division (RND) family, and the small multidrug resistance (SMR) family (85-87).

There were four efflux pump systems identified in *N. gonorrhoeae*: MacAB, NorM, FarAB, and MtrCDE efflux pumps, which belong to the ABC, MATE, MF, and RND families, respectively (88-91). However, the MtrCDE efflux pump has been found to be associated extensively with clinically significant antimicrobial resistance because this pump has been only reported to recognize with antimicrobials currently recommended for treatment of gonorrhea (91). Moreover, the MtrCDE efflux pump can export hydrophobic structure of antimicrobials including penicillin, tetracycline, ciprofloxacin, macrolides (azithromycin and erythromycin) and, ESCs (cefixime, and ceftriaxone) (91-95).

The MtrCDE efflux pump has been investigated in most of antimicrobial resistance mechanisms in *N. gonorrhoeae* isolates (30). The expression of the MtrCDE efflux pump operon occurs under *trans*- and *cis*-acting regulatory element. *Trans*-acting element encoding repressors, while *cis*-acting element encoding activators which are shown in Figure 7. *N. gonorrhoeae* strain expressing intermediate-level resistance to ceftriaxone of the MtrCDE efflux pump commonly have amino acid alterations in DNA-binding domain of the *mtrR* coding region that encodes the multiple transfer resistance repressor (MtrR), which represses the expression of the MtrCDE efflux pump by MtrR repressor bind to the promoter of MtrCDE efflux pump system. Missense mutations in DNA-binding domain coding region of *mtrR* gene by the amino acid alteration at position between 32 to 53 of the helix-turn-helix motif of the MtrR repressor (96) including at position 39 from alanine to threonine (A39T) (43), at position 44 from arginine to histidine (R44H) (97), at position 45 from glycine to aspartic acid (43, 97). These mutations in

DNA-binding domain resulted in the MtrR repressor cannot bound to the target DNA upstream of *mtrCDE* and enhanced to antimicrobial resistance of *N. gonorrhoeae* (96, 98, 99). DNA dimerization domain of MtrR repressor at position 105 from histidine to tyrosine (H105Y) (100) showed increased expression of the mtrCDE efflux pump of ESC and further decreased susceptibility to ESC of *N. gonorrhoeae* (41, 43, 49). The A40D in the MtrR repressor was first reported by Seike *et al.* (40) and confer increased MIC of azithromycin (92, 101-103). However, it is uncertain whether the A40D substitution could contribute to enhance efflux pump activity to ESCs in *N. gonorrhoeae* isolates (40).

    N. gonorrhoeae strain expressing high-level resistance to ceftriaxone have mutations in the overlapping of *mtrR* promoter region. Mutations in *mtrR* promoter, especially a single nucleotide [adenine] deletion in the 13-bp inverted repeat sequence between the -10 and -35 of the *mtrR* promoter region, resulted in overexpression of the efflux pump, and its enhance the capacity to export hydrophobic antimicrobials.

    However, the double mutation of an adenine deletion in the promoter region with the A39T, G45D, or H105Y substitutions resulted in overexpression of the efflux pump and were associated with ceftriaxone susceptibility of *N. gonorrhoea*e. An adenine deletion mutation interrupts the binding of MtrR repressor to promoter of the MtrCDE efflux pump. The A39T and G45D may inhibit DNA-binding domain of MtrR repressor and the H105Y substitution may inhibit DNA dimerization domain of MtrR repressor (96, 99, 100).
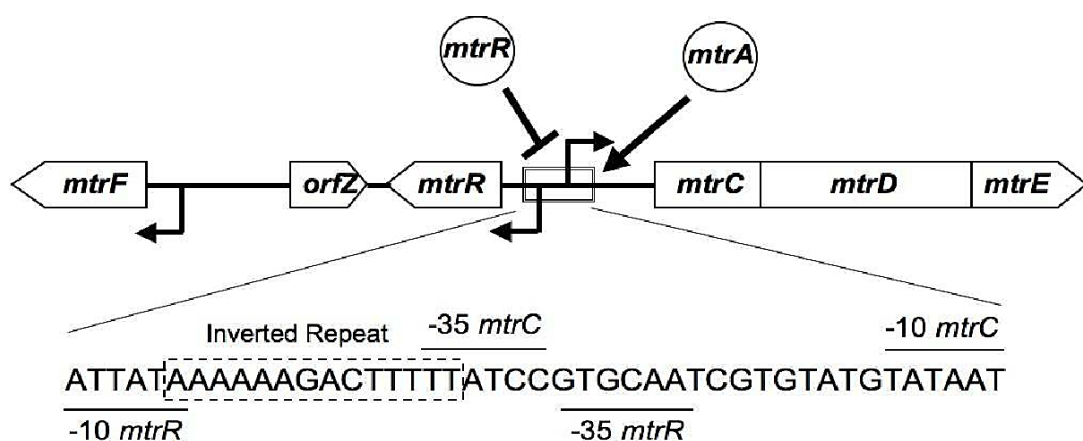
Figure 7. Composition of the *mtr* locus of *N. gonorrhoeae* (104)

3. Loss or reduction of outer membrane porins

The porins are bacterial outer membrane proteins, which have important permeability barrier to uptake of antimicrobials into cells and allow the passage of small molecules into the periplasms of bacteria through outer membrane channels formed by the porins (105). Antimicrobials diffuse into the bacterial cells included penicillin, tetracycline, and extended spectrum cephalosporin (ESCs) such as cefixime and ceftriaxone (106). *N. gonorrhoeae* have two forms of porins, which are as trimeric pore-forming transmembrane porins, namely, PorB1a and PorB1b (107). However, *N. gonorrhoeae* strains expressing PorB1a form were susceptible to penicillin and tetracycline more than *N. gonorrhoeae* strains expressing PorB1b because amino acid alterations in loop 3 of PorB1b caused β-barrel folding in porin and constricted the pore, resulted in decreased penicillin, tetracycline, and ESCs susceptibility (29, 108, 109). The mutations in *porB* gene, or namely, *penB* gene, by the amino acid substitutions at position 120 alone and/or position 120 and position 121 in PorB1b porin, resulted in decreased antimicrobial influx especially penicillin and tetracycline (108). Interestingly, *porB1b* mutation was only presented in strain with *mtrR* mutation and overexpression of MtrCDE efflux pump (106, 109).

There was an association of various genetics of chromosomally mediated intermediate resistance determinants (*penA*, *ponA*, *mtrR* promoter, MtrR repressor, and *porB* genes) and decreased or resistance ceftriaxone susceptibility in *N. gonorrhoeae* (29-33). The resistance determinants and mechanisms of resistance in *N. gonorrhoeae* are summarized in Table 5.

Table 5. The resistance determinants and mechanisms of resistance in *N. gonorrhoeae* (30)

Antimicrobial class	Resistance determinants/mechanisms
Penicillins (e.g., penicillin G and ampicillin)	Mutations in *penA* (encoding the main lethal target PBP2). Traditionally, the mutations were the single amino acid insertion D345 in PBP2 and 4 to 8 concomitant mutations in the PBP2 carboxyl-terminal region, decreasing the PBP2 acylation rate and reducing susceptibility ~6- to 8-fold. In the last decade, many mosaic *penA* alleles with up to 70 amino acid alterations, also reducing PBP2 acylation, were described.
	Mutations in *mtrR*, in the promoter (mainly a single nucleotide [A] deletion in the 13-bp inverted repeat sequence) or coding sequence (commonly a G45D substitution), result in overexpression of and increased efflux from the MtrCDE efflux pump. See the text for rarer mutations resulting in increased MtrCDE efflux.
	porB1b SNPs, e.g., encoding G120K and G120D/A121D mutations in loop 3 of PorB1b, reduce influx (*penB* resistance determinants). Interestingly, the *penB* phenotype is apparent only in strains with the *mtrR* resistance determinant.
	A SNP in *pilQ* (encoding the pore-forming secretin PilQ of the type IV pili), i.e., E666K, reduces influx. Note that this SNP has been found only in the laboratory and is unlikely to be present in clinical isolates, because it disrupts type IV pilus formation, which is essential for pathogenesis.
	A SNP in *ponA* (encoding the second penicillin target, PBP1), i.e., "*ponA1* determinant" (L421P), reduces penicillin acylation of PBP1 ~2- to 4-fold.
	"Factor X," an unknown, nontransformable determinant, increases penicillin MICs ~3- to 6-fold.
	Penicillinase (TEM-1 or TEM-135)-encoding plasmids, i.e., Asian, African, Toronto, Rio, Nîmes, New Zealand, and Johannesburg plasmids, hydrolyze the cyclic amide bond of the β-lactam ring and render the penicillin inactive.
Tetracyclines (e.g., tetracycline and doxycycline)	A SNP in *rpsJ* (encoding ribosomal protein S10), i.e., V57M, reduces the affinity of tetracycline for the 30S ribosomal target.
	mtrR mutations (see above).
	penB mutations (see above).
	A SNP in *pilQ* (see above).
	TetM-encoding plasmids, i.e., American and Dutch plasmids. Evolved derivatives have been described in Uruguay and South Africa. TetM, resembling elongation factor G, binds to the 30S ribosomal subunit and blocks tetracycline target binding.
Spectinomycin	A 16S rRNA SNP, i.e., C1192U, in the spectinomycin-binding region of helix 34, reduces the affinity of the drug for the ribosomal target.
	Mutations in *rpsE* (encoding the 30S ribosomal protein S5), i.e., the T24P mutation and deletions of V25 and K26E, disrupt the binding of spectinomycin to the ribosomal target.
Quinolones (e.g., ciprofloxacin and ofloxacin)	*gyrA* SNPs, e.g., S91F, D95N, and D95G, in the QRDR, reduce quinolone binding to DNA gyrase.
	parC SNPs, e.g., D86N, S88P, and E91K, in the QRDR, reduce quinolone binding to topoisomerase IV.
	Many additional mutations in the QRDR of *gyrA* and *parC* have been described. An overexpressed NorM efflux pump also slightly enhances quinolone MICs.
Macrolides (e.g., erythromycin and azithromycin)	23S rRNA SNPs, i.e., C2611T and A2059G (in 1 to 4 alleles), result in a 23S rRNA target (peptidyltransferase loop of domain V) with a reduced affinity for the 50S ribosomal macrolide target.
	mtrR mutations (see above).
	erm genes (*ermB*, *ermC*, and *ermF*), encoding rRNA methylases that methylate nucleotides in the 23S rRNA target, block the binding of macrolides.
	MacAB efflux pump; its overexpression increases the MICs of macrolides.
	mef-encoded efflux pump exports macrolides out of the bacterial cell and increases the MICs of macrolides.
Cephalosporins (e.g., ceftibuten, cefpodoxime, cefixime, cefotaxime, and ceftriaxone)	Mosaic *penA* alleles encoding mosaic PBP2s with a decreased PBP2 acylation rate. These proteins have up to 70 amino acid alterations and are derived from horizontal transfer of partial *penA* genes from mainly commensal *Neisseria* spp. Mutations in mosaic PBP2s verified to contribute to resistance are A311V, I312M, V316T, V316P, T483S, A501P, A501V, N512Y, and G545S. The resistance mutations need other epistatic mutations in the mosaic *penA* allele.
	penA SNPs, i.e., A501V and A501T, in nonmosaic alleles can also enhance cephalosporin MICs. Some additional SNPs (G542S, P551S, and P551L) were statistically associated with enhanced cephalosporin MICs, but their effects remain to be proven with, e.g., site-directed *penA* mutants in isogenic backgrounds.
	mtrR mutations (see above).
	penB mutations (see above).
	"Factor X," an unknown, nontransformable determinant (see above).

1.7 Combination therapy

The objective of antibiotic combination therapy is to elevate activity of antibiotics against multidrug-resistant bacteria. An effective treatment of *N. gonorrhoeae* infection is antibiotic combination therapy including ceftriaxone plus azithromycin. However, no standard guideline in clinical laboratory is available for *in vitro* testing of antibiotic combination against *N. gonorrhoeae*. The synergistic effect of antibiotic combination has been performed only in research laboratory. There are few studies of antibiotic combinations against *N. gonorrhoeae* as described below.

The combination of ceftriaxone, azithromycin, and fosfomycin by checkerboard method showed no synergism of ceftriaxone plus azithromycin, ceftriaxone plus fosfomycin, and azithromycin plus fosfomycin against *N. gonorrhoeae*. These results showed that ceftriaxone plus fosfomycin had more rapid effects than ceftriaxone plus azithromycin and azithromycin plus fosfomycin. However, fosfomycin alone was bactericidal activity at 2X MIC, but there was regrowth of *N. gonorrhoeae* after >24 hours (110).

Other authors have also obtained consistent results using two Etest strips or agar dilution (111-113). The combinations of the third-generation cephalosporins plus azithromycin, doxycycline, rifampicin, gentamicin, or fosfomycin produced the fractional inhibitory concentration index (FICI) of indifference (111). The *in vitro* combination of azithromycin, cefixime, ceftriaxone, rifampicin, colistin, ertapenem, fosfomycin, gentamicin, minocycline, moxifloxacin, tigecycline, and spectinomycin showed that no synergism and antagonism for all of the 65 tested combinations against *N. gonorrhoeae* (112). The combinations of azithromycin, plus cefixime, ceftriaxone, and gentamicin showed the additive or indifference against 64 tested *N. gonorrhoeae* (113). *In vitro* activity of ceftriaxone, cefodizime, spectinomycin, or gentamicin in combination with azithromycin against 25 clinical isolates of *N. gonorrhoeae* also showed the additive and/or indifference only (114).

CHAPTER IV

MATERIALS AND METHODS

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│   Neisseria gonorrhoeae clinical isolates     │
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┌─────────────────────────────────────────────┐
│       Antimicrobial susceptibility testing     │
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┌─────────────────────────────────────────────┐
│ Isolates with reduced susceptibility or resistance to ceftriaxone │
└─────────────────────────────────────────────┘
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Detection of ceftriaxone resistance mechanisms by PCR	Identification of genetic relationship of *N. gonorrhoeae*	Synergistic activities of antibiotic combination study
Characterization of ceftriaxone resistance mechanisms by DNA sequencing	Detection and characterization of *por* and *tbpB* by PCR and DNA sequencing	Screening of synergistic activities by checkerboard method
	Molecular typing by NG-MAST	Confirmation of synergistic activities by time-kill methods

Table 6. Methodology Scheme

4.1 Bacterial strains

4.1.1 *N. gonorrhoeae* clinical isolates

One hundred and thirty-four *N. gonorrhoeae* isolates from different patients in Thailand during 2016 to 2018 were included in this study. One hundred and seventeen *N. gonorrhoeae* isolates were obtained from The Thai Red Cross Anonymous Clinic (Bangkok, Thailand), and 17 *N. gonorrhoeae* isolates were obtained from the King Chulalongkorn Memorial Hospital (Bangkok, Thailand). The specimens included urethral swab, cervical swab, blood, and conjunctival swab. For culture preservation, all isolates were grown on TM medium [GC agar base (Oxoid, United Kingdom) supplemented with 1% haemoglobin (Oxoid, USA), 1% IsoVitaleX (Oxoid, England), and vancomycin, colistin sulfate, and nystatin selective supplement (Oxoid, England)] in a 5% CO_2 incubator at 35-37ºC for 20-24 hours. The overnight cultures were transferred to cryotubes containing 1 mL of brain heart infusion broth (Oxoid, United Kingdom) with 20% glycerol and then stored at -80 °C (50).

4.1.2 Quality control strain for antimicrobial susceptibility testing and mechanism of resistance

N. gonorrhoeae ATCC 49226 was used as a susceptible control strain for MIC determination and mechanism of ceftriaxone resistance.

4.2 Bacterial identification

All 134 *N. gonorrhoeae* clinical isolates were identified by gram staining, cultivation, and biochemical test (115). Species identification was confirmed by PCR using specific primer for *carA* and *orf1* genes (59, 60).

4.2.1 Gram stain

N. gonorrhoeae is a Gram-negative, diplococcus, approximately 0.8 μm in diameter. Individual diplococci are kidney-or bean- shaped.

4.2.2 Biochemical tests

The biochemical tests used for identification of all *N. gonorrhoeae* isolates included oxidase test, catalase test, carbohydrate utilization test, and nitrate reduction test as described below (Table 7).

4.2.2.1 Oxidase test

Each *N. gonorrhoeae* isolate was applied on a filter paper soaked with N, N, N′, N′-tetramethyl-*p*-phenylenediamine. *N. gonorrhoeae* can produce cytochrome oxidase. So, the color on the filter paper was changed to violet.

4.2.2.2 Catalase test

N. gonorrhoeae colonies were mixed with 3% hydrogen peroxide (H_2O_2) solution. *N. gonorrhoeae* can produce catalase enzyme. So, the rapid elaboration of oxygen bubbles occurs.

4.2.2.3 Carbohydrate utilization test

N. gonorrhoeae colonies were inoculated into tubes containing different sugar such as glucose, maltose, sucrose, and lactose, and incubated in a 5% CO_2 incubator at 35-37°C for 20-24 hours. *N. gonorrhoeae* can utilize glucose, an acid will change the color of phenol red from reddish-orange to yellow.

4.2.2.4 Nitrate reduction test

N. gonorrhoeae colonies were inoculated into nitrate broth and incubated in a 5% CO_2 incubator at 35-37ºC for 20-24 hours. After incubation, reagent A (α-naphthylamine) and reagent B (sulfanilic acid) were added into nitrate tube. If nitrate was reduced to nitrite, color of broth will be changed from colorless to red. However, zinc powder was added into nitrate tube if it was colorless. If nitrate was not reduced to be nitrogen gas, color of broth will change to form a red compound. *N. gonorrhoeae* cannot reduce nitrate to nitrite or nitrogen gas. So, the rapid color of broth changed from colorless to red was not observed.

Table 7. Biochemical reactions of *N. gonorrhoeae* and *Neisseria* species (115)

Species \ Tests	Oxidase	Catalase	Acid production from				Reduction of	
			Glucose	Maltose	Sucrose	Lactose	NO_3	NO_2
N. gonorrhoeae	+	+	+	-	-	-	-	-
N. meningitidis	+	+	+	+	-	-	-	v
N. lactamica	+	+	+	+	-	+	-	v
N. cinerea	+	+	-	-	-	-	-	v
N. polysaccharea	+	+	+	+	-	-	-	v
N. sicca	+	+	+	+	+	-	-	+
N. subflava	+	+	+	+	±	-	-	+
N. mucosa	+	+	+	+	+	-	+	+
N. flavescens	+	+	-	-	-	-	-	+
N. elongate	+	-	-	-	-	-	+	+

Note: +, positive; -, negative; v, weakly positive, ±; some strains are positive

4.2.3 Molecular method for the confirmation of species identification

The presence of *carA* (59) and *orf1* genes (60) were detected by PCR to confirm *N. gonorrhoeae* identification. The amplified PCR products of *carA* and *orf1* genes were observed with all *N gonorrhoeae* isolates but both genes could not be amplified in the other non-gonococcal strains (59, 60).

4.2.3.1 DNA extraction

Five colonies of pure culture *N. gonorrhoeae* isolates were suspended in 200 µl of lysis buffer (1% Triton X-100, 0.5% Tween 20, 1 mM EDTA, 10 mM Tris-HCl, and pH 8) and boiled for 10 min. The boiled suspension was centrifuged at 13,000 rpm at room temperature for 10 min. After centrifugation to remove the cell debris, the supernatant was used as the template DNA and stored at -20 ℃ for the PCR experiments.

4.2.3.2 Primers

Specific primers used for the amplification of *carA* and *orf1* genes of *N. gonorrhoeae* isolates included NgCPS1, NgCPS4, ORF1-F, and ORF1-R primers. All primers are listed in Table 8, and are based on those previously described by Mayta *et al.* (59) and Chaudhry & Saluja (60). All primers were synthesized by BioDesign, Thailand.

Table 8. Specific primers for amplification of *carA* and *orf1* genes

Gene locus	Primer	Sequence (5'-3')	Product size (bp)	Reference
carA	NgCPS1	CGGCATCGTAGCGCGCACAG	412	(59)
	NgCPS4	CGGCTGCTGTCGTCGGCGGAAT		
orf1	orf1-F	CAACTATTCCCGATTGCGA	260	(60)
	orf1-R	GTTATACAGCTTCGCCTGAA		

4.2.3.3 Amplification of *carA* and *orf1* genes

The presence of *carA* genes was amplified using NgCPS1 and NgCPS4 primers as reported previously by Mayta *et al*. (59). The PCR was performed in 25 µl PCR reaction mixture containing 1X *Taq* buffer, 2.0 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Thermo Fisher Scientific), 0.25 mM deoxynucleotide triphosphates (dNTP) (Thermo Fisher Scientific, USA), 0.6 µM of each primer, and 2 µl of DNA template. Distilled water was used as negative control. The parameters of the amplifications were performed using Applied Biosystems Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA) with the following conditions: an initial denaturation step at 94°C for 2 minutes followed by 30 cycles of denaturation step at 94°C for 30 seconds, annealing step at 62°C for 45 seconds and extension step at 72°C for 45 seconds, and final extension step at 72°C for 5 minutes.

The presence of *orf1* genes was amplified using ORF1-F and ORF1-R primers as reported previously by Chaudhry & Saluja (60). The PCR was performed in 25 µl PCR reaction mixture containing 1X *Taq* buffer, 2.0 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase, 0.25 mM dNTP, 0.6 µM of each primer, and 2 µl of DNA template. The parameters of the amplifications were performed with the following conditions: 40 cycles of denaturation step at 94°C for 30 seconds, annealing step at 52°C for 30 seconds, and extension step at 72°C for 1 minute.

4.2.3.4 Analysis of PCR products

The 5 µl amplified PCR products were analyzed on 1.5% agarose gel electrophoresis in 0.5X Tris-Borate-EDTA buffer (TBE) and 0.5 µg/mL of ethidium bromide was added to the solution before pouring the gel into a casting tray. The 6X loading dry buffer (Thermo Fisher Scientific, USA) was mixed with PCR products and loaded into the gel in electrophoresis chamber containing of 0.5X TBE. Electrophoresis was run for 45 min at 100 volts/cm and the gel was visualized using Gel Documentation System (Bio-Rad, USA). The amplified PCR product sizes of *carA* and *orf1* genes were

412 bp and 260 bp, respectively. A 100 bp plus of DNA ladder (Thermo Fisher Scientific, USA) was used as a DNA size marker.

4.2.3.5 Quality control strain

N. gonorrhoeae ATCC 49226 was used as a positive control strain.

4.3 Antibiotic susceptibility testing

All 134 *N. gonorrhoeae* clinical isolates were determined for the minimum inhibitory concentrations (MICs) of 9 antimicrobial agents including penicillin G, tetracycline, ciprofloxacin, azithromycin, cefixime, ceftriaxone, ertapenem, gentamicin, and fosfomycin by agar dilution method. The antimicrobial agents used in this study were supplied by Sigma-Aldrich (St. Louis, MO, USA), with the exception of fosfomycin that was supplied by Meiji Seika pharma CO., LTD. (Tokyo, Japan). *N. gonorrhoeae* ATCC 49226 was used as a susceptible control strain.

The medium used was GC agar base (Oxoid, United Kingdom) supplemented with 1% haemoglobin (Oxoid, USA) and 1% IsoVitaleX (Oxoid, England), with the exception of fosfomycin susceptibility testing which was also supplemented with 25 mg/L of glucose-6-phosphate (G-6-P) (Sigma-Aldrich, USA). The turbidity of *N. gonorrhoeae* clinical isolates and ATCC 49226 suspension was adjusted to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) in 0.85% phosphate buffer saline (PBS) (Sigma-Aldrich, USA), pH 7.2. The adjusted suspension was then inoculated into the wells of a replicator device and transferred to the agar dilution plate. Of the 10^4 CFU per inoculation on each agar plate were the final concentration inoculums. The plates were incubated in a 5% CO_2 incubator at 35-37 ºC for 20-24 hours after which the results were read. The suspension was inoculated on agar plates without antibiotic for the growth control. The MIC is defined as the lowest concentration of antimicrobial agent at which there is no visible growth.

The susceptibilities of penicillin G, tetracycline, ciprofloxacin, cefixime, and ceftriaxone were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (12). For azithromycin susceptibility, the breakpoint criteria by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org) (116) were used because of the absence of the CLSI breakpoints. Ertapenem, fosfomycin and gentamicin susceptibilities were interpreted according to the criteria for *Enterobacteriaceae* from the CLSI because the breakpoints for *N. gonorrhoeae* are not

available (12, 116). The MIC interpretive standards for *N. gonorrhoeae* are detailed in Table 9.

Table 9. MIC interpretive standards (mg/L) for *N. gonorrhoeae*

Antimicrobial agents	MIC interpretive criteria (mg/L)			Reference
	susceptible	intermediate	resistant	
penicillin G	≤ 0.06	0.12-1	≥ 2	
tetracycline	≤ 0.25	0.5-1	≥ 2	
ciprofloxacin	≤ 0.06	0.12-0.5	≥ 1	(12)
cefixime	≤ 0.25	-	-	
ceftriaxone	≤ 0.25	-	-	
azithromycin	≤ 0.25	-	> 0.5	(116)
gentamicin*	≤ 4	8	≥ 16	
fosfomycin*	≤ 64	128	≥ 256	(12)
ertapenem*	≤ 0.5	1	≥ 2	

Note: *Ertapenem, fosfomycin, and gentamicin susceptibilities were interpreted according to criteria for *Enterobacteriaceae* from the CLSI no breakpoint is available for *N. gonorrhoeae*.

4.4 Detection and characterization of ceftriaxone resistance mechanisms among *N. gonorrhoeae* clinical isolates

4.4.1 Detection of *penA, mtrR, ponA,* and *porB* genes by PCR and automated DNA sequencing

The ceftriaxone resistance mechanisms in *N. gonorrhoeae* clinical isolates were randomly investigated for the *penA*, *mtrR*, *ponA*, and *porB* genes

4.4.1.1 DNA extraction

DNA extraction was performed by the boiling method as previously described in Section 4.2.3.1.

4.4.1.2 Primers

Specific primers used for the amplification of *penA, mtrR, ponA*, and *porB* genes of *N. gonorrhoeae* isolates included penA-A1, penA-B1, penA-A2, penA-B2, penA-A3, penA-B3, PonA1-F, PonA1-R, Mtr-F, Mtr-R, Por-F, and Por-R primers (Table 10), previously described by Ito *et al.* (38), Ilina *et al.* (31), and Liao *et al.* (3).

4.4.1.3 Amplification of the full-length *penA* gene by PCR

The nucleotide sequences of full-length *penA* gene (1,749 bp) were amplified using 3 sets of primers including penA-A1 with penA-B1, penA-A2 with penA-B2, and penA-A3 with penA-B3 primers as reported previously by Ito *et al.* (38). The PCR was performed in 100 µl PCR reaction mixture containing 1X *Taq* buffer, 2.5 mM MgCl$_2$, 1 U of *Taq* DNA polymerase, 0.8 mM dNTP, 0.6 µM of primers penA-A1 and penA-B1 or penA-A2 and penA-B2 or penA-A3 and penA-B3, and 8 µl of DNA template. The parameters of the amplifications were performed with the following conditions: an initial denaturation step at 94°C for 5 minutes followed by 35 cycles of denaturation step at 94°C for 1 minute, annealing step at 50°C (penA-A1 with penA-B1), 56°C (penA-A2 with

penA-B2), or 52°C (penA-A3 with penA-B3) for 1 minute and extension step at 72°C for 1 minute, and final extension at 72°C for 10 minutes.

Table 10. Specific primers for amplification and sequencing of ceftriaxone resistance mechanisms

Gene locus (activity)	Primer	Sequence (5'-3')	Product size (bp)	Reference
penA (PBP2)	penA-A1	CGGGCAATACCTTTATGGTGGAAC	668	(38)
	penA-B1	AACCTTCCTGACCTTTGCCGTC		
	penA-A2	AAAACGCCATTACCCGATGGG	583	(38)
	penA-B2	TAATGCCGCGCACATCCAAAG		
	penA-A3	GCCGTAACCGATATGATCGA	862	(38)
	penA-B3	CGTTGATACTCGGATTAAGACG		
ponA (PBP1)	PonA-F	GAGAAAATGGGGGAGGACCG	206	(31)
	PonA-R	GGCTGCCGCATTGCCTGAAC		
mtrR (MtrCDE efflux pump)	MtrR-F	GCCAATCAACAGGCATTCTTA	401	(31)
	MtrR-R	GTTGGAACAACGCGTCAAAC		
porB (outer membrane proteins)	PorB-F	CCGGCCTGCTTAAATTTCTTA	873	(3)
	PorB-R	TATTAGAATTTGTGGCGCAG		

4.4.1.4 Amplification of *mtrR*, *ponA*, and *porB* genes by PCR

The nucleotide sequences of the promoter and coding regions of *mtrR*, *ponA,* and *porB* genes were amplified using specific primers including primers PonA-F and PonA-R, MtrR-F and MtrR-R, PorB-F and PorB-R as reported previously by Ilina *et al.* (31), and Liao *et al.* (3). The PCR was performed in 100 µl PCR reaction mixture containing 1X *Taq* buffer, 2.5 mM MgCl$_2$ 1 U of *Taq* DNA polymerase, 0.8 mM dNTP, 0.6 µM of primers PonA-F and PonA-R, MtrR-F and MtrR-R, PorB-F and PorB-R, and 8 µl of DNA template. The parameters of the amplifications were performed with the following conditions: an initial denaturation step at 94°C for 5 minutes followed by 35 cycles of denaturation step at 94°C for 30 seconds, annealing step at 56°C (*ponA*) (31), 50°C

(*mtrR*) (31), or 52°C (*porB*) (3) for 30 seconds, extension step at 72°C for 1 minute, and final extension step at 72°C for 10 minutes.

4.4.1.5 Analysis of PCR products

Analysis of PCR products was performed by gel electrophoresis as previously described on 4.2.3.4. The amplified PCR product sizes of *penA-1*, *penA-2, penA-3, ponA, mtrR,* and *porB* genes were 668 bp, 583 bp, 862 bp, 206 bp, 401 bp, and 873 bp, respectively.

4.4.1.6 Purification of PCR products

The PCR products of *penA, mtrR*, *ponA*, and *porB* genes were purified using HiYield™Gel/PCR fragments extraction kit as described by manufacturers (RBC Bioscience, Taiwan). Five volumes of DF buffer were added into one volume of PCR product and mixed by vortexing. After that, the sample mixture was placed into DF column and centrifuged at 13,000 rpm for 30 seconds for DNA binding. DF column was washed with 600 µl of wash buffer and centrifuged at 13,000 rpm for 30 seconds for removing salt contamination. The column matrix was dried by centrifugation at 13,000 rpm for 2 minutes. The DF column was placed into a sterile microcentrifuge tube. The pure DNA was eluted with 30 µl of elution buffer into the column matrix and placed at room temperature for matrix absorption of elution buffer. The purified PCR product was eluted by centrifugation at 13,000 rpm for 2 min and stored at -20°C.

4.4.1.7 Preparation of sequencing reaction

Automated DNA sequencing was done at the 1st BASE Inc, Malaysia. DNA samples were sequenced using forward and reverse primers including *penA*, *mtrR*, *ponA* and *porB* (Table 10). Sequencing was conducted under BigDye™ terminator cycling conditions.

4.4.1.8 Sequencing analysis

The nucleotide sequences and deduced amino acid sequences of *penA*, *mtrR*, *ponA,* and *porB* genes were analyzed with the online software available at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/BLAST), and ExPASy (www.expasy.org). Multiple sequence alignment was analyzed by Multalin (http://multalin.toulouse.inra.fr/multalin). The nucleotide and deduced amino acid sequences identified in this study including *penA*, *ponA*, *mtrR*, and *porB* genes were compared with the corresponding sequences in the genome sequenced *N. gonorrhoeae* reference strain FA1090 (GenBank accession number AE004969).

4.4.2 Statistical analysis

Fisher's exact test (two-tailed) was used to verify the association between mutations in resistance determinants and penicillin resistance in *N. gonorrhoeae* isolates by using the statistical package for the social sciences version 22.0 (SPSS Inc, Chicago, USA). Statistically significant association was set at a *P* value of < 0.05 (117).

4.4.3 PBP2 sequence submission

The nucleotide sequence patterns of *penA* gene were analyzed with the online software available at *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR; https://ngstar.canada.ca/allele/email_allele). Furthermore, the novel pattern number of *penA* gene from this study was submitted to NG-STAR database.

4.4.4 Modeling of ceftriaxone bound in the active site of PBP2

The crystal structure of wild type PBP2, mosaic PBP2 pattern XXXIV and the location of the mutations around the active site for ceftriaxone were predicted by using PyMOL Software Version 2.4.

4.5 Molecular typing by *N. gonorrhoeae* multi-antigen sequence typing

The clonal of *N. gonorrhoeae* was determined by *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) using PCR and DNA sequencing. The internal fragments of 2 highly polymorphic antigen-encoding loci including outer membrane proteins (*por*) and transferrin binding protein unit B (*tbpB*) (118) were amplified and sequenced. The specific primers are shown in Table 11.

4.5.1 Detection of *por* and *tbpB* genes by PCR and automated DNA sequencing

4.5.1.1 DNA extraction

DNA extraction was performed by the boiling method as previously described in Section 4.2.3.1.

4.5.1.2 Primers

Specific primers used for the amplification of *por* and *tbpB* genes of *N. gonorrhoeae* isolates included Por-F, Por-R, TbpB-F, and TbpB-R primers (Table 11) previously described by Martin *et al.* (118).

4.5.1.3 Amplification of the *por* and *tbpB* genes by PCR

The DNA fragments of *por* and *tbpB* genes were amplified using Por-F and Por-R, TbpB-F and TbpB-R primers (118). The PCR was performed in 100 µl PCR reaction mixture containing 1X *Taq* buffer, 2.5 mM MgCl$_2$, 2.5 U of *Taq* DNA polymerase, 0.2 mmol/L dNTP, 50 pmol of primers Por-F and Por-R, TbpB-F and TbpB-R, and 8 µl of DNA template. The parameters of the amplifications were performed with the following conditions: an initial denaturation step at 95°C for 4 minutes followed by 25 cycles of denaturation step at 95°C for 30 seconds, annealing step at 58°C (*por*) (118) and 69°C (*tbpB*) for 30 seconds (118), extension step at 72°C for 1 minute, and final extension step at 72°C for 10 minutes.

Table 11. Specific primers for amplification and sequencing of two highly polymorphic antigen-encoding loci

Gene locus	Primer	Sequence (5'-3')	Product size (bp)	Reference
por	Por-F	CAAGAAGACCTCGGCAA	737	(118)
	Por-R	CCGACAACCACTTGGT		
tbpB	TbpB-F	CGTTGTCGGCAGCGCGAAAAC	535	(118)
	TbpB-R	TTCATCGGTGCGCTCGCCTTG		

4.5.1.4 Analysis of PCR products

Analysis of PCR products were performed by gel electrophoresis as previously described on 4.2.3.4. The amplified PCR product sizes of *por* and *tbpB* genes were 737 bp and 535 bp, respectively.

4.5.1.5 Purification of PCR products

Purification of PCR products was performed using HiYield™Gel/PCR Fragments Extraction Kit as previously described on 4.4.1.6.

4.5.1.6 Preparation of sequencing reaction

Preparation of sequencing reaction was performed by the 1st BASE Inc, Malaysia as previously described on 4.4.1.7. DNA samples were sequenced using forward and reverse primers including *por* and *tbpB* (Table 11).

4.5.1.7 Sequencing analysis

The sequences of each strain were aligned, edited, and trimmed to a set length of *por* and *tbpB* sequences. The *por* sequence was trimmed to 490 bp, starting from the conserved region: TTGAA. The *tbpB* sequence was trimmed to 390 bp, starting from the conserved region: CGTCTGAA. The nucleotide sequences of 2 highly polymorphic antigen-encoding loci were analyzed and uploaded onto the NG-MAST database (http://www.ng-mast.net) to obtain the allele number and the sequence type (ST).

Furthermore, the novel allelic number and ST from this study were submitted to NG-MAST database.

4.6 Determination of synergistic activities of antibiotic combinations against _N. gonorrhoeae_ isolates with reduced susceptibility to ceftriaxone

4.6.1 MIC determination by broth microdilution methods

The MICs of ceftriaxone, azithromycin, ertapenem, fosfomycin, and gentamicin against all test isolates with reduced susceptibility to ceftriaxone were evaluated using broth microdilution methods (12, 116).

4.6.1.1 Preparation of antibiotic microdilution plate

Broth microdilution methods were performed in 96-well cell culture plates (SPL, Korea) using fastidious broth (FB) supplemented with 1% IsoVitaleX, with the exception of fosfomycin susceptibility testing which was also added 25 mg/L of G-6-P (119). Microdilution plate of each antibiotic was prepared as follows (Figure 8). Firstly, 50 µl of FB were added in to all wells of microdilution plate, with the exception of wells of column 11 and 12 which added 150 µl and 200 µl of FB, respectively. A volume of 50 µl of each antibiotic stock solution (ceftriaxone, azithromycin, ertapenem, fosfomycin, and gentamicin) was added to all wells of column 1 of microdilution plate and serial two-fold dilution with 50 µl from all wells of column 1 to column 10. 100 µl of FB was adjusted to all wells of microdilution plate, with the exception of column 11 and 12 (12).

4.6.1.2 Preparation of bacterial inoculum

The fresh colonies of _N. gonorrhoeae_ isolates with reduced susceptibility to ceftriaxone which inoculated in FB in a 5% CO_2 incubator at 35 ℃ for 24 hours were diluted with fresh FB (approximately 10^6 CFU/ml). The diluted FBs were incubated in the presence of CO_2 at 37°C for 2 hours with shaking. After 2 hours of pre-incubation, 50 µl of prepared inoculum were added to all wells of antibiotic microdilution plate, with the exception of the wells of column 12. The microdilution plates were incubated in a 5% CO_2 incubator at 35 ℃ for 24 hours after which the results were read. All these

experiments were evaluated. All experiments were performed in duplicate for each strain and each antibiotic testing.
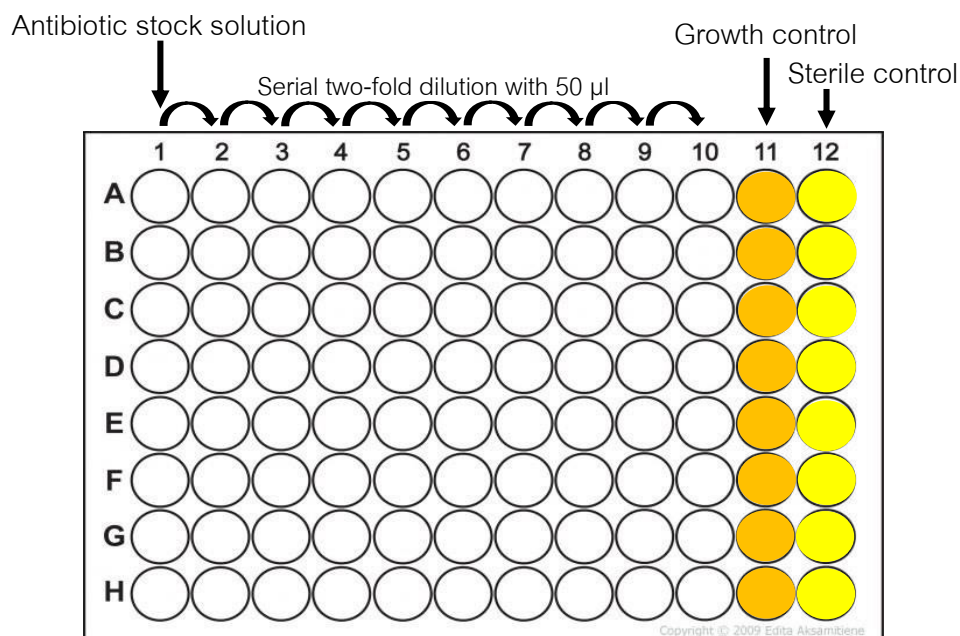
Figure 8. Preparation of antibiotic microdilution plate

4.6.2 Screening of synergistic activities of antibiotic combinations against *N. gonorrhoeae* isolates with reduced susceptibility or resistance to ceftriaxone using checkerboard methods

In this study, the synergistic activities of antibiotics combinations including ceftriaxone plus azithromycin, ceftriaxone plus fosfomycin, ceftriaxone plus gentamicin, and ceftriaxone plus ertapenem were screened against 2 strains of *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone using checkerboard method to determine the FICI (113).

4.6.2.1 Preparation of antibiotic combination by checkerboard plate

Checkerboard method were performed in 96-well cell culture plates using FB supplemented with 1% IsoVitaleX, with the exception of ceftriaxone plus fosfomycin combination which was also added 25 mg/L of G-6-P. Checkerboard plate of antibiotic

A (ceftriaxone) was prepared as follows (Figure 9). First step, 50 µl of FB were added in all wells of checkerboard plate, with the exception of column 12 and well A1. 50 µl of antibiotic A stock solution at 8X MIC concentration were added to all wells of column 1, with the exception of well A1 of checkerboard plate and serial dilution with 50 µl from column 1 to column 11 with the exception of well A1.
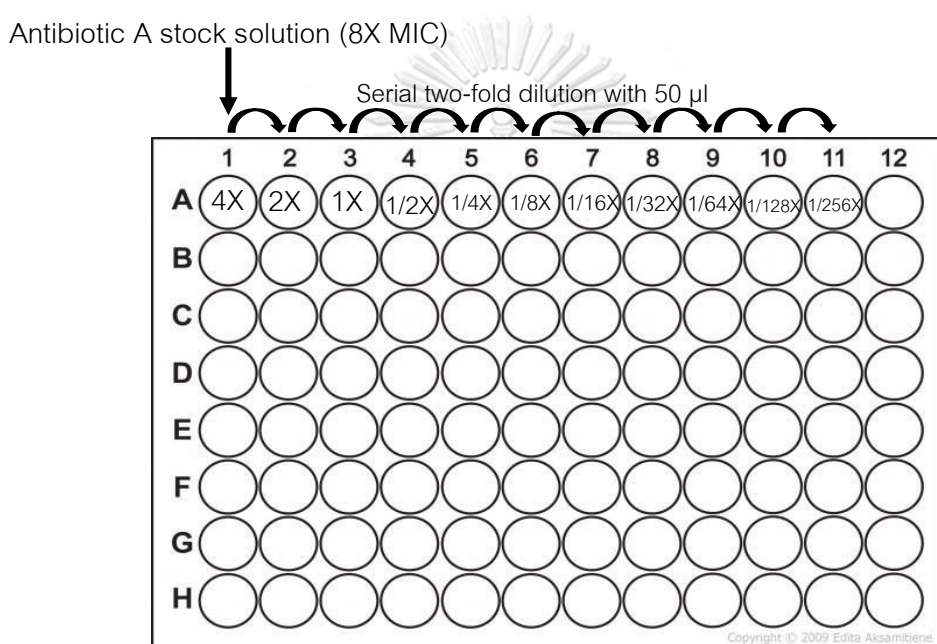
Figure 9. Preparation of checkerboard plate of antibiotic A

Checkerboard plate of antibiotic B (azithromycin, ertapenem, fosfomycin, and gentamicin) was prepared in another plate as follows (Figure 10). Firstly, 100 µl of FB were added in all wells of checkerboard plate, with the exception of wells in row H and well A1. 100 µl of antibiotic B stock solution at 8X MIC concentration were added to all wells of row A, with the exception of well A1 of checkerboard plate and serial dilution with 100 µl from all wells of row A to row G, with the exception of well A1.

Antibiotic B stock solution (8X MIC)
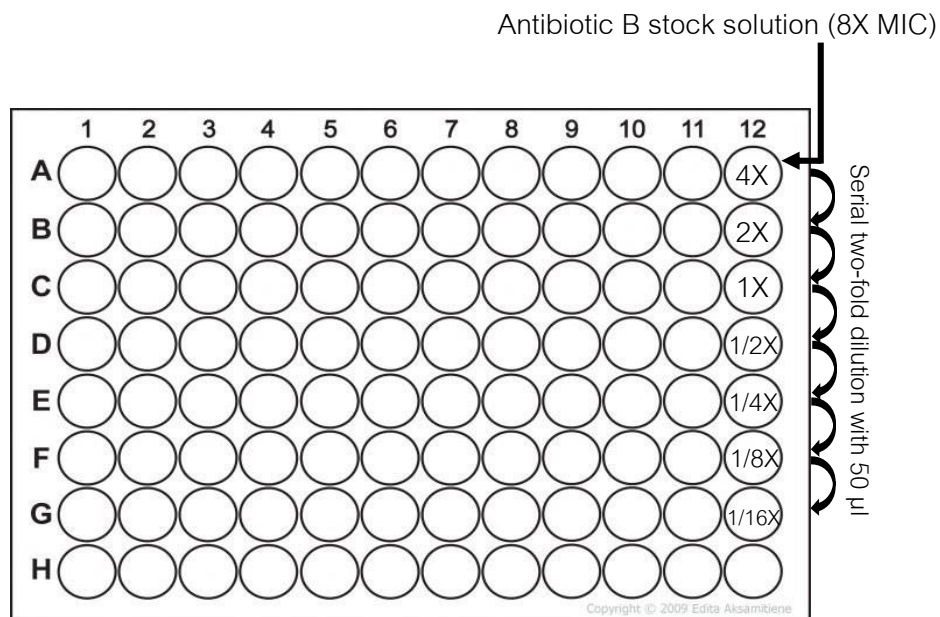
Figure 10. Preparation of checkerboard plate of antibiotic B

Next step, checkerboard plate of antibiotic A plus antibiotic B was prepared as follows (Figure 11). 50 µl of checkerboard plate of diluted antibiotic B in each well were transferred to the same well of checkerboard plate of diluted antibiotic A. After that, FB was adjusted in all well to be 150 µl.

4.6.2.2 Preparation of bacterial inoculum

The fresh colonies of a 24-hours culture of *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone were diluted with fresh FB (approximately 10^6 CFU/ml) and were incubated in the presence of CO_2 at 37°C for 2 hours with shaking. After 2 hours of pre-incubation, 50 µl of prepared inoculum were added to all wells of checkerboard plate of antibiotic A plus antibiotic B, with the exception of wells A1. The checkerboard plate of antibiotic A and B were incubated in a 5% CO_2 incubator at 35 ºC for 24 hours after which the results were read. All experiments were performed at least three times for each strain and each antibiotic combination.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sterile control	A 2X + B 4X	A 1X + B 4X	A 1/2X + B 4X	A 1/4X + B 4X	A 1/8X + B 4X	A 1/16X + B 4X	A 1/32X + B 4X	A 1/64X + B 4X	A 1/128X + B 4X	A 1/256X + B 4X	B 4X
B	A 4X + B 2X	A 2X + B 2X	A 1X + B 2X	A 1/2X + B 2X	A 1/4X + B 2X	A 1/8X + B 2X	A 1/16X + B 2X	A 1/32X + B 2X	A 1/64X + B 2X	A 1/128X + B 2X	A 1/256X + B 2X	B 2X
C	A 4X + B 1X	A 2X + B 1X	A 1X + B 1X	A 1/2X + B 1X	A 1/4X + B 1X	A 1/8X + B 1X	A 1/16X + B 1X	A 1/32X + B 1X	A 1/64X + B 1X	A 1/128X + B 1X	A 1/256X + B 1X	B 1X
D	A 4X + B 1/2X	A 2X + B 1/2X	A 1X + B 1/2X	A 1/2X + B 1/2X	A 1/4X + B 1/2X	A 1/8X + B 1/2X	A 1/16X + B 1/2X	A 1/32X + B 1/2X	A 1/64X + B 1/2X	A 1/128X + B 1/2X	A 1/256X + B 1/2X	B 1/2X
E	A 4X + B 1/4X	A 2X + B 1/4X	A 1X + B 1/4X	A 1/2X + B 1/4X	A 1/4X + B 1/4X	A 1/8X + B 1/4X	A 1/16X + B 1/4X	A 1/32X + B 1/4X	A 1/64X + B 1/4X	A 1/128X + B 1/4X	A 1/256X + B 1/4X	B 1/4X
F	A 4X + B 1/8X	A 2X + B 1/8X	A 1X + B 1/8X	A 1/2X + B 1/8X	A 1/4X + B 1/8X	A 1/8X + B 1/8X	A 1/16X + B 1/8X	A 1/32X + B 1/8X	A 1/64X + B 1/8X	A 1/128X + B 1/8X	A 1/256X + B 1/8X	B 1/8X
G	A 4X + B 1/16X	A 2X + B 1/16X	A 1X + B 1/16X	A 1/2X + B 1/16X	A 1/4X + B 1/16X	A 1/8X + B 1/16X	A 1/16X + B 1/16X	A 1/32X + B 1/16X	A 1/64X + B 1/16X	A 1/128X + B 1/16X	A 1/256X + B 1/16X	B 1/16X
H	A 4X	A 2X	A 1X	A 1/2X	A 1/4X	A 1/8X	A 1/16X	A 1/32X	A 1/64X	A 1/128X	A 1/256X	Growth control

Figure 11. Preparation of checkerboard plate of antibiotic A plus antibiotic B

4.6.2.3 Interpretation

The MIC of antibiotic alone and in combination were read to calculate for the FICI. The FICI data were interpreted using the following criteria: synergism, FICI ≤ 0.5; additive, FICI 0.5 to 1.0; indifference, FICI > 1.0 to 4.0; and antagonism, FICI ≥ 4.0 (113).

The FICI was calculated as following formula:

$$FICI = \frac{\text{MIC drug A combination}}{\text{MIC drug A alone}} + \frac{\text{MIC drug B combination}}{\text{MIC drug B alone}}$$

4.6.3 Confirmation of synergistic activities of antibiotic combinations against *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone using time-kill methods

Two strains of *N. gonorrhoeae* with reduced susceptibility to ceftriaxone that showed the best effectives by checkerboard method were confirmed using time-kill methods. In this study, the best of synergistic activities of antibiotic combinations against *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone was ceftriaxone plus azithromycin. So, *in vitro* activity of ceftriaxone combined with azithromycin were investigated against *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone.

4.6.3.1 Preparation of antibiotics

Time-kill methods were tested against no antibiotic for growth control curve, each antibiotic at concentration 0.125X, 0.25X, and 0.5X for ceftriaxone and at concentration 0.5X and 1X MIC for azithromycin, and antibiotic combination were 0.125X MIC of ceftriaxone plus 0.5X MIC of azithromycin, 0.125X MIC of ceftriaxone plus 1X MIC of azithromycin, 0.25X MIC of ceftriaxone plus 0.5X MIC of azithromycin, and 0.25X MIC of ceftriaxone plus 1X MIC of azithromycin.

4.6.3.2 Preparation of bacterial inoculum

The fresh colonies of a 24-hour culture of *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone were diluted with fresh FB (approximately 10^6 CFU/ml) and were incubated in the presence of CO_2 at 37°C for 2 hours with shaking. After 2 hours of pre-incubation, the 100 μl of prepared inoculum were transferred to test for growth control, antibiotic alone (ceftriaxone, azithromycin), and antibiotic combination (ceftriaxone plus azithromycin). The final volume of all flasks were adjusted to be 10 ml with FB. The viable bacterial cells were determined at 0, 2, 4, 6, 8, 12, and 24 h after incubation with shaking at 37 °C, 5% CO_2. The 20 μl of all tested flasks were removed and diluted in PBS from $1x10^{-1}$ to $1x10^{-7}$. Each dilution was spotted on GC agar plate. The GC agar plates with each dilution spot were incubated in a 5% CO_2 incubator at 35

°C for 24 hours after which the results were read. Colony were counted and calculated for CFU/ml. All experiments were performed at least three times (110). The mean and standard deviation (SD) values of the numbers of CFU/mL in each condition were plotted on a semi-log graph.

4.6.3.3 Interpretation

Bactericidal activity was defined as the viable cells were decreased $\geq$ 3log10 CFU/ml when compared with start cell. The synergistic activities were interpreted as follows (120): Synergism: the viable cells were decreased $\geq$ 2log10 CFU/ml when compared with the most single active antibiotic. Antagonism: the viable cells were increased $\geq$ 2log$_{10}$ CFU/ml when compared with the most single active antibiotic.
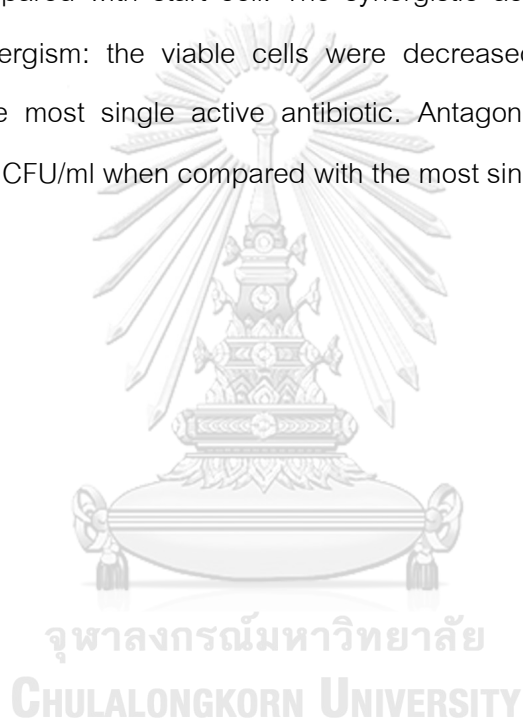
CHAPTER V

RESULTS

5.1 Bacterial strains

A total of 134 *N. gonorrhoeae* isolates were included in this study. There were 117 clinical isolates from The Thai Red Cross Anonymous Clinic and 17 clinical isolates from King Chulalongkorn Memorial Hospital, Thailand during 2016 to 2018. All 117 *N. gonorrhoeae* from Anonymous Clinic were isolated from urethral swab of male patients. Seventeen *N. gonorrhoeae* were isolated from 9 males (52.94%) and 8 females patients (47.06%) at King Chulalongkorn Memorial Hospital. The specimens included pus from cervical swab (47.06%), followed by the urethral swab (35.29%), blood (11.76%), and conjunctival swab (5.88%). The most common specimen in this study was urethral swab which accounted for 91.79% of all test isolates. The types of specimens of the 134 *N. gonorrhoeae* clinical isolates are shown in Table 12.

Table 12. Types of specimens of the 134 *N. gonorrhoeae* clinical isolates

Clinical specimens	*N. gonorrhoeae* (*n*=134)		Totals (*n*=134) (%)
	Anonymous Clinic (*n*=117) (%)	King Chulalongkorn Memorial Hospital (*n*=17) (%)	
urethral swab	117 (100%)	6 (35.29%)	123 (91.79%)
cervical swab	0 (0%)	8 (47.06%)	8 (5.97%)
blood	0 (0%)	2 (11.76%)	2 (1.49%)
conjunctival swab	0 (0%)	1 (5.88%)	1 (0.75%)

5.2 Bacterial identification

5.2.1 Gram stain and biochemical test

All 134 *N. gonorrhoeae* isolates were Gram-negative diplococci (Appendix D, Figure 18) and showed biochemical test results of *N. gonorrhoeae* including oxidase positive, catalase positive, acid production from glucose only, and nitrate reduction test negative (Appendix D, Figure 19).

5.2.2 Identification of *N. gonorrhoeae* by PCR

The presence of *carA* and *orf1* genes were used to confirm for species identification of 134 *N. gonorrhoeae* isolates by PCR. The results showed that all isolates carried both *carA* and *orf1* genes (Appendix D, Figure 20).
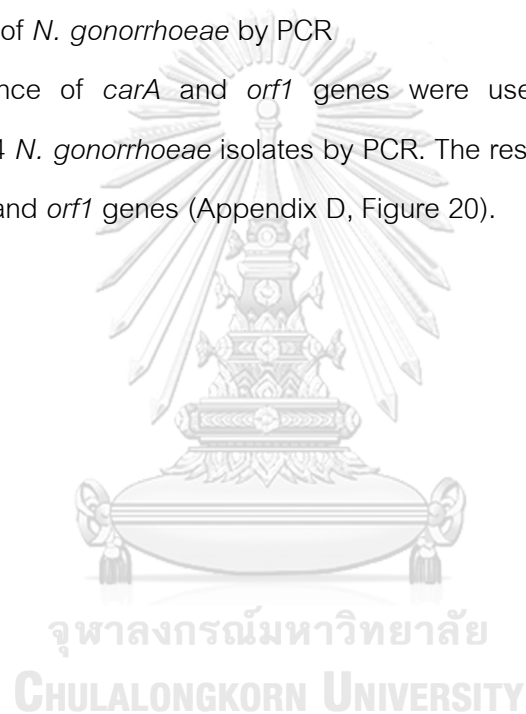
5.3 Antimicrobial susceptibility testing

The results of susceptibility profiles and antimicrobial resistance rates of *N. gonorrhoeae* isolates are summarized in Table 13. Of the 134 *N. gonorrhoeae* clinical isolates, 111 isolates (82.84%) were resistant to at least 3 antimicrobial subclass whereas only 2 isolates (1.49%) were susceptible to all antimicrobial agents tested. The most prevalent antimicrobial resistance was found in ciprofloxacin (90.30%), followed by tetracycline (82.09%), penicillin G (73.13%), gentamicin (59.70%), azithromycin (13.43%), and ertapenem (1.49%). These isolates had MICs of ciprofloxacin, tetracycline, penicillin G, gentamicin, azithromycin, and ertapenem ranging from 0.001 to 32, 0.25 to 128, 0.25 to 512, 0.5 to 64, 0.002 to 4, and 0.002 to 2 mg/L, respectively, with $MICs_{50}/MICs_{90}$ of 2/4, 16/32, 16/128, 16/32, 0.06/1, and 0.015/0.25 mg/L, respectively. All of *N. gonorrhoeae* isolates were susceptible to fosfomycin, cefixime, and ceftriaxone with MICs ranging from 4 to 64, 0.001 to 0.125, and 0.001 to 0.125 mg/L, respectively, and $MICs_{50}/MICs_{90}$ of 16/32, 0.008/0.015, and 0.002/0.015 mg/L, respectively. *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone (MIC of 0.125 mg/L) were found in 2 isolates (1.49%) including NG-083 and NG-091, which were isolated from urethral swab from patients at Anonymous Clinic. Both isolates were resistant to penicillin G, tetracycline, ciprofloxacin, and gentamicin. NG-083 was also resistant to azithromycin (MIC of 1 mg/L) but NG-091 remained susceptible to azithromycin (MIC of 0.5 mg/L). The antimicrobial susceptibility profiles of NG-083 and NG-091 strains are shown in Table 14.

Table 13. The antimicrobial susceptibility testing and antimicrobial resistance rates of the 134 *N. gonorrhoeae* clinical isolates

Antimicrobial agents	No. of resistant isolates (%)	MIC (mg/L)		
		MIC_{50}	MIC_{90}	MIC ranges
penicillin G	98 (73.13%)	16	128	0.25-512
tetracycline	110 (82.09%)	16	32	0.25-128
ciprofloxacin	121 (90.30%)	2	4	0.001-32
azithromycin	18 (13.43%)	0.06	1	0.002-4
cefixime	0 (0%)	0.008	0.015	0.001-0.125
ceftriaxone	0 (0%)	0.002	0.015	0.001-0.125
ertapenem	2 (1.49%)	0.015	0.25	0.002-2
fosfomycin	0 (0%)	16	32	4-64
gentamicin	80 (59.70%)	16	32	0.5-64

Table 14. Antimicrobial susceptibility profiles against the 2 *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone

MIC (mg/L) of antimicrobial agents	Strain	
	NG-083	NG-091
penicillin G	2	2
tetracycline	4	4
ciprofloxacin	4	4
azithromycin	1	0.5
cefixime	0.125	0.125
ceftriaxone	0.125	0.125
gentamicin	16	32
fosfomycin	16	16
ertapenem	1	1

Table 15. Patterns of antimicrobial resistance of the 134 *N. gonorrhoeae* isolates

Antimicrobial agents[a]	No. of isolates (%)
Resistant to 5 antibiotics (*n*=4, 2.99%)	
PEN^R TET^R CIP^R AZT GEN^R	4 (2.99%)
Resistant to 4 antibiotics (*n*=57, 42.54%)	
PEN^R TET^R CIP^R GEN^R	53 (39.55%)
PEN^R TET^R CIP^R AZT^R	2 (1.49%)
PEN^R TET^R CIP^R ETP^R	1 (0.75%)
TET^R CIP^R AZT^R GEN^R	1 (0.75%)
Resistant to 3 antibiotics (*n*=50, 37.31%)	
PEN^R TET^R CIP^R	28 (20.90%)
TET^R CIP^R GEN^R	8 (5.97%)
CIP^R AZT^R GEN^R	4 (2.99%)
PEN^R CIP^R GEN^R	3 (2.24%)
PEN^R TET^R GEN^R	2 (1.49%)
TET^R CIP^R AZT^R	2 (1.49%)
PEN^R CIP^R AZT^R	1 (0.75%)
TET^R AZT^R GEN^R	1 (0.75%)
TET^R AZT^R ETP^R	1 (0.75%)
Resistant to 2 antibiotics (*n*=10, 7.46%)	
TET^R CIP^R	3 (2.24%)
CIP^R GEN^R	3 (2.24%)
PEN^R TET^R	2 (1.49%)
CIP^R AZT^R	2 (1.49%)
Resistant to 1 antibiotic (*n*=11, 8.21%)	
CIP^R	6 (4.48%)
PEN^R	2 (1.49%)
TET^R	2 (1.49%)
GEN^R	1 (0.75%)
Susceptible to all antimicrobial agents tested	2 (1.49%)

[a]AZT, azithromycin; CIP, ciprofloxacin; ETP, ertapenem; GEN, gentamicin; PEN, penicillin G; TET, tetracycline

Antimicrobial resistance patterns are shown in Table 15. There were 22 resistance patterns. The most common patterns of antimicrobial resistance among the *N. gonorrhoeae* isolates in this study were PEN^R TET^R CIP^R GEN^R (39.55%) and PEN^R TET^R CIP^R (20.90%). The prevalence of multidrug-resistance isolates (defined as resistance to at least 3 antimicrobial classes) was found in 111 isolates (82.84%) whereas only 2 isolates (1.49%) were sensitive to all antimicrobial agents tested.

5.4 Characterization of ceftriaxone resistance mechanisms among *N. gonorrhoeae* clinical isolates with various ceftriaxone MICs

Mechanisms of ceftriaxone resistance in *N. gonorrhoeae* isolates with reduced susceptibility or resistance to ceftriaxone have been reported to be associated with alterations in PBP2, PBP1, PorB, MtrR, and *mtrR* promoter region.

Among the 134 *N. gonorrhoeae* clinical isolates, none of them were resistant to ceftriaxone. However, 2 isolates (NG-083 and NG-091) had reduced susceptibility to ceftriaxone (MIC of 0.125 mg/L). These isolates were also resistant to all antimicrobial previously recommended for treatment of gonorrhea such as penicillin G, tetracycline, and ciprofloxacin. Ceftriaxone resistance mechanisms and the result of antimicrobial susceptibility testing of these 2 isolates with reduced susceptibility to ceftriaxone and 29 representative strains of ceftriaxone-susceptible with various ceftriaxone MICs are summarized in Table 16.

Of 31 representative strains of *N. gonorrhoeae*, all of them had mutations in PBP2 encoded by *penA* gene. The nonmosaic PBP2 patterns II, VII, XIV, XIX, XVI, XVIII, XXXXIV, mosaic XXXIV, and new mosaic pattern (Figure 12) were found in 19 isolates (61.29%), 1 isolate (3.23%), 1 isolate (3.23%), 1 isolate (3.23%), 2 isolates (6.45%), 1 isolate (3.23%), 3 isolates (9.68%), 2 isolates (6.45%), and 1 isolate (3.23%), respectively. The most frequently detected mutation in 28 ceftriaxone-susceptible *N. gonorrhoeae* isolates (90.32%) was aspartate insertion at position 345 (D345) in the nonmosaic PBP2 patterns II, VII, XIV, XIX, XVI, XVIII, and XXXXIV. The L421P substitution in PBP1 encoded by *ponA* gene was found in 10 isolates (32.26%). The overexpression of MtrCDE efflux pump associated with an adenine deletion in the 13-bp inverted repeat sequences of the *mtrR* promoter region with amino acid alteration in MtrR repressor were also found in 9 isolates (29.03%). The single substitutions at positions A39T, H105Y, and G45D in the MtrR repressor were found in 11 isolates (35.48%), 4 isolates (12.90%), and 1 isolate (3.23%), respectively. The A39T with H105Y substitution, A39T with R44H substitution, A39T with G45D substitution, A39T with F96S substitution, and D79N, T86A with H105Y substitutions in the MtrR repressor were found in 9 isolates

(29.03%), 3 isolates (9.68%), 1 isolate (3.23%), 1 isolate (3.23%), and 1 isolate (3.23%), respectively. The loss or reduction of outer membrane porin was linked to the amino acid alteration at positions 120 and 121 in PorB porin encoded by *porB* gene. The single substitutions at positions A121G, A121S, and G120D in PorB porin were found in 4 isolates (12.90%), 1 isolate (3.23%), and 1 isolate (3.23%), respectively. The G120D with A121G substitution, G120K with A121G substitution, G120K with A121N substitution, G120K with A121D substitution in PorB porin were found in 4 isolates (12.90%), 3 isolates (9.68%), 3 isolates (9.68%), and 2 isolates (6.45%), respectively.

Moreover, mutations associated with all ceftriaxone resistance mechanisms including the alterations in PBP2, PBP1, PorB, MtrR, and *mtrR* promoter region were also found in 6 isolates (19.35%). Two of the six isolates (NG-083 and NG-091) had reduced susceptibility to ceftriaxone (MIC of 0.125 mg/L) and possessed all specific ceftriaxone resistance patterns. NG-083 and NG-091 had L421P substitution in PBP1, the mosaic PBP2 patterns XXXIV (Figure 12), an adenine deletion in the 13-bp inverted repeat sequence of the *mtrR* promoter region, a H105Y substitution in the MtrR repressor, and G120K and A121N substitutions in PorB porin (Table 16). Four isolates (NG-050, NG-079, NG-090, and NG-097) had low-level ceftriaxone MIC range of 0.008-0.06 mg/L and possessed L421P substitution in PBP1 and an adenine deletion in the 13-bp inverted repeat sequence of the *mtrR* promoter region. NG-050, NG-079, NG-090, and NG-097 had the nonmosaic PBP2 patterns II, VII, XVIII, and XXXXIV, respectively. NG-090 and NG-097 had D79N, T86A with H105Y substitutions and H105Y substitution in the repressor of MtrR and G120K with A121G substitution in PorB porin. NG-050 and NG-079 had A39T with G45D substitution and G45D substitution in the repressor of MtrR and G120K with A121D and G120D substitution in PorB porin.

Table 16. Mutations in resistance determinants and the β-lactam susceptibility among the 31 representative strains of *N. gonorrhoeae*

Isolates no.	MIC (mg/L)			Resistance determinants				
	PEN	CFM	CRO	PBP2	PBP1	*mtrR* promoter	MtrR	PorB
NG-018	512	0.008	0.008	II	WT	WT	A39T	G120D, A121G
NG-019	512	0.008	0.015	II	WT	WT	A39T	G120K, A121N
NG-085	256	0.015	0.015	II	WT	WT	A39T, H105Y	WT
GC-011	128	0.008	0.004	II	WT	WT	A39T, H105Y	WT
NG-056	128	0.015	0.015	XIX	WT	WT	A39T	WT
GC-002	128	0.015	0.008	II	WT	WT	A39T, H105Y	A121G
NG-017	128	0.004	0.004	II	WT	WT	A39T, R44H	A121S
GC-007	64	0.03	0.03	XVIII	WT	WT	A39T	WT
GC-008	64	0.008	0.004	II	WT	WT	A39T, H105Y	A121G
GC-012	64	0.004	0.004	XIV	WT	WT	A39T	G120D, A121G
GC-004	64	0.015	0.015	II	WT	WT	A39T	G120K, A121G
GC-006	64	0.015	0.03	II	WT	WT	A39T, H105Y	WT
GC-005	64	0.008	0.002	XVI	WT	WT	A39T	WT
GC-003	32	0.008	0.004	II	L421P	WT	A39T	G120D, A121G
GC-009	16	0.008	0.004	II	WT	WT	A39T, H105Y	A121G
GC-013	16	0.008	0.004	new	WT	WT	A39T, F96S	A121G
GC-001	16	0.008	0.015	XIX	L421P	WT	A39T	G120K, A121G
GC-017	8	0.004	0.004	II	WT	WT	A39T, H105Y	G120D, A121G
GC-010	8	0.015	0.03	II	WT	WT	A39T, H105Y	WT
NG-090	4	0.06	0.06	XXXXIV	L421P	A del	D79N, T86A, H105Y	G120K, A121D
NG-050	2	0.008	0.008	II	L421P	A del	A39T, G45D	G120K, A121G
NG-097	2	0.03	0.03	XVIII	L421P	A del	H105Y	G120K, A121D
NG-083	2	0.125	0.125	XXXIV	L421P	A del	H105Y	G120K, A121N
NG-091	2	0.125	0.125	XXXIV	L421P	A del	H105Y	G120K, A121N
NG-079	0.5	0.06	0.03	VII	L421P	A del	G45D	G120D
NG-007	0.5	0.015	0.015	II	WT	A del	A39T	WT
NG-025	0.5	0.004	0.004	II	WT	WT	A39T, R44H	WT
NG-051	0.25	0.001	0.001	II	WT	A del	H105Y	WT
NG-015	0.25	0.03	0.03	II	L421P	WT	A39T, R44H	WT
NG-016	0.25	0.03	0.03	XVIII	L421P	WT	A39T	WT
NG-027	0.25	0.015	0.015	II	WT	A del	A39T, H105Y	WT

Note: PEN, penicillin G; CFM, cefixime; CRO, ceftriaxone; WT, wild type; new, new mosaic pattern

Table 17. Mutations in resistance determinants and the β-lactam MIC ranges among the 31 representative strains of *N. gonorrhoeae*

No. of isolates	Isolate no.	MIC (mg/L)			PBP2 pattern	PBP1	*mtrR* promoter	MtrR	PorB porin
		PEN	CFM	CRO					
4	GC-006, GC-010, GC-011, NG-085	8-256	0.008-0.014	0.004-0.03	II	WT	WT	A39T, H105Y	WT
3	GC-002, GC-008, G-009	16-128	0.008-0.015	0.004-0.008	II	WT	WT	A39T, H105Y	A121G
2	NG-083, NG-091	2	0.125	0.125	XXXIV	L421P	A del	H105Y	G120K, A121N
1	NG-019	512	0.008	0.015	II	WT	WT	A39T	G120K, A121N
1	NG-018	512	0.008	0.008	II	WT	WT	A39T	G120D, A121G
1	NG-056	128	0.015	0.015	XIX	WT	WT	A39T	WT
1	NG-017	128	0.004	0.004	II	WT	WT	A39T, R44H	A121S
1	GC-007	64	0.03	0.03	XVIII	WT	WT	A39T	WT
1	GC-004	64	0.015	0.015	II	WT	WT	A39T	G120K, A121G
1	GC-012	64	0.004	0.004	XIV	WT	WT	A39T	G120D, A121G
1	GC-005	64	0.008	0.002	XVI	WT	WT	A39T	WT
1	GC-003	32	0.008	0.004	II	L421P	WT	A39T	G120D, A121G
1	GC-001	16	0.008	0.015	XIX	L421P	WT	A39T	G120K, A121G
1	GC-013	16	0.008	0.004	new	WT	WT	A39T, F96S	A121G
1	GC-017	8	0.004	0.004	II	WT	WT	A39T, H105Y	G120D, A121G
1	NG-090	4	0.06	0.06	XXXXIV	L421P	A del	D79N, T86A, H105Y	G120K, A121D
1	NG-097	2	0.03	0.03	XVIII	L421P	A del	H105Y	G120K, A121D
1	NG-050	2	0.008	0.008	II	L421P	A del	A39T, G45D	G120K, A121G
1	NG-079	0.5	0.06	0.03	VII	L421P	A del	G45D	G120D
1	NG-007	0.5	0.015	0.015	II	WT	A del	A39T	WT
1	NG-025	0.5	0.004	0.004	II	WT	WT	A39T, R44H	WT
1	NG-015	0.25	0.03	0.03	II	L421P	WT	A39T, R44H	WT
1	NG-016	0.25	0.03	0.03	XVIII	L421P	WT	A39T	WT
1	NG-027	0.25	0.015	0.015	II	WT	A del	A39T, H105Y	WT
1	NG-051	0.25	0.001	0.001	II	WT	A del	H105Y	WT

Note: PEN, penicillin G; CFM, cefixime; CRO, ceftriaxone; WT, wild type; new, new mosaic pattern

Resistance determinants and the β-lactam MIC ranges are shown in Table 17. There were 25 resistance determinant patterns. The most common resistance determinance patterns of *N. gonorrhoeae* isolates in this study were PBP2 pattern II and the A39T and H105Y substitutions in MtrR (12.90%).

Among mutations in resistance determinants of the 31 representative strains of *N. gonorrhoeae* (Table 18), only mutation in PorB were associated with resistance to penicillin. The overall percentage of isolates carrying mutation in PorB was significantly higher in penicillin-resistant isolates (17/24; 70.83%) than in penicillin-susceptible isolate (1/7 isolates; 14.29%) (p=0.02).

Table 18. Association between mutations in resistance determinants and resistance to penicillin among the 31 representative strains of *N. gonorrhoeae*

Resistance determinants		PEN^R isolates n (%)	PEN^S isolates n (%)	P value
PBP2	Mutations	24 (100%)	7 (100%)	1
	WT	0 (0%)	0 (0%)	
PBP1	Mutations	7 (29.17%)	3 (42.86%)	0.40
	WT	17 (70.83%)	4 (57.14%)	
mtrR promoter	Mutations	5 (20.83%)	4 (57.14%)	0.08
	WT	19 (79.17%)	3 (42.86%)	
MtrR	Mutations	24 (100%)	7(100%)	1
	WT	0 (0%)	0 (0%)	
PorB porin	Mutations	17 (70.83%)	1 (14.29%)	0.02*
	WT	7 (29.17%)	6 (85.71%)	

Note: PEN^R, resistance to penicillin (MIC of 2–512 mg/L); PEN^S, susceptible to penicillin (MIC of 0.25–0.5 mg/L); WT, wild type. *P* values were determined by chi-square tests. **P* value for differences in percentages of mutation in PorB porin and wild type isolates among PEN^R and PEN^S isolates.

The amino acid sequence alignments of different PBP2 patterns of 9 *N. gonorrhoeae* isolates including nonmosaic PBP2 pattern II, VII, XIV, XIX, XVI, XVIII, XXXXIV, mosaic XXXIV, and new mosaic pattern (GC-013) were compared with the wild type PBP2 of *N. gonorrhoeae* strain LM306 (GenBank accession no. AAA25463) (Figure 12).
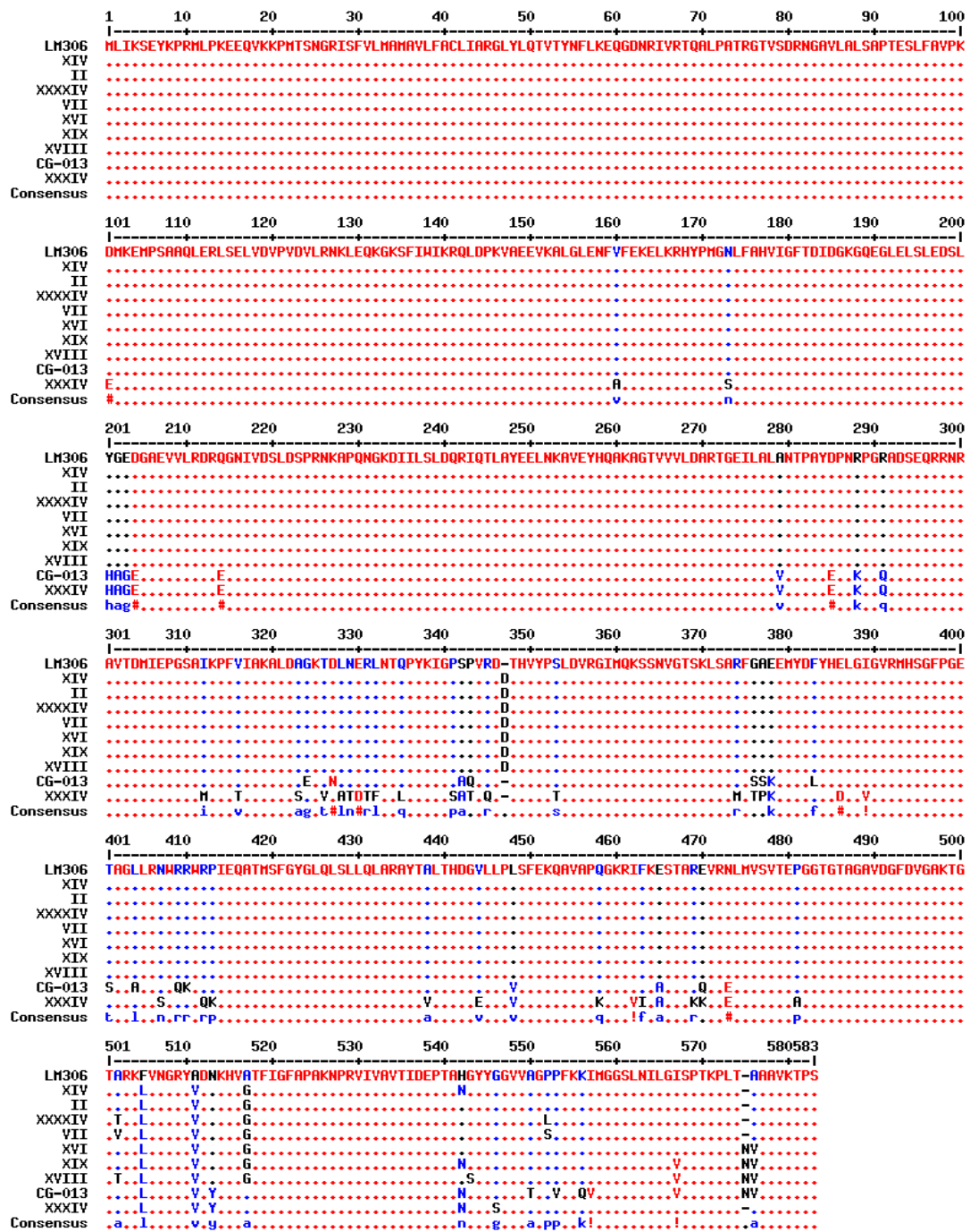
Figure 12. Alignment for different PBP2 patterns of *N. gonorrhoeae*

However, GC-013 had the new mosaic PBP2 pattern. This mosaic-like structure is similar to the PBP2 of *N. meningitidis* strain M38900 (GenBank accession no. WP_118824975.1). As shown in Figure 13, the amino acid sequences of PBP2 of *N. gonorrhoeae* strain GC-013 shared 94% similarity with those of wild type PBP2 of *N. gonorrhoeae* strain LM306 (GenBank accession no. AAA25463) but these sequences was 99% similarity to those of PBP2 pattern of *N. meningitidis* strain M38900 (GenBank accession no. WP_118824975.1) (Figure 14). A novel PBP2 pattern belonged to mosaic PBP2 pattern CXVIII.
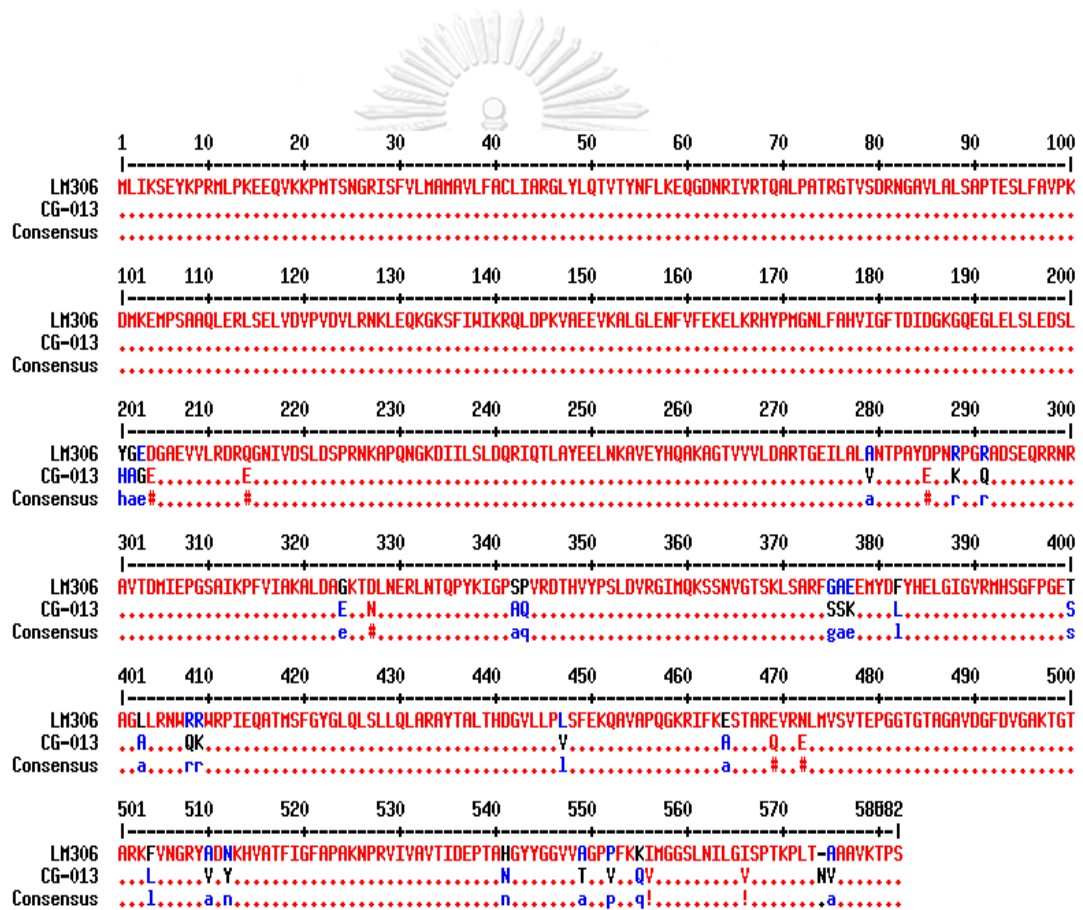
Figure 13. Alignment for amino acid sequences of PBP2 of *N. gonorrhoeae* strain CG-013 and *N. gonorrhoeae* strain LM306 (GenBank accession no. AAA25463)
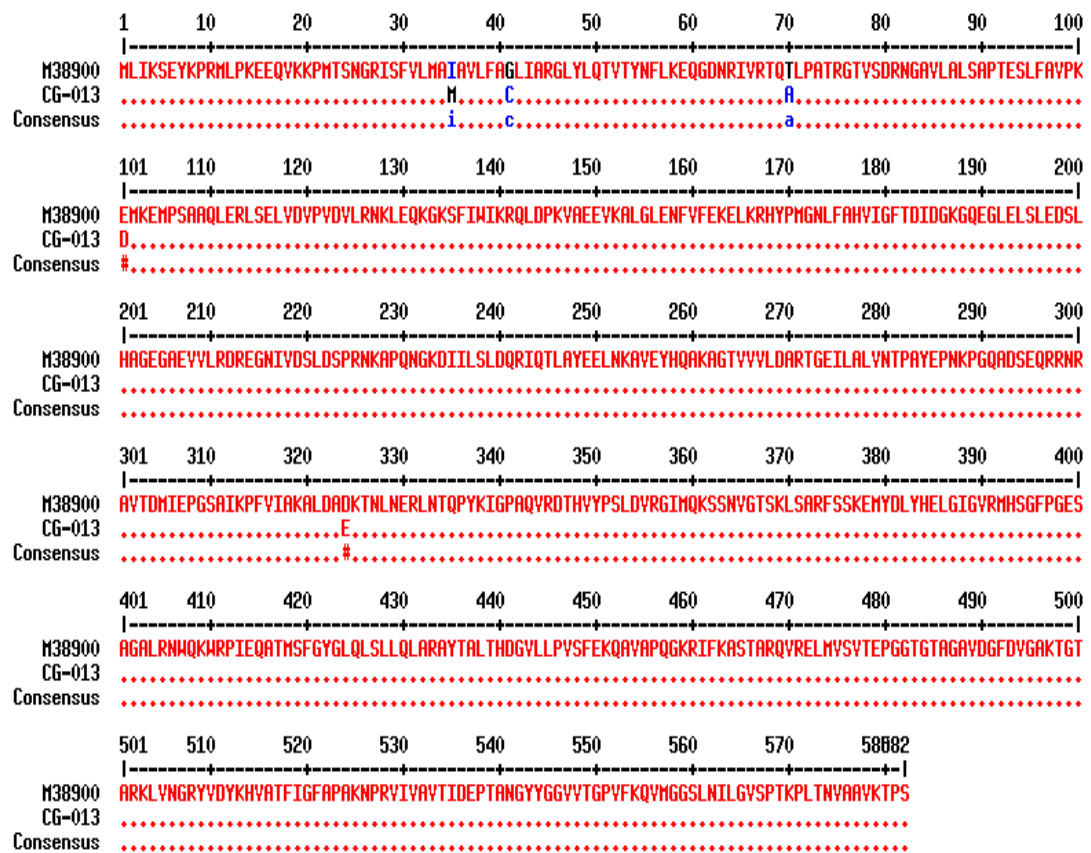
Figure 14. Alignment for amino acid sequences of PBP2 of *N. gonorrhoeae* strain CG-013 and *N. meningitidis* strain M38900 (GenBank accession no. WP_118824975.1)

The crystal structure of a soluble form of wild type PBP2 and mosaic PBP2 pattern XXXIV (amino acid residues of 1–581) from NG-083 and NG-091 with reduced susceptibility to ceftriaxone are shown in Figure 15. PBP2 contains two domains, N-terminal domain and C-terminal transpeptidase domain. A ribbon represents the structure of the C-terminal transpeptidase domain of PBP2 showing the location of the mutations around the active site for ceftriaxone by using PyMOL software version 2.4 (Figure 16). Mutations in PBP2 are represented in pink colors.

Figure 15. The crystal structure of wild type PBP2 and mosaic PBP2 pattern XXXIV from NG-083 and NG-091 strains

(A) Wild type PBP2, (B) mosaic PBP2 pattern XXXIV. The crystal structure of these PBP2 by using PyMOL software version 2.4. Mutations represented by pink color.
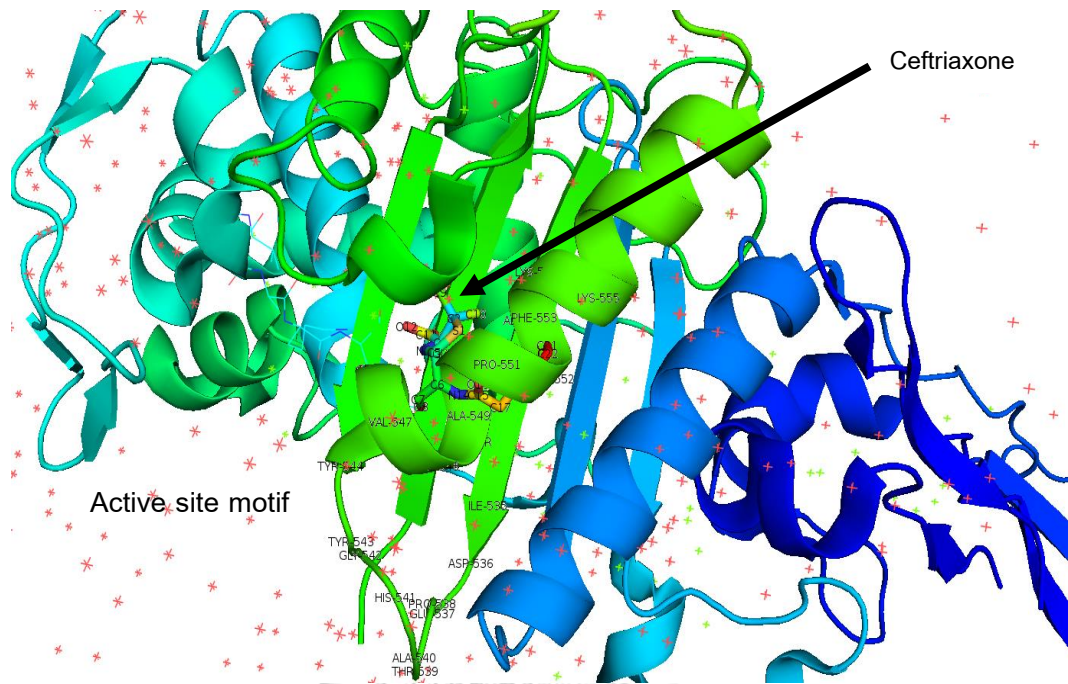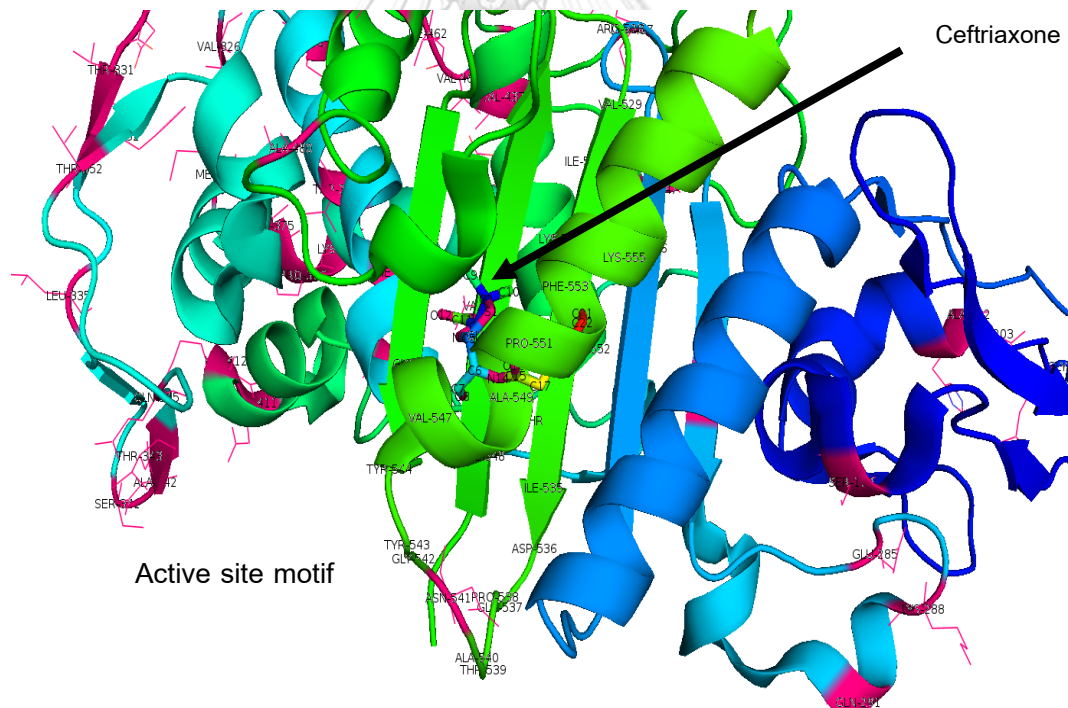
Figure 16. Modeling of β-Lactam antibiotic bound in the active site of wild type PBP2 and mosaic PBP2 pattern XXXIV

    (A) Wild type PBP2, (B) mosaic PBP2 pattern XXXIV. The crystal structure of these PBP2 by using PyMOL software version 2.4. Mutations represented by pink color.

5.5 Identification of genetic relationship among *N. gonorrhoeae* clinical isolates by NG-MAST

The 31 representative strains of *N. gonorrhoeae* with various ceftriaxone MICs were investigated for the genetic relationship by *Neisseria gonorrhoeae* multi-antigen sequence typing (NG-MAST). The allele numbers and sequence types (STs) of all *N. gonorrhoeae* tested isolates are summarized in Table 19.

The 31 representative strains of *N. gonorrhoeae* belonged to various STs. There were 14 known STs and 15 new STs. The most common STs were ST9208 and ST16101 (Table 20). The most common allele numbers of *por* and *tbpB* were *por*16 and *tbpB*18, respectively. In this study, 8 novel allele numbers of *por*, 10 novel allele numbers of *tbpB*, and 15 novel STs were found.

NG-083 and NG-091 with reduced susceptibility to ceftriaxone belonged to different STs (Table 19-21). Isolate NG-083 belonged to ST7235. However, isolate NG-091 had new allele number of *tbpB* and new ST. New allele and new ST have not been published in NG-MAST database. The results suggested that *N. gonorrhoeae* isolates in this study were heterogeneous.

Table 19. MICs, and STs of the 31 representative strains of *N. gonorrhoeae*

Isolates	MIC (mg/L)									NG-MAST		
no.	PEN	TET	CIP	AZT	CFM	CRO	FOS	GEN	ETP	*por*	*tbpB*	ST
NG-018	512	32	32	0.06	0.008	0.008	32	32	0.004	New allele	1161	New ST
NG-019	512	4	8	0.06	0.008	0.015	32	16	0.015	908	953	ST8426
NG-085	256	16	1	0.5	0.015	0.015	32	8	2	New allele	602	New ST
GC-002	128	32	1	0.06	0.015	0.008	16	32	0.015	161	33	ST16101
GC-011	128	64	1	0.06	0.008	0.004	16	16	0.015	New allele	New allele	New ST
NG-017	128	32	2	0.25	0.004	0.004	16	32	0.008	852	1412	New ST
NG-056	128	64	8	0.125	0.015	0.015	8	16	0.008	New allele	New allele	New ST
GC-004	64	8	2	0.06	0.015	0.015	32	32	0.015	6649	New allele	New ST
GC-005	64	2	1	0.03	0.008	0.002	16	16	0.008	30	2583	ST16039
GC-006	64	4	2	0.06	0.015	0.03	32	16	0.015	New allele	New allele	New ST
GC-007	64	32	1	0.06	0.03	0.03	16	16	0.03	8472	156	ST14568
GC-008	64	32	1	0.06	0.008	0.004	16	16	0.004	161	New allele	New ST
GC-012	64	32	1	0.015	0.004	0.004	32	32	0.004	90	98	ST2485
GC-003	32	32	2	0.06	0.008	0.004	8	8	0.008	90	137	ST1691
GC-001	16	2	1	0.06	0.008	0.015	64	64	0.004	543	137	ST1612
GC-009	16	4	1	0.06	0.008	0.004	16	16	0.004	161	New allele	New ST
GC-013	16	4	1	0.06	0.008	0.004	16	8	0.008	1840	1127	ST5334
GC-010	8	1	0.5	0.03	0.015	0.03	16	8	0.015	New allele	New allele	New ST
GC-017	8	2	0.5	0.004	0.004	0.004	8	8	0.004	New allele	New allele	New ST
NG-090	4	4	8	0.5	0.06	0.06	32	16	0.125	4440	563	ST7437
NG-050	2	128	8	0.125	0.008	0.008	16	32	0.03	997	75	ST3047
NG-083	2	4	4	1	0.125	0.125	16	16	1	908	1180	ST7235
NG-091	2	4	4	0.5	0.125	0.125	32	16	1	1914	New allele	New ST
NG-097	2	8	4	1	0.03	0.03	16	32	0.25	242	566	New ST
NG-007	0.5	2	2	0.5	0.015	0.015	16	16	0.008	New allele	18	New ST
NG-025	0.5	1	4	0.25	0.004	0.004	32	16	0.008	1808	893	ST4244
NG-079	0.5	4	8	1	0.06	0.03	4	8	0.125	1132	New allele	New ST
NG-015	0.25	0.5	2	0.25	0.03	0.03	16	4	0.008	161	33	ST16101
NG-016	0.25	0.5	2	0.125	0.03	0.03	8	4	0.008	952	156	ST6284
NG-027	0.25	0.5	8	0.5	0.015	0.015	8	8	0.008	1808	18	ST9208
NG-051	0.25	2	4	0.5	0.001	0.001	16	16	0.015	1808	18	ST9208

Note: PEN, penicillin G; TET, tetracycline; CIP, ciprofloxacin; AZT, azithromycin; CFX, cefixime; CRO, ceftriaxone; ETP, ertapenem; GEN, gentamicin; FOS, fosfomycin; NG-MAST, *N. gonorrhoeae* multi-antigen sequence typing; ST, sequence type

Table 20. STs by NG-MAST of the 31 representative strains of *N. gonorrhoeae*

NG-MAST	No. of isolates	Isolates no.
ST9208	2	NG-027, NG-051
ST16101	2	GC-002,NG-015
ST8426	1	NG-019
ST7437	1	NG-090
ST7235	1	NG-083
ST6284	1	NG-016
ST5334	1	GC-013
ST4244	1	NG-025
ST3047	1	NG-050
ST2485	1	GC-012
ST1691	1	GC-003
ST1612	1	GC-001
ST16039	1	GC-005
ST14568	1	GC-007
New ST	1	GC-004
New ST	1	GC-006
New ST	1	GC-008
New ST	1	GC-009
New ST	1	GC-010
New ST	1	GC-011
New ST	1	GC-017
New ST	1	NG-007
New ST	1	NG-017
New ST	1	NG-018
New ST	1	NG-056
New ST	1	NG-079
New ST	1	NG-085
New ST	1	NG-091
New ST	1	NG-097

Note: ST, sequence type

Table 21. MICs, mutations in resistance determinants, and STs of the 31 representative strains of *N. gonorrhoeae*

Isolates no.	MIC (mg/L)									Resistance determinants					NG-MAST
	PEN	CFM	CRO	ETP	TET	CIP	AZT	FOS	GEN	PBP2	PBP1	*mtrR*	MtrR	PorB	
NG-018	512	0.008	0.008	0.004	32	32	0.06	32	32	II	WT	WT	A39T	G120D, A121G	New ST
NG-019	512	0.008	0.015	0.015	4	8	0.06	32	16	II	WT	WT	A39T	G120K, A121N	ST8426
NG-085	256	0.015	0.015	2	16	1	0.5	32	8	II	WT	WT	A39T, H105Y	WT	New ST
GC-002	128	0.015	0.008	0.015	32	1	0.06	16	32	II	WT	WT	A39T, H105Y	A121G	ST16101
GC-011	128	0.008	0.004	0.015	64	1	0.06	16	16	II	WT	WT	A39T, H105Y	WT	New ST
NG-017	128	0.004	0.004	0.008	32	2	0.25	16	32	II	WT	WT	A39T, R44H	A121S	New ST
NG-056	128	0.015	0.015	0.008	64	8	0.125	8	16	XIX	WT	WT	A39T	WT	New ST
GC-004	64	0.015	0.015	0.015	8	2	0.06	32	32	II	WT	WT	A39T	G120K, A121G	New ST
GC-005	64	0.008	0.002	0.008	2	1	0.03	16	16	XVI	WT	WT	A39T	WT	ST16039
GC-006	64	0.015	0.03	0.015	4	2	0.06	32	16	II	WT	WT	A39T, H105Y	WT	New ST
GC-007	64	0.03	0.03	0.03	32	1	0.06	16	16	XVIII	WT	WT	A39T	WT	ST14568
GC-008	64	0.008	0.004	0.004	32	1	0.06	16	16	II	WT	WT	A39T, H105Y	A121G	New ST
GC-012	64	0.004	0.004	0.004	32	1	0.015	32	32	XIV	WT	WT	A39T	G120D, A121G	ST2485
GC-003	32	0.008	0.004	0.008	32	2	0.06	8	8	II	L421P	WT	A39T	G120D, A121G	ST1691
GC-001	16	0.008	0.015	0.004	2	1	0.06	64	64	XIX	L421P	WT	A39T	G120K, A121G	ST1612
GC-009	16	0.008	0.004	0.004	4	1	0.06	16	16	II	WT	WT	A39T, H105Y	A121G	New ST
GC-013	16	0.008	0.004	0.008	4	1	0.06	16	8	new	WT	WT	A39T, F96S	A121G	ST5334
GC-010	8	0.015	0.03	0.015	1	0.5	0.03	16	8	II	WT	WT	A39T, H105Y	WT	New ST
GC-017	8	0.004	0.004	0.004	2	0.5	0.004	8	8	II	WT	WT	A39T, H105Y	G120D, A121G	New ST
NG-090	4	0.06	0.06	0.125	4	8	0.5	32	16	XXXXIV	L421P	A del	D79N, T86A, H105Y	G120K, A121D	ST7437
NG-050	2	0.008	0.008	0.03	128	8	0.125	16	32	II	L421P	A del	A39T, G45D	G120K, A121G	ST3047
NG-083	2	0.125	0.125	1	4	4	1	16	16	XXXIV	L421P	A del	H105Y	G120K, A121N	ST7235
NG-091	2	0.125	0.125	1	4	4	0.5	32	16	XXXIV	L421P	A del	H105Y	G120K, A121N	New ST
NG-097	2	0.03	0.03	0.25	8	4	1	16	32	XVIII	L421P	A del	H105Y	G120K, A121D	New ST
NG-007	0.5	0.015	0.015	0.008	2	2	0.5	16	16	II	WT	A del	A39T	WT	New ST
NG-025	0.5	0.004	0.004	0.008	1	4	0.25	32	16	II	WT	WT	A39T, R44H	WT	ST4244
NG-079	0.5	0.06	0.03	0.125	4	8	1	4	8	VII	L421P	A del	G45D	G120D	New ST
NG-015	0.25	0.03	0.03	0.008	0.5	2	0.25	16	4	II	L421P	WT	A39T, R44H	WT	ST16101
NG-016	0.25	0.03	0.03	0.008	0.5	2	0.125	8	4	XVIII	L421P	WT	A39T	WT	ST6284
NG-027	0.25	0.015	0.015	0.008	0.5	8	0.5	8	8	II	WT	A del	A39T, H105Y	WT	ST9208
NG-051	0.25	0.001	0.001	0.015	2	4	0.5	16	16	II	WT	A del	H105Y	WT	ST9208

Note: AZT, azithromycin; CIP, ciprofloxacin; CFX, cefixime; CRO, ceftriaxone; ETP, ertapenem; GEN, gentamicin; FOS, fosfomycin; PEN, penicillin G; TET, tetracycline; NG-MAST, PBP2; penicillin binding protein 2; PBP1; penicillin binding protein 1; *mtrR*, *mtrR* promoter; MtrR, MtrR repressor; PorB, PorB porin; NG-MAST, *N. gonorrhoeae* multi-antigen sequence typing; ST, sequence type; WT, wild type; A del, adenine deletion; new, new mosaic pattern.

There were 29 different STs among 31 *N. gonorrhoeae* isolates. Fifteen of these 29 STs (51.72%) were found to be novel, indicating high genetic variability in the bacterial strains studied. ST9208 and ST16101 were the most prevalent ST (n =2; 6.90%), followed by other 27 different STs with 1 isolate each (3.45%). As shown in Table 22, 25 different STs were related to MDR: 2 STs were resistant to 5 antimicrobials (PEN^R TET^R CIP^R AZT^R GEN^R), 18 STs were resistant to 4 antimicrobials (17 STs were PEN^R TET^R CIP^R GEN^R and 1 ST was PEN^R TET^R CIP^R ETP^R), and 5 STs were associated with resistance to 3 antimicrobials (2 STs were PEN^R TET^R CIP^R, 2 STs were TET^R CIP^R GEN^R, and 1 ST was TET^R CIP^R AZT^R). Because of the high diversity of STs, there was no correlation between STs and resistance phenotypes.

Table 22. Resistance patterns and STs of the 31 representative strains of *N. gonorrhoeae*

Resistance pattern	No. of ST	ST
PEN TET CIP AZT GEN	2	ST7235, New ST
PEN TET CIP GEN	17	ST1612, ST2485, ST3047, ST7437, ST8426, ST16039, ST16101, ST14568, 9 New ST
PEN TET CIP ETP	1	New ST
PEN TET CIP	2	ST1691, ST5334
TET CIP GEN	2	ST9208, New ST
TET CIP AZT	1	New ST
CIP GEN	1	ST4244
PEN TET	1	New ST
CIP	3	ST6284, ST9208, ST16101
PEN	1	New ST

Note: PEN, penicillin G; TET, tetracycline; CIP, ciprofloxacin; AZT, azithromycin; ETP, ertapenem; GEN, gentamicin; ST, sequence type

5.6 Screening of synergistic activities of antibiotic combinations against *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone using checkerboard methods

       In vitro activities of antibiotic combinations against NG-083 and NG-091 which had reduced susceptibility to ceftriaxone were screened by using checkerboard method to evaluate whether the combination of ceftriaxone plus azithromycin, ceftriaxone plus fosfomycin, ceftriaxone plus gentamicin, or ceftriaxone plus ertapenem had synergistic effects (FICI of < 0.5) against both isolates. The results of antibiotic combinations are shown in Table 23.

       Synergistic and antagonistic effects were not found in all antibiotic combinations against both isolates. Additive was found in ceftriaxone plus azithromycin, ceftriaxone plus fosfomycin, and ceftriaxone plus gentamicin. Indifference was found in ceftriaxone plus ertapenem (Appendix D, Table 25-28).

       Although synergistic effects was not found in all antibiotic combination against both isolates, the FICI of ceftriaxone plus azithromycin showed the most effective combination against both isolates. The combination of ceftriaxone plus azithromycin was further confirmed by time-kill methods.

Table 23. Summary of antibiotic combinations against NG-083 and NG-091

Isolate no.	PBP2 pattern	PBP1	*mtrR* promoter	MtrR	PorB porin	NG-MAST	FICI			
							CRO:AZT	CRO:FOS	CRO:GEN	CRO:ETP
NG-083	XXXIV	L421P	A del	H105Y	G120K, A121N	ST7235	0.75 (A)	1.00 (A)	1.00 (A)	1.50 (I)
NG-091	XXXIV	L421P	A del	H105Y	G120K, A121N	New ST	0.74 (A)	1.00 (A)	1.00 (A)	1.25 (I)

Note: AZT, azithromycin; CRO, ceftriaxone; ETP, ertapenem; FOS, fosfomycin; GEN, gentamicin; A, additive; I, indifference

5.7 Confirmation of synergistic activities of antibiotic combinations against *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone using time-kill methods

Ceftriaxone plus azithromycin showed the best result, being additive, by checkerboard methods against 2 *N. gonorrhoeae* isolates with reduced susceptibility to ceftriaxone. The time-kill method was tested to confirm the best activity.

The concentrations of ceftriaxone at 0.125X, 0.25X, and 0.5X MIC and azithromycin at 0.5X and 1X MIC were used alone and in combination against 2 *N. gonorrhoeae* isolates, NG-083 and NG-091.

As shown in Figure 17, at 0.125X, 0.25X, and 0.5X MIC of ceftriaxone alone showed bactericidal effect against both isolates which showed ≥3log10 decrease when compared to growth control curve. The 0.5X MIC of ceftriaxone rapidly inhibited bacterial growth within 8 to 12 hours in both isolates, but the concentration at 0.125X and 0.25X MIC was unable to inhibit bacterial growth within 24 hours. At 0.5X and 1X MIC of azithromycin alone was unable to inhibit bacterial growth within 24 hours. Time-kill results of ceftriaxone plus azithromycin combination against NG-083 and NG-091 are shown in Figure 17. The results showed that at 0.125X MIC of ceftriaxone plus 0.5X MIC of azithromycin, 0.125X MIC of ceftriaxone plus 1X MIC of azithromycin, 0.25X MIC of ceftriaxone plus 0.5X MIC of azithromycin, and 0.25X MIC of ceftriaxone plus 1X MIC of azithromycin showed effective killing against NG-083 and NG-091. All combinations had no synergistic activity but had bactericidal activity.

In conclusion, the results of *in vitro* activities of antibiotic combination using checkerboard method were correlated with the results of time-kill method. Therefore, the combination of ceftriaxone plus azithromycin was effective, no synergistic activity, and had bactericidal activity against NG-083 and NG-091 with reduced susceptibility to ceftriaxone.
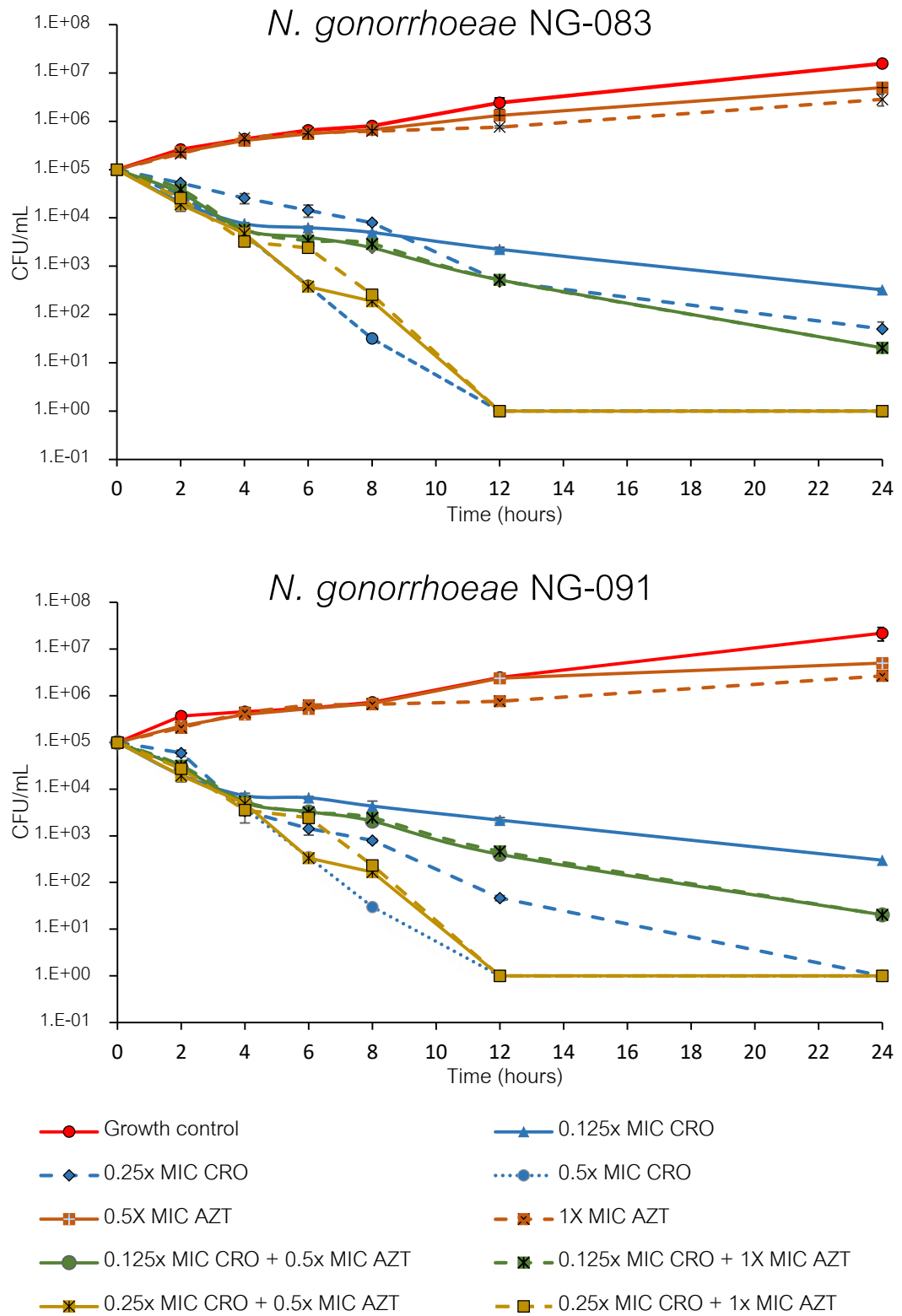
Figure 17. Time-kill curve of ceftriaxone plus azithromycin against *N. gonorrhoeae*

CHAPTER VI

DISCUSSION

       N. gonorrhoeae is the major pathogen of the sexually transmitted diseases. It is the causative agent of gonorrhea. There were 555,608 cases of gonorrhea reported in the United States with the rate of 171.9 cases per 100,000 population in 2017. The rate of reported gonorrhea cases increased 18.6% in 2016-2017, and increased 75.2% since the historic low in 2009 (121). The emergence and spread of multidrug-resistant *N. gonorrhoeae* has been a major public health concern worldwide (27, 53, 122). At present, ceftriaxone–resistant *N. gonorrhoeae* is emerging in some countries including France, Spain, China, and Japan (19-21, 66). In this study, we investigated 134 *N. gonorrhoeae* clinical isolates from Thai patients during 2016 to 2018. Pus from the urethral swab was the most common clinical isolates, accounted for 91.79% of all isolates. Identification of *N. gonorrhoeae* by biochemical tests correlate well with PCR results using specific primers for *carA* (59) and *orf1* genes (60). Therefore, amplifications of these genes can be used for rapid detection of *N. gonorrhoeae*.

       Our results showed high resistance rates of *N. gonorrhoeae* to penicillin G (73.13%), tetracycline (82.09%), and ciprofloxacin (90.30%) which were similar to those reported from Siriraj Hospital, Thailand (27), Switzerland (67), Laos (69), and Canada (45). The data from the National Antimicrobial Resistance Surveillance Center, Thailand (NARST) showed the resistance rates of 83.5% for tetracycline and 92.2% for ciprofloxacin during 2016 (26). Our results showed that the antibiotics which were previously used to treat gonorrhea had high resistance rates. However, all *N. gonorrhoeae* isolates were still susceptible to cefixime and ceftriaxone in this study. Consistently, the data reported from Bangrak Hospital in 2013 (123), the NARST in 2016 (26), and Siriraj Hospital in 2017 (27) showed that 100% of *N. gonorrhoeae* isolates were susceptible to cefixime and ceftriaxone. The surveillance of antimicrobial susceptibility to *N. gonorrhoeae* in Japan identified 5 isolates with ceftriaxone MICs of 0.5 mg/L

among 2126 isolates tested during 2000 to 2014 (40). The ceftriaxone-resistant *N. gonorrhoeae* isolates were also reported in France (20), Spain (21), Canada (22), Denmark (23), Argentina (24), China (66), and Australia (74). In this study, the prevalence of multidrug-resistant *N. gonorrhoeae* was found in 82.84%, which was a little bit lower than those reported from Laos (84.8%) (69), Siriraj Hospital (89.7%) (27), but higher than the report from Switzerland (70.6%) (67). Interestingly, we reported that 1.46% of *N. gonorrhoeae* had reduced susceptibility to ceftriaxone (MIC of 0.125 mg/L). The reduced susceptibility to ceftriaxone isolates were reported in Australia (5.4%) (124), Korea (13.94%) (44), Japan (6.85%) (125). Sweden (22.22%) (32), and Canada (4.73%) (45). Reduced susceptibility to extended-spectrum cephalosporins (cefixime MIC of 0.25 mg/L and ceftriaxone MIC of 0.125-0.25 mg/L) was also reported from Singapore (1.3%), Australia (4.8%), China (55.8%), Korea (29.3%), Japan (20.3%), Hong Kong (23.3%), India (10.8%) (126), and Canada (9.4%) (45).

Our results showed resistance rate of *N. gonorrhoeae* to azithromycin (13.43%) which were similar to those reported from Japan (102), USA (101), and Germany (127), but much lower than those reported from China (28.6%) (71). Interestingly, *N. gonorrhoeae* isolates with high-level resistance to azithromycin (azithromycin MIC of $\geq$ 256 mg/L) were reported in China (71), Germany (127), Sweden (128), United Kingdom, Italy, Argentina, and the United States (36). These reports indicate that azithromycin resistance in *N. gonorrhoeae* has been increasing worldwide. The high MIC of azithromycin may be the possible cause for the recommended combination therapy with ceftriaxone plus azithromycin.

Ceftriaxone resistance mechanisms in *N. gonorrhoeae* are due to modification of PBP2 target, an increased efflux from MtrCDE efflux pump, loss or reduced influx of PorB porin, and modification of PBP1 target (29-33). Specific alterations in PBP2 encoded by *penA* gene has been reported to be the main mechanism of ceftriaxone resistance (19-21, 36). Our data showed that 31 representative strains with difference ceftriaxone MIC had nonmosaic PBP2 pattern II (61.29%), VII (3.23%), XIV (3.23%), XIX (6.45%), XVI (3.23%), XVIII (9.68%), XXXXIV (3.23%), mosaic XXXIV (6.45%), and new

mosaic pattern (3.23%). The nonmosaic PBP2 pattern II, VII, XIV, XIX, XVI, XVIII, and XXXXIV showed intermediate resistance and resistance to penicillin but were susceptible to cefixime and ceftriaxone (MICs of cefixime and ceftriaxone ranging from 0.001 to 0.06 and 0.001 to 0.06 mg/L, respectively) (Table 16). The seven nonmosaic PBP2 patterns including pattern II, VII, XIV, XIX, XVI, XVIII, and XXXXIV containing an insertion of an aspartate at position 345 (D345) in the transpeptidase domain of PBP2 and point mutations at 4-8 positions in the carboxyl terminal region have been reported to cause reduced binding affinity of β-lactam antibiotics to PBP2 and decreased susceptibility or resistance to penicillin (35). However, nonmosaic PBP2 pattern II, XIV, XIX, and XVI were reported to have little effect against ceftriaxone MIC (MIC of $\leq$ 0.008 mg/L). Nonmosaic PBP2 pattern VII, XVIII, and XXXXIV had effect against ceftriaxone MIC (MIC range of 0.06 to 0.12 mg/L) (33). In this study, 5 of the 31 representative strains containing a substitution at position 501 (nonmosaic patterns VII, XVIII with A501V substitution and nonmosaic pattern XXXXIV with A501T substitution) were less susceptible to ceftriaxone, with MICs of 0.06, 0.03, and 0.03 mg/L, respectively. Whiley *et al*. showed that the nonmosaic PBP2 pattern VII, XVIII, and XXXXIV had ceftriaxone MIC ranging from 0.06 to 0.125, ≤0.008 to 0.06, and 0.06 to 0.12 mg/L, respectively (33, 38). The substitution of A501T in PBP2 was reported to be associated with decreased ceftriaxone susceptibility (36, 40). A previous transformation results by Takahata *et al*. (129) indicated that the amino acid substitution at position 501 increased ceftriaxone MICs in a ceftriaxone-susceptible strain. Furthermore, an amino acid substitution at position 501 was also found in some ceftriaxone-less susceptible strain (32, 33, 38, 40). Therefore, this suggests that the substitution at position A501 of PBP2 may have significant contribution to ceftriaxone susceptibility more than the mosaic PBP2 (32, 33, 38, 129).

In this study, the isolates containing mosaic PBP2 pattern XXXIV showed resistance to penicillin but reduced susceptibility to ceftriaxone. This is consistent with previous studies which showed that *N. gonorrhoeae* isolates containing mosaic PBP2 pattern XXXIV from USA (130), Austria (74), Japan (40), France (20), Spain (21), and

Canada (45) had reduced susceptibility to ceftriaxone. A new mosaic PBP2 pattern was different from the wild type PBP2 of *N. gonorrhoeae* strain LM306 (GenBank accession no. AAA25463) and did not correspond to the report from PBP2 sequences of *N. gonorrhoeae*. Most regions in the transpeptidase domain in this new PBP2 were 99% similarity to those in the PBP2 sequences *N. meningitidis* (GenBank accession no. WP_118824975.1). This mosaic-like structure was considered to emerge by recombination or direct horizontal transfer with entire *penA* genes from *N. meningitidis* (37). A novel PBP2 pattern belonged to mosaic PBP2 pattern CXVIII.

NG-083 and NG-091 strains with penicillin and ceftriaxone MICs of 2 and 0.125 mg/L, respectively, were isolated from urethral swab from Anonymous Clinic in 2017. The reduced susceptibility to ceftriaxone of both strains were confirmed by agar dilution method for three different experiments. Interestingly, these isolates had mosaic PBP2 pattern XXXIV which was similar to previously reported strains with resistance to penicillin (MIC of $\geq$ 2 mg/L) and reduced susceptibility or resistance to ceftriaxone (20, 33, 40, 45, 74). The Austrian strain with a ceftriaxone MIC of 0.5 mg/L had mosaic PBP2 pattern XXXIV with T535A (74). GU110332 strain from Japan with a ceftriaxone MIC of 0.5 mg/L had mosaic PBP2 pattern XXXIV with P551S (40). F89 strain from France and Spain with a ceftriaxone MIC of 1-2 mg/L had mosaic PBP2 pattern XXXIV with A501P (20, 21). The Canadian strain with a ceftriaxone MIC of 0.063-0.125 mg/L had mosaic PBP2 pattern XXXIV (45). Overall, these data suggests that the mosaic PBP2 pattern XXXIV containing an additional mutation at A501P or T535A or P551S conferred decreased ceftriaxone susceptibility more than the mosaic PBP2 pattern XXXIV.

Furthermore, previous studies reported that amino acid substitutions in PBP2 at A311V, I312M, V316T/P, T483S, A501P/T/V, N512Y, and G545S were also associated with increased ESC MIC (39, 44, 83, 129). G542S and P551S/L were also associated with enhanced ceftriaxone MIC in *N. gonorrhoeae* (33). These I312M, V316T, N512Y, and G545S mutations were detected in the mosaic PBP2 pattern XXXIV, suggesting that these 4 mutations in the mosaic PBP2 pattern XXXIV might be important for the reduced susceptibility to ceftriaxone. A previous transformation results by Tomberg *et al.*

demonstrated the reversion of I312M, V316T, and G545S substitutions in PBP2 of *N. gonorrhoeae* isolate with reduced susceptibility or resistance to ceftriaxone to those in wild-type PBP2, dramatically decreased the MIC of ceftriaxone (16-fold) (131). All three amino acid substitutions are located near the β-lactam active site. The I312 and V316 are located opposite S310 and K313 in the SxxK active site motif on the α-helix. They pack into a hydrophobic pocket. These interactions might be disrupted and the position of the SxxK motif active site altered by mutation, leading to decreased β-lactam acylation of PBP2 (131). The main chain amides of G545 and G546 can interact with the side chain of T498 and T500, respectively, within the KTG(T) active site motif. Mutation of G545S could interfere the interactions with the two threonine on β3 with the main chain of helix α11 (131).

A β-lactams, penicillin G was docked into the active site by superimposing the active site motifs of mutated PBP2 structure from *N. gonorrhoeae* clincial isolates with those of a crystal structure of PBP5 (a D-alanine carboxypeptidase) from *E. coli* in complex with penicillin G (33). The 10 residues corresponding to the three conserved motifs in the active sites of these two PBPs overlapped with an root mean square deviation of 6.79 Å, further emphasizing the high degree of similarity of the active sites within PBPs (84).

The peptidoglycan cross-linking activity of PBPs is a construction and repair mechanism that act on a terminal D-alanyl-D-alanine moiety on a pentapeptide unit and catalyze the formation of a peptide bond between the penultimate D-alanine and a glycine unit on an adjacent peptidoglycan strand, releasing the terminal D-alanine unit in the process. However, the structure of β-lactam ring in ceftriaxone mimics the D-alanyl-D-alanine moiety, and the PBP attacks the β-lactam ring in ceftriaxone as if it was its normal D-alanyl-D-alanine substrate (11, 76, 84). The location of mutations within PBP2 in clincial isolates with reduced ceftriaxone suceptibility was due to mutation in transpeptidase domain of PBP2 followed by mutation among three of the active-site motif; I312M, V316P, and G545S substitution, and the 37 mutations in the C-terminal region of the protein. Therefore, in structural terms, the C-terminal mutations must alter

the architecture at or around the active site in such a way as to lower the reactivity of the enzyme for β-lactam antibiotics without significantly affecting the ability of the enzyme to catalyze transpeptidation of its natural peptide substrate. Also, the C-terminal mutations lead to the decrease in penicillin acylation rate contibuting to the reduced ceftriaxone suceptibility.

N512 is located relatively distant from the active site. The N512Y has been reported to be associated with decreased susceptibility to ESCs without affecting penicillin susceptibility. The reversion of N512Y substitution in PBP2 of *N. gonorrhoeae* isolate with reduced susceptibility to ceftriaxone to the wild-type PBP2 decreased the MIC ceftriaxone (2-fold) (39, 131).

Our study showed that the prevalence of L421P mutation in PBP1 associated with intermediate and resistance to penicillin (32.26%) was lower than those reported from Canada (68.18%) (45), Switzerland (69.23%) (67), Japan (86.30%) (125), Republic of Korea (91.67%) (44), and Sweden (94.44%) (32). PBP1 encoded by *ponA* gene is the secondary penicillin target of β-lactam antibiotics. The substitution at L421P in PBP1 reduced penicillin acylation of PBP1 and was reported to confer high level penicillin resistance in *N. gonorrhoeae* (30, 39), but did not affect MICs of ESCs including cefixime and ceftriaxone (118). These results suggested that mutations in PBP1 does not play an important role in ceftriaxone resistance mechanism.

MtrR represses the expression of the MtrCDE operon that encodes an MtrCDE efflux pump. Both mutations in the MtrR repressor and *mtrR* promoter were observed in 9 isolates (29.03%). The most common single and double substitution in MtrR repressor were A39T (35.48%) and A39T with H105Y (29.03%). Interestingly, the A39T and G45D substitutions in the MtrR repressor were reported to confer increased resistance to hydrophobic antimicrobials. These A39T and G45D substitutions inhibit the binding of MtrR to *mtrCDE* promoter, resulting in the overexpression of the MtrCDE efflux pump (43). Nine isolates showed an adenine deletion in the 13-bp inverted-repeat sequence in the *mtrR* promoter, resulting in increased expression of high-level resistance to tetracycline. The amino acid alteration at position 120 and 121 in PorB porin were

observed in 18 isolates (58.06%). The most common single and double substitution in PorB porin were A121G (12.90%) and G120D with A121G (12.90%). Mutations in these two positions were reported to be associated with decreased β-lactam entry and increased resistance to β-lactam and tetracycline (45, 108), especially in isolates containing mutations in PorB together with MtrR which conferred overexpression of the MtrCDE efflux pump (109). Our results showed that both mutations in *mtrR* and *porB* were identified in 6 isolates (19.35%) which were associated with intermediate or resistance to penicillin and azithromycin, resistance to tetracycline, and enhanced MICs of cefixime and ceftriaxone. One of the important azithromycin resistance mechanism was mutation in *mtrR* gene by an adenine deletion in the *mtrR* promoter region and/or amino acid substitution in MtrR repressor which is consistent with ceftriaxone resistance mechanism (23, 92, 103). An adenine deletion with G45D or H105Y mutation in the *mtrR* gene was identified in azithromycin-resistant isolates in this study. Shigemura *et al.*, showed that mutation by A deletion was significantly found in isolates with azithromycin MIC of ≥ 0.5 mg/L (102).

The 31 representative strains of *N. gonorrhoeae* with various ceftriaxone MIC were investigated by NG-MAST. There were 29 different STs, 15 STs of which (51.72%) were found to be novel, indicating high genetic variability in the bacterial strains studied. ST9208 and ST16101 were the most prevalent STs (*n*=2; 6.90%). Seventeen STs had the most prevalent resistance pattern (PEN[R], TET[R], CIP[R], and GEN[R]). Because of the high diversity of STs, the correlation between STs, and resistance patterns was not specific evidence (45). NG-083 and NG-091 with reduced susceptibility to ceftriaxone belonged to different STs. NG-083 belonged to ST7235 but NG-091 belonged to the new ST. There were heterogeneity of *N. gonorrhoeae* strains with reduced susceptibility to ceftriaxone has been observed in a recent study (33, 37, 44, 45, 125). ST7235 has not been reported to be correlated with reduced susceptibility to ceftriaxone. Therefore, we have few data to compare with previous studies. High-level ceftriaxone resistance was reported to be associated with ST4220 (19), ST1407 (20, 21), ST1901, ST9751, ST7363 (40), ST3435 (5), ST15925 (80), ST1614 (22, 23, 80), and ST4015 (78). These STs were

not found in this study. The limitation of this study was that there were only two isolates with reduced susceptibility to ceftriaxone and no ceftriaxone-resistant isolates.

In vitro activity of the combination in ceftriaxone plus azithromycin, ceftriaxone plus fosfomycin, ceftriaxone plus gentamicin, and ceftriaxone plus ertapenem was evaluated against 2 strains with reduced susceptibility to ceftriaxone including NG-083 and NG-091. Synergistic effects was not found in all antibiotic combinations against both isolates. Consistently, previous studies showed no synergism of ceftriaxone plus other antibiotics e.g., azithromycin, fosfomycin cefixime, colistin, ertapenem, gentamicin, minocycline, moxifloxacin, rifampicin, spectinomycin, or tigecycline against all tested *N. gonorrhoeae* isolates (110-114). The most effective combination was ceftriaxone plus azithromycin which were bactericidal activity for both isolates. This combination showed additive against both isolates (FICI range of 0.74 to 0.75). The results were similar to the study by Furuya *et al.*, which also showed that the combination of ceftriaxone plus azithromycin was additive (mean FICIs of 0.81) (114). Hauser *et al.*, Barbee *et al.*, Wind *et al.*, and Pereira *et al.*, showed that the combination of ceftriaxone plus azithromycin was indifferent with FICI ranges of 1.3 to 2.4 (110), 1.5 to 2.0 (111), 0.6 to 2.0 (112), and 1.7 to 2.3 (113), respectively. However, there are only a few studies on antibiotic combination against *N. gonorrhoeae* by checkerboard and time-kill methods (110, 114). This may be because ceftriaxone resistance rates of *N. gonorrhoeae* isolates was still low.

The study of mutations in resistance determinants of *N. gonorrhoeae* with reduced susceptibility to ceftriaxone is crucial for the development of diagnostic tools for rapid detection of ceftriaxone-nonsusceptible *N. gonorrhoeae*, leading to effective treatment of gonorrhea.

CHAPTER VII

CONCLUSION

The present study demonstrated the high rate of antimicrobial resistance among 134 *N. gonorrhoeae* isolates during 2016 to 2018. *N. gonorrhoeae* isolates showed high resistance rates of antimicrobial agents including ciprofloxacin (90.30%), followed by tetracycline (82.09%), and penicillin G (73.13%), and azithromycin (13.43%). The high prevalence of multidrug-resistant *N. gonorrhoeae* isolates was found in 111 isolates (82.84%). Resistance to cefixime and ceftriaxone which are currently used to treat gonorrhea was not detected in this study. Reduced susceptibility to ceftriaxone (MIC of 0.125 mg/L) was found in 2 isolates (1.49%) including NG-083 and NG-091 isolates.

Ceftriaxone resistance mechanisms have been reported to be related to the mutations in PBP2, PBP1, *mtrR* promoter, MtrR repressor, and PorB porin which resulted in reduced susceptibility or resistance to ceftriaxone. Mutations in PBP2 were found in all 31 tested isolates, including 29 susceptible and 2 reduced susceptible isolates. The most prevalent PBP2 type was the pattern II (19 isolates, 61.29%). The most frequently detected mutation was D345 insertion in the nonmosaic PBP2 patterns II, VII, XIV, XIX, XVI, XVIII, and XXXXIV which conferred intermediate resistance or resistance to penicillin in *N. gonorrhoeae* isolates. However, NG-083 and NG-091 with reduced susceptibility to ceftriaxone had PBP2 pattern XXXIV. The new mosaic PBP2 pattern which showed 99% amino acids similarity those of PBP2 of *N. meningitidis* (NCBI Reference Sequence: WP_118824975.1). A novel PBP2 pattern belonged to mosaic PBP2 pattern CXVIII. The L421P alteration in PBP1 was found in 10 isolates (32.26%). Mutation in PBP1 conferred decreased penicillin MIC (2- to 4-fold) (46). The overexpression of MtrCDE efflux pump associated with an adenine deletion in the 13-bp inverted repeat sequence of the promoter region and amino acid alterations in MtrR repressor were also found in 9 isolates (29.03%) which were associated with intermediate or resistance to penicillin, tetracycline, and azithromycin and enhanced

MICs of cefixime and ceftriaxone. The most common amino acid alterations in MtrR repressor were A39T (35.48%) and the A39T with H105Y (29.03%). Mutation in MtrR was associated with elevated MICs of penicillin, tetracycline, azithromycin, cefixime, and ceftriaxone. The common amino acid alterations in PorB porin were positions A121G (12.90%) and G120D with A121G (12.90%). Mutation in PorB was associated with resistance to penicillin and tetracycline and decreased cefixime and ceftriaxone susceptibility.

This study reported the first two *N. gonorrhoeae* strains with reduced susceptibility to ceftriaxone in Thailand. These two isolates also had mosaic PBP2 pattern XXXIV, an L421P substitution in PBP1, an adenine deletion in the 13-bp inverted repeat sequence of promoter region, a H105Y substitution in the MtrR repressor, and G120K and A121N substitutions in PorB porin. These mosaic PBP2s pattern XXXIV, was commonly associated with decrease ceftriaxone susceptibility (20, 33, 40, 45, 74). However, NG-083 and NG-091 belonged to different STs. NG-083 belonged to ST7235 but NG-091 belonged to new ST in NG-MAST database. Therefore, two strains were heterogeneous populations.

The result from NG-MAST showed the heterogeneous populations among 31 representative strains of *N. gonorrhoeae* in Thailand. The 8 novel allele numbers of *por*, 10 novel allele numbers of *tbpB*, and 15 novel STs were identified among all tested isolates. The most common STs were ST9208 and ST16101.

NG-083 and NG-091 strains with reduced susceptibility to ceftriaxone were tested in ceftriaxone plus azithromycin, fosfomycin, ertapenem, or gentamicin and no synergy was found for any of them. The result from checkerboard and time-kill methods showed that the best combination was ceftriaxone plus azithromycin (FICI ranges of 0.74 to 0.75) which showed bactericidal effects against both isolates.

In conclusion, although ceftriaxone-resistant *N. gonorrhoeae* was not detected in this study, the emergence and spread of multidrug-resistant *N. gonorrhoeae* strains with reduced susceptibility to ceftriaxone are a major public health concern in Thailand.

Therefore, continuous antimicrobial resistance surveillance is important to limit the emergence and spread of antimicrobial-resistant *N. gonorrhoeae* strains.

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APPENDIX

APPENDIX A

REAGENTS AND INSTRUMENT

Reagents

Absolute ethanol	(Merck, Germany)
Agarose	(Biorad, USA)
Azithromycin	(Sigma, USA)
Boric acid	(USB, Japan)
Cefixime	(Sigma, USA)
Ceftriaxone	(Sigma, USA)
Ciprofloxacin	(Sigma, USA)
Columbia broth base	(BBL, USA)
DNA gel loading dye (6x)	(Thermo Fisher Scientific, USA)
DNTPs	(Thermo Fisher Scientific, USA)
EDTA	(Pacific Science, Thailand)
Ertapenem	(Sigma, USA)
Ethidium bromide	(Sigma, USA)
Fosfomycin	(Meiji Seika pharma, Japan)
GC agar base	(Oxoid, United Kingdom)
Gene ruler 100 bp plus DNA ladder	(Thermo Fisher Scientific, USA)
Gentamicin	(Sigma, USA)
Glucose-6-phosphate	(Sigma, USA)
Haemoglobin powder	(Oxoid, USA)
Hematin	(Sigma, USA)
Hiyield Gel/PCR DNA mini kit	(RBCBioscience, Taiwan)
Hydrochloric acid	(Merck, Germany)
IsovitaleX	(Oxoid, England)
NAD	(Sigma, USA)
Neopeptone	(Sigma, USA)

Penicillin G	(Sigma, USA)
Phosphate buffer saline	(Sigma, USA)
Pyridoxal	(Sigma, USA)
Skimmed milk broth	(BD, France)
Sodium hydroxide	(Merck, Germany)
Taq DNA polymerase	(Thermo Fisher Scientific, USA)
Tetracycline	(Sigma, USA)
Tris	(Pacific Science, Thailand)
Tris-HCl	(Pacific Science, Thailand)
Triton X-100	(Pacific Science, Thailand)
Tween 20	(Biorad, USA)
Tween 80	(Biorad, USA)
VCN selective supplement	(Oxoid, England)
Yeast extract	(Sigma, USA)

Instruments

Applied Biosystems Veriti 96-Well Thermal Cycler	(Thermo Fisher Scientific, USA)
Autoclave	(Hirayama, Japan)
Automatic pipette	(Gilson, France)
Camera Gel Doc™ MZL	(BIO-RAD, USA)
CO_2 Incubator	(Thermo Fisher Scientific,, USA)
Forma Orbital shaker incubator	(Thermo Fisher Scientific, USA)
Hot air oven	(Memmert, Germany)
Microcentrifuge	(Eppendrof, Germany)

APPENDIX B

MEDIA AND ANTIBIOTIC SOLUTION PREPARATION

1. Thayer-Martin agar

Suspend 36 grams of GC agar base in 500 mL of distilled water. Suspend 10 grams of soluble haemoglobin powder in 500 ml of distilled water and shake with glass beads to dissolve the haemoglobin powder. Sterilize by autoclaving at 121°C for 15 minutes. Once the medium was prepared, 1% IsoVitaleX and 2% VCN selective supplement, were added and stored at 4°C.

2. Chocolate agar (BBL, USA)

Suspend 36 grams of GC agar base in 500 mL of distilled water. Suspend 10 grams of soluble haemoglobin powder in 500 ml of distilled water and shake with glass beads to dissolve the haemoglobin powder. Sterilize by autoclaving at 121°C for 15 minutes. Once the medium was prepared, 1% IsoVitaleX was added and stored at 4°C.

3. Fastidious broth

Suspend 35 grams of columbia broth base, 5 grams of glucose, 5 grams of yeast extract, 2 grams of neopeptone, and 0.75 grams of agarose in 960 mL of distilled water. Sterilize by autoclaving at 121°C for 15 minutes. Once the medium is prepared, 1% IsoVitaleX, 30 mL of 0.05% hematin were added in 0.1 M NaOH, 5 mL of Tween 80 (10% [v/v]), 6 mL of pyridoxal (0.1% [w/v]), and 1.5 mL of NAD (1% [w/v]) with sterilization, were added and stored at 4°C.

APPENDIX C

REAGENT PREPARATION

1. 0.5 M EDTA (pH 8.0)

Disodium ethylene diamine tetra-acelate $2H_2O$ 186.1 g/L. Distilled water 1 L. Adjust pH to 8.0 and volume to 1 liter. The reagent was stored at room temperature.

2. 10X Tris-Borate buffer (TBE)

Tris base 108 g/L, Boric acid 55 g/L, and 0.5 M EDTA (pH 8.0) 40 ml. Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at $121^{o}C$ for 15 min.

3. 10 mM Tris-HCl (pH 8)

6 g of Tris base was suspended and completely dissolved in 60 mL distilled water. Adjust the pH by using HCl and the volume to be 100 mL. The solution was mixed and sterilized by autoclaving at $121^{o}C$ for 15 min.

4. Lysis buffer (pH 8)

1% Triton X-100, 0.5% Tween 20, 1 mM EDTA, and 10mM Tris-HCl. Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at $121^{o}C$ for 15 min.

5. Phosphate buffer saline (pH 7.4)

1 pouch of phosphate buffered saline (PBS) powder was dissolved in distilled water. Adjust the volume to 1000 mL. The PBS was sterilized by autoclaving at $121\ ^{o}C$ for 15 minutes and stored at room temperature.

6. 1.5% agarose gel

1.5 g of agarose was suspended and dissolved by heating in 100 mL of 0.5X TBE buffer.

7. Antibiotic solution preparation

7.1 Azithromycin, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 50 μL of 95% ethanol and 4.95 mL sterile distilled water.

7.2 Cefixime, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 50 μL of 0.1 mol/L phosphate buffer, pH 7.0, and 4.95 ml sterile distilled water.

7.3 Ceftriaxone, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 5 mL sterile distilled water.

7.4 Ciprofloxacin, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 5 ml sterile distilled water.

7.5 Ertapenem, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 50 μL of 0.01 mol/L phosphate buffer, pH 7.2 and 4.95 mL sterile distilled water.

7.6 Fosfomycin, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 5 mL sterile distilled water.

7.7 Gentamicin, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 5 mL sterile distilled water.

7.8 Penicillin G, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 5 mL sterile distilled water.

7.9 Tetracycline, stock concentration 5120 mg/L. Prepare a stock solution; dissolve 0.0256 g in 5 mL sterile distilled water.

APPENDIX D

THE RESULTS OF ALL TESTS IN THIS STUDY

1. Gram stain
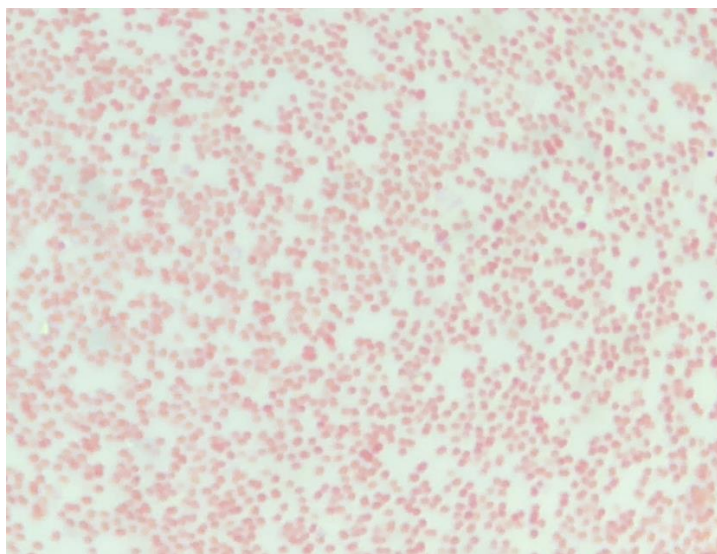
Figure 18. Gram stain of *N. gonorrhoeae* isolates

2. Biochemical tests

(A)   (B)   (C)
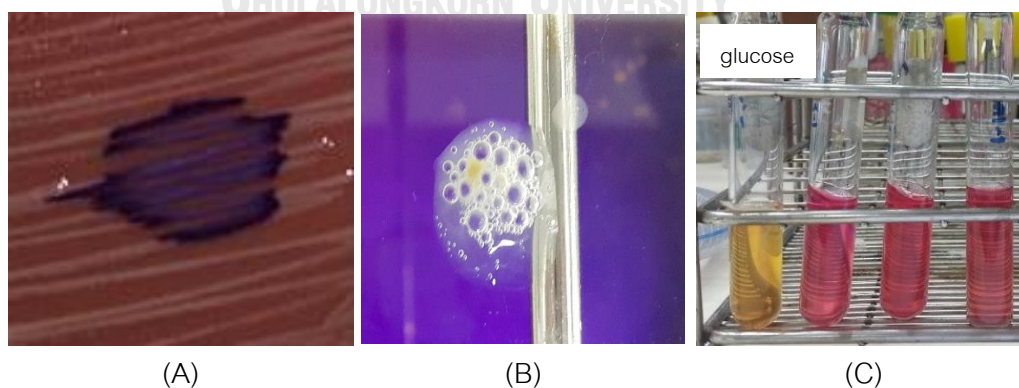
Figure 19. Biochemical tests

(A), oxidase- positive; (B), catalase-positive; (C), glucose-positive.

3. PCR screening for the presence of *carA* and *orf1* genes

(A)   (B)
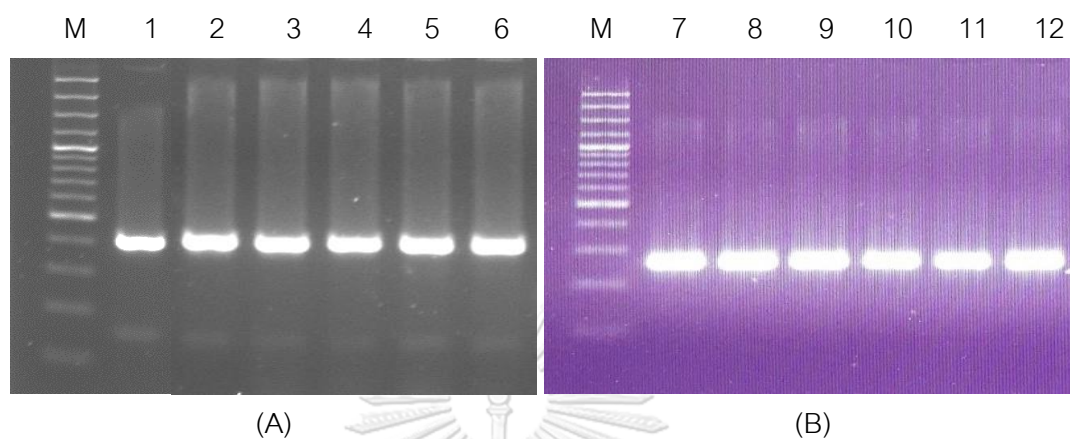
Figure 20. PCR screening for the presence of *carA* and *orf1* genes

(A), PCR screening for the presence of *carA* gene: M, 100 bp plus DNA ladder; Lanes 1, Templates, *carA*-like (412 bp) of *N. gonorrhoeae* ATCC 49226; Lanes 2-6, *N. gonorrhoeae* isolates harboring the *carA*-like gene.

(b), PCR screening for the presence of *orf1* gene: M, 100-bp plus DNA ladder; Lanes 7, Templates, *orf1*-like (280 bp) of *N. gonorrhoeae* ATCC 49226; Lanes 8-12, *N. gonorrhoeae* isolates harboring the *orf1*-like gene.

4. MICs of 9 antimicrobial agents against the 134 *N. gonorrhoeae* isolates

Table 24. MICs of 9 antimicrobial agents against the 134 *N. gonorrhoeae* isolates

No.	Isolates no.	MIC (mg/L)								
		PEN	TET	CIP	AZT	CFM	CRO	FOS	GEN	ETP
1	GC-001	16	2	1	0.06	0.008	0.015	64	64	0.004
2	GC-002	128	32	1	0.06	0.015	0.008	16	32	0.015
3	GC-003	32	32	2	0.06	0.008	0.004	8	8	0.008
4	GC-004	64	8	2	0.06	0.015	0.015	32	32	0.015
5	GC-005	64	2	1	0.03	0.008	0.002	16	16	0.008
6	GC-006	64	4	2	0.06	0.015	0.03	32	16	0.015
7	GC-007	64	32	1	0.06	0.03	0.03	16	16	0.03
8	GC-008	64	32	1	0.06	0.008	0.004	16	16	0.004
9	GC-009	16	4	1	0.06	0.008	0.004	16	16	0.004
10	GC-010	8	1	0.5	0.03	0.015	0.03	16	8	0.015
11	GC-011	128	64	1	0.06	0.008	0.004	16	16	0.015
12	GC-012	64	32	1	0.015	0.004	0.004	32	32	0.004
13	GC-013	16	4	1	0.06	0.008	0.004	16	8	0.008
14	GC-014	4	2	1	0.015	0.004	0.004	8	8	0.004
15	GC-015	2	2	1	0.004	0.002	0.002	4	4	0.002
16	GC-016	2	2	1	0.004	0.004	0.002	4	4	0.002
17	GC-017	8	2	0.5	0.004	0.004	0.004	8	8	0.004
18	NG-001	256	32	2	0.004	0.002	0.002	32	32	0.008
19	NG-002	256	32	2	0.004	0.001	0.001	16	16	0.004
20	NG-003	64	32	4	0.008	0.002	0.002	16	32	0.008
21	NG-004	64	16	4	0.004	0.001	0.001	16	16	0.004
22	NG-005	0.25	16	4	0.015	0.008	0.004	32	16	0.008
23	NG-006	0.5	32	1	0.03	0.004	0.002	8	16	0.008
24	NG-007	0.5	2	2	0.5	0.015	0.015	16	16	0.008
25	NG-008	128	0.5	1	0.008	0.002	0.002	16	16	0.004
26	NG-009	128	32	0.5	0.008	0.001	0.001	16	16	0.015
27	NG-010	0.25	16	0.5	0.008	0.001	0.001	4	4	0.004
28	NG-011	0.5	16	0.5	0.004	0.001	0.001	16	8	0.008

Table 24. MICs of 9 antimicrobial agents against the 134 *N. gonorrhoeae* isolates (continue)

No.	Isolates no.	MIC (mg/L)								
		PEN	TET	CIP	AZT	CFM	CRO	FOS	GEN	ETP
29	NG-012	0.25	0.25	2	0.015	0.004	0.004	16	16	0.002
30	NG-013	0.5	2	2	0.03	0.015	0.015	8	16	0.008
31	NG-014	128	21	1	0.06	0.004	0.004	16	16	0.008
32	NG-015	0.25	0.5	2	0.25	0.03	0.03	16	4	0.008
33	NG-016	0.25	0.5	2	0.125	0.03	0.03	8	4	0.008
34	NG-017	128	32	2	0.25	0.004	0.004	16	32	0.008
35	NG-018	512	32	32	0.06	0.008	0.008	32	32	0.004
36	NG-019	512	4	8	0.06	0.008	0.015	32	16	0.015
37	NG-020	0.5	1	2	0.125	0.004	0.004	8	4	0.008
38	NG-021	4	2	0.5	0.008	0.002	0.002	4	4	0.002
39	NG-022	64	32	2	0.06	0.004	0.004	32	16	0.015
40	NG-023	0.5	2	2	0.015	0.004	0.004	16	16	0.008
41	NG-024	32	16	2	0.015	0.002	0.002	32	8	0.008
42	NG-025	0.5	1	4	0.25	0.004	0.004	32	16	0.008
43	NG-026	4	4	2	0.015	0.002	0.002	4	4	0.002
44	NG-027	0.25	0.5	8	0.5	0.015	0.015	8	8	0.008
45	NG-028	0.5	32	2	0.015	0.004	0.004	16	8	0.008
46	NG-029	4	2	1	0.03	0.002	0.002	4	4	0.004
47	NG-030	512	32	1	0.06	0.002	0.002	16	16	0.008
48	NG-031	64	32	2	0.015	0.004	0.004	32	32	0.015
49	NG-032	64	32	1	0.03	0.002	0.002	16	16	0.008
50	NG-033	0.25	1	0.25	0.015	0.002	0.002	16	16	0.008
51	NG-034	0.5	1	1	0.015	0.002	0.002	8	16	0.008
52	NG-035	64	16	1	0.015	0.004	0.004	32	16	0.008
53	NG-036	32	32	1	0.03	0.008	0.008	16	8	0.015
54	NG-037	16	32	1	0.03	0.008	0.008	32	16	0.015
55	NG-038	8	1	1	0.03	0.002	0.002	32	16	0.008
56	NG-039	16	32	2	0.03	0.001	0.001	16	8	0.008
57	NG-040	64	32	2	0.125	0.008	0.008	16	16	0.015
58	NG-041	128	32	1	0.03	0.002	0.002	16	8	0.008

Table 24. MICs of 9 antimicrobial agents against the 134 *N. gonorrhoeae* isolates (continue)

No.	Isolates no.	MIC (mg/L)								
		PEN	TET	CIP	AZT	CFM	CRO	FOS	GEN	ETP
59	NG-042	128	1	2	0.125	0.002	0.002	16	16	0.008
60	NG-043	32	32	1	0.06	0.004	0.004	16	16	0.03
61	NG-044	32	32	1	0.06	0.015	0.015	8	16	0.06
62	NG-045	8	4	1	0.03	0.002	0.002	8	8	0.008
63	NG-064	16	32	1	0.125	0.002	0.002	32	16	0.008
64	NG-047	64	16	2	0.015	0.001	0.001	4	4	0.008
65	NG-048	64	16	4	0.125	0.001	0.001	8	4	0.008
66	NG-049	64	32	1	0.06	0.004	0.004	16	16	0.008
67	NG-050	2	128	8	0.125	0.008	0.008	16	32	0.03
68	NG-051	0.25	2	4	0.5	0.001	0.001	16	16	0.015
69	NG-052	32	32	2	0.03	0.001	0.001	16	8	0.015
70	NG-053	0.25	2	1	0.015	0.001	0.001	16	16	0.015
71	NG-054	4	16	1	0.03	0.004	0.004	16	16	0.008
72	NG-055	64	32	2	0.03	0.004	0.004	16	32	0.008
73	NG-056	128	64	8	0.125	0.015	0.015	8	16	0.008
74	NG-057	64	64	8	0.06	0.008	0.008	16	8	0.015
75	NG-058	256	16	2	0.06	0.002	0.002	16	16	0.008
76	NG-059	32	64	4	0.25	0.015	0.008	16	8	0.25
77	NG-060	256	32	1	0.25	0.004	0.002	16	16	0.25
78	NG-061	64	32	2	0.06	0.004	0.004	32	16	0.25
79	NG-062	64	32	1	0.06	0.004	0.002	32	16	0.125
80	NG-063	8	32	2	1	0.002	0.001	32	8	1
81	NG-064	8	64	4	0.125	0.004	0.002	16	16	0.125
82	NG-065	64	16	1	0.125	0.015	0.002	16	32	0.125
83	NG-066	4	32	2	0.06	0.015	0.008	16	16	0.125
84	NG-067	2	2	4	0.5	0.03	0.015	8	16	0.125
85	NG-068	0.25	1	2	1	0.015	0.002	16	16	0.5
86	NG-069	8	8	1	0.125	0.015	0.001	16	8	0.125
87	NG-070	0.5	2	2	0.25	0.03	0.008	16	8	0.5
88	NG-071	128	32	2	0.125	0.015	0.004	32	32	0.125

Table 24. MICs of 9 antimicrobial agents against the 134 *N. gonorrhoeae* isolates (continue)

No.	Isolates no.	MIC (mg/L)								
		PEN	TET	CIP	AZT	CFM	CRO	FOS	GEN	ETP
89	NG-072	256	16	1	0.25	0.008	0.002	16	8	0.25
90	NG-073	0.5	16	4	0.004	0.002	0.002	32	8	0.125
91	NG-074	0.5	2	2	4	0.008	0.004	16	16	0.06
92	NG-075	0.25	1	4	0.25	0.004	0.002	32	8	0.125
93	NG-076	0.25	2	2	1	0.002	0.001	16	8	0.125
94	NG-077	32	32	4	0.125	0.015	0.002	8	16	0.125
95	NG-078	32	64	1	0.125	0.008	0.002	16	16	0.06
96	NG-079	0.5	4	8	1	0.06	0.03	4	8	0.125
97	NG-080	0.5	1	0.002	0.125	0.015	0.002	16	0.5	0.125
98	NG-081	32	32	4	0.002	0.002	0.001	16	8	0.125
99	NG-082	64	32	4	1	0.008	0.004	16	16	0.06
100	NG-083	2	4	4	1	0.125	0.125	16	16	1
101	NG-084	32	4	4	0.5	0.008	0.002	8	8	0.06
102	NG-085	256	16	1	0.5	0.015	0.015	32	8	2
103	NG-086	0.5	32	4	0.5	0.008	0.004	32	32	0.06
104	NG-087	0.25	1	2	2	0.008	0.002	16	16	0.06
105	NG-088	2	1	0.25	0.5	0.008	0.001	32	8	1
106	NG-089	32	4	1	0.125	0.015	0.004	16	16	0.25
107	NG-090	4	4	8	0.5	0.06	0.06	32	16	0.125
108	NG-091	2	4	4	0.5	0.125	0.125	32	16	1
109	NG-092	16	16	2	0.25	0.008	0.002	16	8	0.06
110	NG-093	8	4	2	0.125	0.008	0.004	16	16	0.06
111	NG-094	0.25	1	2	1	0.004	0.002	16	8	0.25
112	NG-095	0.25	1	4	2	0.002	0.002	16	8	0.25
113	NG-096	0.5	2	0.008	1	0.004	0.002	16	8	2
114	NG-097	2	8	4	1	0.03	0.03	16	32	0.25
115	NG-098	64	32	1	0.5	0.015	0.002	32	8	0.03
116	NG-099	128	8	2	0.5	0.008	0.002	16	8	0.06
117	NG-100	0.25	1	2	2	0.008	0.001	16	16	0.25
118	NG-101	32	32	0.5	0.5	0.008	0.002	32	16	0.125

Table 24. MICs of 9 antimicrobial agents against the 134 *N. gonorrhoeae* isolates (continue)

No.	Isolates no.	MIC (mg/L)								
		PEN	TET	CIP	AZT	CFM	CRO	FOS	GEN	ETP
119	NG-102	64	32	1	0.002	0.002	0.001	64	32	0.25
120	NG-103	0.25	1	2	2	0.002	0.002	16	16	0.03
121	NG-104	64	32	2	0.125	0.008	0.002	16	16	0.06
122	NG-105	64	16	1	0.25	0.004	0.002	32	16	0.25
123	NG-106	0.25	1	4	0.25	0.002	0.004	16	4	0.06
124	NG-107	0.25	0.25	0.001	0.5	0.015	0.004	16	8	0.03
125	NG-108	256	32	1	0.002	0.015	0.001	16	4	0.125
126	NG-109	512	1	2	1	0.015	0.004	16	8	0.06
127	NG-110	64	64	2	0.5	0.015	0.008	32	16	0.06
128	NG-111	64	32	2	0.5	0.008	0.004	32	8	0.06
129	NG-112	256	32	2	0.5	0.008	0.002	16	4	0.06
130	NG-113	4	2	2	1	0.008	0.004	8	8	0.008
131	NG-114	2	2	4	1	0.015	0.004	16	16	0.06
132	NG-115	0.25	2	0.002	2	0.002	0.002	32	16	0.25
133	NG-116	128	32	1	0.25	0.008	0.002	32	4	0.03
134	NG-117	128	64	8	0.125	0.004	0.002	8	32	0.03

Note: AZT, azithromycin; CFX, cefixime; CRO, ceftriaxone; CIP, ciprofloxacin; ETP, ertapenem; GEN, gentamicin; FOS, fosfomycin; PEN, penicillin G; TET, tetracycline.

5. The antibiotic combinations against NG-083 and NG-091 using checkerboard methods

Table 25. The combination of ceftriaxone plus azithromycin against NG-083 and NG-091

Strains	ceftriaxone (A) + azithromycin (B) (mg/L)					
	MIC A combination	MIC A alone	MIC B combination	MIC B alone	FICI	Interpretation
NG-083	0.03	0.06	0.5	2	0.75	additive
NG-091	0.03	0.06	0.06	0.25	0.74	additive

Table 26. The combination of ceftriaxone plus fosfomycin against NG-083 and NG-091

Strains	ceftriaxone (A) + fosfomycin (B) (mg/L)					
	MIC A combination	MIC A alone	MIC B combination	MIC B alone	FICI	Interpretation
NG-083	0.03	0.06	16	32	1.00	additive
NG-091	0.03	0.06	16	32	1.00	additive

Table 27. The combination of ceftriaxone plus gentamicin against NG-083 and NG-091

Strains	ceftriaxone (A) + gentamicin (B) (mg/L)					
	MIC A combination	MIC A alone	MIC B combination	MIC B alone	FICI	Interpretation
NG-083	0.03	0.06	16	32	1.00	additive
NG-091	0.03	0.06	4	8	1.00	additive

Table 28. The combination of ceftriaxone plus ertapenem against NG-083 and NG-091

Strains	ceftriaxone (A) + ertapenem (B) (mg/L)					
	MIC A combination	MIC A alone	MIC B combination	MIC B alone	FICI	Interpretation
NG-083	0.03	0.06	0.25	0.25	1.50	indifference
NG-091	0.06	0.06	0.015	0.03	1.25	indifference

APPENDIX E

DNA CODON

Table 29. Abbreviations for the amino acids

Amino acids	Three-letter symbols	One-letter symbol
Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

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