PREVALENCE OF GONOCOCCAL INFECTION IN HIV-POSITIVE PATIENTS AND ANTIMICROBIAL RESISTANCE OF *NEISSERIA GONORRHOEAE*

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Thesis
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ABSTRACT

Neisseria gonorrhoeae is the causative agent of gonorrhea, one of the classical sexually transmitted diseases (STDs), humans being the only host. In this study, a total of 1,131 endocervical swabs from 797 HIV-positive patients were collected during June, 2005 to September, 2006 to detect *N. gonorrhoeae* and another common infection organism, *Chlamydia trachomatis* by Gen-probe system. The prevalence of gonococcal and chlamydial infections was 1.1% and 8.9%, respectively. There was only one co-infected patient. The mean age of the HIV-positive patients with a positive result for *N. gonorrhoeae* and *C. trachomatis* was 26.9 ± 6.2 and 29.9 ± 9.5 years, respectively.

This study also provides the antimicrobial susceptibility pattern of 122 other gonococcal isolates collected from June 2005 to May 2007. The drugs tested by the disk diffusion method were penicillin, tetracycline, ciprofloxacin, ofloxacin, cefotaxime and ceftriaxone. 86.1% of the isolates were resistant and 13.9% were intermediate resistant to penicillin. Among the 122 isolates, 4.1% were penicillinase-producing *N. gonorrhoeae* (PPNG), 10.7% were tetracycline-resistant *N. gonorrhoeae* (TRNG) and 79.5% were PPNG-TRNG. For tetracycline, 95.1% of the isolates were resistant and 4.9% were intermediate resistant. None of the isolates was susceptible to penicillin and tetracycline. 90.2% and 91% of the isolates were resistant to ciprofloxacin and ofloxacin, respectively. No gonococcal isolate with resistance to cefotaxime and ceftriaxone was detected. 83.6% of 122 isolates produced beta-lactamase enzyme and all of these were positive for bla_{TEM} gene by polymerase chain reaction (PCR) which was 100% identical to the bla_{TEM} gene. Its product was 100% identical to the beta-lactamase TEM protein of *Salmonella enterica* identified as TEM-135.

The major clinical implication of this report is that *N. gonorrhoeae* and *C. trachomatis* are still detectable among HIV-positive patients in Thailand and the routine screening of both organisms should be done. There is an increasing potential for treatment failure with most recommended drugs for the treatment of gonorrhea.

KEYWORDS: PREVALENCE/ *NEISSERIA GONORRHOEAE*/ ANTIMICROBIAL RESISTANCE/ PENICILLINASE-PRODUCING *N. GONORRHOEAE*

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ความชุกของการติดเชื้อก่อโรคหนองในในผู้ป่วยติดเชื้อเอดส์และการดื้อต่อยาต้านจุลชีพของเชื้อไนซีเรีย โกโนเรีย (PREVALENCE OF GONOCOCCAL INFECTION IN HIV-POSITIVE PATIENTS AND ANTIMICROBIAL RESISTANCE OF *NEISSERIA GONORRHOEAE*)

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บทคัดย่อ

เชื้อ *Neisseria gonorrhoeae* เป็นเชื้อสาเหตุของโรคหนองในซึ่งเป็นโรคติดต่อทางเพศสัมพันธ์ที่สำคัญ โดยสามารถก่อโรคเฉพาะในมนุษย์เท่านั้น ในการศึกษานี้ทำการศึกษาในตัวอย่างตรวจจาก 1,131 endocervical swab จากผู้ป่วยติดเชื้อเอดส์จำนวน 797 ราย ในช่วงเดือนมิถุนายน 2548 ถึงเดือนกันยายน 2549 เพื่อตรวจหาเชื้อ *N. gonorrhoeae* และ *Chlamydia trachomatis* ด้วยชุดตรวจ Gen-probe พบว่าความชุกของการติดเชื้อโกโนเรียและคลามัยเดียคือ 1.1% และ 8.9% ตามลำดับ และผู้ป่วย 1 รายติดเชื้อทั้งสองชนิด อายุเฉลี่ยของผู้ป่วยติดเชื้อเอดส์ที่ให้ผลบวกต่อเชื้อ *N. gonorrhoeae* และ *C. trachomatis* คือ 26.9 ± 6.2 และ 29.9 ± 9.5 ปี ตามลำดับ

การศึกษานี้ยังให้ผลความไวต่อยาต้านจุลชีพของเชื้อ *N. gonorrhoeae* อีก 122 สายพันธ์ที่เก็บในช่วงเดือนมิถุนายน 2548 ถึงเดือนพฤษภาคม 2550 ยาที่ใช้ในการทดสอบด้วยวิธี disk diffusion ได้แก่ penicillin, tetracycline, ciprofloxacin, ofloxacin, cefotaxime และ ceftriaxone พบว่า 86.1% ของเชื้อดื้อและ 13.9% ดื้อปานกลางต่อ penicillin และ 4.1% ของเชื้อจัดเป็น penicillinase-producing *N. gonorrhoeae* (PPNG) 10.7% จัดเป็น tetracycline-resistant *N. gonorrhoeae* (TRNG) และ 79.5% จัดเป็น PPNG-TRNG สำหรับ tetracycline 95.1% ของเชื้อให้ผลดื้อและ 4.9% ให้ผลดื้อปานกลาง และไม่พบสายพันธุ์ที่ไวต่อ penicillin และ tetracycline พบว่า 90.2% และ 91% ของเชื้อดื้อต่อ ciprofloxacin และ ofloxacin ตามลำดับ ไม่พบสายพันธุ์ที่ดื้อต่อ cefotaxime และ ceftriaxone จากการทดสอบหาเอ็นไซม์ beta-lactamase พบว่า 83.6% ของเชื้อสร้างเอ็นไซม์นี้ และตรวจพบ bla_{TEM} gene ในเชื้อที่สร้างเอ็นไซม์ beta-lactamase ทุกสายพันธุ์ด้วยวิธี polymerase chain reaction (PCR) ซึ่งมีลำดับเบสคล้ายคลึงกับ bla_{TEM} gene และ beta-lactamase TEM protein ซึ่งพบว่าเหมือนกับ TEM-135 beta-lactamase

จากรายงานนี้ถือได้ว่ายังคงตรวจพบเชื้อ *N. gonorrhoeae* และ *C. trachomatis* ในผู้ป่วยติดเชื้อเอดส์ในประเทศไทย และควรมีการตรวจกรองเชื้อทั้งสองชนิด และพบมีการเพิ่มขึ้นของความล้มเหลวในการรักษาโรคหนองในด้วย recommended drugs ส่วนใหญ่

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LIST OF ABBREVIATIONS

ABBREVIATION	TERM
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bla	Structure gene of beta-lactamase
BLAST	Basic Local Alignment Search Tool
bp	base pair
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
cm	centimeter (10^{-2} meter)
CMRNG	Chromosomally resistant *N. gonorrhoeae*
CO_2	Carbon dioxide
DGI	Disseminated gonococcal infection
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside 5´-triphosphate
EDTA	ethylenediamine tetracetic acid
FDA	Food and Drug Administration
GISP	Gonococcal Isolate Surveillance Project
HIV	Human immunodeficiency virus
kb	kilobase pair (10^3 base pair)
kDa	kilodalton (10^3 Dalton)
LOS	Lipo-oligosaccharides
LPS	Lipopolysaccharide
M	Molar

LIST OF ABBREVIATIONS (CONTINUED)

ABBREVIATION	TERM
ml	milliliter (10^{-3} liter)
mm	milimeter (10^{-3} meter)
mM	millimolar (10^{-3} molar)
M.W.	molecular weight
nm	nanometer (10^{-9} meter)
No.	number
PACE	Probe assay-chemiluminescence enhanced
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
PPNG	Penicillinase-producing *N. gonorrhoeae*
PID	Pelvic inflammatory disease
RLU	Relative light units
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
STDs	Sexually transmitted diseases
TAE	Tris acetate ethylenediamine tetracetic acid
TEM	Ambler class A enzyme first isolated from a patient 'Temoniera'
Tm	Melting temperature
TRNG	Tetracycline-resistant *N. gonorrhoeae*
U	unit(s)
USA	United States of America
UV	ultraviolet
V/V	volume by volume
W/V	weight by volume
µg	microgram (10^{-6} gram)

LIST OF ABBREVIATIONS (CONTINUED)

ABBREVIATION	TERM
μm	micrometer (10^{-6} meter)
μM	micromolar (10^{-6} molar)
%	percent
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
°C	degree(s) Celsius
Ala, A	Alanine
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartate
Cys, C	Cysteine
Gln, Q	Glutamine
Glu, E	Glutamate
Gly, G	Glycine
His, H	Histidine
Ile, I	Isoleucine
Leu, L	Leucine
Lys, K	Lysine
Met, M	Methionine
Phe, F	Phenylalanine
Pro, P	Proline
Ser, S	Serine
Thr, T	Threonine
Trp, W	Tryptophan
Tyr, Y	Tyrosine
Val, V	Valine

CHAPTER I
INTRODUCTION

Neisseria gonorrhoeae is the causative agent of gonorrhea, one of the sexually transmitted diseases (STDs) with human as the only host. The estimated number of cases globally is 200 million every year (1). A recent study (2) showed that untreated cases exceed treated cases, suggesting that the number of gonococcal infections is much greater than reported. In adults, complications include infertility, pelvic inflammatory disease (PID), ectopic pregnancy, premature rupture of membranes, preterm birth, puerperal infection, and facilitation of human immunodeficiency virus (HIV) type 1 transmission. Complications of congenital infections in newborns include gonococcal conjunctivitis (3). It has been known that patients with gonococcal infections may also be simultaneously infected with other sexually transmissible agents. One common association is between *N. gonorrhoeae* and *Chlamydia trachomatis.* The highest rate of gonorrhea and chlamydial infection are in adolescent women aged 15 to 19 years and in men aged 20 to 24 years (4). However, the vast majority of these infections go undetected because of the asymptomatic nature of the infections in women, which increases the likelihood that the pathogens will be spreaded to others before treatment is initiated. Furthermore, undiagnosed and untreated *C. trachomatis* and *N. gonorrhoeae* infections often lead to more serious and more costly complications. Lifetime medical costs associated with gonococcal and chlamydial infections in patients aged 15 to 24 years in 2000 were estimated at $325.4 million. Screening for both diseases has been shown to be a cost-effective strategy for preventing adverse reproductive health consequences (5). The Centers for Disease Control and Prevention (CDC) in the USA recommends *Chlamydia* screening for pregnant women, annual screening for sexually active adolescents, for women aged 20 to 25 years, and for older women with risk factors (e.g., those who report new or multiple sex partners) (4). Early identification and treatment of *C. trachomatis* infection during the early stages of the sequelae can greatly reduce the

onset of PID and the overall cost of treatment (6). STDs including gonococcal and chlamydial infections are important because HIV infection is becoming more prevalent (7) and there is a greater realization that STDs enhanced the transmission of HIV (8). Data from studies have documented that STDs enhance the transmission of HIV and were known cause of gynaecological and pregnancy-related morbidity (8, 9). Effective management of STDs can reduce the incidence of HIV transmission and underscores the need for early detection and treatments of STDs. The laboratory diagnosis of *N. gonorrhoeae* and *C. trachomatis* can be difficult, particularly for asymptomatic women and in developing countries. Although the culture has long been regarded as the "gold standard" for the detection of these organisms, culture is not well suited as a screening tool for *C. trachomatis* infections. In addition, both organisms require that great care be taken to ensure the viability of the organisms during transport of the specimen from the collection site to the testing laboratory (10). To overcome the shortcomings of culture, nucleic acid hybridization tests have been developed to aid in the diagnosis of *N. gonorrhoeae* and *C. trachomatis* infections. The use of DNA probe test is a relatively simple and reliable method for detecting gonococcal and chlamydial infections and provides analytical specificities equivalent to that of culture (11).

Gonococcal resistance to antimicrobial agents is an increasing problem in the treatment of gonorrhea. The organism acquires resistance by spontaneous mutations or acquires new DNA via conjugation or transformation. The resistant genes may be chromosomal or extrachromosomal (12). A single organism may have both mechanisms of resistance, and resistance to multiple antimicrobial agents is often seen. Resistance against almost all antimicrobial agents is spreading now in Southeast Asian Region countries (13). Surveillance was therefore considered mandatory to develop strategy and policy for proper therapy. From 1945 to 1988, penicillin was drug of choice for treatment of gonococcal infections. During this time, the resistance of gonococci gradually increased until treatment failure with penicillin became widespread and penicillin was discontinued as the first-line drug (14). The antimicrobial agents currently recommended for treatment of gonococcal infections

are expanded-spectrum cephalosporins or fluoroquinolones (4). Even though penicillin is still used in some countries, the prevalence of gonococci resistant to this drug remains high worldwide (15). Therefore, global surveillance of *N. gonorrhoeae* resistant to penicillin remains important. Plasmid-mediated resistance to penicillin is due to the production of a penicillinase (TEM-1 beta-lactamase) that can hydrolyze the beta-lactam ring in penicillin structure (16). The TEM-1 beta-lactamase spread worldwide throughout the family Enterobacteriaceae and by the 1970s, plasmid encoding the TEM-1 enzyme had spread in *N. gonorrhoeae* (17). Penicillinase-producing *N. gonorrhoeae* (PPNG) isolates have become prevalent world wide.

This study provided the surveillance data in Thailand on the rate of *N. gonorrhoeae* and *C. trachomatis* infections in HIV-positive patients in Rajavithi Hospital and Siriraj Hospital using Gen-probe system. The antimicrobial susceptibility of gonococcal isolates from Siriraj Hospital and Bangrak Hospital were determined by using disk diffusion method and preliminary molecular study of gonococcal resistance to penicillin will be determined by polymerase chain reaction of bla_{TEM} gene coding for beta-lactamase TEM enzyme. The antimicrobial susceptibility pattern and molecular characterization will be useful for the physicians to select the appropriate drug for treatment and control the spreading of resistance genes.

CHAPTER II
LITERATURE REVIEW

Gonorrhea is a sexually transmitted disease caused by *Neisseria gonorrhoeae* for which humans are the only natural host. It is transmitted by human-to-human contact and is highly adapted to the genital tract, surviving poorly outside the human body. Nevertheless, it is versatile in resisting attack, for example in its ability to develop resistance to antimicrobials and in the antigenic variability by which it evades host defense, thus persisting and often causing asymptomatic (and undetected) infection (18). The symptoms of gonorrhea are similar to those caused by other agents, most notably *Chlamydia trachomatis*. Gonococcal infections and their complications are among the most frequent communicable diseases in many countries (19). Gonococci can develop resistance to several commonly used antimicrobials. The failure to cure a case of gonorrhea has public health implication due to the potential to continue transmission and rapid emergence of antimicrobial resistance. Moreover, a number of sexually transmitted infections have been identified as facilitating the spread of human immunodeficiency virus (HIV) (20).

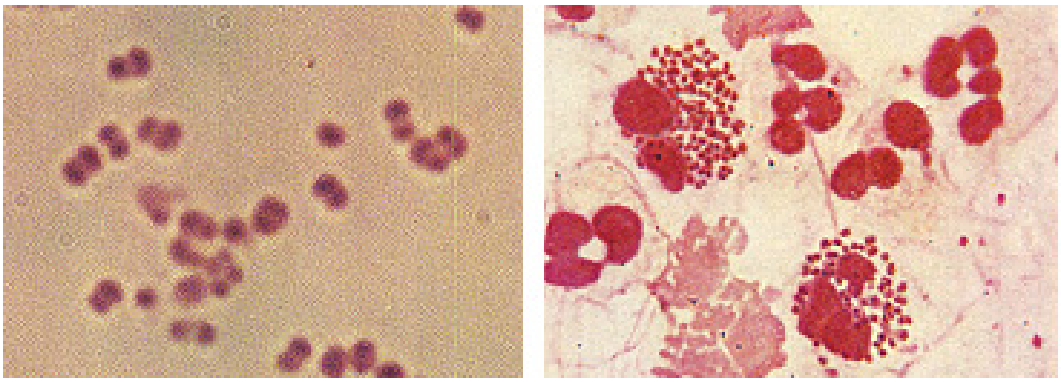
Taxonomy of the Genus *Neisseria*

The genus *Neisseria* belongs to the family Neisseriaceae which includes a group of closely related gram-negative diplococci that are primarily commensals of the mucous membrane of mammals (21). Several species of *Neisseria* are opportunistic pathogens, but two species, *N. meningitidis* and *N. gonorrhoeae*, are important human pathogens (22). Since the publication of of Systematic Bacteriology in 1984, the taxonomy of *Neisseria* species has undergone substantial revision. At that time, the Neisseriaceae included four genera: *Neisseria, Moraxella, Kingella,* and *Acinetobacter. Neisseria* consisted of 11 species that were considered "true neisseria" and three animal species (*N. caviae, N. ovis* and *N. cunuculi*). The application of molecular techniques to these and related organisms has resulted in several taxonomic modification to the family (23). In the forthcoming edition of Bergey's Manual, the family Neisseriaceae is classified in the β-subgroup of the *Protobacteria*, which includes the "true neisseria", *Kingella, Eikenella*, *Simonsiella*, *Alysiella,* the former Centers for Disease Control and Prevention (CDC) groups M-5, M-6 and EF-4. CDC groups M-5 and M-6 now have been reclassified as *N. weaveri* and *N. elongate* subsp. *nitroreducens,* respectively (24, 25).

General Characteristics of *N. gonorrhoeae*

1. Morphology and structure

N. gonorrhoeae is a gram-negative coccus, 0.6 to 1.0 μm in diameter, usually seen in pairs with adjacent flattened sides (Figure 1A and Figure 2). The organism is frequently found intracellularly in polymorphonuclear leukocytes (neutrophils) of the gonorrheal pustular exudate (Figure 1B) (26). Pili, which play a major role in adherence, extend several micrometers from the cell surface (Figure 2) (27). *N. gonorrhoeae* is aerobic bacteria, nonmotile and do not form spores. The optimum growth temperature is 35° to 37°C and its growth is stimulated by CO_2 and humidity. Gonococci produce several colony types in culture that are related to their piliation in the colony. Typical colonies tend to be small (about 0.5 mm in diameter), glistening and raised. *N. gonorrhoeae* possesses a typical gram-negative outer membrane composed of proteins, phospholipids, and lipopolysaccharide (28). The bacterium characteristically releases outer membrane fragments called "blebs" during growth. These blebs consist of outer-membrane components including LPS and protein; they are produced by rapidly growing cells (29, 30) and probably have a role in pathogenesis if they are disseminated during infection.



(A) **(B)**

Figure 1. *Neisseria gonorrhoeae* in gram stain of (A) pure culture and (B) a pustular exudate (26)

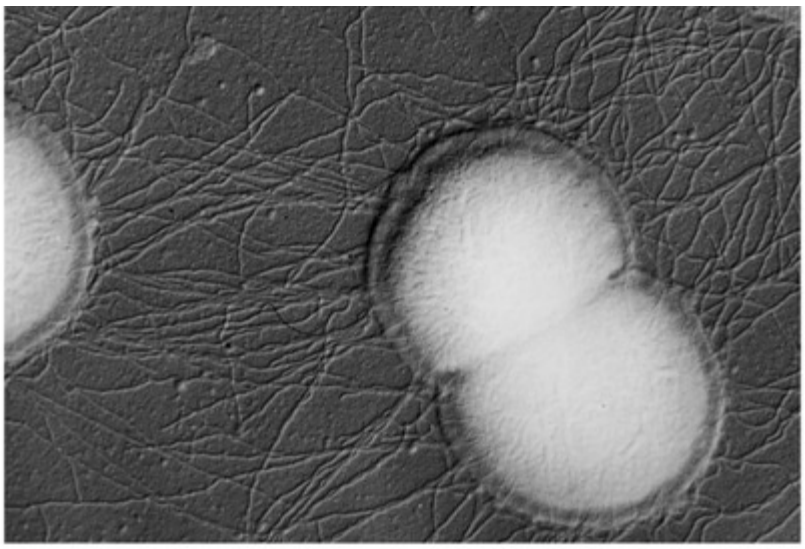


Figure 2. Electron micrograph of *N. gonorrhoeae* (27)

2. Growth requirements

It is a fastidious and will not grow in the absence of energy source such as glucose, pyruvate or lactate (28). All strains require cystine for growth; glutamine and cocarboxylase are required by a significant number of strains during primary isolation. For these reasons a growth factor supplement is added to media used for isolation of *N. gonorrhoeae.* Many strains of *N. gonorrhoeae* have additional genetic defects in the pathway used for the biosynthesis of amino acids, purines, pyrimidines and vitamins. This results in specific growth requirements and reinforces the need to use complex media for isolation of this organism.

3. Metabolism

It dissimilates glucose via the Entner-Doudoroff (ED) pathway with acetate and CO_2 as the primary end products (31). Fermentative pathways such as the Embden-Meyerhoff pathway do not appear to function in the dissimilation of glucose by this organism. The CO_2 and bicarbonate ion stimulate gonococcal growth by reducing the lag phase (32, 33).

4. Antigenic structure

N. gonorrhoeae is antigenically heterogeneous and capable of changing its surface structure in vivo to avoid host defense. Surface structures include the following:

4.1 Pili

Pili are the hair-like appendages that extend up to several micrometers from the gonococcal surface. They enhance attachment to host cells and resistance to phagocytosis. They consist of stacked pilin proteins (M.W. 17,000-21,000). The amino terminal of the pilin molecule, which contains a high percentage of hydrophobic amino acids, is conserved. The pilins of almost all strains of *N. gonorrhoeae* are antigenically different, and a single strain can make many antigenically distinct forms of pilin (34).

4.2 Por (Protein I)

Por extends through the gonococcal cell membrane. It occurs in trimers to form pores in the surface through which some nutrients enter the cell. The M.W. of Por varies from 34,000 to 37,000. Each strain of gonococcus expresses only one type of Por, but the Por of different strains is antigenically different (35). From serologic typing of Por by agglutination reactions with monoclonal antibodies, there are 18 serovars of PorA and 28 serovars of PorB. (Serotyping is done only in reference laboratories.)

4.3 Opa (Protein II)

This protein functions in adhesion of gonococci within colonies and attachment to host cells. One portion of the Opa molecule is in the gonococcal outer membrane, and the rest is exposed on the surface. The M.W. of Opa ranges from 24,000 to 32,000. A strain of gonococcus can express no, one, two or occasionally three types of Opa, though each strain has ten or more genes for different Opas. Opa is present in gonococci from opaque colonies but may not be present in those from transparent colonies (36).

4.4 Rmp (Protein III)

This protein (M.W. about 33,000) is antigenically conserved in all gonococci. It is a reduction-modifiable protein (Rmp) and changes its apparent M.W. when in a reduce state. It associates with Por in the formation of pores in the cell surface (36).

4.5 Lipo-oligosaccharide (LOS)

In contrast to that of the enteric gram-negative rod, gonococcal LPS does not have long O-antigen side chain and is called a lipooligosaccharide. Its M.W. is 3,000-7,000. Gonococci can express more than one antigenically different LOS chain simultaneously. Toxicity in gonococcal infections is largely due to the endotoxic effects of LOS (37).

4.6 Other proteins

Several other proteins of gonococci have poorly defined roles in pathogenesis. Lip (H8) is surface-exposed protein. The Fbp (iron-binding protein), similar in M.W. to Por, is expressed when the available iron supply is limited.

5. Genetics and antigenic heterogeneity

Gonococci develop mechanisms for frequently switching from one antigenic form (pilin, Opa or lipopolysaccharide) to another antigenic form of the same molecule. This switching takes place in one in every $10^{2.5}$-10^{3} gonococci, and extremely rapid rate of change for bacteria (34). Since pilin, Opa and lipopolysaccharide are surface exposed antigen, they are important in the immune response. The molecules' rapid switching from one antigenic form to another helps the gonococci elude the immune system.

The switching mechanism for pilin, which has been the most thoroughly studied, is different from the mechanism for Opa. Gonococci have multiple genes that code for pilin, but only one gene is inserted into the expression site. Gonococci can remove all or part of this pilin gene and replace it with all or part of another pilin gene. This mechanism allows gonococci to express many antigenically different pilin molecules over time. The switching mechanism of Opa involves, at least in part, the addition or removal from the DNA of one or more of pentameric coding repeats preceding the sequence that codes for the structural Opa gene. The switching mechanism of lipopolysaccharide is unknown (37). The antigens and heterogeneity of types are shown in Table 1.

Gonococci contain several plasmids; 95% of strains have a small "cryptic" plasmid (M.W. 2.4×10^{6}) of unknown function. Two other plasmids (M.W. 3.4×10^{6} and 4.7×10^{6}) contain genes that code for β-lactamase production, which causes resistance to penicillin. These plasmids are transmissible by conjugation among gonococci. They are similar to a plasmid found in penicillinase-producing *Haemophilus* or other gram-negative organisms (17). Five to 20% of gonococci contain a plasmid (24.5×10^{6}) with the genes that code for conjugation; the incidence is highest in geographic areas where penicillinase-producing gonococci are common (12). High-level tetracycline resistance has developed in gonococci by the insertion of a streptococcal gene coding for tetracycline resistance into the conjugative plasmid.

Table 1. Antigenic heterogeneity of *N. gonorrhoeae*

Antigens	Number of types
Pilin	Hundreds
Por (protein I) (US system)	PorA with 18 subtypes
	PorB with 28 subtypes
Opa (protein II)	Many (perhaps hundreds)
Rmp (protein III)	One
Lipo-oligosaccharide	Eight or more
Fbp (iron-binding protein)	One
Lip (H8)	One
IgA1 protease	Two

6. Virulence factors

Like the other pyogenic bacteria, *N. gonorrhoeae* has a wide range of virulence determinants, although it does not produce exotoxin (Table 2).

6.1 Pili

The bacterium first attaches to epithelial cells by using pili, specifically N-methylphenylalanine (type 4) pili, the main subunit of which is pilE (39). After initial attachment, the bacteria enter a second stage of binding mediated by the outer membrane protein (also known as Opa) which is needed for tight binding and invasion of epithelial cells. Also, Opa from one bacterium will bind to lipo-oligosaccharides (LOS) of an adjacent bacterium forming a microcolony which may be functionally analogous to a biofilm (40). However, the invasion of a cell involves a single bacterium, not whole microcolony.

6.2 Outer membrane porin

The outer membrane porin of *N. gonorrhoeae* (also known as Por) is equivalent to the *ompC* and *ompF* porins of *E. coli* that are involved in the passage of solutes through the outer membrane. However, Por apparently has a role in virulence that allows gonococci to survive inside phagocytes (41). Purified Por inhibits the ability of phagocytes to kill ingested bacteria.

6.3 Lipo-oligosaccharide (LOS)

It may be responsible for the symptoms of gonorrhea. LOS triggers an intense inflammatory response. Subsequent activation of complement, attraction and feeding by phagocytes, and the lysis of the phagocytes themselves, contributes to the purulent discharge (42). The local production of tumor necrosis factors (TNFs), elicited by LOS, may be the main cause of damage to the fallopian tubes. In addition, in strains causing systemic infection, LOS binds sialic acid from the serum forming a microcapsule of sialylated LOS (42), which allows gonococci to resist host immune response and serum bactericidal reaction.

6.4 Reduction-modifiable proteins (Rmp)

LOS and Por on the bacterial surface are known to be effective targets for bactericidal antibodies. However, if antibodies produced against Rmp react with their antigenic site on the gonococcal surface, the effect is to block bactericidal antibodies against LOS and Por and to protect the bacterium from complement-mediated lysis (26, 43).

6.5 Iron-regulated proteins

N. gonorrhoeae has a well-developed iron acquisition system that permits it to extract iron from its host during growth, which is necessary to support bacterial invasion. Basically, the bacterium is able to form two transferrin receptors (Tbp1 and Tbp2) and one lactoferrin receptor (Lbp) in its outer membrane (44, 45), which are induced under low-iron conditions, and which are able to directly extract iron from transferrin and lactoferrin, respectively. The proteins can also extract iron from heme and hemoglobin (26).

6.6 Immunoglobulin A1 proteases

Gonococci produce an IgA1 protease that splits and inactivates IgA1, a major mucosal immunoglobulin of human. Menigococci, pneumococci and *Haemophilus influenzae* produce a similar IgA1 protease (38).

Table 2. Surface components of *N. gonorrhoeae* that may play a role in virulence (26)

Designation	**Location**	**Contribution**
PilE	Major fimbrial protein	Initial binding to epithelial cells
Opa	Outer membrane protein	Contributes to invasion
Por	Outer membrane porin	May prevent phagolysosome formation in neutrophils and/or reduce oxidative burst
LOS	Outer membrane lipo-oligosaccharide	Elicits inflammatory response, triggers release of TNF
Rmp	Outer membrane protein	Elicits formation of infective antibodies that block bactericidal antibodies against protein I and LOS
Tbp1 and Tbp2	Outer membrane receptors for transferrin	Iron acquisition for growth
Lbp	Outer membrane receptors for lactoferrin	Iron acquisition for growth

7. Pathogenesis

A classic review of gonorrhea (46) describes the clinical manifestation of gonococcal urethritis. Sexually transmitted gonococcal infections typically occur on the mucosal epithelia of the male urethra or the female uterine cervix. *N. gonorrhoeae* can also infect the rectum, throat, and conjunctiva of the eye. Gonococcal transmission generally occurs through direct sexual contact but indirect modes of transmission have been reported (47). Infections are often associated with a characteristic inflammatory response and a massive infiltration of polymorphonuclear leukocytes and mononuclear phagocytes into the infected tissue of urethra, endocervix, and other mucosal sites (48, 49) (Figure 3). As a result, the infected epithelial layer is destroyed providing the bacteria access to deeper tissues (50). An essential first step in the infection of mucosal surface is the anchoring of gonococci via the pili (51). Subsequent to the binding via pili, the colony opacity-associated (Opa) proteins, a family of phase-variable outer membrane proteins, confer intimate binding to target cells (52, 53). Infection is frequently accompanied by an intense focal inflammatory response, and shedding of infected epithelial cells is common. In female, gonococci can ascend the urogenital tract, resulting in pelvic inflammatory disease (PID). Disseminated gonococcal infection (DGI) causes acute arthritis and, less often, myocarditis (54)

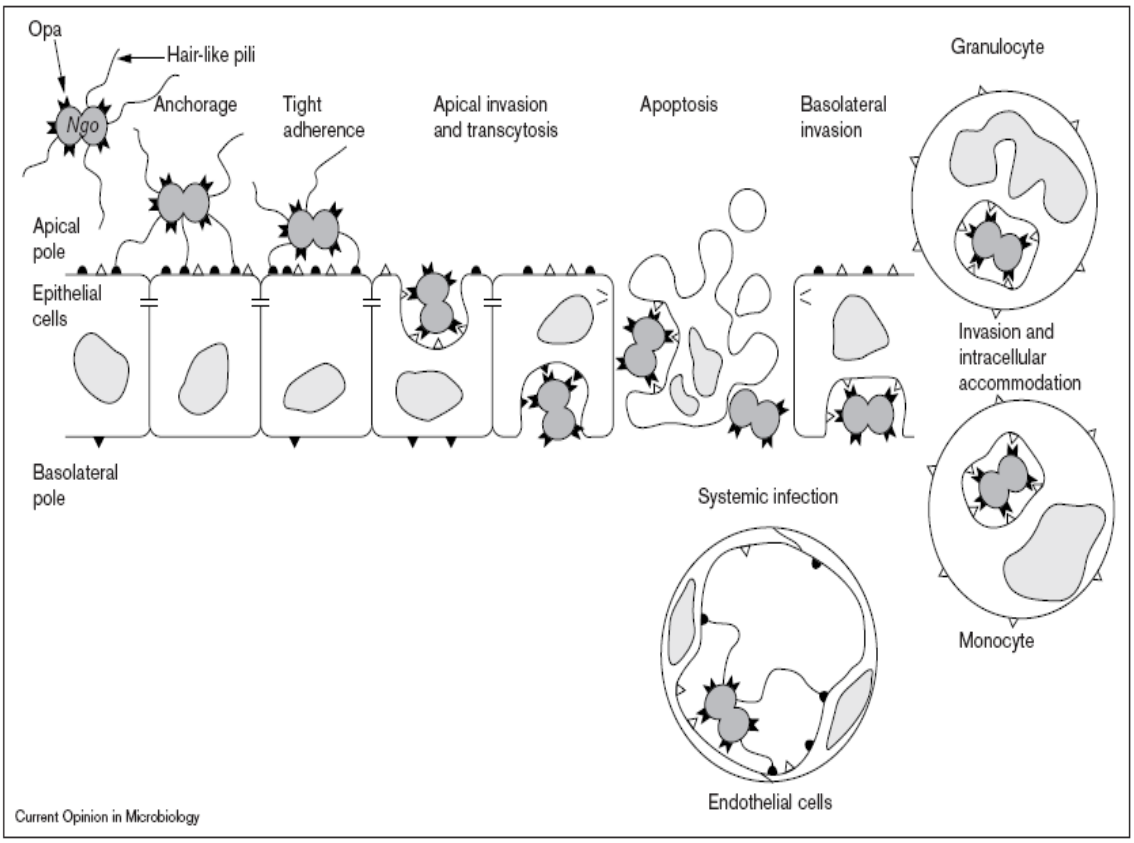


Figure 3. Putative events during infection of the mucosal tissue by *N. gonorrhoeae* (49).

8. Genetic mechanisms

8.1 Transformation

In transformation, naked DNA is taken up by recipient cell and ultimately integrated into the chromosome. *N. gonorrhoeae* is competent for genetic transformation throughout its entire growth cycle (55). Competence for transformation is much greater in piliated (Pil^+) colonial forms than in their isogenic non-piliated (Pil^-) colonial variants. Pilin (PilE), the major pilus subunit, and PilC, the minor pilus-associated protein, are required for DNA uptake (56). A competence factor that is involved in a transformation step subsequent to the initial DNA uptake has also been identified (57). Gonococci can be transformed by either chromosome or plasmid DNA, although the efficiency of transformation with plasmid DNA is approximately 1,000-fold lower than with chromosomal DNA. Electroporation of both chromosomal and plasmid DNA has also been used to introduce DNA into gonococci (58). Transformation may be how gonococci exchange DNA in vivo. *N. gonorrhoeae*, like many other naturally competent bacteria, is highly autolytic, which results in the release of biologically active transforming DNA into the environment (59).

8.2 Conjugation

Conjugation in *N. gonorrhoeae* is different from that of *Escherichia coli*, in that a plasmid-encoded pilus has not been identified and transfer only occurs when cells are on a solid surface. Two gonoccoal conjugal plasmids of 24,500 and 25,200 kDa mediate the transfer of R plasmids to strains of *N. gonorrhoeae*, as well as to commensal *Neisseria*, *E.coli* and to *Haemophilus influenzae*. Chromosomal genes are not mobilized by conjugation. The mobilization of R plasmid is often accompanied by transfer of the conjugal plasmids; however, no evidence of stable cointegrated formation has been found (60).

9. Clinical aspects

9.1 Gonococcal infections in adults

9.1.1 Male

In male, *N. gonorrhoeae* causes an acute urethritis with dysuria and urethral discharge (61). The incubation period ranges from 1 to 14 days or longer, with an average of 2 to 7 days. 95-99% of infected men will experience a discharge at some time. The discharge is purulent in 75% of cases, cloudy in 20% and mucoid in about 5%. About 2.5% of men presenting to sexually transmitted disease clinics are truly asymptomatic, and it is estimated that the prevalence of asymptomatic gonorrhea in men in the general population may be as high as 5%. This pattern of disease is often associated with infection by certain Por IA gonococcal serovars and with arginine, hypoxanthine and uracil (AHU) and certain other auxotypes of *N. gonorrhoeae* (62). If left untreated, most cases of gonorrhea in men resolve spontaneously, but in less than 10% of cases, ascending infection may result in gonococcal epididymitis, epididymo-orchitis, prostatitis, periurethral abscess and urethral stricture. These complications are rarely seen in clinical practice in USA.

9.1.2 Female

In female, the primary gonococcal infection is present in the endocervix, with concomitant urethral infection occurring in 70-90% of cases. After an incubation period of 8-10 days, patients may present with cervicovaginal discharge, abnormal or intermenstrual bleeding and abdominal or pelvic pain; the latter suggests the presence of upper genital tract disease (63). The presence of dysuria indicates significant urethral involvement. Gonococcal infection of the vaginal squamous epithelium of postpubertal women is uncommon, and in women who have had hysterectomies, the urethra is the most common primary site of infection. Although it has often been stated that most women with genital gonococcal infection are asymptomatic, this is probably not true. This assertion was based on the detection of infected women during widespread screening such as family planning clinics, and did not account for women who presented to physicians or emergency rooms with a spectrum of symptoms referable to the genital tract (e.g., vaginal discharge, menorrhagia) or the lower abdomen (61). Symptoms of uncomplicated endocervical infection often resemble those of other conditions, such as cystitis or

vaginal infections and the symptomatology of gonococcal endocervicitis is often coinfected with *C. trachomatis*, *Trichomonas vaginalis* and/or *Candida albicans.* Although the genital tract may appear normal, careful endocervical examination often reveals areas of cervical mucosa that bleed on swabbing. Only 10-20% of infected women, however, will present with an obvious mucopurulent endocervical discharge. Infection of the Bartholin's and Skene's glands may be seen in about one third of women with genital tract infection. Careful manipulation of these areas can sometimes provide purulent material for direct examination and culture. Endocervical gonorrhea may also complicate pregnancy and is a recognized cofactor for spontaneous abortion, chorioamnionitis, premature rupture of membrane and premature delivery (61). Infants born to women with genital tract infection are at risk for gonococcal conjunctivitis ("opthalmia neonatorum") or pharyngeal gonococcal infection. Assending gonococcal infection may occur in 10-20% of infected women and can result in acute pelvic inflammatory disease (PID) that is manifested as salpingitis (infection of the fallopian tubes), endometritis and tubo-ovarian abscess, all of which can lead to scarring of the fallopian tubes, ectopic pregnancies, sterility and chronic pelvic pain.

9.1.3 Disseminated gonococcal infection (DGI)

In a small percentage (approximately 0.5-3%) of infected individuals, *N. gonorrhoeae* may invade the bloodstream, resulting in disseminated gonococcal infection (DGI) (64). This infection is characterized by low-grade fever (rarely above 39 °C), hemorrhagic skin lesion, tenosynovitis, migratory polyarthralgias and arthritis. Women appear to be a greater risk for DGI, particularly during menstruation and during the second and third trimesters of pregnancy. The skin lesions are generally painful and appear as a papule that evolves into a necrotic pustule on an erythematous base. Usually there are as few as 5 or up to 30 lesions present, and the majority of them are on the extremities (i.e., toes, fingers) (64). In 30-40% of cases, *N. gonorrhoeae* from the bloodstream may localize in one or more joints to cause a purulent and destructive gonococcal arthritis. Complications of DGI include permanent joint damage, endocarditis and rarely meningitis. (61). Gonococcal endocarditis is a rare clinical entity, with fewer than 60 cases reported in the literature since 1938. Gonococcal endocarditis may develop in about 1-2% of patients with disseminated infection, usually involves the aortic valve and follows a rapid and

destructive course (65). Gonococcal meningitis is a rare complication of disseminated infection that has features typical of meningitis causes by other organisms.

9.2 Gonococcal infections among children

Historically, gonococcal infections in children included only opthalmia neonatorum, which is ocular gonorrhea transmitted to the infant during passage through the infected cervix (66). Almost all cases of gonorrhea in children during the newborn period are the result of ocular contamination, although more serious infections with gonococci in newborns have been reported (67). Transmission of gonorrhea from adults to children by fomites (e.g., shared towels) was proposed as a model of transmission in older children. However, it is now recognized that gonococcal infections, including conjunctivitis and other sexually transmitted diseases in children beyond the immediate neonatal period are indicators of sexual abuse (68). With a careful, multidisciplinary approach, histories of sexual contacts can usually be obtained from older children. When a child with gonorrhea is identified, investigation of both adult caretakers and other siblings frequently reveals infected adults and other infected children. Gonococcal infections in children resemble those in adults, with some notable differences. *N. gonorrhoeae* causes a vaginitis, rather than a cervicitis, in prepubertal girls. The epithelium of the prepubertal vagina is composed of columnar epithelial cells, which are the cell types that *N. gonorrhoeae* preferentially infects. With the onset of puberty, these cells are replaced by a stratified aquamous epithelium that is not susceptible to gonococcal infection. Female children with genital gonococcal infection generally present with a vaginal discharge. Urethral infection in male children, if present, resembles that seen in adults. Pharyngeal and rectal gonococcal infections, as in adults, are usually asymptomatic in children (61).

10. Laboratory diagnosis

10.1 Direct Gram stain smears

The diagnosis of gonococcal urethritis in adult male is frequently made by the observation of gram-negative diplococci within or closely associated with polymorphonuclear leukocytes (Figure 1, Right) on a smear prepared from the urethral discharge. When properly performed, the Gram stain has a sensitivity of 90-95% and specificity of 95-100% for diagnosing genital gonorrhea in symptomatic man (61). In female, Gram stain of endocervical specimen collected under direct visualization of the cervix may also be very helpful in diagnosis. The sensitivity and specificity of the Gram stain for rectal specimens are lower than with cervical specimens (69). Gram-stained smears should not be relied on for diagnosis of gonorrhea and should be used adjunctively along with more specific tests.

10.2 Cultivation

As with other pathogenic microorganisms, successful isolation depends on the collection of proper specimens and this is particularly important for the recovery of *N. gonorrhoeae*. The appropriate sites for culture are summarized in Table 3. Ideally, specimens should be inoculated onto appropriate media and incubated immediately after collection. Urethral, cervical and pharyngeal specimens are inoculated onto selective media such as Thayer-Martin or Martin-Lewis, which contain antimicrobial and antifungal agents to inhibit the growth of normal flora. Specimens collected from normally sterile sites such as blood, synovial fluid and conjunctiva may be inoculated onto a non-selective medium such as chocolate agar (69).

Table 3. Body sites to culture for *N. gonorrhoeae* (61)

Patient	**Primary site (s)**	**Secondary site (s)**
Female	Endocervix	Rectum, urethra, pharynx
Male, heterosexual	Urethra	Pharynx
Male, homosexual	Urethra, rectum, pharynx	
Female, disseminated infection	Blood, endocervix, rectum	Pharynx, skin lesions[a], joint fluid[b]
Male, disseminated infection	Blood, urethra	Pharynx, rectum, skin lesions, joint fluid[b]

[a] If present

[b] Culture if arthritis present

10.3 Identification

All isolates of oxidase-positive, gram-negative diplococci that are recovered from urogenital sites and that grow on selective media may be presumptively identified as *N. gonorrhoeae*. However, confirmatory identification tests are recommended for all isolates and are required for identification of isolates from extragenital sites. These may include carbohydrate-utilization tests, immunogenic method (e.g., monoclonal antibody fluorescence tests; coagglutination tests), enzymatic procedures (e.g., chromogenic detection of specific enzyme activities) or the DNA probe culture confirmation test (61).

10.4 Direct probe and amplified probe detection

Probe and nucleic acid amplification tests (NAATs) permit the direct detection of *N. gonorrhoeae* in clinical specimens. These tests have gained popularity because they permit the concurrent detection of *C. trachomatis.* An advantage of these tests is that specimens may be transported and stored for several days before being tested in the laboratory; they don not require viable organisms. A disadvantage is that isolates are not available for ongoing surveillance or for antimicrobial susceptibility testing if question of treatment efficacy arise (70).

10.4.1 Nucleic acid hybridization (DNA probe) tests

Two nucleic acid probe assays, the Gen-probe PACE 2 and PACE 2C assays (Gen-probe Inc., USA) and the Hybrid capture II assay (Digene Corp., USA) are approved by the Food and Drug Administration (FDA) for detecting *N. gonorrhoeae).*

10.4.1.1 Gen-probe tests

In the Gen-probe tests, an acridinium ester labeled DNA probe for a specific sequence of *N. gonorrhoeae* or *C. trachomatis* rRNA is allowed to hybridize with any complementary rRNA in the specimen (71). An acridinium ester hybridization protection assay detects any DNA-RNA hybrids which are adsorbed to magnetic particles. The acridinium ester label is hydrolyzed from any unhybridized DNA probe. Chemilumunescence generated by the acridinium ester-DNA-RNA hybrids is then detected with a luminometer that gives a numerical readout (70, 71). The PACE 2C test detects both *N. gonorrhoeae* and *C. trachomatis* in a single test. If a positive result is obtained in this test, separate tests for the individual organisms must be performed. An unlabeled probe is incubated with the initially positive specimen; the unlabeled probe competitively inhibits binding of the labeled probe. A prescribed reduction in the signal obtained for the assay performed with and without the unlabeled probe verifies the initial positive test result.

10.4.1.2 Hybrid capture II assay

The Digene assay uses RNA hybridization probes which are specific for both genomic DNA and cryptic plasmid DNA sequences of *N. gonorrhoeae* and *C. trachomatis* (72). In this test, RNA-DNA hybrids are captured in the wells of microtiter plates by hybrid-specific antibodies. The hybrids are detected with alkaline phosphatase-labeled anti-RNA-DNA hybrid antibodies, and the signal is amplified by using chemiluminescent substrate detected by a luminometer.

10.4.2 Nucleic acid amplification tests (NAATs)

NAATs are designed to amplify *N. gonorrhoeae*-specific sequences; positive results may be obtained from as little as a single copy of target DNA or RNA. NAATs from three manufacturers are currently FDA approved for the detection of *N. gonorrhoeae*: the Abbott LCx (Abbott Laboratories Inc., USA), the Roche AMPLICOR (Roche Diagnostics, Germany) and the Becton Dickinson

BDProbeTec (BD Biosciences, USA) tests. Most NAATs have been cleared by the FDA to detect *N. gonorrhoeae* in endocervical swabs from women, urethral swabs from men and urine from men and women. NAATs are not recommended for the detection of *N. gonorrhoeae* in either rectal or pharyngeal specimens (70).

Treatment and Prevention

A relatively high proportion of gonococci now bear a plasmid that encodes a β-lactamase, an enzyme that destroys penicillin. Gonococci bearing drug resistance plasmids can cause serious invasive diseases such as pelvic inflammatory disease as well as disseminated gonococcal infection. As a consequence of widespread penicillin resistance, the recommended initial therapy for gonorrhea is no longer penicillin, but a β-lactamase-resistant cephalosporin-cefixime or ceftriaxone- given orally or intramuscularly, respectively. Single-dose oral therapy is effective and offers the distinct advantage of observed therapy (i.e., no treatment failure due to lack of compliance and no injection required). Both quinolones (ciprofloxacin, ofloxacin and levofloxacin) and cefixime are available for this purpose, but gonococci resistant to quinolones have become a serious problem in Southeast Asia and resistant isolates are being reported with increasing frequency in Hawaii, California and elsewhere among men who have sex with men (73).

Prevention efforts must be based on a multiple approach as follows:

- Behavior including condom use and decreasing the number of sexual partners
- Early diagnosis and treatment
- Partner notification
- Screening and case-finding
- Vaccine development

Attempts at vaccine development have proved to be difficult because gonococci, as a strict human pathogen, have a long-standing and sophisticated relationship with their host. They have managed to survive the host's immune response by antigenic variation or phase variation. Acquired immunity following infection is inadequate and repeat infections are common among individuals with repeat exposures (73).

Human Immunodeficiency Virus (HIV) and Sexually Transmitted Diseases (STDs)

Since the detection of the first case of HIV infection during 1981 in USA, this epidemic has been the most devastating. After Africa, the South East Asia region happens to be the most affected. Among various factors associated with sexual transmission of HIV, STDs seem to contribute significantly. Though, several advances have been made in the management of HIV infection, development of safe and potent vaccine still remains a big challenge. Thus, the efforts for prevention and control of HIV largely rely on STD control measures (9). Several research studies carried out globally have provided ample evidence that STDs remain as the single largest factor tremendously enhancing the spread of HIV (74-76).

HIV/ STDs link and interaction

Enormous evidence indicating that both ulcerative and inflammatory STDs increase the risk of HIV infection has become available in the last two decades. Initially, in Africa (77) and subsequently in other places (78), it was reported that individuals with recent genital ulcerative disease were at risk of acquiring HIV infection from an infected partner. Studies on the role of STDs in HIV transmission were reviewed by Wasserheit (79) and concluded that both ulcerative and non-ulcerative STDs increase the risk of HIV transmission by 3 to 5 fold and due to higher frequency of non-ulcerative STDs, these infections may be responsible for more HIV transmission compared to genital ulcers. Gonococcal, *Chlamydia* and *Trichomonas* infections are associated with a relative increase of 60 to 340% in the prevalence of HIV infection in both men and women (80). In 1999, Fleming and Wasserheit (81) reported that both ulcerative and non-ulcerative STDs promote HIV transmission via a variety of biological mechanisms. This various mechanisms were studied which could explain higher probability of HIV transmission. STDs facilitate HIV shedding in the genital tract. Detection of HIV-1 virus or viral DNA from genital ulcer exudates was also reported (82, 83). Similarly, an evidence of HIV-1 virus was also found in HIV-infected individuals suffering from urethritis/cervicitis due to gonococcal/chlamydial etiology. These infections were shown to increase the viral

load in genital secretions and after treatment, HIV viral load in the genital secretions was reduced (84). A study conducted among female sex workers (85) concluded that the genital HIV virus shedding was associated with STDs such as gonorrhea or *Chlamydia* infection.

Genital ulcers facilitate acquisition of HIV infection, by causing disruption of normal epithelial barrier, which provides a portal of entry to HIV virus. Genital discharge due to non-ulcerative STDs contains cells expressing molecules to which HIV can attach, such as CD4 and chemokine receptor CCR5, making these patients more susceptible to HIV infection. The median concentration of endocervical CD4 T-lymphocytes was shown to be higher in patients with gonorrhea, *Chlamydia* infection or trichomoniasis compared to patients without these diseases, which indicates that the non-ulcerative STDs may facilitate HIV acquisition by recruiting HIV target cells to the endocervix (9, 86).

Effect of STD treatment on HIV spread

STD treatment reduces an individual's ability to transmit HIV by decreasing the amount and frequency of HIV shedding (81). New evidence indicates that STD detection and treatment can substantially reduce HIV transmission (87). For example:

- STD treatment reduces the prevalence and magnitude of HIV shedding

Treatment of gonorrhea in HIV-infected men resulted in a reduction in the number of men who shed HIV, as well as a lower concentration of HIV shed. With STD treatment, the level of shedding among co-infected men returns to the level seen in men who are not co-infected.

- STD treatment reduces the spread of HIV infection in communities

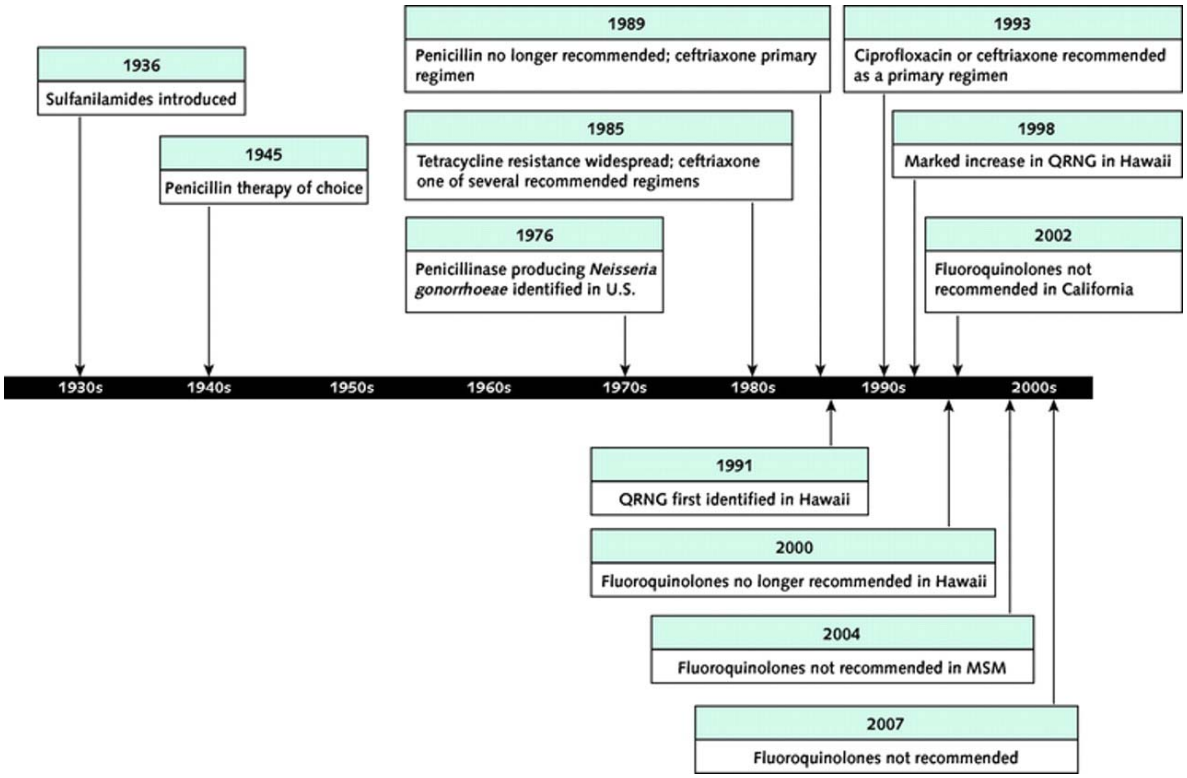
A community-level, randomized trial in a rural African community in Tanzania (88) demonstrated a 42% decrease in new, heterosexually transmitted HIV infections in communities with improved STD treatment. An ongoing study in Uganda (89) is further exploring the impact of mass STD treatment in slowing the spread of HIV. These studies will be critical in more clearly defining the role STD treatment can play in HIV prevention efforts in the developing world and in industrialized nations.

Antimicrobial Resistance

Over the past 60 years *N. gonorrhoeae* has developed resistance to multiple classes of antimicrobial agents (Figure 4) (90). Sulfanilamides were used for gonococcal treatment after their introduction in 1936, but their efficacy was short-lived because of the rapid emergence of resistance by 1945. Penicillin became the recommended antimicrobial regimen for the next 40 years. The progressive decline in susceptibility—initially associated with chromosomally mediated resistance and later by the acquisition and spread of plasmids containing genes for penicillinase production. In 1985, because of emerging penicillin resistance, ceftriaxone became a recommended regimen for the treatment of uncomplicated gonococcal infections. At the same time, tetracycline resistance (both plasmid and chromosomally mediated) was spreading to the extent that tetracycline was no longer a viable treatment option. By 1989, resistance to penicillin was sufficiently widespread that penicillin was no longer effective. Ceftriaxone then became the recommended regimen for gonorrhea therapy, with ciprofloxacin as an alternative treatment option. By 1993, on the basis of data regarding high efficacy, safety, and convenience as single-dose therapies, oral fluoroquinolones (ciprofloxacin, ofloxacin) were recommended as oral regimens for gonorrhea treatment, as was the oral third-generation cephalosporin, cefixime (90). In 1986, concerns about emerging gonococcal antimicrobial resistance lead to the development of the Gonococcal Isolate Surveillance Project (GISP), a national sentinel surveillance system that monitors antimicrobial susceptibility in USA. The GISP findings of notable importance include the continued high prevalence of penicillin and tetracycline resistance, which has remained greater than 15%; the increasing prevalence of isolates with decreased susceptibility to macrolides; the appearance of a limited number of multidrug-resistant isolates with decreased susceptibility to cefixime; and most important, the dramatic spread of quinolone-resistant *N. gonorrhoeae*.

Based in part on data from GISP, the emergence of quinolone-resistant *N. gonorrhoeae* was first identified in Hawaii in 1991, at about the same time that it was recognized as a problem in Asia (91). Thereafter, sporadic occurrences of quinolone-resistant *N. gonorrhoeae* were noted in USA throughout the 1990s. By

2000, quinolone-resistant *N. gonorrhoeae* was increasingly observed in persons who became infected in Asia, the Pacific Islands (including Hawaii), or California. As a result, fluoroquinolones were no longer recommended for treating gonorrhea acquired in those locales. Over the next several years, GISP identified increased rates of quinolone-resistant *N. gonorrhoeae* among men who have sex with men. This finding prompted an advisory in 2004 that fluoroquinolones were no longer recommended for treating gonorrhea in men who have sex with men, regardless of locale. On the basis of these cumulative data, the Centers for Disease Control and Prevention (CDC) announced that fluoroquinolones are no longer recommended for treating gonococcal infections and associated conditions, such as pelvic inflammatory disease or epididymitis, in any population in USA (92). The rapid spread throughout USA of quinolone-resistant *N. gonorrhoeae* in heterosexual men, as well as among men who have sex with men, makes therapy of *N. gonorrhoeae* problematic. These limitations highlight the difficulty of defining an appropriate sentinel population and a frequency of sampling with sufficient representation to assure susceptibility in a particular locale. Thus, there is a need to monitor antimicrobial resistance at the local level, which would provide a more in-depth understanding of resistance trends and contribute to decisions that affect treatment recommendations in various locations (93).



QRNG = quinolone-resistant *Neisseria gonorrhoeae*; MSM = men who have sex with men

Figure 4. Historical perspective on antimicrobial resistance in USA (90)

Mechanisms of Antimicrobial Resistance

1. Penicillin resistance

The targets of β-lactam agents are the penicillin binding proteins (PBPs) which are enzymes located in the cell membrane involving in cell wall synthesis. Alterations in PBP-2 and PBP-1 decrease their affinity for penicillin, and thus the susceptibility of the organism. PBP-2 is encoded by the *penA* locus. Changes in other loci such as *mtr* and *penB* produce additive effects. The *mtr* locus mediates resistance to a wide range of drugs, detergents and dyes through an active efflux system (12). Mutations in the *penB* locus, which affect a porin, result in reduced permeability of the cell membrane to hydrophilic compounds. Gonococci exhibiting these changes are termed chromosomally resistant *N. gonorrhoeae* (CMRNG). Reduced susceptibility to cephalosporin, tetracycline and other drugs is also mediated by chromosomal mechanisms.

Resistance to penicillin is also mediated by a plasmid-borne, inducible TEM-1 type β-lactamase. This enzyme hydrolyses the β-lactam ring of penicillin, thus inactivating them. In contrast to the slow evolution and incremental increase in resistance associated with chromosomal changes, acquisition of the plasmid confers resistance in a single step. Transmission of the resistance by conjugation required the presence of another mobilizing plasmid which was already present in the Asian PPNG (94). It is possible that the plasmid was initially acquired from *Haemophilus* species (17). β-Lactamase production (PPNG) and chromosomal changes (CMRNG) can coexist in the same isolate. This is relevant because of the clinical use of penicillin in combination with β-lactamase inhibitor. In theory, and sometimes in practice they represent an effective oral therapy for PPNG infections, but more commonly single-dose regimens of penicillin/inhibitor combinations have failed.

2. Cephalosporin resistance

Altered gonococcal susceptibility to cephalosporin is chromosomally mediated and is due to the same changes that account for decreased penicillin susceptibility (95). There is cross-resistance between penicillins and early generation cephalosporins such as cefuroxime. However, this is not the case for the later generation cephalosporins such as ceftriaxone and cefixime. Not all cephalosporins are hydrolysed by the TEM-1 type β-lactamase, and therefore some of these compounds are active against PPNG. Other β-lactamases (cephalosporinases), which are constitutively expressed by many other gram-negative genera, have thus far not been detected in gonococci and there has been no transfer of genetic material encoding production of extended spectrum β-lactamases into pathogenic *Neisseria*. If such an event were to occur it would be devastating for gonorrhea treatment programmes that rely heavily on the third-generation cephalosporins (12).

3. Quinolone resistance

The quinolone most widely used for the treatment of gonorrhoea are 'second generation' agents such as ciprofloxacin and ofloxacin. Similar to the development of chromosomal penicillin resistance, resistance to quinolone has developed incrementally over a number of years and multiple chromosomal changes are involved. Access of quinolone to its targets is reduced by changes in cell permeability and possibly by efflux mechanisms. These events produce low-level quinolone resistance. The targets of quinolone are topoisomerases, including DNA gyrase. High-level clinically relevant resistance is mediated by alteration of the target sites, initially via mutation in the *gyrA* gene. Multiple amino acid substitutions are described which, when combined, result in high-level resistance. Multiple mutations also occur in the *parC* gene which codes for the production of topoisomerase IV, a secondary target for quinolone in gonococci. Quinolone resistance is almost exclusively mediated by chromosomal mutations, which affect either the target sites or access of quinolone to the cell (96).

4. Tetracycline resistance

Both chromosomal and plasmid-borne resistance mechanisms are found in gonococci, the latter being responsible for high-level resistance. Chromosomal resistance is linked to the *mtr* and *penB* alterations which also reduce susceptibility to penicillin. The combination of these and other chromosomal mutations results in clinically significant resistance. High-level tetracycline resistance in gonococci (TRNG) results from acquisition of the *tetM* determinant and was first reported in 1986 (12). The *tetM* plasmid is widely dispersed in the normal genital tract flora; the mobility of the plasmid and the selective pressure created by the use of tetracycline to treat other STDs has contributed to the increase of TRNG phenotype (97).

Control of Antimicrobial Resistance in *N. gonorrhoeae*

Control of antimicrobial resistance in gonococci relies, to a significant extent, on control of the disease itself. Other factors involved are generally applicable to control of antimicrobial resistance in other bacteria (98). Antimicrobial resistance in gonococci rarely arises first in settings with an established regulatory framework that oversees drug evaluation and approval, enforcement of prescription-only drug access, reliable drug delivery systems, an informed prescriber base, and laboratory systems with good and evaluative diagnostic standards. Rather, antimicrobial resistance in gonococci most often arises, and resistant gonococci spread most frequently, in situations in which drugs are readily available (often in the informal health sector), drug quality is suspect (as a result of poor manufacturing, adulteration, or degradation), and compliance with standard treatment regimens is poor—the latter often because of the unaffordability of the recommended dose (99). All of the above factors must be continuously addressed for controlling antimicrobial resistance in gonococci.

PART A

THE PREVALENCE OF *N. GONORRHOEAE* AND *C. TRACHOMATIS* IN HIV-POSITIVE PATIENTS

CHAPTER III
MATERIALS AND METHODS: PART A

1. Specimen collection

One thousand one hundred and thirty-one endocervical swabs were obtained from 797 HIV-positive patients during June 2005 to September 2006. All specimens were obtained from 470 patients (59%) visited Siriraj Hospital and 327 patients (41%) visited Rajavithi Hospital. Each endocervical swab was collected by using the Gen-probe specimen collection kit (Gen-probe Inc., USA) and transported to clinical microbiology laboratory at Siriraj Hospital in the Gen-probe transport medium. All specimens were stored at 2°C to 25°C until tested.

2. Chemicals and equipments

The Gen-probe system test kits (Gen-probe Inc., USA) for *N. gonorrhoeae* and *C. trachomatis* consist of lyophilized probe reagent for *N. gonorrhoeae* or *C. trachomatis*, hybridization buffer, selection reagent, separation reagent, wash solution, detection reagents I and II. The tests were performed in disposable polystyrene reaction tubes (12 × 75 mm). The magnetic separation unit and luminometer were provided by the manufacture to be used in the reaction. This method required only standard laboratory equipments such as vortex mixer, covered water-bath, micropipette and pipette tips.

3. Gen-probe system for *N. gonorrhoeae* and *C. trachomatis*

Each specimen was tested for *N. gonorrhoeae* and *C. trachomatis* by the PACE 2 Gen-probe system (Gen-probe Inc., USA).

3.1 Specimen preparation

Each specimen transported tube was allowed to reach room temperature before testing. Each transport tube was vortexed for at least 5 seconds. Most liquid from swab was obtained by pressing the swab against wall of tube and the swab was discarded. The transport tube was vortexed for 5 seconds to ensure homogeneity of the liquid before testing.

Some specimens may be too viscous to pipette, the FAST Express reagent (Gen-probe Inc., USA) was used to improve the specimen to use easily. Prior to discard the swab, one drop of FAST Express reagent was added into the transport tube. The treated specimen was incubated in 60°C water-baht for 10 minutes. The treated specimen was removed from water-bath, vortexed, squeezed and the swab was discarded.

3.2 Reagent preparation

All reagents except probe reagent, hybridization buffer, and separation reagent were reached room temperature before using. Probe reagent and separation reagent were maintained at 4°C until used.

3.2.1 Probe reagent

Hybridization buffer was vortexed for 10 seconds after removed from refrigerator. After votexing, the reagent was warmed by swirling the vial in a water-bath at 60°C ± 1°C for 3 to 4 minutes and vortexed again for 10 seconds to ensure a homogeneous solution. Six ml of hybridization buffer were pipetted into lyophilized probe reagent. The reagent was incubated at room temperature for 2 minutes and then vortexed for 10 seconds before use. The reagent was visually inspected to ensure that

it was completely hydrated and homogeneous. The reconstituted probe reagent was stable for 3 weeks at 2°C to 8°C.

3.2.2 Separation suspension

It was necessary to determine the number of tests to be performed and calculate the volumes of selection reagent and separation reagent as follows:

$$\text{Volume of selection reagent (ml)} = \text{Number of tests + 2 extra tests}$$

$$\text{Volome of separation reagent (ml)} = \frac{\text{Volume of selection reagent (ml)}}{20}$$

The required volume of selection reagent was poured into a clean dry container. The required separation reagent was added into the selection reagent and mixed well. The prepared separation suspension was stored at room temperature and was stable for 6 hours.

3.3 Detection of *N. gonorrhoeae* and *C. trachomatis*

3.3.1 Hybridization

The test was carried out by adding 100 µl of one positive, one negative and three proficiency controls to bottom of each labeled tube. Then 100 µl of each specimen was pipetted to bottom of each labeled tube. Either *N. gonorrhoeae* or *C. trachomatis* probe reagent in a volume of 100 µl was pipetted into bottom of each tube. The tubes were then covered with sealing card and shaken 3 to 5 times. The tubes were incubated in a covered water-bath at 60°C ± 1°C for 1 hour.

3.3.2 Separation

Tubes were removed from water-bath. The sealing card was then removed. One ml of the well mixed separation suspension was pipetted into each tube. All tubes were covered with sealing card and shaken vigorously until bubble was visible. They were immediately incubated in a covered water-bath at 60°C ± 1°C for 10 minutes. Tubes were removed from water-bath. The sealing card was then removed and the rack was set on magnetic base for 5 minutes at room temperature. Tube rack

and base were holded together and the contents were decanted, shaken 2 to 3 times and bloted 3 times (for 5 seconds each) on absorbent paper. Each tube was filled with wash solution and tube rack was put on magnetic base for 20 minutes at room temperature. Tube rack and base were holded together, decanted but were not blotted. Approximately 50 to 100 µl of wash solution were remaining in each tube. The tube rack was separated from the magnetic base and the tube rack was shaken to resuspend the pellets.

3.3.3 Detection

The damp tissue was used to wipe each tube to ensure that no residue was presented on the outside of the tube. The tube was inserted and read by the luminometer within 60 minutes of the final decant. Tubes should be maintained at 20°C to 25°C before reading. The tubes were read for *N. gonorrhoeae* and *C. trachomatis* separately and read in the following order:

Negative reference : 3 tubes
Positive control : 1 tube
Standard proficiency control : 3 tubes
Specimen tubes

3.4 Results

3.4.1 Calculation of results

The results of the Gen-probe system for *N. gonorrhoeae* and *C. trachomatis* were calculated according to the manufacturer's recommendation (100) which was based on the difference between the response in Relative Light Units (RLU) of the specimen and the mean response of the negative reference.

$$\text{Mean response of the negative reference} = \frac{\text{Sum of the three negative references}}{3}$$

Example: For *N. gonorrhoeae*

Mean of negative reference = $\frac{(55 \text{ RLU} + 60 \text{ RLU} + 50 \text{ RLU})}{3} = 55 \text{ RLU}$

Specimen response = 894 RLU

Difference = 894 RLU – 55 RLU = 839 RLU ------➢ Positive

3.4.2 Interpretation of results

- *N. gonorrhoeae*

Positive: The difference was greater than or equal to 300 RLU

Negative: The difference is less than 300 RLU

- *C. trachomatis*

Positive: The difference was greater than or equal to 350 RLU

Negative: The difference is less than 350 RLU

3.5 Quality control

The tests were performed simultaneously with negative reference, positive control and 3 standard proficiency controls in everyday testing. The results were read when all control values were correct and samples were repeated if there was a low positive or indeterminate result.

- Negative reference

The response of each negative reference value should be ≤ 200 RLU.

- Positive control

The difference between the response of the positive control and the mean response of the negative reference should be greater than 600 RLU.

- Standard proficiency controls

The response of each standard proficiency controls should be in the range as shown in Table 4.

Table 4. The range of three standard proficiency controls for *N. gonorrhoeae* and *C. trachomatis* in Relative Light Units (RLU)

Control	***N. gonorrhoeae***	***C. trachomatis***
Proficiency 1	2,000 - 6,000	20 - 200
Proficiency 2	500 - 1,500	500 - 1,500
Proficiency 3	20 - 200	2,000 - 6,000

CHAPTER IV
RESULTS: PART A

1. Source of Clinical Specimens

A total of 1,131 endocervical swabs of 797 HIV-positive patients were collected consecutively from patients at Siriraj Hospital and Rajavithi Hospital during June 2005 to September 2006. There were 717 endocervical swabs from Siriraj Hospital and 414 endocervical swabs from Rajavithi Hospital as shown in Figure 5. The endocervical swabs were collected from 470 patients from Siriraj Hospital and 327 patients from Rajavithi Hospital. These are because some patients visited the hospital more than one time. There were 133 (16.7%), 56 (7%) and 1 (0.1%) patients visited 2, 3 and 4 times, respectively at Siriraj Hospital and 74 (9.3%) and 6 (0.8%) patients visited 2 and 3 times, respectively at Rajavithi Hospital and 280 (35.1%) and 247 (31.0%) patients visited at Siriraj Hospital and Rajavithi Hospital, respectively were collected endocervical swabs only one time (Figure 6). The age distribution of patients ranged from 14 to 66 years. There were 5 (0.8%) HIV-positive patients aged less than or equal to 15 years, followed by 116 (19.5%) patients aged 16 to 25 years, 327 (55.1%) patients aged 26 to 35 years, 123 (20.7%) patients aged 36 to 45 years, 20 (3.4%) patients aged 46 to 55 years and 3 (0.5%) patients aged more than 55 years as shown in Figure 7. Ages of 203 patients were unknown.

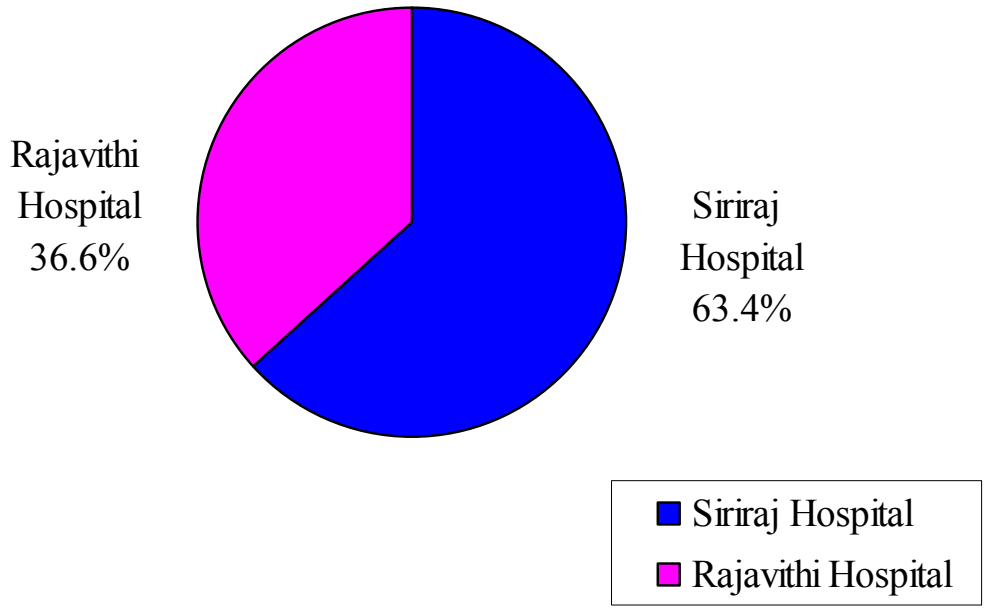


Figure 5. Distribution of specimens obtained from HIV-positive patients visited Siriraj Hospital and Rajavithi Hospital

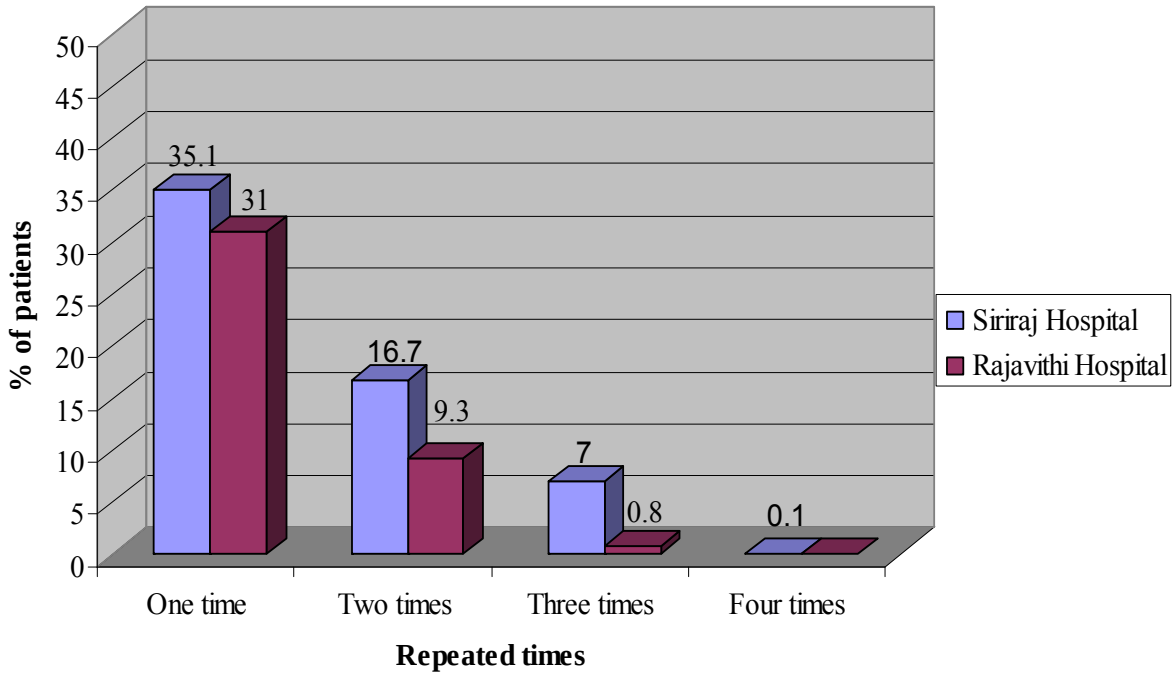


Figure 6. Distribution of specimens obtained from HIV-positive patients more than one time

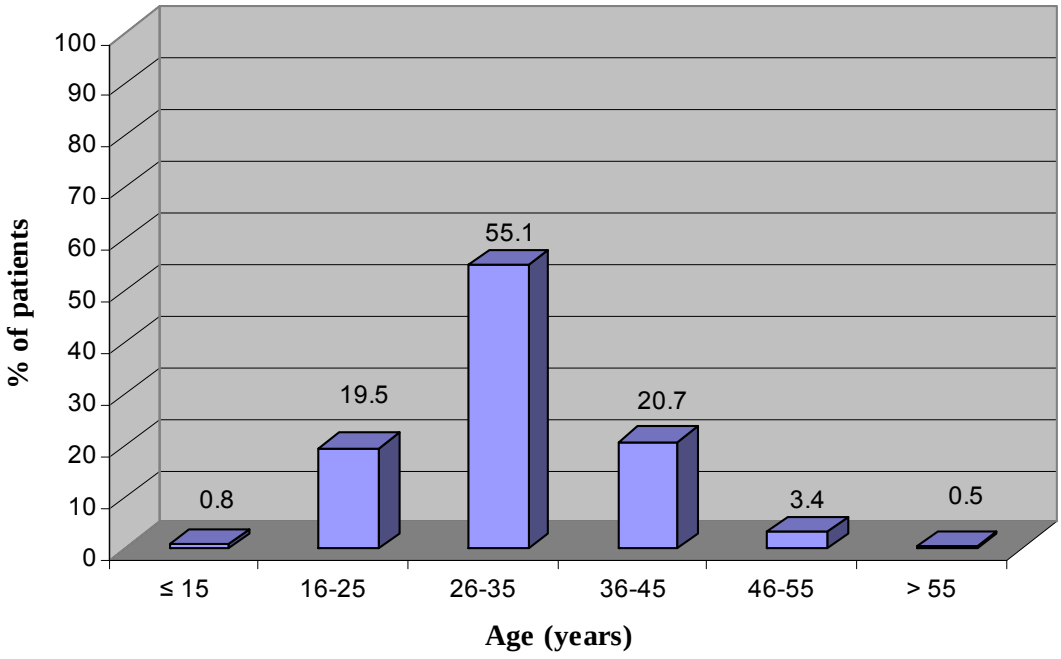


Figure 7. The HIV-positive patients from Siriraj Hospital and Rajavithi Hospital according to ages

2. Detection of *N. gonorrhoeae* and *C. trachomatis*

Each endocervical swab was tested for *N. gonorrhoeae* and *C. trachomatis* using the Gen-probe system (Gen-probe Inc., USA) for *N. gonorrhoeae* and *C. trachomatis,* respectively. For the specimen preparation step, some specimens were too viscous to pipett, therefore the using of FAST Express reagent (Gen-probe Inc., USA) could improve the specimen to be more liquid. For every testing of Gen-probe system, the standard proficiency test using standard proficiency 1, 2 and 3 was conducted to ensure consistency in performance of laboratory procedures. Table 6 shows the value of negative, positive and 3 standard proficiency controls in Gen-probe system for *N. gonorrhoeae* and *C. trachomatis.* For standard proficiency control of *N. gonorrhoeae*, the value must range from 2,000-6,000, 500-1,500 and 20-200 Relative Light Unit (RLU) for standard proficiency 1, 2 and 3 respectively. For standard proficiency control of *C. trachomatis*, the value must range from 20-200, 500-1,500 and 2,000-6,000 RLU for standard proficiency 1, 2 and 3 respectively (100). The quality controls were performed simultaneously with every testing using three negative controls and one positive control. The tested results were read only when all control results were valid. The response of each negative control value should be less than or equal to 200 RLU and the value of positive control which calculated from the difference between the response of the positive control and the mean response of the negative control should be greater than 600 RLU for both *N. gonorrhoeae* and *C. trachomatis.* The results of standard proficiency controls, negative control and positive control in this study were in accordance with the manufacturer's guidelines as shown in Table 5.

Of 1,131 endocervical swabs from 797 HIV-positive patients, nine patients (1.1%) were positive for gonococcal infection and 71 patients (8.9%) were positive for chlamydial infection. Of these, there was only 1 patient positive for both organisms (Figure 8). Table 6 and 7 show the distribution of gonococcal and chlamydial infected patients, respectively by age range. The mean age of HIV-positive patients with positive result for *N. gonorrhoeae* was 26.9 ± 6.2 years with the ages of 2 positive patients were unknown. The mean age of HIV-positive patients with positive result for *C. trachomatis* was 29.9 ± 9.5 years which the ages of 27 positive patients were

unknown. The gonococcal infection was found highest prevalence in aged 16-25 years (1.7%), followed by aged 26-35 years (1.5%). The prevalence of chlamydial infection in aged > 55 years was 33.3% (1/3). This is because the number of tested patients was very low. The most chlamydial infection was found in the lower age range which was 20%, 10.3% and 6.7% in aged ≤15, 16-25 and 26-35 years, respectively (Table 7). The aged of patient who was positive for both *N. gonorrhoeae* and *C. trachomatis* was unknown.

For the repeated sending specimens from each HIV-positive patient visited Siriraj Hospital, there were 2.3% of patients who were two times repeating of specimen were positive for *N. gonorrhoeae* and 1.8% of patients which was three times repeating of specimen were positive. Whereas, only 0.01% of patients which no repeating of specimen were positive for *N. gonorrhoeae* (Table 8). There were 10.7% and 8.3% of patients which was three and two times repeating of specimen, respectively, were positive for *C. trachomatis* and 5.4% of patients which no repeating of specimen were positive (Table 9). For the HIV-positive patients visited Rajavithi Hospital, there were 2.7% of patients which was two times repeating of specimen were positive for *N. gonorrhoeae* and none of patients which no repeating of specimen was positive (Table 8). There were 23% of patients which was two times repeating of specimen were positive for *C. trachomatis* and 8.5% of no repeating of specimen were positive (Table 9).

Table 5. The value of negative, positive and 3 standard proficiency controls in Gen-probe system for *N. gonorrhoeae* and *C. trachomatis* in relative light units

Control	*N. gonorrhoeae*		*C. trachomatis*	
	Range	Mean ± SD	Range	Mean ± SD
Negative	34-110	64.47 ± 28.32	34-205	93.76 ± 48.05
Positive	514-2,530	1,291.82 ± 621.33	656-2,452	1,432.59 ± 588.21
Proficiency 1	2,094-4,598	2,880.33 ± 718.55	38-188	84.29 ± 43.19
Proficiency 2	501-1,388	898.94 ± 236.61	561-1,229	938.57 ± 199.55
Proficiency 3	32-178	72.94 ± 37.21	2,164-4,785	3,673.94 ± 853.39

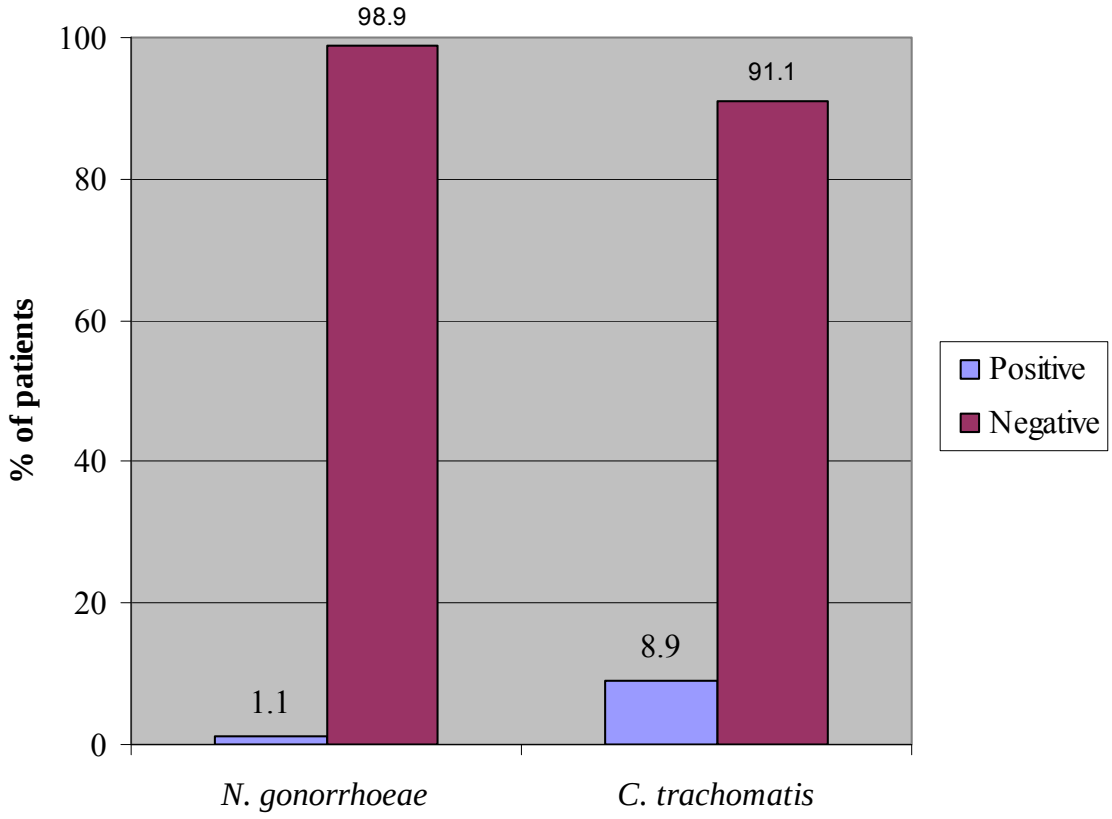


Figure 8. Prevalence of *N. gonorrhoeae* and *C. trachomatis* in 797 HIV-positive patients

* One patient was positive for both *N. gonorrhoeae* and *C. trachomatis*.

Table 6. Prevalence of patients who were positive for *N. gonorrhoeae* by Gen-probe method according to age

Age (years)	Number of tested patients	Number of *N. gonorrhoeae* -positive patients	Prevalence (%)
≤ 15	5	0	0
16-25	116	2	1.7
26-35	327	5	1.5
36-45	123	0	0
46-55	20	0	0
> 55	3	0	0
Unknown*	203	2	1.0
Total	797	9	-

* One patient was positive for both *N. gonorrhoeae* and *C. trachomatis.*

Table 7. Prevalence of patients who were positive for *C. trachomatis* by Gen-probe method according to age

Age (years)	Number of tested patients	Number of *C.trachomatis* -positive patients	Prevalence (%)
≤ 15	5	1	20.0
16-25	116	12	10.3
26-35	327	22	6.7
36-45	123	7	5.7
46-55	20	1	5.0
> 55	3	1	33.3
Unknown*	203	27	13.3
Total	797	71	-

* One patient was positive for both *N. gonorrhoeae* and *C. trachomatis.*

Table 8. Percentage of patients who were positive for *N. gonorrhoeae* according to repeated specimens

Repeated times	Number of patients	Positive for *N. gonorrhoeae*	
		Number	Percentage
Siriraj Hospital			
One time	280	3	0.01
Two times	133	3	2.3
Three times	56	1	1.8
Four times	1	0	0
Rajavithi Hospital			
One time	247	0	0
Two times	74	2	2.7
Three times	6	0	0
Four times	-	-	-
Total	797	9	-

Table 9. Percentage of patients who were positive for *C. trachomatis* according to repeated specimens

Repeated times	Number of patients	Positive for *C. trachomatis*	
		Number	Percentage
Siriraj Hospital			
One time	280	15	5.4
Two times	133	11	8.3
Three times	56	6	10.7
Four times	1	1	-
Rajavithi Hospital			
One time	247	21	8.5
Two times	74	17	23.0
Three times	6	0	0
Four times	-	-	-
Total	797	71	-

CHAPTER V
DISCUSSION: PART A

N. gonorrhoeae infection is generally limited to superficial mucosal surface lined with columnar epithelium. The areas most frequently involved are urethra, cervix, rectum, pharynx and conjunctiva. Endocervical infection is the most common form of uncomplicated gonorrhea in women (103). Therefore, clinical specimens tested in this study were collected as endocervical swabs to detect gonorrhea and chlamydial infection in female. The screening of *N. gonorrhoeae* and *C. trachomatis* infections in HIV-positive patients was performed, because among the various factors associated with the sexual transmission of HIV, sexually transmitted diseases (STDs) seem to contribute significantly (9). Prevalence rates of STDs up to 75% and incidence rates of 38% per year have been reported from Nairobi and Thailand, respectively (104, 105). STDs are also known to enhance the transmission of HIV; thus, their control may be one of the most cost-effective approaches in the fight against HIV in the developing countries (106, 107). In Israel (108), the HIV-positive patients had a greater prevalence of STDs including *N. gonorrhoeae* and *C. trachomatis* than the high risk HIV-negative patients (79.5% and 37.5%, respectively). In this study it was found that the prevalence of cervical infection with *N. gonorrhoeae* and *C. trachomatis* in female HIV-positive patients in two large hospitals in Thailand was low. Chlamydial infection occurred approximately 8 times higher than gonococcal infection i.e., 1.1% and 8.9% of patients were infected with *N. gonorrhoeae* and *C. trachomatis*, respectively. Infection with *N. gonorrhoeae* is less common, but most infections may be undiagnosed, because these infections are generally asymptomatic. The *N. gonorrhoeae* prevalence of 1.1% and *C. trachomatis* prevalence of 8.9% for HIV-positive patients in this study were less than those detected by Gen-probe PACE2 System in 1997 in Thailand (109), which showed that the prevalence of *N. gonorrhoeae* was 2.7% and of *C. trachomatis* was 16.2% in HIV-positive pregnant women. However, the studies from South Africa (110) and Peru (111) reported that

the prevalence of *N. gonorrhoeae* in HIV-positive patients was higher than those of *C. trachomatis*. There were 66% of *N. gonorrhoeae* and 30% of *C. trachomatis* in South Africa and 20% of *N. gonorrhoeae* and no chlamydial infection was detected in Peru. The prevalence rates of gonorrhea and chlamydial infection was very high in Papua New Guinea (112). The number simultaneously infected with both organisms in this study was very low (0.1%), although the co-infection is still high e.g., 17.6% and 16.1% as reported in Eastern Sydney and United Kingdom, respectively (113, 114). Like other investigators (115, 116), it was found that the infection rate of both *N. gonorrhoeae* and *C. trachomatis* was high in younger age group (aged ≤ 35 years).

Reports of sexually transmitted disease surveillance in Thailand have included gonorrhea, syphilis, chancroid, lymphogranuloma venereum and granuloma inguinale since 1952. Gonorrhea, the most common STD, accounts for approximately half of reported cases (117). The highest incidences of all STDs (7.9 per 1,000 adults) and of gonorrhea (4.5 per 1,000 adults) were reported in 1986. By 1990, the incidences dropped to 4.5 per 1,000 adults for total STDs and to 2.4 per 1,000 adults for gonorrhea (109). More recently, the number of cases reported in Thailand has continued to decrease (118, 119), thought to be related mostly to increase in condom use for men (120, 121). The prevalence of *N. gonorrhoeae* reported in this study was nearly one third of the gonorrhea prevalence of 2.7% reported for pregnant women in Bangkok 9 years ago (109) which is consistent with the dramatic decrease in STDs reported in Thailand (119). However, the high prevalence of *C. trachomatis* infection found in this study illustrates the need for routine screening. The relatively low rate of gonococcal and chlamydial infection among HIV-positive individuals from this study may come from the routine prophylaxis aimed at preventing STD infections and may from low risk sexual behavior such as greater condom use and abstinence. However, the routinely screening HIV patients for STDs including gonococcal and chlamydial infection, regardless of the presence or absence of urogenital symptoms remain important.

Standard diagnosis of *C. trachomatis* and *N. gonorrhea* infections is made by culture. Culture for chlamydial cervicitis, when properly performed, has a sensitivity of 70% to 90%, and a specificity of nearly 100% (10, 122). However, *C. trachomatis* culture is technically difficult and time-consuming. The enzyme immunoassays and

direct fluorescent antibody tests, which can be used for screening, are less sensitive and specific (123). Studies have demonstrated that early identification and treatment of *C. trachomatis* infections during the early stages of the disease can greatly reduce the onset of PID (6) and the overall cost of treatment (124). For gonorrhea, culture in 5% CO_2 environment is the diagnostic standard method (125) and depending on appropriate media and specimen handling. However, such culturing is not possible in many developing countries. Direct smear of clinical specimen and Gram stain for gram-negative diplococci intracellularly in polymorphonuclear leukocytes is the most common diagnostic tool, especially for symptomatic infection. However, the sensitivity of Gram stain is 40% to 60%; and the specificity is 95% to 100%. Although a positive Gram stain is usually valid, a negative result has little diagnostic utility (126). In addition, both *N. gonorrhoeae* and *C. trachomatis* require the great care taken to ensure the viability of the organisms during transport of specimen from the collection site to the testing laboratory. Recent laboratory efforts have focused on DNA hybridization and polymerase chain reaction techniques, which detect infection more reliably.

DNA probe (Gen-probe) testing for the detection of *N. gonorrhoeae* and *C. trachomatis* has been evaluated for diagnostic utility in many studies (72, 127). For both organisms, the overall sensitivity compared with culture ranged from 77.1% to 99.4% and the specificity from 97.6% to 99.6%. Most recent studies have concluded that the DNA probe is a useful screening and diagnostic test (72, 128). An advantage of using this methodology is that the same specimen can be used to test for both *C. trachomatis* and *N. gonorrhoeae.* A study using DNA probe testing in northern Thailand indicated that it improved diagnosis in female patients (129). However, for developing countries, this test may not be affordable for routine use because the reagent cost of DNA probe testing remains high (109). New screening methods such as urine testing by nucleic acid amplification assays (LCR/PCR) have the advantage of not requiring pelvic exams (123), but currently are more expensive than DNA probe (Gen-probe). If the cost of these tests is further reduced, they may have an important role in STD screening and surveillance in developing countries. Finally, *N. gonorrhoeae* and *C. trachomatis* is still detected among HIV-positive female patients in Thailand and the routine screening of both organisms should be done.

PART B

STUDY OF ANTIMICROBIAL SUSCEPTIBILITY AND PENICILLIN RESISTANCE IN *N. GONORRHOEAE*

CHAPTER VI
MATERIALS AND METHODS: PART B

1. Bacteria

1.1 Clinical isolates

One hundred and twenty-two isolates of *N. gonorrhoeae* from clinical specimens were studied. They were collected from patients visited Sirirraj Hospital and Bangrak Hospital during June 2005 – May 2007.

1.2 Quality control

N. gonorrhoeae American Type Culture Collection (ATCC) 49226 was used as a quality control for antimicrobial susceptibility testing and negative control for beta-lactamase test. Beta-lactamase producing *Staphylococcus aureus* was used as a positive control for beta-lactamase test.

2. Storage of isolates

1) To prepare an isolate for freezing, each isolate was grown overnight (18-20 hours) on GC medium or blood agar. A heavy suspension of growth was made in the stock medium i.e., 5% W/V trypticase soy broth plus 20% V/V glycerol (stock medium) by using a small volume of stock medium (approximately 1 ml). All isolates of *N. gonorrhoeae* were kept frozen at -80°C in stock medium until used.

2) Isolates were recovered from storage when it was needed by subcultured from frozen stock medium on GC medium and incubated overnight (18-20 hours) at 35°C in 5% CO_2 incubator.

3. Identification of *N. gonorrhoeae*

N. gonorrhoeae was isolated and identified according to standard microbiological techniques (70).

3.1 Typical characteristics

The colonies with typical characteristics of *N. gonorrhoeae* included small size (about 0.5-1 mm in diameter), beige to gray-brown colored, translucent and smooth and raised colony on GC medium for pathogenic *Neisseria.*

3.2 Gram staining

The Gram stain of *N. gonorrhoeae* from colonies showed uniformly with characteristic gram-negative diplococci or short chains and had adjacent sides that were flattened and formed like a "coffee bean" appearance.

3.3 Biochemical tests

All isolates were tested for the biochemical characteristics as follows (70):

Biochemical tests	**Characteristics of *N. gonorrhoeae***
Oxidase test	Positive
Sugar fermentation	
- glucose	Positive
- maltose	Negative
- sucrose	Negative
- lactose	Negative
- fructose	Negative

4. Antimicrobial susceptibility test

All gonococcal isolates were tested for antimicrobial susceptibility by using disk diffusion method as described by Clinical and Laboratory Standards Institute (101).

4.1 Colony count

This method was used to ensure that the final inoculum concentration of bacteria was approximately to 1×10^8 CFU/ml. This was performed by subculture *N. gonorrhoeae* ATCC 49226 from stock culture on GC medium and incubated overnight at 35°C in CO_2 incubator. Colonies on GC medium were suspended in tryptone water (1% W/V) and adjusted to a turbidity of 0.5 and 1.0 McFarland standard using a nephelometer. The suspension was then diluted to 1:10, 1:100, 1:1000 and 1:10000 with tryptone water. After mixing, a 0.1 ml aliquot was spreaded over the surface of blood agar, incubated at 35°C in 5% CO_2 incubator for 72 hours. The colonies was counted and calculated as CFU/ml.

4.2 Disk diffusion method

All gonococcal isolates were tested for antimicrobial susceptibility against 6 antimicrobial agents, which were penicillin (10 units: Oxoid Ltd.,England), cefotaxime (30 µg: Becton-Dickinson BBL Co., Ltd., USA), ceftriaxone (30 µg: Oxoid Ltd.,England), tetracycline (30 µg: Oxoid Ltd.,England), ciprofloxacin (5 µg: Oxoid Ltd.,England) and ofloxacin (5 µg: Becton-Dickinson BBL Co., Ltd., USA).

4.2.1 Inoculum preparation

The direct colony suspension procedure was used for testing *N. gonorrhoeae*. Inoculation of gonococci was prepared from the colonies grown overnight on chocolate agar at 35°C in 5% CO_2 incubator. Colonies were suspended in tryptone water (1% W/V) and adjusted to a turbidity of 1.0 McFarland standard using a nephelometer.

4.2.2 Plate inoculation

Within 15 minutes after adjusting of the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension and rotated several times. Excess inoculum from the swab was removed by pressing the swab on the inside wall of the tube. The entire surface of GC medium was swabbed in three separated direction (turn 60° after each pass) to achieve smooth, confluent growth on the agar surface.

4.2.3 Applying the antimicrobial disks

Within 15 minutes after inoculating plates, but after the inoculum was dried, the 6 mm antimicrobial disks were applied with slight pressure to ensure that they will not become detached from the agar surface. Three disks were placed onto the agar surface of 100 mm agar plate and disks were not removed once they touched the agar surface.

4.2.4 Incubation

Within 15 minutes after disks placement, the plates were inverted and placed in an incubator at 35°C in 5% CO_2 incubator for 20-24 hours.

4.2.5 Measurement of inhibition zone

The diameter of complete inhibition zone was measured as judged by naked eyes. If a confluent lawn of growth was not achieved, the test would be repeated.

4.2.6 Interpretation

The interpretation criteria of disk diffusion method were according to CLSI (101), as show in Table 10.

Table 10. Zone diameter interpretive standards of antimicrobial agents for *N. gonorrhoeae*

Antibacterial agent	Disk content	Diameter of inhibition zone (mm)*		
		Resistance	Intermediate	Susceptible
Penicillin	10 unit	≤ 26	27-46	≥ 47
Cefotaxime	30 μg	-	-	≥ 31
Ceftriaxone	30 μg	-	-	≥ 35
Tetracycline	30 μg	≤ 30	31-37	≥ 38
Ciprofloxacin	5 μg	≤ 27	28-40	≥ 41
Ofloxacin	5 μg	≤ 24	25-30	≥ 31

* Diameter of disk is 6 mm.

5. Detection of beta-lactamase

All gonococcal isolates were tested for the production of beta-lactamase by using chromogenic cephalosporin method (13).

5.1 Inoculum preparation

Each gonococcal isolate was incubated on GC medium overnight at 35°C in 5% CO_2 incubator. Freshly isolated colonies are used for testing.

5.2 Procedure

250 μg of nitrocefin (Glaxo Group Research Ltd., England) were dissolved in 500 μl of dimethyl sulfoxide (DMSO; Oxoid, USA). One drop of the solution was placed on the glass slide. The full loop of gonococcal culture was taken from GC medium and mixed well with the solution. Beta-lactamase producing *S. aureus* and *N. gonorrhoeae* ATCC 49226 were used as positive and negative quality controls, respectively.

5.3 Reading and interpreting results

The mixture was examined for changing color by naked eyes. The results were read within 5 minutes after testing. The result was interpreted as follows and shown in Figure 9.

Color of suspension	Interpretation of result
Yellow	Negative
Pink	Positive

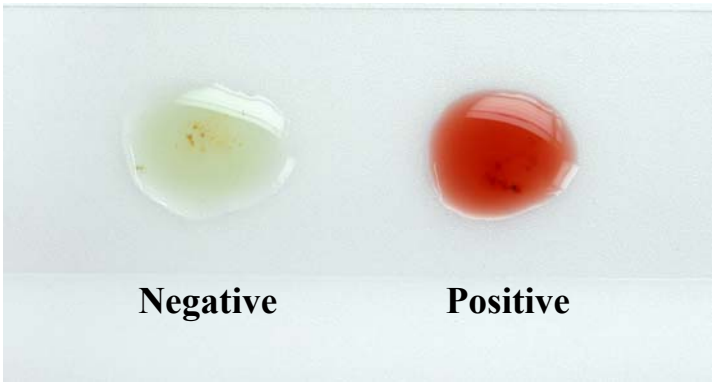


Figure 9. The result of the detection of enzyme beta-lactamase in *N. gonorrhoeae* ATCC 49226 and beta-lactamase producing *S.aureus*

6. Detection of beta-lactamase TEM (*bla*$_{TEM}$) gene by polymerase chain reaction (PCR)

All isolates of *N. gonorrhoeae* were used to extract plasmid by using NucleoSpin Plasmid DNA Purification kit (Macherey-Nagel Inc., USA) as follows:

6.1 Plasmid DNA extraction

A loopful colony of each gonococcal isolate from 24 hours on blood agar was suspended in 250 µl of resuspension buffer A1 in a 1.5 ml microcentrifuge tube and vortexed until there was no cell clump in the suspension. 250 µl of lysis buffer A2 was added into the suspension and mixed gently by inverting the tube 6-8 times. The suspension was incubated at room temperature for a maximum of 5 minutes. After incubation, 300 µl of neutralization buffer A3 was then added and mixed gently by inverting the tube 6-8 times. The cell lysate was centrifuged at 11,000 × g for 10 minutes at room temperature. A new NucleoSpin Plasmid column was placed in a new 2 ml collecting tube. The supernatant from previous step was loaded onto the setting column. The column was centrifuged at 11,000 × g for 1 minute and the flow-through liquid was discarded. The NucleoSpin Plasmid column was placed back into the 2 ml collecting tube. 600 µl of wash buffer A4 was added into the column and centrifuged at 11,000 × g for 1 minute. The flow-through liquid was discarded. The NucleoSpin Plasmid column was reinserted into the 2 ml collecting tube and the column was then centrifuged at 11,000 × g for 2 minutes to dry the silica membrane completely. For elution of highly pure DNA, the NucleoSpin Plasmid column was placed into a new 1.5 ml microcentrifuge tube and 50 µl of elution buffer AE was added and the column was incubated at room temperature for 1 minute. After incubation, the column was centrifuged at 11,000 × g for 1 minute. The purify plasmid DNA was stored at -20°C until used.

6.2 Preparation of pUC19 plasmid DNA

pUC19 plasmid DNA containing ampicillin resistant gene (bla_{TEM}) was used as a positive control in the amplification of bla_{TEM} gene by PCR method. The pUC19 plasmid was transformed into TOP10 *E. coli* to increase the amount of plasmid as follows:

3 µl of pUC19 plasmid DNA was added into the tube containing TOP10 *E. coli* and mixed gently by using the end of pipette tip. The tube was placed on ice for 5-30 seconds. The tube was then heated shock by placing in 42°C water-bath for 30 seconds and immediately on ice for 1-2 minutes. 200 µl of S.O.C medium was added into the tube and incubated at 37°C with shaking for 1 hour. 50 µl of the transformants was spreaded on a surface of prewarmed Luria-Bertani (LB) plate containing 100 µg/ml of ampicillin and incubated overnight at 37°C. An efficient transforming reaction should produce several hundred colonies. Approximately 5 colonies were picked to extract plasmid DNA by using NucleoSpin Plasmid DNA Purification kit (Macherey-Nagel Inc., USA) in the same method as gonococcal plasmid DNA isolation.

6.3 PCR amplification of bla_{TEM} gene from *N. gonorrhoeae*

The gene coding for transferable beta-lactamase (bla_{TEM}) gene was detected by using the PCR amplification from plasmid DNA. The oligonucleotide primers, i.e., TEM F and TEM R specific for the beta-lactamase gene were used. The melting temperature and PCR product length were shown in Table 11.

The PCR amplification of bla_{TEM} was performed in a 25 µl polymerase chain reaction mixture containing plasmid DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5 µM each primer, 400 µM each deoxynucleoside triphosphate and 1 U of *Taq* DNA polymerase (DyNAzyme^{TM} II DNA Polymerase, Roche Co., Ltd., USA). The concentration and volume of each reagent in polymerase chain reaction mixture were shown in Table 12. The PCR cycling was performed in the PCR Sprint ThermoHybaid (Hausen Berstein Co., Ltd, UK) by using the following conditions: predenaturation at 95°C for 5 minutes; 25 cycles of 1 minute at 95 °C, 1 minute at 55 °C and 1 minute at 72 °C; and final extension at 72 °C for 7 minutes. The amplified products were detected by electrophoresis in a 2% agarose gel (Ultra pure agarose electrophoresis grade, Life Technologies Inc., USA) containing 1X Tris-acetate-EDTA buffer. Prior to electrophoresis, samples were mixed with loading dye. The gel

was run in 1X Tris-acetate-EDTA buffer at a consistant voltage of 100 volts for 30 minutes and a standard 100 bp DNA ladder was used as a size marker. Following electrophoresis, the gel was stained with ethidium bromide and photographed by a geldoc system (Biorad Co., Ltd., USA).

Table 11. Nucleotide sequences and melting temperature (Tm) of the primers used to detect the *bla*$_{TEM}$ genes and the PCR product length of each primer

Primers	Nucleotide sequences (5′→3′)	Tm (°C)	Product length	Source or reference
TEM F	ATGAGTATTCAACATTTCCG	54	868 bp	102
TEM R	CTGACAGTTACCAATGCTTA	56		

Table 12. Concentration of each reagent in a PCR mixture (total volume 25 µl) for the *bla*$_{TEM}$ gene

Reagent	Volume (µl): 1 sample	Final concentration
10X PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1% Triton X-100 and 15 mM $MgCl_2$]	2.5	1X PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100 and 1.5 mM $MgCl_2$]
25 mM $MgCl_2$	1	1 mM
Forward primer (100 µM)	0.125	0.5 µM
Reverse primer (100 µM)	0.125	0.5 µM
10 mM dNTP	1	400 µM
DyNAzyme^{TM} II DNA polymerase (2U/ µl)	0.5	1 U
Total genomic DNA (1:100)	1	-
MilliQ water	18.75	-

6.4 Confirmatory of PCR product of *bla*$_{TEM}$ gene by DNA sequencing

One PCR product was chosen for DNA sequencing to ensure that the obtained PCR product was actually *bla*$_{TEM}$ gene.

6.4.1 Purification of PCR product

The PCR product was directly purified by using the NucleoSpin Extract II PCR clean-up kit (Macherey-Nagel Inc., USA) as follows:

Two volumes of buffer NT were mixed with 1 volume of PCR product. The new NucleoSpin Extract II column was placed into a 2 ml collecting tube and the suspension were loaded onto the column. The column was centrifuged at 11,000 × g for 1 minute. The flow-through was discarded and the NucleoSpin Extract II column was placed back into the collecting tube. 600 µl of buffer NT3 was added into the column and centrifuged at 11,000 × g for 1 minute. The flow-through was discarded. The column was placed back into the collecting tube and centrifuged at 11,000 × g for 2 minutes to removed residual buffer NT3. For DNA elution, the column was placed into a new 1.5 ml microcentrifuge tube. 20 µl of elution buffer NE was added into the column and incubated at room temperature for 1 minute to increase the yield of eluted DNA. After incubation, tube was centrifuged at 11,000 × g for 1 minute. The purified PCR product was stored at -20°C until used.

6.4.2 DNA sequencing of *bla*$_{TEM}$ gene

The purified PCR product was verified by DNA sequencing with TEM-F and TEM-R primers at Ward Medic Ltd. in order to confirm the sequence of *bla*$_{TEM}$ gene.

6.4.3 BLAST sequence with GenBank database and program of bioinformatics tools

The obtained sequences of *bla*$_{TEM}$ gene were performed nucleotide blast (blastn) and protein blast (blastx) in GenBank database from website: http://ncbi.nlm.nih.gov/blast/Blast.cgi.

CHAPTER VII
RESULTS: PART B

1. Sources of *N. gonorrhoeae* isolates

A total of 122 isolates from clinical specimens were collected consecutively during June 2005 to May 2007 from different patients visited Siriraj Hospital and Bangrak Hospital. There were 30 patients (24.6%) from Siriraj Hospital and 92 patients (75.4%) from Bangrak Hospital as shown in Figure 10. There were 75.4% male and 24.6% female. The patients' ages varied from 3 days to 68 years old. The isolates were cultured from patients aged less than 15 years (4.9%), followed by patients aged 16 to 25 years (40.2%), 26 to 35 years (30.3%), 36 to 45 years (11.5%), 46 to 55 years (0.8%), and more than 56 years (0.8%) as shown in Figure 11. Ages of 14 patients were unknown.

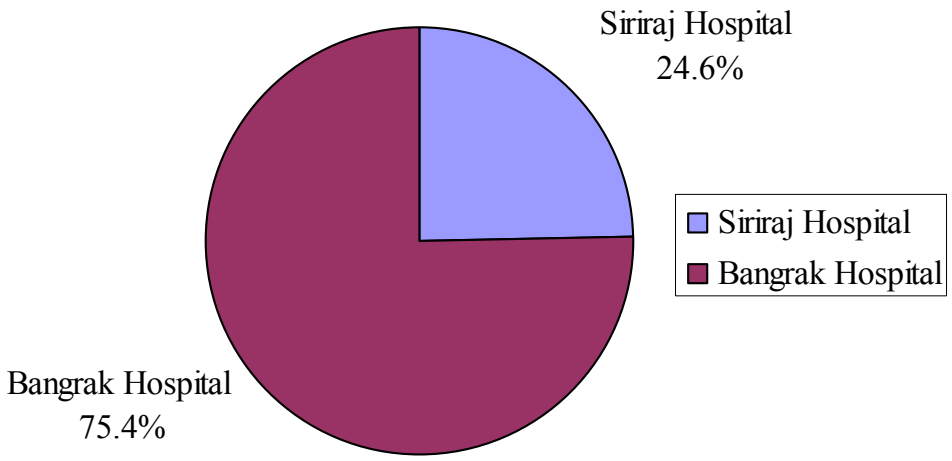


Figure10. Distribution of gonococcal isolates obtained from patients visited Siriraj Hospital and Bangrak Hospital

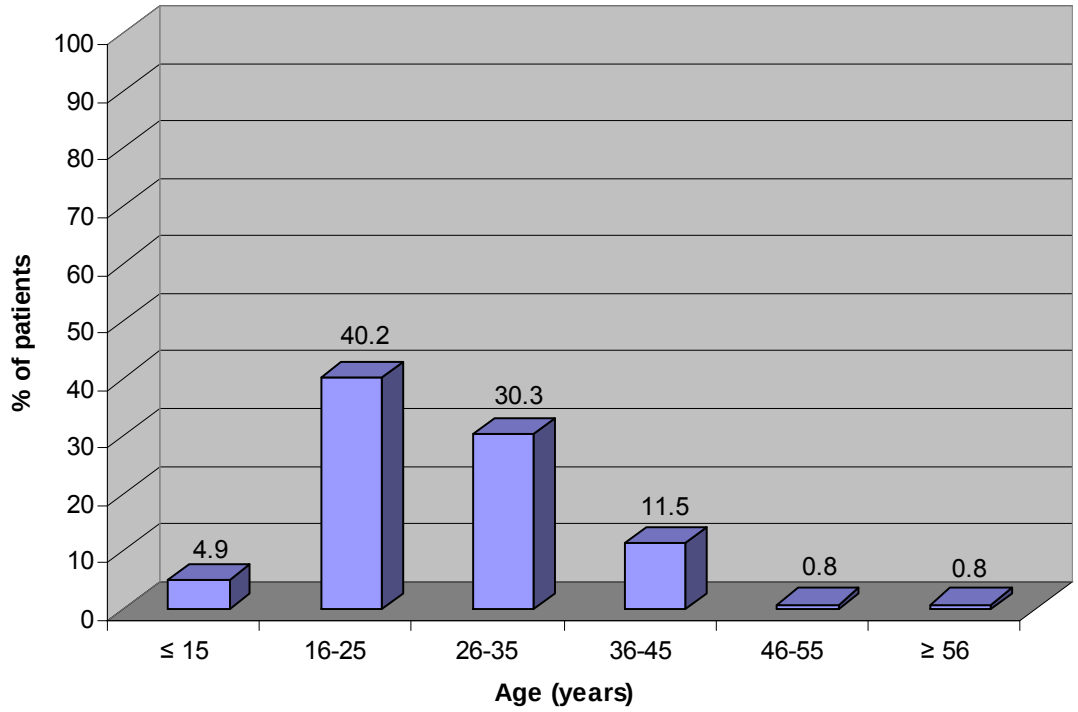


Figure 11. Gonococcal infected patients from Siriraj Hospital and Bangrak Hospital according to ages

2. Antimicrobial susceptibility test

For antimicrobial susceptibility test, the accuracy of the McFarland standard was verified by adjusting a suspension of a control strain, *N. gonorrhoeae* ATCC 49226, to the turbidity of 0.5 McFarland standard. The suspension was then prepared serial 10-fold dilutions and performed colony counts on blood agar plates. The plates were incubated at 35°C in CO_2 incubator for 72 hours. It was found that the mean bacterial density of 0.5 McFarland standard suspension was less than 1.5×10^8 CFU/ml (data not shown). Subsequently, the turbidity was increased to 1.0 McFarland standard and the colony count of *N. gonorrhoeae* ATCC 49226 on blood agar and incubated at 35 °C in CO_2 incubator for 72 hours was performed. The result was shown in Table 13. The mean bacterial density of 1.0 McFarland standards suspension was higher than the turbidity of 0.5 McFarland standard but also less than 1.5×10^8 CFU/ml. When prepared 1.0 McFarland standard of bacterial suspension to test for all 6 antimicrobial agents used in this study with *N. gonorrhoeae* ATCC 49226 in GC base with 1% growth supplement and incubated at 35°C in CO_2 incubator for 20 - 24 hours, it was found that the inhibition zones of all antimicrobial agents were shown clearly and ranged within the limitation zone recommended by CLSI (101). Therefore, all antimicrobial susceptibility tests were performed by preparing 1.0 McFarland standard suspension and using *N. gonorrhoeae* ATCC 49226 for quality control in everyday testing and the results were read only when the controls were in the acceptable range.

Table 13. Colony count of *N. gonorrhoeae* ATCC 49226 at turbidity of 1.0 McFarland standard for each dilution on blood agar plates incubated at 37 °C in CO_2 incubator for 72 hours

Dilution	Number of colonies			Mean ± S.D.
	Plate No. 1	Plate No. 2	Plate No. 3	
10^{-3}	> 300	> 300	> 300	-
10^{-4}	87	139	149	125 ± 33.3
10^{-5}	26	23	31	26.7 ± 4
10^{-6}	7	8	12	9 ± 2.6
10^{-7}	2	1	0	1 ± 1

Overall, the susceptibility of *N. gonorrhoeae* to 6 antimicrobial agents was summarized in Table 14 and Figure 12. Among 122 isolates tested by disk diffusion method, none was susceptible to penicillin. For non-susceptible isolates, 17 isolates (13.9%) were in intermediate category and 105 isolates (86.1%) were resistant. Interestingly, all isolates were susceptible to cefotaxime and ceftriaxone. For ceftriaxone, there was 1 isolate not available for antimicrobial susceptibility test. Therefore, the result was covered only 121 isolates. For tetracycline, 116 isolates (95.1%) were resistant, 6 isolates (4.9%) were intermediate resistant and none was susceptible. For ciprofloxacin and ofloxacin, 110 isolates (90.2%) and 111 isolates (91%) were resistant. Only 2 isolates (1.6%) were susceptible to ciprofloxacin and 6 isolates (4.9%) were susceptible to ofloxacin. Other 10 (8.2%) and 5 (4.10%) isolates were intermediate resistant to ciprofloxacin and ofloxacin, respectively. Figure 13 show the distribution of the inhibition zone diameter of the isolates to all 6 antimicrobial agents which the diameter of each antimicrobial disk was 6 mm. The inhibition zone diameter of penicillin-resistant *N. gonorrhoeae* ranged 6-25 mm. There was 32 isolates (26.2%) exhibit no inhibition zone and 101 isolates (82.8%) exhibit inhibition zone diameter $\leq$ 19 mm for penicillin. For 17 penicillin intermediate-resistant isolates, the inhibition zone diameter ranged 28-40 mm. For cefotaxime and ceftriaxone, all isolates produced zone diameter $\geq$ 35 mm to cefotaxime and ceftriaxone. For tetracycline, the resistant isolates produced inhibition zone diameter ranged 6-30 mm and 18 isolates (14.8%) produced inhibition zone diameter of 14 mm and 110 isolates (90.2%) produced inhibition zone diameter $\leq$ 19 mm. For ciprofloxacin, 21 isolates (17.2%) produced no inhibition zone and 31 isolates (25.4%) produced no inhibition zone to ofloxacin. The inhibition zone diameter to ciprofloxacin and ofloxacin ranged 6-44 mm and 6-38 mm, respectively. There were 97 isolates (79.5%) resistant to penicillin, tetracycline, ciprofloxacin and ofloxacin but remain susceptible to cefotaxime and ceftriaxone. Two ciprofloxacin-susceptible isolates were also susceptible to ofloxacin but resistant to peniciliin and tetracycline. One isolate was susceptible to ofloxacin but resistant to ciprofloxacin and intermediate resistant to penicillin and tetracycline. Other 3 ofloxacin-susceptible isolates were intermediate resistant to ciprofloxacin.

Table 14. Antimicrobial susceptibility of *N. gonoorhoeae* by disk diffusion method

Antimicrobial agents	**Number (%) of susceptibility**		
	Susceptible	**Intermediate**	**Resistant**
Penicillin	-	17 (13.9)	105 (86.1)
Cefotaxime	122 (100)	-	-
Ceftriaxone [a]	121 (100)	-	-
Tetracycline	-	6 (4.9)	116 (95.1)
Ciprofloxacin	2 (1.6)	10 (8.2)	110 (90.2)
Ofloxacin	6 (4.9)	5 (4.1)	111 (91)

[a] One isolate was excluded from analysis because it was non-viable.

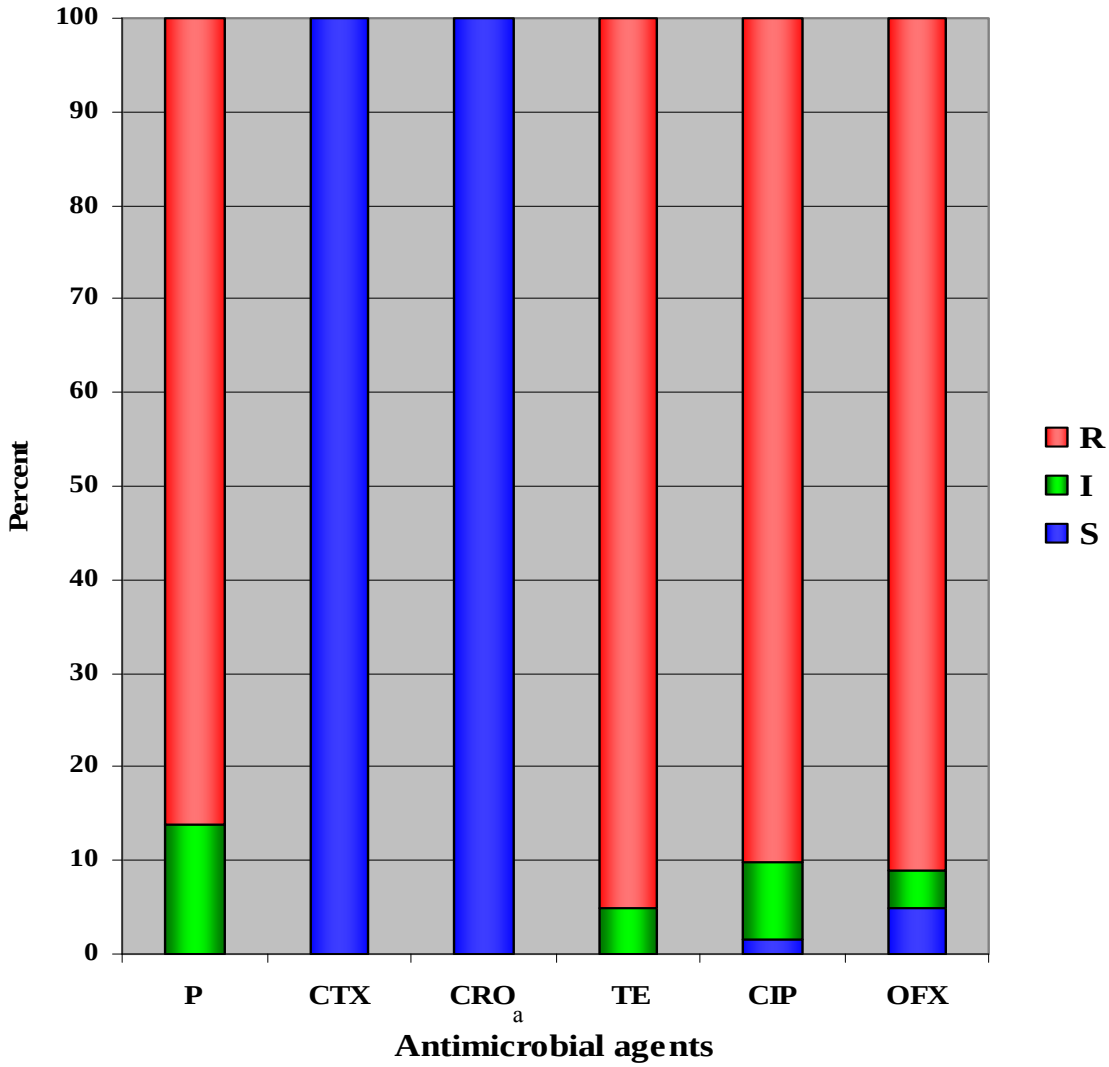
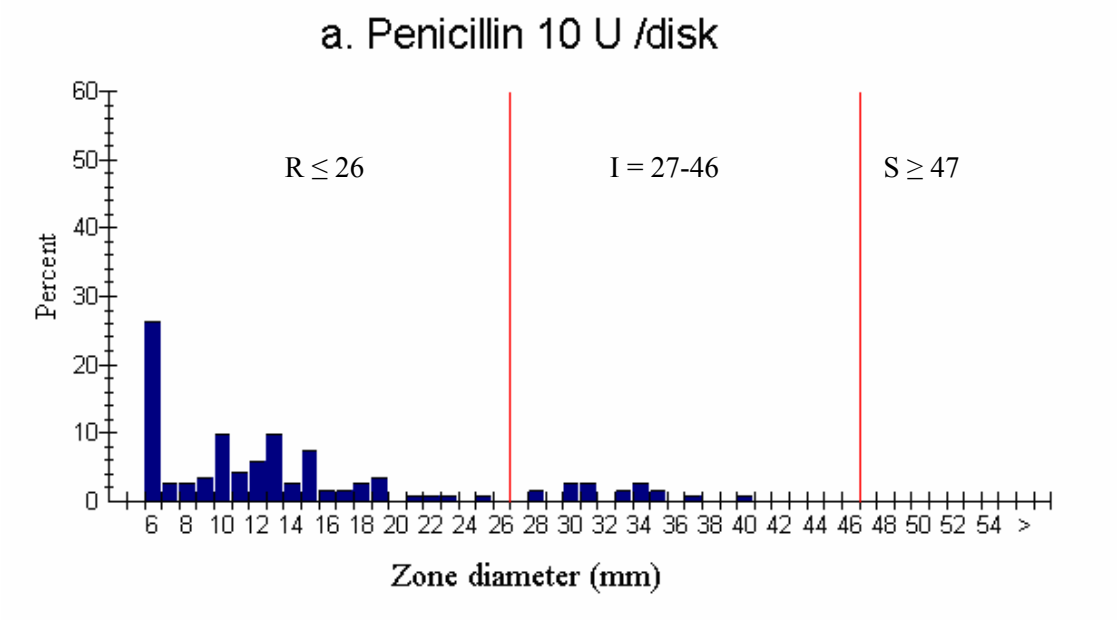


Figure 12. Antimicrobial susceptibility of *N. gonorrhoeae* by disk diffusion method

[a] One isolate was excluded from analysis because they were non-viable.

S, susceptible; I, intermediate resistant; R, resistant. Breakpoints used to define percentages of susceptible, intermediate resistant and resistant categories are those recommended by CLSI (101).



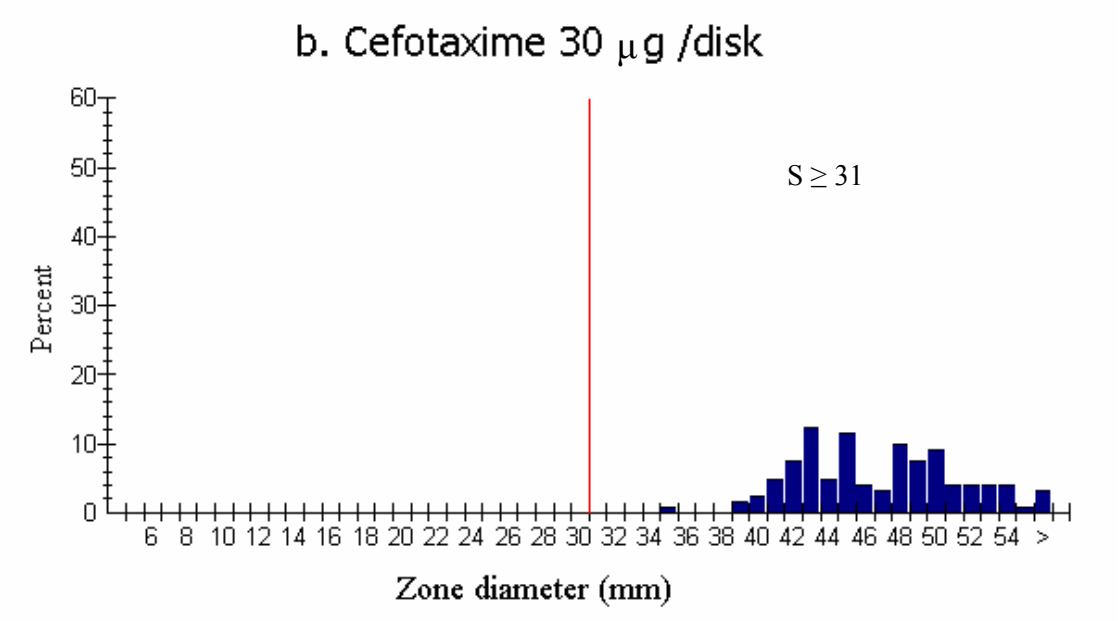
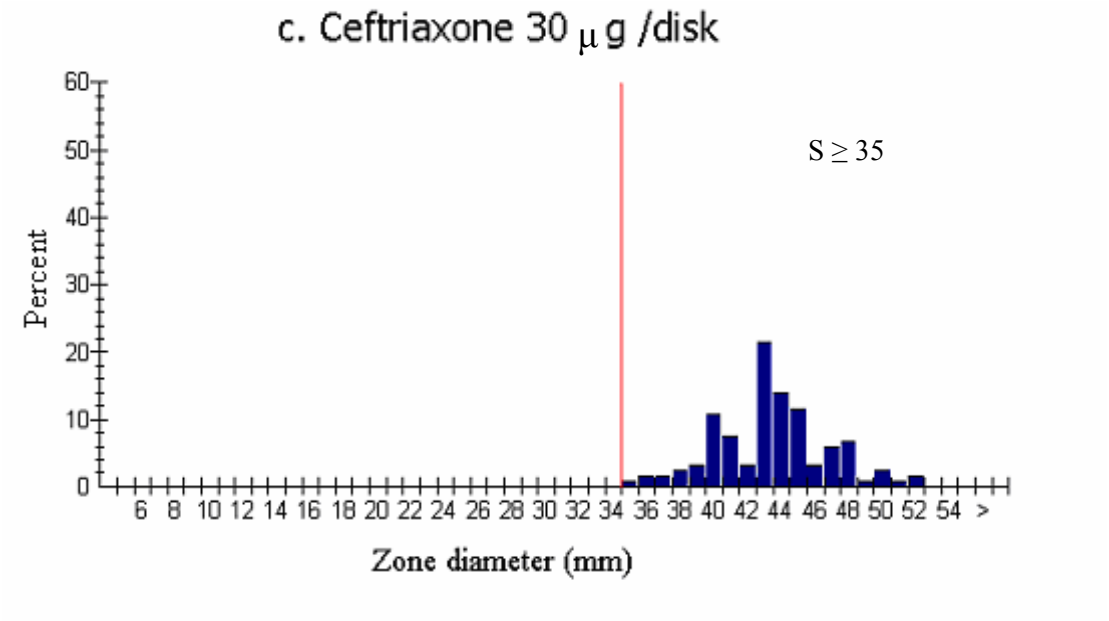


Figure 13. Histogram and susceptibility results of *N. gonorrhoeae* against 6 antimicrobial agents by disk diffusion method



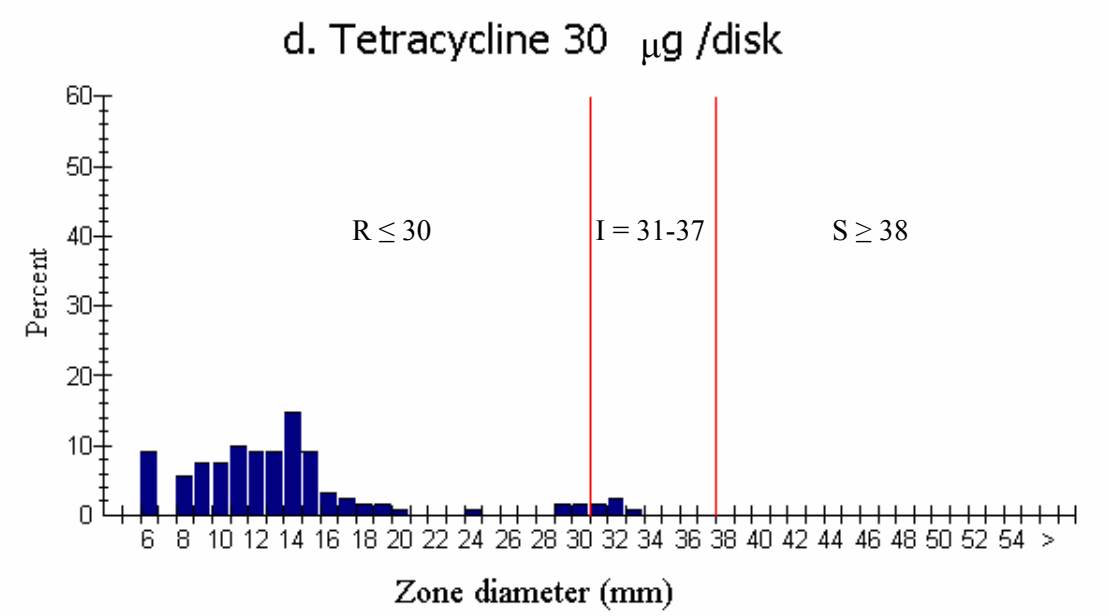
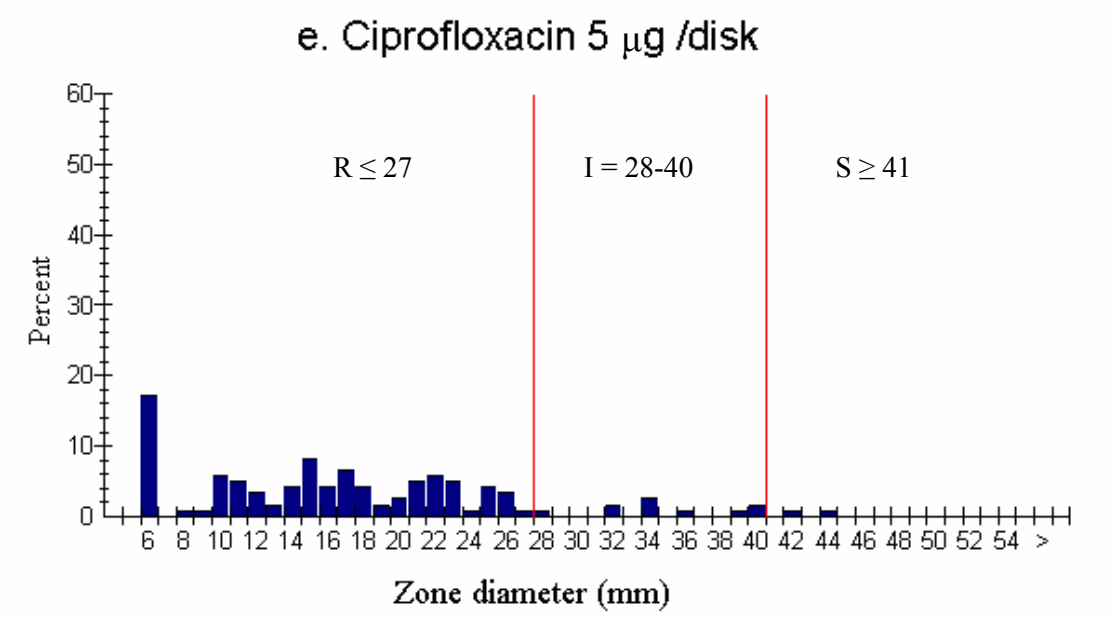


Figure 13. Histogram and susceptibility results of *N. gonorrhoeae* against 6 antimicrobial agents by disk diffusion method (Continued)



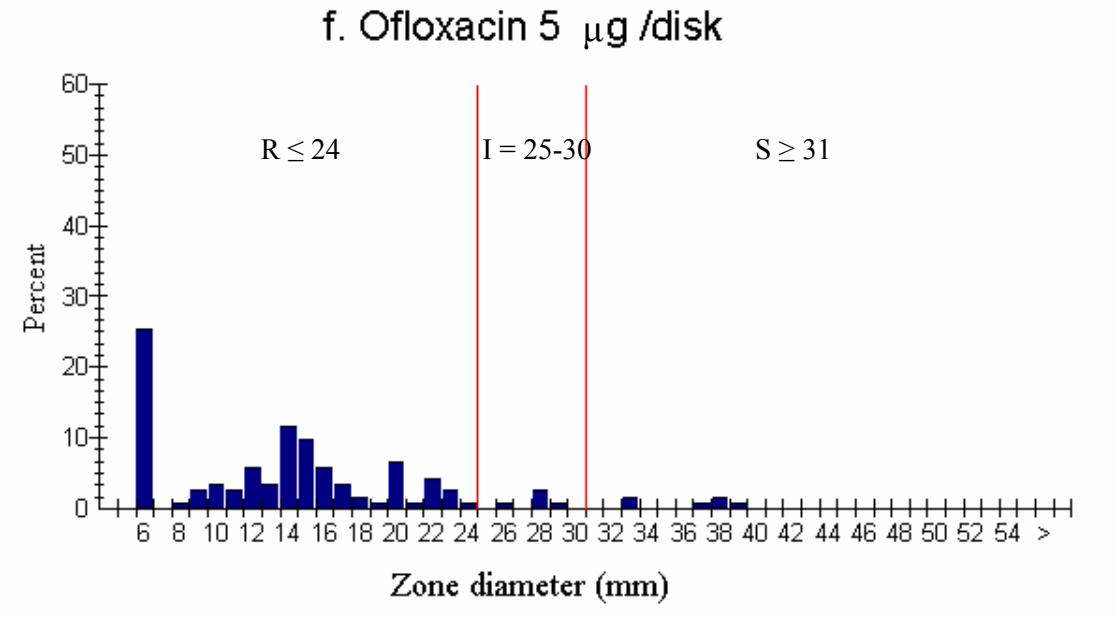


Figure 13. Histogram and susceptibility results of *N. gonorrhoeae* against 6 antimicrobial agents by disk diffusion method (Continued)

3. Detection of beta-lactamase production in *N. gonorrhoeae*

All isolates of *N. gonorrhoeae* were tested for beta-lactamase production by using chromogenic cephalosporin method (13). Of the 122 isolates tested, 102 isolates (83.6%) were resistant to penicillin by beta-lactamase production (Figure 14). All beta-lactamase positive isolates demonstrated a high level of penicillin resistance. Of the 20 beta-lactamase negative isolates, 3 isolates and 17 isolates were resistant and intermediate resistant to penicillin, respectively. None of beta-lactamase negative isolates was susceptible to penicillin. The antimicrobial susceptibility of the isolates which were separated into beta-lactamase positive and beta-lactamase negative is shown in Table 15 and Figure 15. The percentages of resistance to penicillin for beta-lactamase-positive isolates were 6.7-fold higher than those for beta-lactamase-negative isolates. For cefotaxime and ceftriaxone, both beta-lactamase positive and negative isolates remained susceptible.

Table 16 shows the inhibition zone diameters obtained with the 6 antimicrobial agents when 102 beta-lactamase positive and 20 beta-lactamase negative isolates of *N. gonorrhoeae* were tested. The inhibition zone diameters obtained with penicillin for beta-lactamase positive isolates were smaller than for the beta-lactamase negative isolates. Most beta-lactamase positive isolates produced inhibition zone diameter $\leq$ 19 mm except one isolate which produced inhibition zone diameter of 23 mm. For tetracycline, ciprofloxacin and ofloxacin, the number of isolates with beta-lactamase negative produced inhibition zone diamerters > 30 mm were more than those for beta-lactamase positive isolates. The inhibition zone diameters of beta-lactamase positive and negative isolates were high for both cefotaxime and ceftriaxone.

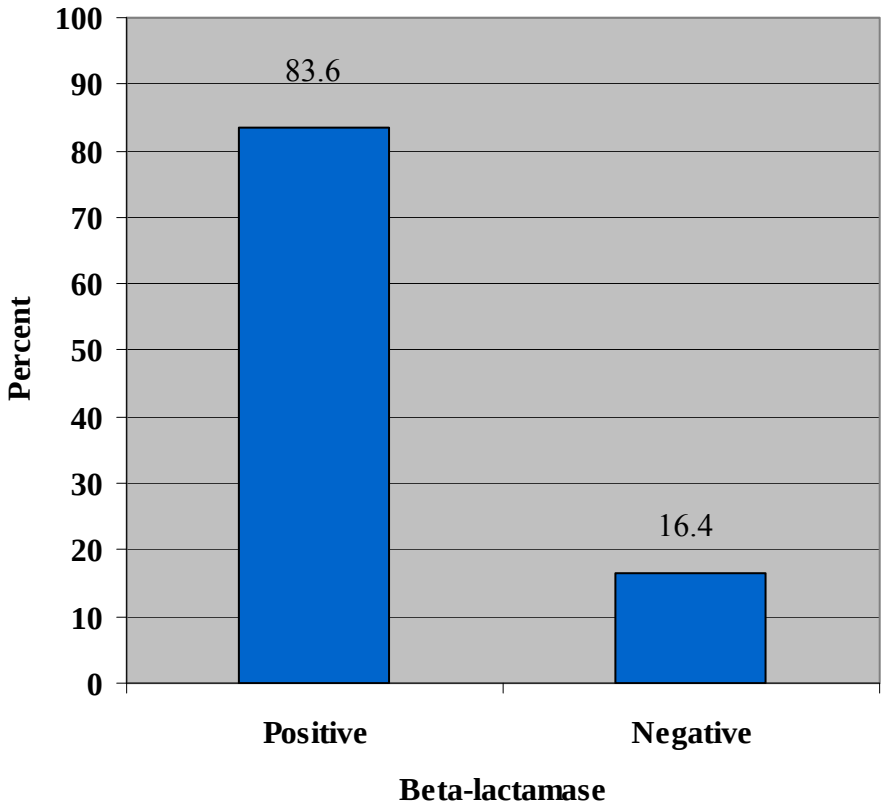


Figure 14. Beta-lactamase production in122 isolates of *N. gonorrhoeae*

Table 15. Antimicrobial susceptibility of 102 beta-lactamase positive and 20 beta-lactamase negative *N. gonorrhoeae*

Antimicrobial agents	**Isolate type***	**Number (%) of susceptibility**		
		Susceptible	**Intermediate**	**Resistant**
Penicillin	BL-pos	-	-	102 (100)
	BL-neg	-	17 (85)	3 (15)
Cefotaxime	BL-pos	102 (100)	-	-
	BL-neg	20 (100)	-	-
Ceftriaxone	BL-pos	102 (100)	-	-
	BL-neg	20 (100)	-	-
Tetracycline	BL-pos	-	3 (2.9)	99 (97.1)
	BL-neg	-	3 (15)	17 (85)
Ciprofloxacin	BL-pos	2 (2)	4 (3.9)	96 (94.1)
	BL-neg	-	5 (25)	15 (75)
Ofloxacin	BL-pos	2 (2)	3 (2.9)	97 (95.1)
	BL-neg	4 (20)	2 (10)	14 (70)

* BL-pos, beta-lactamase positive; BL-neg, beta-lactamase negative.

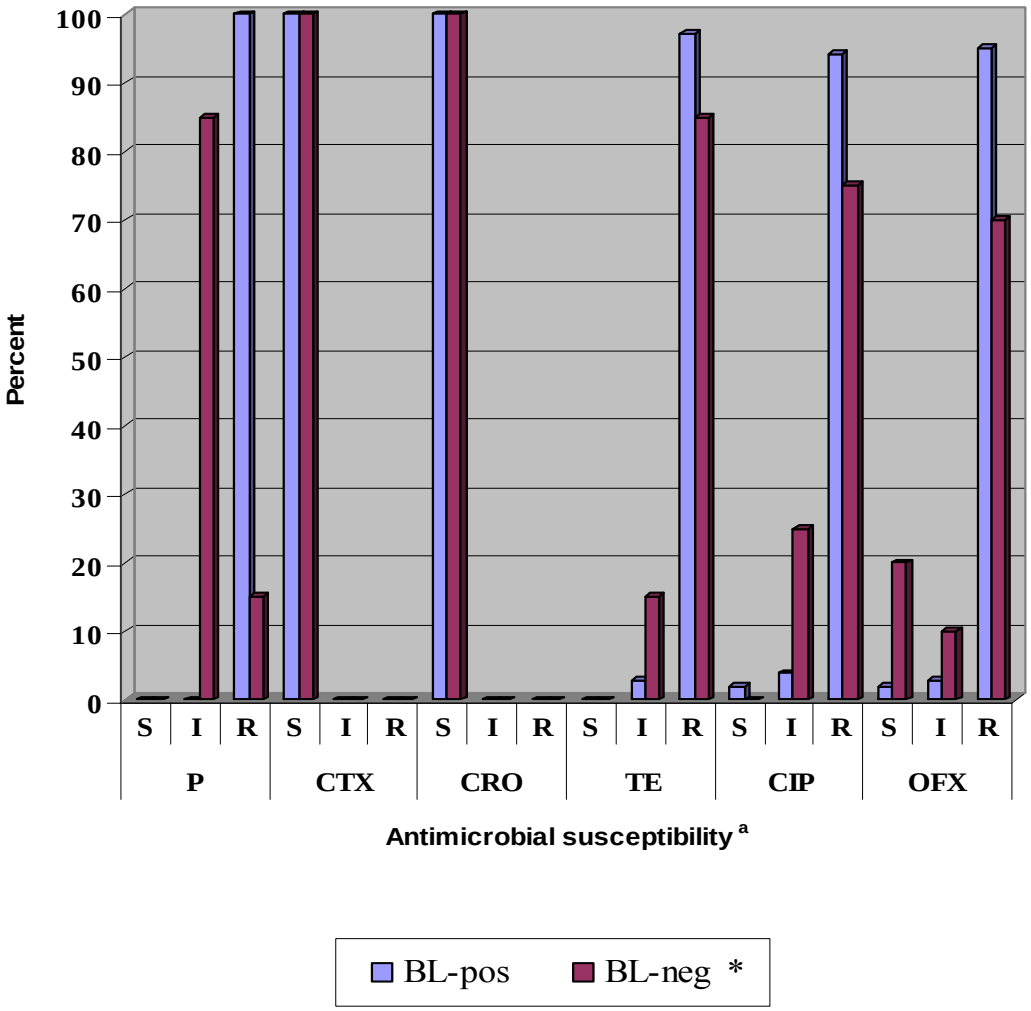


Figure 15. Antimicrobial susceptibility of 102 beta-lactamase positive and 20 beta-lactamase negative *N. gonorrhoeae*

* BL-pos, beta-lactamase positive; BL-neg, beta-lactamase negative.

[a] P, penicillin; CTX, cefotaxime; CRO, ceftriaxone; TE, tetracycline; CIP, ciprofloxacin; OFX, ofloxacin.

S, susceptible; I, intermediate resistant; R, resistant. Breakpoints used to define percentages of susceptible, intermediate resistant and resistant categories are those recommended by CLSI (101).

Table 16. Inhibition zone diameter from 6 antimicrobial agents tested with 102 beta-lactamase positive and 20 beta-lactamase negative isolates of *N. gonorrhoeae*

Antimicrobial agents	Beta-lactamase [b]	Number of isolates with zone diameters (mm) of					
		6-12	13-19	20-30	31-40	41-50	> 50
Penicillin	-ve	-	-	8	12	-	-
	+ve	66	35	1	-	-	-
Cefotaxime	-ve	-	-	-	3	14	3
	+ve	-	-	-	3	77	22
Ceftriaxone [a]	-ve	-	-	-	5	15	-
	+ve	-	-	-	20	78	3
Tetracycline	-ve	7	6	4	3	-	-
	+ve	52	45	2	3	-	-
Ciprofloxacin	-ve	8	4	2	6	-	-
	+ve	32	32	33	3	2	-
Ofloxacin	-ve	11	2	3	4	-	-
	+ve	38	43	19	2	-	-

[a] One isolate was excluded from analysis because it was non-viable.

[b] +ve, positive; -ve, negative.

4. Distribution of PPNG and/or TRNG

Based on plasmid mediated resistance to penicillin and tetracycline, the isolates were phenotypically categorized into three different groups (Table 17). Of 122 gonococcal isolates, it was found that 5 isolates (4.1%) were in the categories of penicillinase-producing *N. gonorrhoeae* (PPNG), 13 isolates (10.7%) and 96 isolates (79.5%) were in the catgories of tetracycline-resistant *N. gonorrhoeae* (TRNG) and PPNG-TRNG, respectively as shown in Table 18. The inhibition zone diameters of PPNG, TRNG and PPNG-TRNG to 6 antimicrobial agents were summarized in Table 19. For penicillin, the PPNG and PPNG-TRNG isolates show small inhibition zone diameter of 11 ± 4.6 and 10.7 ± 4.2 mm, respectively but the TRNG isolates show the inhibition zone diameter of 32.7 ± 3.4 mm. For tetracycline, the TRNG and PPNG-TRNG isolates show small inhibition zone diameter of 11.8 ± 3.1 and 11.9 ± 3.2 mm, respectively but the PPNG isolates show the inhibition zone diameter of 31.6 ± 15.2 mm. For ciprofloxacin and ofloxacin, the PPNG-TRNG isolates show small inhibition zone diameter of 16 ± 6.7 and 13.5 ± 5.5 mm, respectively. However, the TRNG isolates show the small inhibition zone diameter only to ofloxacin (16.5 ± 10.6 mm). The PPNG isolates show the inhibition zone diameter of 31.6 ± 15.2 and 27.6 ± 12.9 mm to ciprofloxacin and ofloxacin, respectively.

Table 17. Phenotypic categories of *N. gonorrhoeae* based on plasmid mediated resistance to penicillin and tetracycline

Category	Criteria [a] (101)
1. Penicillinase-producing *N. gonorrhoeae* (PPNG)	β-lactamase +ve; tetracycline zone diameter >19 mm
2. Tetracycline-resistant *N. gonorrhoeae* (TRNG)	β-lactamase –ve; tetracycline zone diameter ≤19 mm
3. PPNG-TRNG	β-lactamase +ve; tetracycline zone diameter ≤19 mm

[a] +ve, positive; -ve, negative

Table 18. Distribution of penicillinase-producing (PPNG) and/ or tetracycline-resistant *Neisseria gonorrhoeae* (TRNG) in 122 gonococcal isolates

Clinical isolates	Number of isolates	%
PPNG	5	4.1
TRNG	13	10.7
PPNG-TRNG	96	79.5

Table 19. Inhibition zone diameter of PPNG, TRNG and PPNG-TRNG to 6 antimicrobial agents

Antimicrobial agents	Inhibition zone diameter (mm)	
	Range	Mean ± S.D.
Penicillin		
PPNG	6-15	11.0 ± 4.6
TRNG	28-40	32.7 ± 3.4
PPNG-TRNG	6-23	10.7 ± 4.2
Cefotaxime		
PPNG	44-59	50.2 ± 3.6
TRNG	39-53	46.1 ± 4.3
PPNG-TRNG	40-61	47.2 ± 4.5
Ceftriaxone		
PPNG	40-50	45.2 ± 3.6
TRNG	38-40	42.2 ± 2.0
PPNG-TRNG	36-52	43.7 ± 3.3
Tetracycline		
PPNG	20-32	29.0 ± 5.1
TRNG	6-15	11.8 ± 3.1
PPNG-TRNG	6-19	11.9 ± 3.2
Ciprofloxacin		
PPNG	6-44	31.6 ± 15.2
TRNG	10-40	46.1 ± 4.3
PPNG-TRNG	6-32	16.0 ± 6.7
Ofloxacin		
PPNG	6-38	27.6 ± 12.9
TRNG	6-38	16.5 ± 10.6
PPNG-TRNG	6-28	13.5 ± 5.5

5. Detection of beta-lactamase TEM (*bla*$_{TEM}$) gene

All gonococcal isolates except one nonviable isolate were used for analysis of the presence of beta-lactamase TEM (*bla*$_{TEM}$) gene by polymerase chain reaction (PCR) method with TEM forward and TEM reverse primers. In this study, the chromosomal DNA was prepared by boiling the watered-suspension of colonies from *N. gonorrhoeae* or total genomic DNA extraction by using the Puregene DNA purification kit (Gentra Systems, USA) did not provide the consistent results from the PCR amplification, because the *b1a*$_{TEM}$ gene fragments were amplified in only some PCR reactions (data not shown). Therefore, the plasmid DNA extraction using NucleoSpin Plasmid DNA Purification kit (Macherey-Nagel Inc., USA) was used in this study. The 868 bp *b1a*$_{TEM}$ gene fragments amplified and separated in a 2% agarose gel were shown in Figures 16. The pUC19 plasmid DNA containing ampicillin resistance gene (*b1a*$_{TEM}$) was used as a positive control in the PCR amplification reaction. The extracted plasmid from *N. gonorrhoeae* ATCC 49226, the penicillin susceptible isolate, did not contain the *b1a*$_{TEM}$ gene (Figue 16, Lane 1). The results from the detection of *b1a*$_{TEM}$ genes showed that 101 out of 121 tested isolates were positive for *b1a*$_{TEM}$ genes detection (Table 20). All of isolates were positive for beta-lactamase production and were highly resistance to penicillin with the inhibition zone diameters ≤19 mm except one isolate had inhibition zone diameter >19 mm. All beta-lactamase negative isolates were negative for *b1a*$_{TEM}$ genes detection and had inhibition zone diameter >19 mm.

The PCR product of isolate number 22 was chosen for DNA sequencing of *bla*$_{TEM}$ gene. This isolate produced beta-lactamase and it was highly resistant to penicillin with the inhibition zone diameter of 6 mm. It was resistant to other antimicrobial agents, but remained susceptible to cefotaxime and ceftriaxone. The antimicrobial susceptibility of this isolate is shown in Table 21. The purified PCR product of *bla*$_{TEM}$ gene fragments of the isolate number 22 and separated in 2% agarose gel is shown in Figure 17. The PCR product was sent for DNA sequencing with TEM forward and TEM reverse primers. The obtained eight hundred and sixty-one base pairs of nucleotide sequences and translated amino acid sequence of *bla*$_{TEM}$ gene are shown in Figure 18. From nucleotide analysis of obtained nucleotide

sequence with the GenBank database by blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the *bla*$_{TEM}$ gene of *N. gonorrhoeae* isolate number 22 was 100% identical to *bla*$_{TEM}$ gene of *Salmonella enterica* strain AJ634602 and 99% identity to *bla*$_{TEM}$ gene of *Salmonella enterica* strain CP001120.1, *Escherichia coli,* e.g. strain EU491958.1, AB263754.2, *Psychrobacter pulmonis* strain EU433997.1, to *bla*$_{TEM-1}$ gene of *Haemophilus parainfluenzae* strain AM849806.1, *E. coli* strain e.g., EU213261.1, EF035622.1 and Enterobacter sp., e.g. strain EF035620.1, EF035619.1. The protein sequence alignment using the translated Genbank database showed 100% similarity to amino acid sequences with Accession number CAG25427 from *Salmonella enterica* but the official name of this beta-lactamse has not been reported. However, according to the publish data by Pasquali F (130), which indicated that mutation in amino acid position 182 from methionine to threonine in amino acid of TEM-1 sequence will change this sequence into TEM-135 that is still a restricted spectrum beta-lactamase. The amino acid in other positions such as 39, 104 and 238 (Ambler numbering system, (131)) in our beta-lactamse sequence is still identical to restricted spectrum TEM-1.

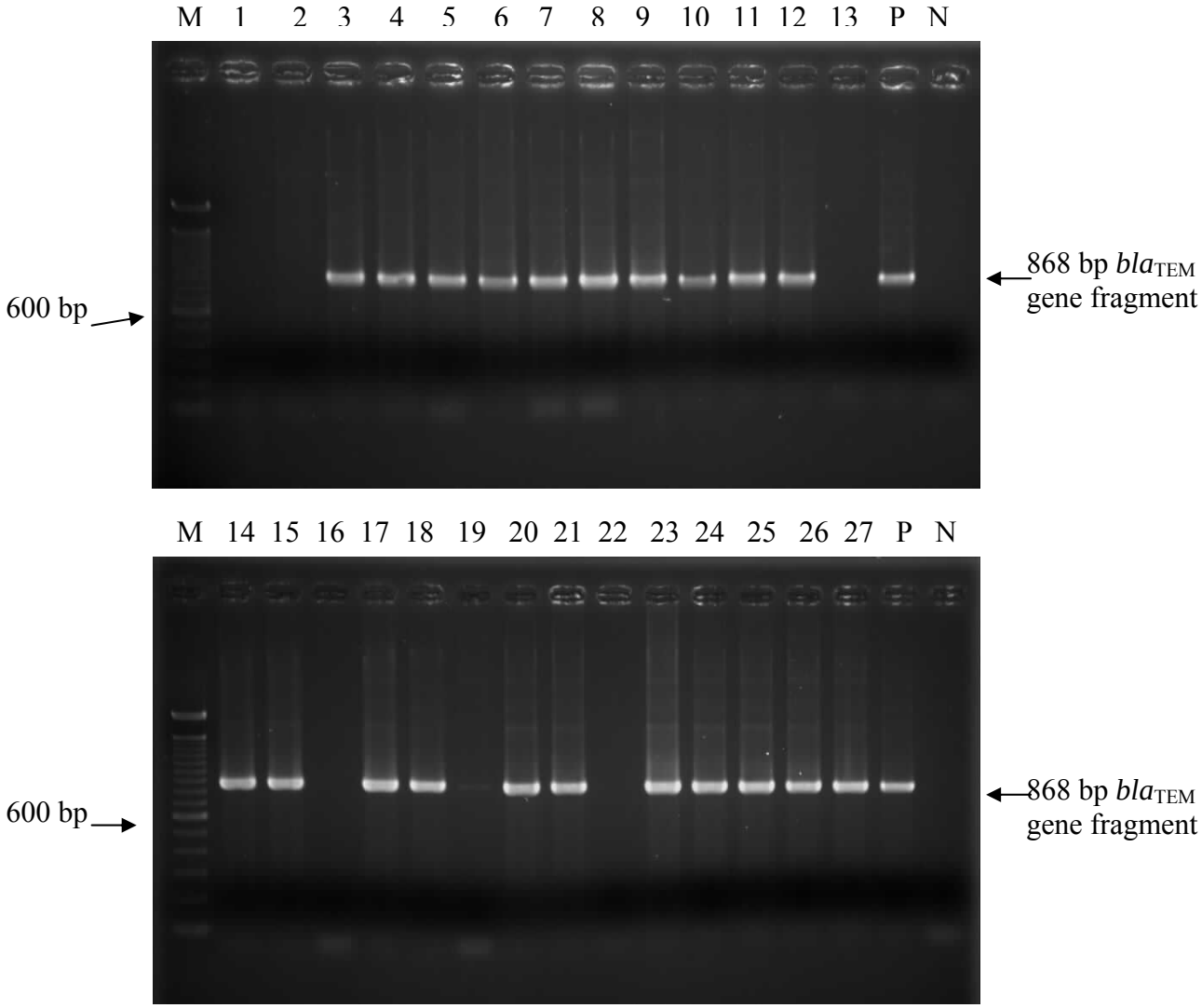


Figure 16. The 868 bp bla_{TEM} gene fragment was amplified by using the TEM forward and TEM reverse primers

Lane M was the standard 100 bp DNA ladder (Invitrogen Co., Ltd., USA), which was used as a size marker.

Lane 3-12, 14, 15, 17, 18, 20, 21 and 23-27 show 867 bp bla_{TEM} gene fragments from beta-lactamase positive isolates.

Lane 1, 2, 13, 16, 19 and 22 show no amplified 867 bp $bla_{TEM\ gene}$ fragment from *N. gonorrhoeae* ATCC 49226 and beta-lactamase negative isolates.

Lane P was a pUC 19 plasmid DNA which was used as a positive control.

Lane N was a negative control.

Table 20. Beta-lactamase production and the presence of *bla*$_{TEM}$ gene in 121 isolates of *N. gonorrhoeae*

Isolate number [a]	Beta-lactamase	*bla*$_{TEM}$ gene	Isolate number [a]	Beta-lactamase	*bla*$_{TEM}$ gene
* 2 (I)	-	-	35 (R)	+	+
3 (R)	+	+	36 (R)	+	+
4 (R)	+	+	37 (R)	+	+
5 (R)	+	+	38 (R)	+	+
7 (R)	+	+	* 39 (I)	-	-
8 (R)	+	+	40 (R)	+	+
9 (R)	+	+	41 (R)	+	+
10 (R)	+	+	42 (R)	+	+
11 (R)	+	+	43(R)	+	+
12 (R)	+	+	* 44 (I)	-	-
13 (R)	+	+	45 (R)	+	+
* 14 (I)	-	-	46 (R)	+	+
15 (R)	+	+	* 47 (R)	-	-
19 (R)	+	+	48 (R)	+	+
* 20 (R)	-	-	49 (R)	+	+
21 (R)	+	+	50 (R)	+	+
22 (R)	+	+	* 51 (R)	+	+
* 24 (I)	-	-	* 52 (I)	-	-
25 (R)	+	+	53 (R)	+	+
26 (R)	+	+	54 (R)	+	+
* 27 (R)	-	-	55 (R)	+	+
28 (R)	+	+	56 (R)	+	+
29 (R)	+	+	* 57 (I)	-	-
30 (R)	+	+	58 (R)	+	+
31 (R)	+	+	* 59 (I)	-	-
32 (R)	+	+	60 (R)	+	+
33 (R)	+	+	61 (R)	+	+

Table 20. Beta-lactamase production and the presence of *bla*$_{TEM}$ gene in 121 isolates of *N. gonorrhoeae* (continued)

Isolate number [a]	Beta-lactamase	*bla*$_{TEM}$ gene
* 62 (I)	-	-
63 (R)	+	+
64 (R)	+	+
65 (R)	+	+
66 (R)	+	+
67 (R)	+	+
* 68 (I)	-	-
69 (R)	+	+
70 (R)	+	+
71 (R)	+	+
72 (R)	+	+
73 (R)	+	+
74 (R)	+	+
75 (R)	+	+
76 (R)	+	+
77 (R)	+	+
78 (R)	+	+
79 (R)	+	+
80 (R)	+	+
81 (R)	+	+
* 82 (I)	-	-
83 (R)	+	+
84 (R)	+	+
85 (R)	+	+
* 86 (I)	-	-
87 (R)	+	+
88 (R)	+	+
89 (R)	+	+
90 (R)	+	+
91 (R)	+	+
* 92 (I)	-	-
93 (R)	+	+
94 (R)	+	+
95 (R)	+	+
96 (R)	+	+
97 (R)	+	+
98 (R)	+	+
100 (R)	+	+
101 (R)	+	+
102 (R)	+	+
105 (R)	+	+
106 (R)	+	+
* 107 (I)	-	-
109 (R)	+	+
110 (R)	+	+
111 (R)	+	+
112 (R)	+	+
* 113 (I)	-	-
* 114 (I)	-	-
115 (R)	+	+
116 (R)	+	+
117 (R)	+	+
118 (R)	+	+
119 (R)	+	+

Table 20. Beta-lactamase production and the presence of *bla*$_{TEM}$ gene in 121 isolates of *N. gonorrhoeae* (continued)

Isolate number [a]	**Beta-lactamase**	***bla*$_{TEM}$ gene**
120 (R)	+	+
121 (R)	+	+
124 (R)	+	+
* 125 (I)	-	-
126 (R)	+	+
127 (R)	+	+
128 (R)	+	+
135 (R)	+	+
136 (R)	+	+
138 (R)	+	+
139 (R)	+	+
140 (R)	+	+
141 (R)	+	+
Total number of positive result [b] (%)	101 (83.5%)	101 (83.5%)

[a] The parentheses was the susceptibility of each isolate to penicillin.

[b] One isolate was excluded from analysis because it was non-viable.

* Inhibition zone diameter to penicillin >19 mm.

Other isolates had inhibition zone diameter ≤19 mm.

Table 21. Antimicrobial susceptibility of the isolate number 22 selected for DNA sequencing of *bla*$_{TEM}$ gene

Antimicrobial agents	Inhibition zone diameter	Interpretation
Penicillin	6	Resistant
Cefotaxime	50	Susceptible
Ceftriaxone	40	Susceptible
Tetracycline	12	Resistant
Ciprofloxacin	10	Resistant
Ofloxacin	6	Resistant

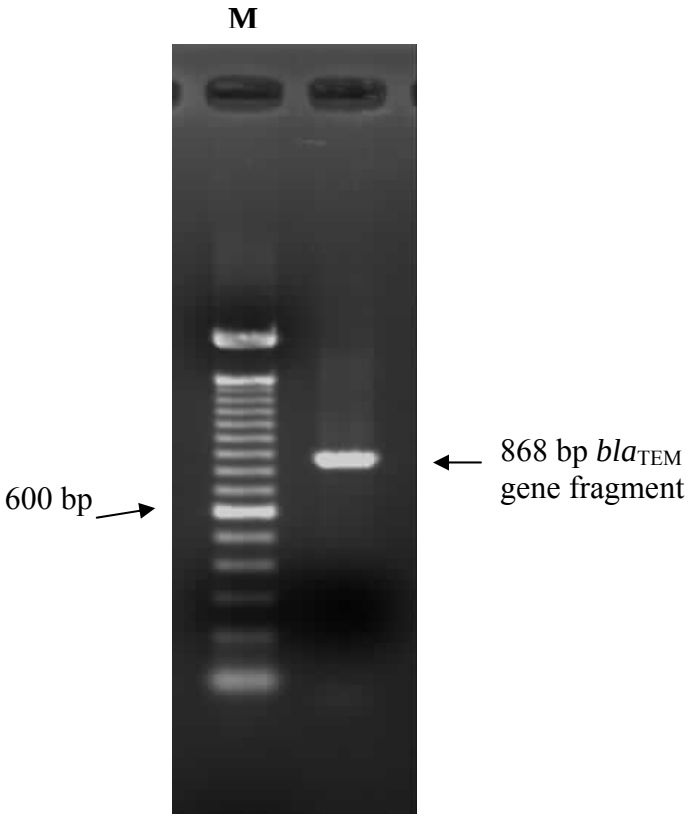


Figure 17. The purified PCR product of the isolate number 22 selected for DNA sequencing of bla_{TEM} gene

Lane M was the standard 100 bp DNA ladder (Invitrogen Co., Ltd., USA), which was used as a size marker.

>Number 22

```
   |→
1  ATGAGTATTC AACATTTCCG TGTCGCCCTT ATTCCCTTTT TTGCGGCATT
   M  S  I  Q  H  F  R  V  A  L  I  P  F  F  A  A  F

51 TTGCCTTCCT GTTTTTGCTC ACCCAGAAAC GCTGGTGAAA GTAAAAGATG
    C  L  P  V  F  A  H  P  E  T  L  V  K  V  K  D  A

101 CTGAAGATCA GTTGGGTGCA CGAGTGGGTT ACATCGAACT GGATCTCAAC
     E  D  Q  L  G  A  R  V  G  Y  I  E  L  D  L  N

151 AGCGGTAAGA TCCTTGAGAG TTTTCGCCCC GAAGAACGTT TTCCAATGAT
    S  G  K  I  L  E  S  F  R  P  E  E  R  F  P  M  M

201 GAGCACTTTT AAAGTTCTGC TATGTGGTGC GGTATTATCC CGTGTTGACG
    S  T  F  K  V  L  L  C  G  A  V  L  S  R  V  D  A

251 CCGGGCAAGA GCAACTCGGT CGCCGCATAC ACTATTCTCA GAATGACTTG
     G  Q  E  Q  L  G  R  R  I  H  Y  S  Q  N  D  L

301 GTTGAGTACT CACCAGTCAC AGAAAAGCAT CTTACGGATG GCATGACAGT
    V  E  Y  S  P  V  T  E  K  H  L  T  D  G  M  T  V

351 AAGAGAATTA TGCAGTGCTG CCATAACCAT GAGTGATAAC ACTGCTGCCA
     R  E  L  C  S  A  A  I  T  M  S  D  N  T  A  A  N

401 ACTTACTTCT GACAACGATC GGAGGACCGA AGGAGCTAAC CGCTTTTTTG
     L  L  L  T  T  I  G  G  P  K  E  L  T  A  F  L

451 CACAACATGG GGGATCATGT AACTCGCCTT GATCGTTGGG AACCGGAGCT
    H  N  M  G  D  H  V  T  R  L  D  R  W  E  P  E  L

501 GAATGAAGCC ATACCAAACG ACGAGCGTGA CACCACGACG CCTGCAGCAA
     N  E  A  I  P  N  D  E  R  D  T  T  T  P  A  A  M

551 TGGCAACAAC GTTGCGCAAA CTATTAACTG GCGAACTACT TACTCTAGCT
     A  T  T  L  R  K  L  L  T  G  E  L  L  T  L  A

601 TCCCGGCAAC AATTAATAGA CTGGATGGAG GCGGATAAAG TTGCAGGACC
    S  R  Q  Q  L  I  D  W  M  E  A  D  K  V  A  G  P

651 ACTTCTGCGC TCGGCCCTTC CGGCTGGCTG GTTTATTGCT GATAAATCTG
     L  L  R  S  A  L  P  A  G  W  F  I  A  D  K  S  G
```

[To be continued in the next page]

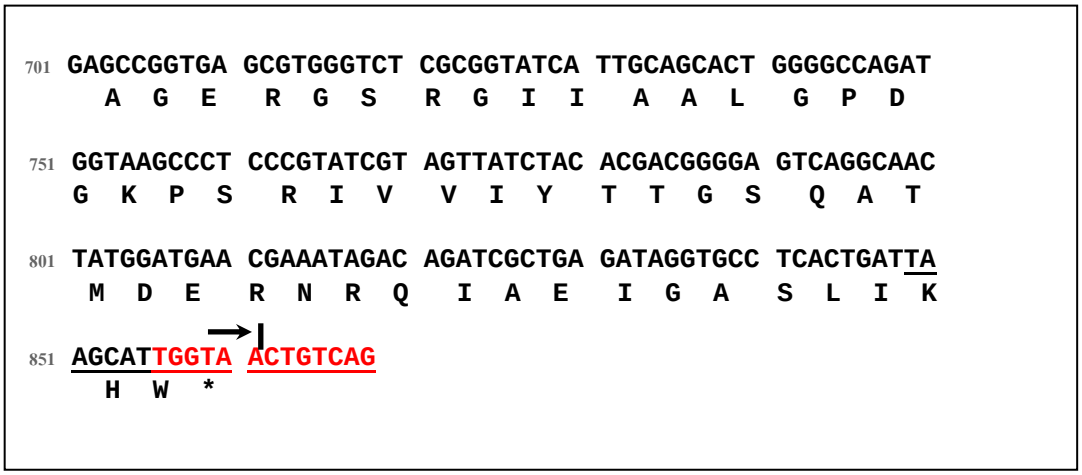


Figure 18. The 868 base pairs of nucleotide sequences and deduced amino acid sequence of *bla*$_{TEM}$ gene of *N. gonorrhoeae* isolate number 22

The vertical bars indicated start or stop of the coding sequences of *bla*$_{TEM}$ gene. The underlined letters indicate sequence for primer annealing. The red letter indicated the sequence of primers which did not obtained from the real sequencing. The red boxes indicated the 4 beta-lactamase active domain and the blue box indicated the M182T that leads to changing from TEM-1 beta-lactamase to TEM-135 beta-lactamase.

CHAPTER VIII
DISCUSSION: PART B

The control of gonococcal infection is important considering the high incidence of acute infections, complications and the role of *N. gonorrhoeae* in facilitating HIV acquisition and antimicrobial resistance has become a major global public health concern. The organism acquires resistance by spontaneous mutation or acquisition of new DNA via conjugation and transformation, thus resistance may be chromosomal or extrachromosomal (132). Since the introduction of sulfonamides in the 1930s, *N. gonorrhoeae* isolates have developed resistance to many of the antimicrobial agents used in the treatment of gonococcal infections. There has been a remarkable increase in antimicrobial resistance among *N. gonorrhoeae* isolates in many developing countries in recent years (133, 134). As antimicrobial susceptibility testing of *N. gonorrhoeae* provides guidance for appropriate treatment, there is a need for simple, reliable and cost-effective method for susceptibility testing. The antimicrobial susceptibility method used in this study was disk diffusion method which has been widely used and perfectly reproducible and comparable with the dilution agar method, MIC, if standardized parameters are observed (135, 136). Although, the MIC testing was more reliable as it has the advantage of producing a more defined estimation of the level of resistance in less sensitive strains (135, 137) but the disk diffusion method was simple, cost-effective and therefore, was more feasible in routine diagnostic laboratories.

Penicillin was widely used for the treatment of gonorrhea for many years and still being used in some countries (13). Appearance of chromosomal resistance produced a slow stepwise decline in penicillin sensitivity until clinically significant levels were reached (138). Chromosomal penicillin resistance in *N. gonorrhoeae* is the result of a series of changes in penicillin-binding proteins and/or outer membrane permeability by mutations at multiple loci, including *penA, mtr* and *penB* (15). The other mechanism for stable, heritable penicillin resistance is

the mediated resistance due to production of beta-lactamase enzyme. This mechanism was first reported in 1976, when two different plasmid-bearing strains were discovered to have very high penicillin MICs, the capacity to produce a TEM-1 type beta-lactamase and an almost universal failure to respond to high-dose penicillin therapy. The initial epidemiology of these beta-lactamase-producing strains showed a spread from Asia and Africa throughout the world (139). The beta-lactamase enzyme was identical to the one in *Haemophilus influenzae* and was thought to be acquired from that organism, which had only a few years earlier acquired this enzyme from an enteric gram-negative bacillus (17). A Japanese study showed that there can be an interaction between chromosomal and plasmid-mediated resistance that can influence the final MICs (140). The transformation of a penicillin-susceptible isolate with a beta-lactamase-encoding plasmid results in a 32-fold increase in MIC, whereas the same plasmid caused a 128-fold increase in MIC when transformed into a chromosomal-mediated resistance isolate. Previous researchers have reported the resistance of *N. gonorrhoeae* to penicillin in Thailand, 89.1% in 1997(141) and 67.1% in 2005 (142). In this study, 86.1% of isolates were resistant to penicillin and no gonococcal isolate with susceptible to penicillin was detected. The high-level penicillin resistance observed in our study may be due to a result of drug therapy for unrelated illness or self-medication with penicillin or other beta-lactam drugs. The prevalences of penicillin-resistant isolates vary considerably geographically. Recent reports have indicated prevalences of penicillin resistance of approximately 6.5% in USA. (143), 81.8% in China (144), 66.7% in New Dehli (145), 66.7% in Bangladesh (146) and 68% in Indonesia (147). For the detection of beta-lactamase production in *N. gonorrhoeae*, this study used the chromogenic cephalosporin (nitrocefin). The action of the penicillinase changes the color of the reagent from yellow to pink/red. This was the most commonly used methods (93%) in the routine laboratories (136). Reasons for the popularity of this test system are commercial availability, logistical simplicity, availability of results within seconds to minutes and reliability (148). In the present study, 102 of 122 (83.6%) isolates produced beta-lactamse which was higher than those of the report in 1992 in Thailand (149) which 28.2% of isolates produced beta-lactamase. All beta-lactamase positive isolates found in this study demonstrated a high level of resistance to penicillin which is in accordance to the report in 2005 in

Thailand (142) and 15% of beta-lactamase negative isolates were resistant to penicillin. Penicillinase-producing *N. gonorrhoeae* (PPNG) was first described in 1970 and have now spread worldwide (146). Subsequent abandonment of penicillin for the treatment of gonorrhea did not lead to substantial decreases in the prevalences of this strain (150). PPNG strains are characterized by a sudden and marked increase in their minimum inhibitory concentrations (MICs) of penicillin, or a sudden and marked decrease in their inhibition zone diameters with a 10U penicillin disk in the disk diffusion test (139). Penicillin-susceptible isolates have an MIC of ≤1 μg/ml and inhibition zone diameter of ≥47 mm. PPNG isolates, on the other hand, have MIC of ≥16 μg/ml and inhibition zone diameter of ≤19 mm, and also are resistant to ampicillin and amoxicillin (101). There were 4.1% of isolates tested in this study were PPNG and most PPNG isolates in this study produced inhibition zone diameter ≤ 19 mm. High prevalence of PPNG was observed in other countries that there were 31% and 44.4% PPNG prevalence in Bangladesh and Nepal, respectively (13), while the percentage of PPNG reported in United States was only 1% (143).

Moreover, this study also determined the molecular mechanism of resistance to penicillin by plasmid-mediated resistance of bla_{TEM} gene encoding the beta-lactamase TEM enzyme. This was performed in all gonococcal isolates by polymerase chain reaction method using the universal primers for bla_{TEM} gene (102) which covered the start codon to stop codon of bla_{TEM} gene (Appendix C). It was found that 101 of 121 tested isolates containing the bla_{TEM} gene in their genome and all PCR-positive isolates also were positive for beta-lactamase test. These finding was in agreement with the report of the high-level of resistance to penicillin in *N. gonorrhoeae* by plasmid containing bla_{TEM}, which can be transferred by conjugation (97). The result was also consistent with the study in the isolates from Russia during the period 2004-2005 (151) which the presence of the bla_{TEM} gene was observed in the isolates with high-level resistance to penicillin (MICs of 4.0 to 16.0 μg/ml). The PCR product from the penicillin resistant isolate was confirmed by DNA sequencing using the amplified primers. The sequence of bla_{TEM} gene obtained in this study was 100% identity with the bla_{TEM} gene of *Salmonella enterica* (Genbank Accession No. AJ634602) and 100% similarity with the beta-lactamase TEM protein of *Salmonella enterica* (Genbank Accession No. CAG25427). Interestingly, the amino acid sequence found in

this study was identified to be the TEM-135 beta-lactamase, because of a single amino acid substitution at position 182 (M182T in TEM-135) (130). However, this data requires further investigation. TEM-1 beta-lactamase is a class A enzyme and the epidemiological study has shown that TEM-1 is the most common plasmid-mediated beta-lactamase and is a major determinant of bacterial resistance to beta-lactam drugs (152). Four motifs, S^{70}-X-X-K, S^{130}-X-N, the Ω-loop and K^{234}-T/S-G, are found in the vicinity of the active-site pocket of class A beta-lactamase (153). The amino acid Q39, E104 and G238 make the beta-lactamase in this study identical to restricted spectrum beta-lactamase (131). This data showed new finding that the *N. gonorrhoeae* isolates with TEM-135 gene was found in Thailand. This is one interesting point on the discovery of TEM-135. This variant is very rare determination of TEM-135 in other isolates would be worth to elucidate the clonal spread.

Tetracycline, a cheap and widely used antimicrobial agent, is an adjunct therapy for *C. trachomatis* in syndromic management of STDs, as well as in some other illnesses (13). The tetracyclines are no longer recommended for treatment of gonorrhea because of increasing resistance, as well as the need for multiple dose therapy, which decreases compliance (99). Both chromosomal and plasmid mediated resistance are widespread. Chromosomal tetracycline resistance is mainly by prevention of accumulation of drug within cell by either decreasing the influx or increasing the ability of cell to export drug (154). High-level tetracycline resistance was first reported in 1985 from CDC Atlanta, USA (13). Plasmid-borne tetracycline resistance in gonococci was characterized by a sudden and marked increase in the MIC of tetracycline or decrease in the inhibition zone diameter in the disk diffusion test (139). These strains are called tetracycline-resistant *N. gonorrhoeae* (TRNG) strains and they have an MIC of ≥16 μg/ml and an inhibiton zone diameter of ≤19 mm (101). In a study by Gonococcal Isolate Surveillance Project (GISP) in USA (143), it was found that the TRNG prevalence, which was 3.7% in 2003, has varied little since 1988, while chromosomally mediated tetracycline resistance declined modestly. Tetracycline resistance was reported to be high in Indonesia in 2001 (100%) (147) and Bangladesh in 2005 (57%) (13). In the present study, 95.1% of isolates were resistant to tetracycline and 10.7% was TRNG which was higher than those of the study in

1999 in Thailand (141) that there was 89.1% of isolates were resistant to tetracycline and the prevalence of TRNG was only 7.9%.

The fluoroquinolones were introduced and widely used clinically in the 1980s because of their effectiveness and convenience in single-dose oral administration, together with lower cost (155). Resistance to fluoroquinolone in *N. gonorrhoeae* results primarily from point mutations in DNA gyrase (*gyrA*) gene and topoisomerase (*parC*) gene. High-level quinolone resistance indicates adaptive mutations of *N. gonorrhoeae* under selective antibiotic pressure (133). After 10 years of clinical use, an increasing trend of fluoroquinolone resistance in *N. gonorrhoeae* has been reported in Southeast Asia (10.6-100%) (13), Japan (73.5%) (156), China (48.8%) (157) and Canada (4.8%) (158). The ciprofloxacin resistance in Thailand was increase from 0.3% in 1992 (149), 21.8% in 1997 (141) and up to 49% in 2002 (142). There were 90.2 and 91% resistance to ciprofloxacin and ofloxacin, respectively observed in this study which increased further than those previous data (141, 142 and 149). These appearances of highly resistance to ciprofloxacin and ofloxacin may result from inappropriate therapy due to self-medication and poor compliance which is common among patients with gonorrhea.

Very high level of activity of the broad spectrum cephalosporin (i.e., cefotaxime and ceftriaxone) against *N. gonorrhoeae* was observed in most of the laboratories (13). Reduced susceptibility to ceftriaxone (2.4%) was reported in India (159) and neighboring countries should be considered with caution as this antimicrobial is used as a drug of choice in most of the centers for management of acute gonorrhea (13). However, gonococcal isolates in this study remained 100% susceptibility to both cefotaxime and ceftriaxone but the inhibition zone diameter to ceftriaxone of 3 isolates were close to break point (i.e., 36 mm). This result was similar to that obtained from the report in Indonesia (147).

The data presented here indicated that *N. gonorrhoeae* has developed resistance to the older and the commonly used antimicrobial agents. Continuing surveillance of gonococcal susceptibility testing not only alerts us to control the resistance but also allow appropriate and cost-effective implementation of decision of antibiotic therapy.

CHAPTER IX
CONCLUSIONS

The present study can be concluded as follows:

1. The using of FAST Express reagent (Gen-probe Inc., USA) in the viscous specimen could improve the specimen to be more watery and easily to pipette.
2. Of 1,131 endocervical swabs from 797 HIV-positive patients, nine patients (1.1%) were positive for gonococcal infection and 71 patients (8.9%) were positive for chlamydial infection. There was only 1 patient positive for both organisms.
3. The gonococcal infection was found at highest prevalence in age group of 16-25 years (1.7%), followed by aged 26-35 years (1.5%).
4. The most chlamydial infection was found in the lower age range which was 20%, 10.3% and 6.7% in age group of ≤15, 16-25 and 26-35 years, respectively.
5. A collection of 122 gonococcal isolates were obtained from Siriraj Hospital and Bangrak Hospital during June 2005 to May 2007. By using antimicrobial disk diffusion, the susceptibilities to cefotaxime, ceftriaxone, ciprofloxacin and ofloxacin were 100%, 100%, 1.6% and 4.9%, respectively. None was susceptible to penicillin and tetracycline.
6. There were 97 isolates (79.5%) resistant to penicillin, tetracycline, ciprofloxacin and ofloxacin but remain susceptible to cefotaxime and ceftriaxone.
7. Of the 122 isolates tested, 102 isolates (83.6%) were resistant to penicillin by beta-lactamase production and all beta-lactamase positive isolates demonstrated a high level of penicillin resistance.

8. Based on plasmid mediated resistance to penicillin and tetracycline, 4.1% were penicillinase-producing *N. gonorrhoeae* (PPNG), 10.7% were tetracycline-resistant *N. gonorrhoeae* (TRNG) and 79.5% were PPNG-TRNG.
9. The extraction plasmid from *N. gonorrhoeae* ATCC 49226, the penicillin susceptible isolate, did not contain the $b1a_{\text{TEM}}$ gene fragment.
10. The $b1a_{\text{TEM}}$ gene was detected in 101 out of 121 tested isolates (83.5%) by polymerase chain reaction (PCR). All beta-lactamase positive isolates were positive for $b1a_{\text{TEM}}$ gene in their plasmid.
11. The 868 base pairs of nucleotide sequences were obtained when performed the $b1a_{\text{TEM}}$ gene sequencing and it was 100% identical to bla_{TEM} gene and 100% similarity to beta-lactamase TEM protein of *Salmonella enterica* when performed nucleotide analysis with the GenBank database.
12. From the deduced amino acid sequence in this study, the mutation in amino acid position 182 from methionine to threonine in amino acid of TEM-1 sequence will change this sequence into TEM-135 that is still a restricted spectrum beta-lactamase.

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APPENDIX

APPENDIX A

Culture media

1. Trypticase Soy Broth

Dissolve 30 grams of the powder in one liter of distilled water. Mix thoroughly and then dispense exactly 5 ml in 15x150 ml test tube and then autoclave at 121°C 15 pounds / $inch^2$ pressure for 15 minutes.

2. GC medium

Disolve 36 grams of GC agar base in 990 ml of distilled water. Boil to dissolve the agar and sterilize by autoclaving at 120°C for 15 minutes. Immediately after autoclaving, allow it to cool in a 50° to 60°C water bath. Add 1% growth supplement into cooled medium and gently mix. The mixture is poured into glass, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 25 ml for plates with a diameter of 100 mm. The agar medium is allowed to cool to room temperature and stored in a refrigerator (2° to 8°C).

APPENDIX B

The pUC19 plasmid containing ampicillin resistance gene (Ap)

The plasmid pUC19 offers the following advantages:

1. It has a high copy number.

2. The plasmid has a polylinker of 50 nucleotides that contain several different restriction sites.

3. “Blue-white” screening for recombinants. The polylinker sequence overlaps with the *lacZ* gene.

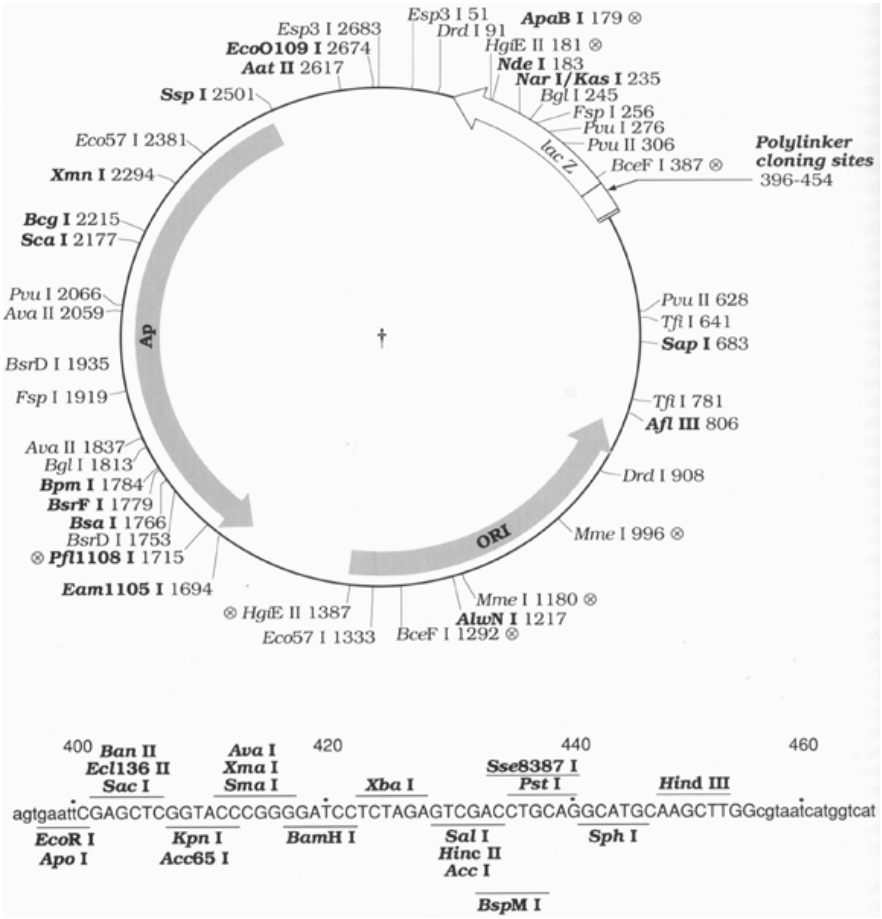

Figure 19. pUC19 plasmid

APPENDIX C

Nucleotide sequence of *bla*$_{TEM}$ gene (23941-24960 nucleotides of *E. coli* plasmid pC15-1a; *bla*$_{TEM-1}$ gene, GenBank Accession No. NC005327). Nucleotide sequences complementary with TEM primers are boldface. The start codon of *bla*$_{TEM-1}$ is indicated by horizontal arrow. The stop codon of *bla*$_{TEM-1}$ is indicated by asterisks.

```
//
23941 tgtgcgcgga acccctattt gtttattttt ctaaatacat tcaaatatgt atccgctcat
                                                    TEM F
24001 gagacaataa ccctggtaaa tgcttcaata atattgaaaa aggaagagt|a tgagtattca
24061 acattttcgt gtcgccctta ttccctttt tgcggcattt tgccttcctg tttttgctca
24121 cccagaaacg ctggtgaaag taaaagatgc tgaagatcag ttgggtgcac gagtgggtta
24181 catcgaactg gatctcaaca gcggtaagat ccttgagagt tttcgccccg aagaacgttt
24241 tccaatgatg agcactttta aagttctgct atgtggtgcg gtattatccc gtgttgacgc
24301 cgggcaagag caactcggtc gccgcataca ctattctcag aatgacttgg ttgagtactc
24361 accagtcaca gaaaagcatc ttacggatgg catgacagta agagaattat gcagtgctgc
24421 cataaccatg agtgataaca ctgctgccaa cttacttctg acaacgatcg gaggaccgaa
24481 ggagctaacc gctttttttgc acaacatggg ggatcatgta actcgccttg atcgttggga
24541 accggagctg aatgaagcca taccaaacga cgagcgtgac accacgatgc ctgcagcaat
24601 ggcaacaacg ttgcgcaaac tattaactgg cgaactactt actctagctt cccggcaaca
24661 attaatagac tggatggagg cggataaagt tgcaggacca cttctgcgct cggcccttcc
24721 ggctggctgg tttattgctg ataaatctgg agccggtgag cgtgggtctc gcggtatcat
24781 tgcagcactg gggccagatg gtaagccctc ccgtatcgta gttatctaca cgacggggag
24841 tcaggcaact atggatgaac gaaatagaca gatcgctgag ataggtgcct cactgattaa
      TEM R *
24901 gcattggtaa ctgtcagacc aagtttactc atatatactt tagattgatt taaaacttca
//
```

APPENDIX D

Reagents for gel electrophoresis

1. 0.5M EDTA

Dissolve 146.12 grams of EDTA in 200 ml of distilled water. Adjust the pH to 8.0, autoclave. Store at room temperature for no longer than 1 year.

2. 50X TAE buffer

Dissolve 242 grams of Tris base in 500 ml of distilled water. Mix thoroughly and then add 57.1 ml of glacial acetic acid and 100 ml of 0.5M EDTA, pH 8.0. Adjust the volume to 1 liter, autoclave. Store at room temperature for no longer than 1 year.

3. Loading dye (10X)

Dissolve 8 grams of sucrose (40% final) in 10 ml of 0.5M EDTA (250 mM final). Add distilled water to 20 ml, dissolve sucrose completely and then add 0.15 gram of bromphenol blue (1.5% final), autoclave, aliquot 1 ml into eppendorfs and store at room temperature.

BIOGRAPHY

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